TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. AII OSA - TOM. 241

BIOLOGICA - GEOGRAPHICA - GEOLOGICA

Differential Regulation of c-FLIP Isoforms Through Post-translational Modifications

by

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Turku 2009

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ISBN 978-951-29-4069-1 (PRINT)
ISBN 978-951-29-4070-7 (PDF)
ISSN 0082-6979
Painosalama Oy, Turku, Finland 2009

To my family

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LIST OF ORIGINAL PUBLICATIONS

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I Poukkula M, Kaunisto A, Hietakangas V, Denessiouk K, Katajamäki T, Johnson MS, Sistonen L, Eriksson JE. 2005. Rapid turnover of c-FLIPshort is determined by its unique C-terminal tail. J Biol Chem 280(29): 27345-27355.

II Meinander A, Söderström TS, Kaunisto A, Poukkula M, Sistonen L, Eriksson JE. 2007. Fever-like hyperthermia controls T lymphocyte persistence by inducing degradation of cellular FLIPshort. J Immunol 178(6): 3944-3953.

III Kaunisto A, Kochin V, Asaoka T, Mikhailov A, Poukkula M, Meinander A, Eriksson JE. 2009. PKC-mediated phosphorylation regulates c-FLIP ubiquitylation and stability. Cell Death Differ 16(9): 1215-1226.

ABBREVIATIONS

AICD	Activation-induced cell death
ALPS	Autoimmune lymphoproliferative syndrome
AP-1	Activator protein 1
Apaf-1	Apoptotic protease-activating factor-1
APC	Anaphase-promoting complex
ASK	Apoptosis signal-regulating kinase
Bad	Bcl-2-associated death protein
Bak	Bcl-2 homologous antagonistic/killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma gene 2
BH	Bcl-2 homology
Bid	BH3-interacting domain death agonist
Bim	BH3-interacting mediator of cell death
BIR	Baculoviral IAP repeat
Bok	Bcl-2-related ovarian killer
BRUCE	BIR repeat containing ubiquitin-conjugating enzyme
BSA	Bovine serum albumin
CAD	Caspase-activated DNase
CaMK	Ca^{2+}-calmodulin-dependent protein kinase
CARD	Caspase recruitment domain
CARMA	Caspase-recruitment domain MAGUK protein 1
Cbl	Casitas B-lineage lymphoma
ced	Cell death abnormality
CHIP	Carboxyl terminus of Hsc70-interacting protein
CHX	Cycloheximide
CML	Chronic myelogenous leukemia
CUE	Cue1-homogous domain
CYLD	Cylindromatosis tumor-suppressor
DcR	Decoy receptor
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP-binding protein with low pI
DISC	Death inducing signaling complex
DMSO	Dimethyl sulfoxide
DR	Death receptor
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
E4	Poly-ubiquitin chain conjugation factor
E6-AP	E6-associated protein
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
Erk	Extracellular signal-regulated protein kinase
FADD	Fas-associated protein with death domain
FAT10	Human leukocyte antigen F-associated transcript 10
FCS	Fetal calf serum
FLICE	FADD-like ICE
FLIP	FLICE-inhibitory protein
GAT	Gamma-ear-containing ADP-ribosylation-factor binding protein
GFP	Green fluorescent protein
GSK3	Glycogen synthase kinase 3
GST	Glutathione-S-transferase
HDAC	Histone deacetylase
HECT	Homologous to E6-AP carboxyterminus
HPV	Human papillomavirus
HRP	Horse radish peroxidase
Hsc70	Heat shock cognate protein 70
HtrA2	High temperature requirement A2
IAP	Inhibitor of apoptosis
ICAD	Inhibitor of CAD
ICE	Interleukin-converting enzyme
IκB	Inhibitor κB
IKK	IκB kinase
IL	Interleukin
JNK	c-Jun N-terminal kinase
KSHV	Kaposi's sarcoma-associated herpesvirus
LUBAC	Linear ubiquitin chain assembly complex
MAGUK	Membrane-associated guanylate kinase
MALT	Mucosa-associated lymphoid tissue lymphoma translocation protein
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MJD	Machado-Joseph disease protease
MOMP	Mitochondrial outer membrane permeabilization
MS	Multiple sclerosis
Nedd8	Neural-precursor-cell-expressed developmentally down-regulated 8
NEMO	NF-κB essential regulator
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa enhancer binding protein
NIK	NF-κB-inducing kinase
OTU	Ovarian tumor-type protease
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLAD	Pre-ligand association domain
PMSF	Phenylmethylsulfonyl fluoride
PPAR	Peroxisome proliferator-activated receptor
Puma	p53-upregulated modulator of apoptosis
RAIDD	RIP-associated ICH-1/CED-3-homologue with death domain
RING	Really interesting new gene
RIP	Receptor-interacting protein
SCF	Skp1-Cullin-F-box protein
SDS	Sodium dodekyl sulphate
SLE	Systemic lupus erythematosus
SMAC	Second mitochondria-derived activator of caspases
SUMO	Small ubiquitin-like modifier
TAK	Transforming growth factor (TGF)-β-activated kinase
tBid	Truncated Bid

TCR T cell receptor
TNF Tumor necrosis factor
TOM Target of Myb
TPA 12-O-tetradecanoyl-phorbol 13-acetate
TRADD TNF receptor-associated factor
TRAF2 TNF receptor-associated factor 2
TRAIL TNF-related apoptosis-inducing ligand
UBA Ubiquitin-associated
UBC Ubiquitin-conjugating enzyme
UBD Ubiquitin-binding domain
UBL Ubiquitin-like domain
U-box Homologous to Ufd2
UCH Ubiquitin C-terminal hydrolase
UEV Ubiquitin E2 variant
Ufd Ubiquitin fusion degradation
UIM Ubiquitin interacting motif
USP Ubiquitin-specific protease
VEGF Vascular endothelial growth factor
XIAP X-linked IAP

ABSTRACT

Cells are constantly responding to signals from the surrounding tissues and the environment. To dispose of infected and potentially dangerous cells, to ensure the optimal execution of developmental processes and to maintain tissue homeostasis, a multicellular organism needs to tightly control both the number and the quality of its cells. Apoptosis is a form of active cellular self-destruction that enables an organism to regulate its cell number by deleting damaged or potentially dangerous cells. Apoptosis can be induced by death ligands, which bind to death receptors on the cell surface. Ligation of the receptors leads to the formation of an intracellular death inducing signaling complex (DISC). One of the DISC components is caspase-8, a protease that triggers the caspase cascade and is thereby a key initiator of programmed cell death. The activation of caspase-8 is controlled by the cellular FLICE-inhibitory proteins (c-FLIPs). Consequently, sensitivity towards receptor-mediated apoptosis is determined by the amount of c-FLIP, and the c-FLIP levels are actively regulated for example during erythroid differentiation of K562 erythroleukemia cells and by hyperthermia in Jurkat leukemia cells. The aim of my thesis was to investigate how c-FLIP is regulated during these processes. We found that c-FLIP isoforms are short-lived proteins, although c-$FLIP_S$ had an even shorter half-life than c-$FLIP_L$. In both experimental models, increased death receptor sensitivity correlated with induced ubiquitylation and consequent proteasomal degradation of c-FLIP. Furthermore, we elucidated how phosphorylation regulates the biological functions and the turnover of c-FLIP, thereby contributing to death receptor sensitivity. We mapped the first phosphorylation sites on c-FLIP and dissected how their phosphorylation affects c-FLIP. Moreover, we demonstrated that phosphorylation of serine 193, a phosphorylated residue common to all c-FLIPs, is primarily mediated by the classical PKC. Furthermore, we discovered a novel connection between the phosphorylation and ubiquitylation of c-FLIP: phosphorylation of S193 protects c-FLIP from ubiquitylation. Surprisingly, although all c-FLIP isoforms are phosphorylated on this conserved residue, the biological outcome is different for the long and short isoforms, since S193 specifically prolongs the half-lives of the short c-FLIP isoforms, but not c-$FLIP_L$. To summarize, we show that c-FLIP proteins are modified by ubiquitylation and phosphorylation, and that the biological outcomes of these modifications are isoform-specifically determined.

INTRODUCTION

The life of a human being begins from a single cell, which through regulated growth and proliferation gives rise to the estimated 10^{14} cells that comprise the body of an adult. Yet already during embryonic development some cells die and give way to new ones. In an adult organism, approximately 60 billion cells are generated daily, and an equal number die in order to maintain homeostasis. In addition, an organism has to defend itself against harmful agents by activating the immune system, which aims at eliminating the danger and the infected cells, after which the excess immune cells have to be cleared from the body. Therefore, the life of an organism depends on its ability to detect and specifically remove damaged or excess cells. Insufficient or excess killing of cells may lead to cancer or autoimmune diseases.

All cells share the ability to die through programmed cell death, or apoptosis. When the death signal comes from outside the cell, apoptosis is usually initiated by death ligands that bind to death receptors on the cell surface. However, the death signal is not perceived as an absolute, and the cellular response to a given death signals is determined by a delicate balance between the activities of pro- and anti-apoptotic proteins. These activities, as well as protein levels and localization, are governed by post-translational modifications, such as phosphorylation and ubiquitylation. If a cell is sensitive to death-inducing stimuli, receptor ligation is followed by intracellular signaling cascades that activate the caspase family of proteases. Caspases subsequently cleave several substrates in an orderly manner, leading to the destruction of proteins, DNA, and organelles and to the cleavage of the cell into smaller particles or apoptotic bodies, which are finally engulfed by neighboring cells.

It is hardly surprising that the cellular life-or-death decision needs to be regulated at multiple levels. For example, a cell may express varying amounts of different death receptors, thereby determining its degree of sensitivity against specific death ligands. Moreover, the activation of caspases is controlled by a plethora of pro- and anti-apoptotic proteins. An additional mode of regulation is achieved through post-translational modification of proteins, resulting in swift and precise changes in protein activity, localization, or stability. Among the various post-translational mechanisms, phosphorylation is perhaps the best known, and it is involved in practically all cellular processes, including programmed cell death. Attaching of a small protein called ubiquitin is known to target proteins for proteasomal degradation, enabling the cell to specifically and dynamically adjust the levels of pivotal regulatory proteins. Furthermore, there is cross-talk between post-translational modifications. Therefore, even subtle changes in protein modification can have profound implications on death receptor sensitivity.

Regulation of the apoptotic machinery is complex, and elaborate mechanisms and signaling pathways are constantly being discovered. The importance of both apoptosis and ubiquitylation is emphasized by the fact that the fundamental discoveries made in these research areas have been awarded with the Nobel Prize. In this thesis, I first introduce how proteins and biological processes are post-translationally regulated by phosphorylation and ubiquitylation. Thereafter I describe the central events and players of cell death, accompanied with examples of how they are affected by post-translational modifications. In the experimental part, I have studied how the stability and biological functions of c-FLIP are regulated by ubiquitylation and phosphorylation in an isoform-specific fashion in two experimental models. Furthermore, I have investigated the functions and biological implications of novel phosphorylation sites.

REVIEW OF THE LITERATURE

1 Post-translational regulation of proteins

The astonishing varieties of biological processes of all organisms are carried out and regulated by proteins. The blueprint of all proteins of prokaryotic and eukaryotic organisms is contained within the genes, the basic functional units of DNA, which are written in a universal genetic code. To extract the protein-coding information, the cell first copies the nucleotide sequence of a gene onto an messenger RNA molecule. This process, called transcription, is regulated by a plethora of general and gene-specific transcription factors, which interact with regulatory DNA sequences and stimulate the transcription of the gene. In the next step, the information contained within messenger RNA copy is used as a template to guide the production of a chain of amino acids. Much like a necklace is built up from sequential pearls, all proteins are built up by amino acids, where the number, order and chemical properties of the amino acids determine the features of the protein.

The genetic code produces one amino acid for each three-letter codon of the messenger RNA, and the resulting polypeptides contain diverse combinations of the twenty amino acids encompassed by the genetic code. Next, the nascent polypeptide chain is folded into secondary and tertiary structures on the basis of the amino acid side chains adopting a specific three-dimensional shape demanding the least energy. The simple secondary structures include α-helices, β-sheets, and random coils, and they are further folded into tertiary structures which usually have a hydrophobic core, while the polar and charged amino acids tend to reside on the outer surface of the protein to mediate interactions. The tertiary structures are further stabilized by weak interactions.

While the three-dimensional structure of the protein largely governs its chemical properties and biological functions, drastic changes in protein function must be rapidly achieved when the cell has to react to environmental cues. The organization, localization, enzymatic activity and interactions of proteins can all be quickly transformed by post-translational modifications. Post-translational modifications include an astounding range of covalent and non-covalent modifications that regulate biological processes in complex ways. Proteins can be covalently modified by adding or removing chemical groups (e.g. phosphorylation, methylation, deamination), fatty acids (e.g. myristoylation, palmitoylation) or sugars (glycosylation), as well as chemical processes such as nitrosylation or oxidation-reduction reactions. In addition, a high degree of versatility is provided by covalent and non-covalent modifications by small proteins, such as ubiquitylation and neddylation. Finally, enzymatic processing of proteins, as well as prolyl isomerization, represents a mode of post-translational regulation. Most post-translational modifications are reversible, allowing more alternatives for protein regulation.

2 Protein phosphorylation enables versatile protein modification

The first observations of protein modification by phosphorylation were made over 100 years ago when Phoebus Levene noted that the vitellin protein contained phosphoserine (Levene and Alsberg 1906). Decades later, Eugene Kennedy described a phenomenon he called “enzymatic phosphorylation of proteins” (Burnett and Kennedy 1954). From this started an era of vigorous endeavor to understand the biochemistry of phosphorylation and its biological significance.

Phosphorylation denotes the covalent attachment of a small, negatively charged phosphate group to the substrate and is probably the most widespread and the best characterized mechanism for post-translational modification of protein function. Phosphorylation targets an estimated one third of all cellular proteins, and is used for signal transduction in all cellular processes, including differentiation, trafficking, metabolism and growth (reviewed by Ubersax and Ferrell 2007). Like most types of post-translational modifications, the outcome of phosphorylation is highly substrate specific, and is known to regulate protein function negatively or positively, provide novel interaction interfaces, or affect protein stability or localization. Most phosphorylation events occur on serine, threonine or tyrosine residues, of which serine phosphorylation is by far the most common. The dynamics of phosphorylation is regulated by the counteracting forces of kinases, which catalyze the addition of the phosphate group to the substrate, and phosphatases, which remove phosphate groups. In eukaryotes, kinase genes make up 1.5-2.5% of the genome and are highly conserved between species. Nevertheless, different kinases display a remarkable diversity in terms of activation mechanisms. For example, whereas some kinases are activated by cellular stresses such as DNA damage or proteotoxic stress, others function as sensors of the nutritional status of the cell. Moreover, the tissue-specific expression of kinases, their subcellular localization and preference for particular substrate sequences allow for the precise targeting of phosphorylation events. Compared to kinases, the number of phosphatases is smaller. Consequently, phosphatases tend to display a greater promiscuity in substrate recognition. Certain phosphatases prefer phosphorylated serine/threonine residues, while some catalyze tyrosine dephosphorylation, and the dual specificity phosphatases are able to strip all of the above from the covalently linked phosphate group.

2.1 The classification and regulation of the protein kinase C

The protein kinase C (PKC) family comprises several serine/threonine kinases that play pivotal roles in cell proliferation, apoptosis, growth and malignant transformation (reviewed by Griner and Kazanietz 2007). For this reason, PKC has been suggested to be a potential target for cancer therapy (reviewed by Mackay and Twelves 2007). Most PKC isoforms are ubiquitously expressed in different types of tissues. The ATP and substrate-binding properties of the PKC family members are mediated by a highly conserved catalytic domain. PKC proteins are regulated by their N-terminal regulatory domains, which target the PKC to the plasma membrane, as well as by their pseudosubstrate domains which are required for autoinhibition. Kinases of the PKC family phosphorylate serine or threonine residues that usually conform to the consensus sequence, R/K^{1-3}-X^{2-0}-S/T-X^{2-0}-R/K^{1-3} (reviewed by Steinberg 2008).

The members of the protein kinase C family are divided into three groups according to their structure and activating stimuli (Figure 1). The classical PKCs include isoforms α, βI, βII and γ. Their regulatory domains contain a C1 region that binds diacylglycerol (DAG) or 12-O-tetradecanoylphorbol-13-acetate (TPA)/phorbol 12-myristate 13-acetate (PMA), and a C2 region that binds to anionic phospholipids in a calcium-dependent manner (Castagna et al. 1982, Kikkawa et al. 1983, Sharkey et al. 1984). Hence, the activation of the cPKCs depends on lipids and calcium. The novel PKCs, in turn, contain two C1 domains and a non-functional C2 domain. Because the C2 domain is unable to bind calcium, nPKCs are activated in a calcium-independent manner by DAG or TPA alone. Finally, the atypical PKCs lack the C2 domain, but contain an atypical C1 domain that binds ceramide and PIP_3, thereby mediating their activation.

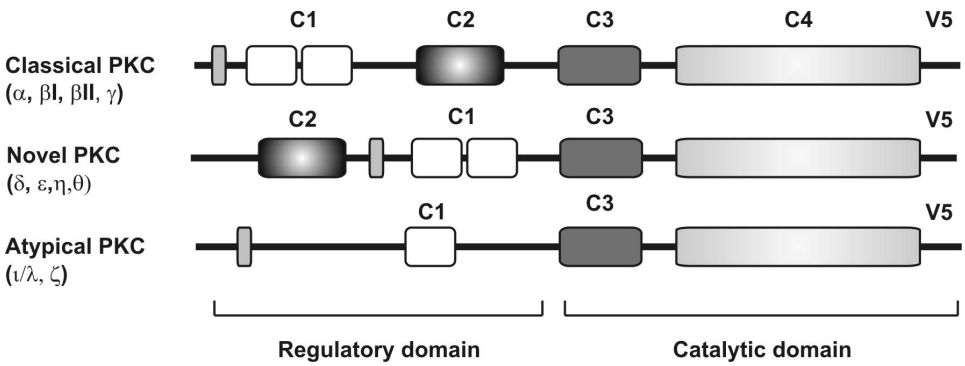

Figure 1. The PKC isozymes are classified on the basis of their structure and method of activation. The classical PKCs include α, βI, βII, and γ, and they bind calcium via their C2 domains. This increases the affinity of the C1 domain for diacylglycerol and phorbol esters. Novel PKC respond to DAG, but not calcium. Because the atypical PKCs lack a C2 domain, as well as a functional C1 domain, they do not respond to calcium or DAG. The catalytic domains (C3 and C4) are highly conserved. The PKC isozymes also contain pseudosubstrate domains which keep them in an inactive conformation in the absence of activating stimuli. Adapted from Churchill et al. (2008).

The PKCs are activated in a multi-step manner including sequential phosphorylations, changes in localization and structural reorganizations (Cazaubon et al. 1994, Orr and Newton 1994, Srinivasan et al. 1996, Hansra et al. 1999, Parekh et al. 1999, Ziegler et al. 1999). First, the PKC undergo maturation by phosphoinositide-dependent kinase 1 (PDK1)-mediated phosphorylation of the activation loop. This reveals the critical domains in the C terminus of the PKC, leading to autophosphorylation and stabilization of the kinase. The mature PKC is then capable of being activated by DAG and calcium. A mature PKC usually resides in the cytosol, where it is kept inactive state by interactions between the kinase domain and the pseudosubstrate domain.

Mature PKCs are recruited to the membrane upon a rise in intracellular DAG or calcium in response to phorbol esters or receptor stimulation. When the intracellular calcium and DAG levels rise, the classical and the novel PKCs respond differently (reviewed by Parekh et al. 2000, Newton 2003). The classical PKC are recruited to the membrane by their calcium-binding C2 domain. When the C1 domain binds DAG, the conformation of the enzyme is massively re-organized, releasing the pseudosubstrate domain and allowing substrate binding. The novel PKCs, in turn, are not recruited to the membrane as quickly as the cPKC, because the nPKCs do not bind calcium. Strong affinity to DAG, however, compensates for this over time (Giorgione et al. 2006). Although binding to DAG is a key step in the activation of the cPKC and nPKC, it does not explain the diversity of PKC subcellular localization. PKC isozymes are distributed around cellular organelles such as the nuclear membrane, mitochondria, and the Golgi, upon phorbol ester treatment or growth factor stimulation. These alterations in localization are likely to explain the differences in substrate specificity, making the underlying mechanisms attracting targets of research. The binding of PKC to DAG results in conformational change that reveals the pseudosubstrate domain of the PKC, thereby enabling substrate binding.

Inactivation of the PKCs occurs by a negative feed-back loop, although the mechanisms seem to differ according to the isoform. The mechanism of PKC downregulation is not yet very well known, but it is partly explained by the short half-life of DAG. Upon prolonged phorbol ester treatment, at least PKCα is known to become dephosphorylated and

subsequently internalized by a caveolae-dependent mechanism into an endosomal compartment (Bornancin and Parker 1996, Hansra et al. 1999, Prevostel et al. 2000). PKCδ, on the other hand, is thought to become prone to degradation by its activating phosphorylation events (Srivastava et al. 2002). Other PKC isoforms have also been shown to be regulated by the ubiquitin-proteasome pathway by a mechanism that depends on their kinase activity (Lee et al. 1996, Lu et al 1998, Leontieva et al. 2004).

The degree of substrate specificity and the level of functional overlap between the PKC isoforms are still incompletely understood. As the PKC isozymes are distributed around cellular organelles, such as nuclear membrane, mitochondria, and the Golgi upon phorbol ester treatment or growth factor stimulation, some features in PKC substrate selection are likely to be explained by changes in PKC localization (reviewed by Jaken and Parker 2000, Schechtman and Mochly-Rosen 2001). In addition, the activity and substrate selection of the PKC are regulated by specific interaction partners. The proteins that recognize particular sequences on active PKCs are collectively referred to as receptors of activated protein kinase C (RACKs). The RACKs are membrane-associated proteins, which anchor and scaffold PKCs to domains in the membrane to co-localize with their activators and substrates. Specific RACKs, RACK1 and RACK2/β-COP, have been characterized for PKCβ and PKCε, respectively (Csukai et al. 1997, reviewed in Mackay and Mochly-Rosen 2001, Schechtman and Mochly-Rosen 2001). In addition, p32/gClqBP has been found to act as a RACK for PKCδ, but as it also binds inactive PKCθ, its role is still somewhat unclear (Robles-Flores et al. 2002). The original hypothesis of Mochly-Rosen and colleagues was that each PKC isoform has its own, specific RACK, and that this accounts for isoform-specific PKC responses. A convincing body of evidence suggests, however, that localization of the PKC is regulated also independently of RACKs, and that interactions with cytoskeletal proteins such as actin and tubulin, as well as other, non-RACK scaffolding proteins, are important in many situations (Jaken and Parker 2000, Schechtman and Mochly-Rosen 2001).

2.2 PKCs as targets for cancer therapy

Since the PKC family plays such multi-faceted roles in various central biological processes, its members have been considered as tempting targets for cancer treatment. However, functional characterization of the PKC isozymes has suffered from the poor specificity of the current PKC inhibitors as well as the frequent use of overexpression studies. Recently, the function of the PKC isozymes has been successfully disrupted by short peptides which target regions of the C2 domain required for RACK binding (reviewed by Churchill et al. 2008). Discrimination between PKC isozymes can also be achieved by targeting the C1 domains for their small structural dissimilarities (Pu et al. 2005). In addition to inhibitors of PKC activity, isozyme-specific activators would also be desirable.

Several pharmacological agents targeting PKC are currently in clinical trials. Two indolocarbazoles, enzastaurin and midostaurin, are in phase I-II trials for various cancer types. Enzastaurin is a macrocyclic bisindolylmaleimide that competes with ATP in binding to PKC, thereby preventing activation. While PKCβ is the primary target of enzastaurin, many other PKC isoforms are inhibited, and in addition, also the phosphatidylinositol 3-kinase (PI3K)/Akt and glycogen synthase kinase 3 (GSK3) pathways are affected (Graff et al. 2005). Enzastaurin is being trialed alone or in combination with conventional therapies for example for breast, ovarian, and peritoneal cavity cancer, as well as for metastasized malignancies of colorectal and pancreatic cancer (reviewed by Podar et al. 2007). Midostaurin is a staurosporine derivative that targets the classical and the novel PKCs. In addition, midostaurin blocks several growth factor receptor tyrosine kinases (Weisberg and Griffin 2001, Growney et al. 2005). Midostaurin has proved promising for example in the

preclinical models for acute myeloid leukemia, mast cell leukemia, peripheral T-cell lymphoma and solid tumors (reviewed by Podar et al. 2007). Current clinical trials focus on the effects of midostaurin in aggressive systemic mastocytosis, mast cell leukemia and acute myeloid leukemia. In addition to these indolocarbazoles, other PKC-targeting drugs like tamoxifen, bryostatin and curcumin are currently in phase I-III trials for treating a wide array of tumors. While these developments demonstrate that PKCs are attractive targets especially for cancer therapy, further information is needed on the specific tasks of individual PKC isozymes. A deeper understanding of their isozyme-specific features and biological roles will help delineate future therapies targeting PKC proteins.

3 Post-translational modification of proteins by ubiquitin

Until recently, ubiquitylation was thought to function primarily as a mediator of proteasomal degradation. However, the biological versatility of ubiquitin signaling has begun to emerge, and today ubiquitylation is considered as one of the most common post-translational regulatory mechanisms. Ubiquitin moieties can be attached onto substrates as single proteins or in chains of variable length. Further complexity is provided by different linkage types, as ubiquitin moieties can be conjugated to different lysine residues of ubiquitin, as well as by non-covalent ubiquitin binding through specialized ubiquitin-binding domains. Ubiquitin contains seven lysines, all of which serve as platforms for chain branching. Recent studies have revealed that the number of genes encoding proteins involved in the enzymatic reactions of ubiquitin and ubiquitin-like proteins is similar to the genes whose products are associated with phosphorylation (reviewed by Herrmann et al. 2007). In the following section, I review the biochemical basis of ubiquitin signaling and discuss its biological implications, with a particular emphasis on proteasomal degradation.

3.1 Ubiquitin and ubiquitin-like proteins

Ubiquitin is a small, 76-amino-acid protein that was originally cloned from bovine thymus and named after its ubiquitous expression (Figure 2, Goldstein et al. 1975). Ubiquitin is one of the most conserved of proteins, and although it is absent in prokaryotes, it is expressed in all eukaryote tissues and cell types. Remarkably, the amino acid sequences of ubiquitin in yeast and humans differ by only three amino acids. The mammalian genome contains multiple ubiquitin genes. Ubiquitin is translated as fusions, and it is processed into mature proteins by ubiquitin C-terminal hydrolases. Ubiquitin is a globular protein that contains a conserved ubiquitin fold (ββαββαβ), shared by other members of ubiquitin-like molecules. The conjugation of ubiquitin to its substrate is mediated by two glycine residues in the C-terminal end of ubiquitin. Although ubiquitin is usually attached to lysine residues of the substrate, also proteins or protein fragments without lysines have been found to be ubiquitylated. In these occasions, the ubiquitylation has been proposed to occur on serine and threonine residues (Cadwell and Coscoy 2005, Wang et al. 2007). In addition, the ubiquitylation of certain substrates is controlled by destabilizing N-terminal amino acids, which attract ubiquitin ligases and mediate the ubiquitylation of internal lysines (reviewed by Varshavsky 1997).

Ubiquitin is not the only small molecule used for signal transfer. To date, more than 10 different ubiquitin-like modifiers have been identified in mammals, including small ubiquitin-related modifier (SUMO), human leukocyte antigen F-associated transcript 10 (FAT10) and neural-precursor-cell-expressed developmentally down-regulated 8 (Nedd8) (Hochstrasser 2009). The ubiquitin-like proteins are classified on the basis of common tertiary structures

and certain pivotal features, such as the ubiquitin fold and the C-terminal glycine residue (reviewed by Herrmann et al. 2007). In addition, several proteins contain ubiquitin-binding motifs or domains that mediate non-covalent ubiquitin binding. The ubiquitin-domain proteins are also capable of modifying ubiquitylation-related processes by acting as cofactors and adaptors, one example being the proteasomal subunit Rpn10 that recognizes polyubiquitylated substrates.

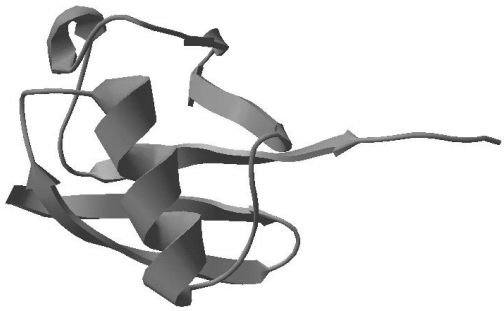

Figure 2. Structure of ubiquitin. The C-terminus of the protein, through which ubiquitin is attached to its substrates, is seen to the right. The image was made using DeepView/Swiss-pdbViewer v. 3.7 and is based on the crystal structure of human ubiquitin (protein data bank code 1UBQ). Structure of ubiquitin refined at 1.8 A resolution (Vijay-Kumar et al. 1987).

3.2 Non-covalent ubiquitin binding

Ubiquitin signaling is mediated by specific domains that bind different forms of ubiquitin in a non-covalent manner. To date, close to 20 ubiquitin-binding domains (UBD) have been identified (reviewed by Hurley et al. 2006). Remarkably, these domains share little similarity with each other, and they also interact with several surfaces of ubiquitin. In addition, some ubiquitin-binding domains specifically bind interfaces of two ubiquitin molecules bound to each other, while some recognize differing ubiquitin chain topologies. The ubiquitin-binding domains are diverse also in size, ranging from 20 to 150 amino acids (Hurley et al. 2006).

Many ubiquitin-binding domains are α-helical. These include the ubiquitin-associated domain (UBA), ubiquitin-interacting motif (UIM), double-sided UIM (DUIM), motif interacting with ubiquitin (MIU), coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE), Golgi-localized, gamma-ear-containing ADP-ribosylation-factor binding protein (GAT), and target of Myb (TOM) (Hurley et al. 2006). All α-helical interaction domains interact with ubiquitin I44 residing in a hydrophobic patch (for example Swanson et al. 2003, Bilodeau et al. 2004, Varadan et al. 2005, Wang et al. 2005). The UIMs are characterized by short stretches of hydrophobic amino acids, and they are common in proteins involved in intracellular trafficking. Ubiquitin serves as a signaling tag that is recognized by proteins containing UIMs, many of which regulate the transport of ubiquitylated cargo. In addition, the proteasomal subunit S5a contains UIMs. Non-covalent interaction via UIM often promotes covalent ubiquitylation of the target protein. As suggested by the name, MIU and DUIM are UIM variants (Hirano et al. 2006).

The UBA domains constitute an important group of ubiquitin-binding domains, which differs from others in recognizing polyubiquitin chains displaying certain types of linkages (Raasi et al. 2005). It is currently unclear how the UBAs distinguish between differently linked ubiquitin chains, but K48-linked di- and tetraubiquitin has been shown to adapt a closed conformation, whereas K63 di- and tetraubiquitin harbors an extended conformation, providing a putative basis for differential recognition (Varadan et al. 2002, Varadan et al.

2004). The interactions mediated by ubiquitin-binding domains are typically weak, although they are essential for many physiological processes. An interesting example of how ubiquitin-binding domains can affect covalent ubiquitylation events is the E3-independent monoubiquitylation (Hoeller et al. 2007). Proteins containing any of a number of ubiquitin-binding domains are able to directly interact with E2 ubiquitin-conjugating enzymes, thereby enabling direct monoubiquitylation without E3 enzymes.

3.3 Three enzymatic steps of ubiquitin conjugation

The covalent conjugation of ubiquitin to its substrate is executed by a three-step enzymatic pathway, composed of an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase (Figure 3, reviewed by Glickman and Ciechanover 2002). At every step, the number of enzymes involved in ubiquitin conjugation increases, as does the specificity of the reactions they catalyze. The ubiquitin-activating enzyme E1 initiates a cascade of enzymatic events that leads to covalent conjugation of ubiquitin to the substrate. From E1 the activated ubiquitin is transferred to the E2 ubiquitin conjugating enzymes. Covalent attachment of ubiquitin to the substrate is either directly executed or indirectly facilitated by the E3 ubiquitin ligases, which fall into two categories, the HECT and RING ligases. Of these, the HECT ligases bind the ubiquitin before transferring it to the substrate, whereas the RING E3 ligases act as scaffolds that mediate direct transfer of the ubiquitin to the substrate.

Most organisms, including humans and the baker's yeast *Saccharomyces cerevisiae*, contain a single ubiquitin E1 enzyme. In mammals, however, the *UBA1* gene gives rise to two transcripts by alternative splicing (Busch and Goldknopf 1981). The initial findings on the physiological importance of ubiquitylation arose from the observation that cells, in which the expression of E1 is temperature-sensitive, undergo cell-cycle arrest (Finley et al. 1984, McGrath et al. 1991). Not surprisingly, the deletion of the single ubiquitin gene is lethal (McGrath et al. 1991).

In the first step of ubiquitin conjugation, ubiquitin is loaded to the E1 ubiquitin-activating enzyme, which activates the ubiquitin moiety in an ATP-dependent reaction. This gives rise to an adenylate ubiquitin intermediate, donating the ubiquitin to the E1 active site cysteine. The binding of ubiquitin to the active cysteine by a thioester bond enables the E1 to bind two ubiquitins simultaneously, one in adenylate and one in thioester form. The high-energy E1-ubiquitin thioester intermediate is the activated ubiquitin, which can be further transferred to the ubiquitin-conjugating enzyme E2.

The ubiquitin-conjugating enzymes share a conserved core domain of ~150 amino acids, consisting of four standard helices, a short helix and a four-stranded antiparallel β-sheet (reviewed by Pickart 2001). The active cysteine is located in a long loop, and the conserved residues surrounding it are likely to mediate interactions with E1, ubiquitin or both. Some E2s have N- or C-terminal extensions, which probably facilitate interactions with the E3 enzymes and provide specificity. The size of individual E2s varies greatly, ranging from the approximately 30 kDa *S. cerevisiae* E2 enzymes to the mammalian 528-kDa ubiquitin-conjugating enzyme (BRUCE; Hauser et al. 1998). In addition to E2-like activity, BRUCE contains baculovirus repeats which provide it with IAP-like properties.

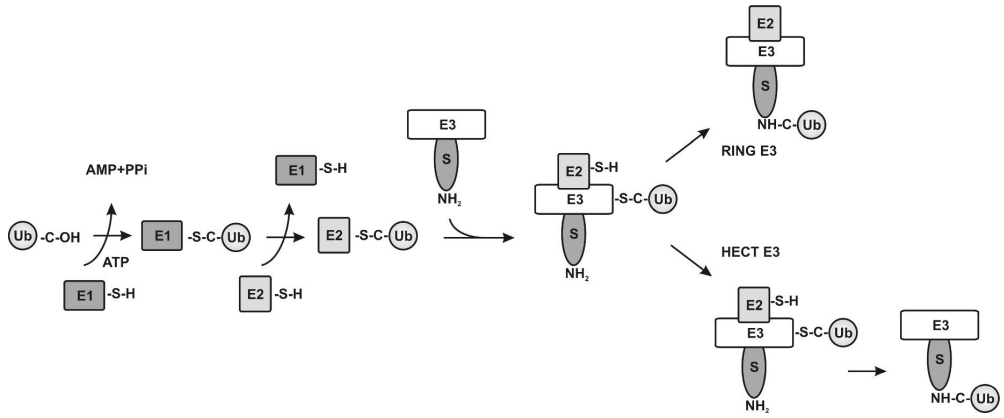

Figure 3. Covalent conjugation of ubiquitin to the substrate requires a three-step enzymatic process. In the first step, ubiquitin is activated in an ATP-dependent manner by the ubiquitin activating enzyme, E1. Thereafter, activated ubiquitin is transferred to the E2 enzyme, which either conjugates the ubiquitin either to a homologous to the E6-AP COOH terminus (HECT) domain ubiquitin ligase E3 or directly to the substrate, if catalyzed by a really interesting new gene (RING) finger E3. E3 ubiquitin ligases are responsible for recognizing and binding the substrate. Adapted from Weissman (2001).

The yeast genome encodes 13 E2 enzymes, Ubc1-13, and at least 25 E2s are encoded by the mammalian genomes. Some of the 25 mammalian enzymes are, however, not dedicated to ubiquitin, but to other ubiquitin-like proteins. Despite the structural similarity among the E2 enzymes, they play diverse biological roles. This is mainly due to the specificity of the E2-E3 interactions. In addition, some E2 enzymes have specialized in mediating conjugation of certain types of ubiquitin chains, for instance chains linked via K63 are often catalyzed by Ubc13. A yeast strain deficient in Ubc13 is viable but sensitive to ultraviolet radiation, indicating a role for K63-linked ubiquitin chains in DNA repair and illustrating the specialization of E2s (Hofmann and Pickart 1999).

The E2-ubiquitin thioester intermediate is labile. However, as the E2-ubiquitin interface includes the C terminus of ubiquitin and some residues surrounding the active cysteine, the ubiquitin C terminus is probably extended and partly surrounds the E2, but does not overlap with its E3 binding regions. The crystal structures of the E2-E3 complexes have revealed that most E2 enzymes contact E3 by the side chains of their C terminus (for example Huang et al. 1999, Zheng et al. 2000).

The third class of enzymes involved in ubiquitin conjugation is the E3 ubiquitin ligases, which constitute the largest and the most diverse group of enzymes in the ubiquitylation pathway. The lack of homology and the greatest variety in structure underlie specific recognition of individual substrates. Despite their variety, all E3 enzymes fall into two major categories, the homologous to the E6-AP COOH terminus (HECT) domain and the really interesting new gene (RING)-finger domain E3s. An estimated >30 HECT ligases exist in mammals, while the number of RING ligases is much larger, over 200 (reviewed by Pickart 2001, Petroski and Deshaies 2005, Bernassola et al. 2008). Many of these ligases are yet to be characterized.

The first identified HECT ligase was E6-AP that targets p53 in the presence of the human papilloma virus (HPV) oncoprotein (Huibregtse et al. 1991). The HECT E3 ligases are called the “true” ubiquitin ligases, because during the conjugation process, they accept the ubiquitin via their active cysteine generating a covalent thioester intermediate with ubiquitin

(Scheffner et al. 1995). The active cysteine is located in the C-terminal HECT domain encompassing about 350 amino acids. The amino terminus of the HECT domain is variable and likely to mediate substrate recognition. In addition to the C-terminal HECT domain, the N termini of E3 ligases often contain conserved domains, such as WW domains, which bind proline-rich sequences and phosphorylated serine and threonine residues, as well as PKC-like C2 domains, which bind lipids in response to calcium influx (Springael et al. 1999, reviewed by Bernassola et al. 2008).

In contrast to HECT-type E3 ligases, members of the RING-finger ligase family do not form thioester intermediates with ubiquitin, but rather serve as scaffolds that bring substrates and the ubiquitin-loaded E2 enzyme together (for example Joazeiro et al. 1999, Lorick et al. 1999, Fang et al. 2000). The RING-finger consists of a conserved pattern of cysteine and histidine residues that cross-brace each other coordinating the binding of two zinc cations. Depending on whether the fifth coordination site is occupied by cysteine or histidine, the RING-finger domains are divided into RING-HC and RING-H2 subgroups, respectively. The RING-finger E3 family is further divided to single and multi-subunit ligases. Well-known examples of the single subunit ligases include Parkin (Shimura et al. 2000) and murine double minute 2 (Mdm2), the E3 for p53 (Fang et al. 2000). These ligases are mono- or homodimeric proteins with a RING finger domain and substrate-recognition regions within one polypeptide.

The multi-subunit RING E3 complexes are classified into three types, the Skp1-cullin-F-box protein (SCF), anaphase promoting complex (APC) and the von Hippel-Lindau-Cul2/elongin B-elongin C (VCB-CBC) type ligases. Some multisubunit RING finger ligases consist of a significant number of subunits. For example, the yeast APC/cyclosome contains 11 subunits and the mammalian as many as 12. Of these components, Apc11 contains a RING finger and performs some of the substrate binding functions (Leverson et al. 2000). The Apc2 subunit contains a Cullin homology domain, resembling the Cdc53/Cullin1 subunits of the SCF complexes. APC/cyclosome is a well characterized cell cycle regulator, whose functions are determined by numerous regulatory subunits and their post-translational modifications (reviewed by Pesin and Orr-Weaver 2008). The SCF complexes, in turn, act in concert with the E2 Cdc34/Ubc3 and the members of the UBCH5 family. The Hrt1/Rbx1/Roc1, Skp1 and members of the Cdc53/Cullin-1 family are thought to be the common components of all SCF ligase complexes (reviewed by Glickman and Ciechanover 2002), which are further organized and complemented by additional subunits (Kamura et al. 1999, Skowyra et al. 1999). The SCF complexes are named after the F-box subunit, which is responsible for substrate recognition. The VCB-Cul2-Rbx1 complex, an archetypal example of the VCB-CBC E3 complex, resembles the SCF complexes. Similarly to SCF, a VCB complex contains a Hrt1/Rbx1/Roc1 RING finger protein, as well as elongins B and C, Cullin 2, and the substrate recognition subunit pVHL (Kamura et al. 1999, Hon et al. 2002). An elongin B/C interaction motif called the Socs box provides additional substrate specificity to the VCB E3 ligases (reviewed in Stebbins et al. 1999). The best-known substrate of the VHL E3 ligase may be the hypoxia-induced factor (HIF) 1α (Ivan et al. 2001, Jaakkola et al. 2001).

In addition to the E1, E2 and E3 enzymes, some proteins involved in ubiquitin conjugation cannot be unambiguously categorized to the above-mentioned groups. The E4 elongation factors help build polyubiquitin chains by binding to substrates modified with short ubiquitin chains and catalyzing chain elongation with the help of the E1-E3 machinery. E4 proteins contain a motif called homologous to ubiquitin fusion degradation 2 or U-box, which resembles the RING finger (Aravind and Koonin 2000, Hatakeyama et al. 2001). Intriguingly, certain U-box proteins have been shown to elongate ubiquitin chains independently of RING or HECT E3s. An interesting example of a U-box protein is the carboxyl terminus of Hsc70-interacting protein (CHIP), which binds substrates with the help of heat shock cognate protein 70 (Hsc70) and heat shock protein 90 (Hsp90), thereby

promoting the ubiquitylation of unfolded or aggregated proteins (Murata et al. 2001). In addition, the ubiquitin E2 variant (UEV) family resembles ubiquitin-conjugating enzymes, but lacks the active cysteine residues required for enzymatic activity (Sancho et al. 1998). The known UEV proteins act in heterodimeric complexes with Ubc13 to assemble K63 ubiquitin chains in an E3-dependent manner. Therefore, the UEV-E2 complexes can be seen as a subgroup of E2 enzymes dedicated to K63-chain assembly. Both U-box and UEV proteins illustrate that ubiquitin chains may be subjected to significant remodeling also after they have been appended to the substrate.

3.4 The deubiquitylating enzymes

The work of the E1-3 enzymes is balanced, counteracted and refined by the deubiquitylating enzymes (DUBs). In addition to removing ubiquitin moieties from substrates, the DUBs are important regulators of ubiquitin maturation and ubiquitin chain remodeling (reviewed by Reyes-Turcu et al. 2009). Of the 100 putative DUBs encoded by the human genome (Nijman et al. 2005), the ubiquitin-specific DUBs are divided into five families on the basis of their structure and conserved domains. Four of them, including ubiquitin C-terminal hydrolases (UCH), ubiquitin-specific proteases (USP), ovarian tumor-type proteases (OTU), and the Machado-Joseph disease proteases (MJD), display papain-like cysteine protease activity. How the activity of DUBs is regulated has remained largely enigmatic, but at least transcriptional regulation, post-translational modifications and scaffold binding contribute to adjusting DUB-like activity (Reyes-Turcu et al. 2009). Intriguingly, some DUBs have been found to operate in a complex with E3 ubiquitin ligases (Ventii and Wilkinson 2008).

The DUBs have an essential role in maturation of ubiquitin from proproteins that are produced as linear polyubiquitin or as fusions of ubiquitin and a ribosomal protein (Wiborg et al. 1985, Baker and Board 1987, Ozkaynak et al. 1987). DUBs process these proproteins into mature ubiquitin monomers. The DUBs are also responsible for recycling ubiquitin from the substrates that are targeted to the proteasome, or from ubiquitin that has remained as a thioester intermediate (Pickart and Rose 1985). There is evidence that DUBs, such as Usp14, Uch37, and POH1/Rpn11, associate and cooperate with the regulatory particle of the proteasome and edit ubiquitin chains (Eytan et al. 1993, Lam et al. 1997, Chernova et al. 2003, Crosas et al. 2006, Hanna et al. 2006, Yao et al. 2006). A20 and cylindromatosis tumor-suppressor (CYLD), on the other hand, have been found to modulate nuclear factor κ enhancer binding protein (NF-κB) signaling by deubiquitylating several proteins of the pathway (Wertz et al. 2004, Wang et al. 2008).

3.5 The diverse outcomes of ubiquitylation

Ubiquitin signaling is extremely diverse. A protein can be ubiquitylated on several lysine residues by single ubiquitins or ubiquitin chains of variable lengths (reviewed by Ikeda and Dikic 2008). In addition, heterologous ubiquitin chains contain ubiquitin together with other ubiquitin-like proteins. Ubiquitin chains can be assembled on all seven lysine residues of ubiquitin, and different linkage types generate distinct chain topologies. Recent reports show the chains may also branch via several different lysines within one chain (Ikeda and Dikic 2008). Yet another level of complexity to the ubiquitin network is provided by the numerous motifs and domains that mediate non-covalent ubiquitin binding. The astonishing variety of biological outcomes of ubiquitin signaling is discussed below using different ubiquitin chain linkage types as examples.

3.5.1 Monoubiquitylation

Formation of a ubiquitin chain is not a prerequisite for ubiquitin signaling. The attachment of single ubiquitin moieties to substrates (monoubiquitylation) takes part in diverse biological processes. For example, monoubiquitylation of histones modulates transcription and DNA repair (reviewed by Weake and Workman 2008), and the mutation of a single histone H2B ubiquitylation site in *S. cerevisiae* ceases the sporulation of the yeast cells, indicating that H2B ubiquitylation is also required for meiosis (Robzyk et al. 2000)

Monoubiquitylation has been ascribed important roles in regulating multiple aspects of membrane trafficking. In addition, several endocytic and biosynthetic transmembrane proteins are known to require ubiquitylation for vesicular transport into lysosome/vacuole compartments in vesicles (reviewed by Katzmann et al. 2002, Hicke and Dunn 2004). The biological significance of monoubiquitylation was originally demonstrated with membrane proteins such as permeases and transporters, which depend on ubiquitylation for rapid internalization. Conclusive evidence for the role of monoubiquitylation in endosomal trafficking was later obtained by extensive yeast studies (Nakatsu et al. 2000, Roth and Davis 2000, Shih et al. 2000, Katzmann et al. 2002, Haglund et al. 2003). Monoubiquitylation also serves as a sorting signal during later stages of endosomal trafficking and regulates the formation of multivesicular bodies. In addition, monoubiquitylation is involved in protein sorting by the trans-Golgi network (Beck et al. 1999, Helliwell et al. 2001, Soetens et al. 2001). Finally, monoubiquitylation of the gag polyprotein has been found fundamental for viral budding (Patnaik et al. 2000, Schubert et al. 2000, Strack et al. 2000).

3.5.2 Polyubiquitylation

K63-linked ubiquitin chains regulate a variety of processes, most of which are unrelated to proteasomal degradation, including endocytosis of cell surface receptors (reviewed by Hicke 1999, Strous and Govers 1999), DNA repair (Spence et al. 1995), stress responses (Arnason and Ellison 1994), ribosome function (Spence et al. 2000), as well as signaling pathways (Deng et al. 2000, Wang et al. 2001). In contrast to K63 ubiquitin chains, biological functions mediated by K29 chains are not well understood. Nonetheless, K29 polyubiquitylation by the E3 ligase Itch has been shown to mediate lysosomal degradation of Deltex transcription factor (Chastagner et al. 2006). In addition, the K6- and K11-linked ubiquitin chains have not yet been studied in a great detail, but the proteasome subunit Rpn10/S5a is known to bind both K6- and K11-linked ubiquitin chains (Baboshina and Haas 1996). K11 polyubiquitin chains also signal endoplasmic reticulum-associated degradation (ERAD; Xu et al. 2009). It is not currently known if K6 and K11 chains can serve other signaling purposes.

K48-linked polyubiquitylation efficiently targets proteins for proteasomal degradation. For efficient degradation, a K48 chain of at least four ubiquitin moieties is required (Thrower et al. 2000). The Rpn10 and Rpn13 subunits of the proteasome recognize both the K48-linked diubiquitin and the ubiquitin-like domains (UBLs) of ubiquitin-associated proteins (Husnjak et al. 2008). However, it was recently reported that in yeast all polyubiquitin chains, apart from K63, mediate proteosomal degradation, and they can be disassembled by the proteasome (Xu et al. 2009).

3.6 Proteasomal degradation of ubiquitylated proteins

By far, the best-known consequence of protein polyubiquitylation is proteasomal degradation. Carefully regulated degradation of proteins allows the cell to adapt to differing stimuli by modulating the half-lives of key regulatory proteins. Since the life span of a protein may vary from less than a minute to several days, a cell must be capable of dynamic degradation of pivotal proteins, as well as steady bulk degradation of the less seminal components. The former is operated by the ubiquitin-proteasome pathway, in which proteins are tagged by ubiquitin chains, recognized by the proteasome, cleaved, and finally released as short peptides. Although this machinery is crucial for regulating the stability of important signaling molecules, it is also responsible for protecting the cells from proteotoxic stress by eliminating damaged and misfolded proteins in close collaboration with the chaperone proteins (reviewed by Goldberg 2003). Remarkably, the proteasome has been found to degrade also non-ubiquitylated proteins, such as p21, indicating that multiple signals are capable of targeting proteins to the proteasome (Sheaff et al. 2000).

3.6.1 Structure of the 26S proteasome

The 26S proteasome is a ~2.5 MDa holoenzyme that consists of more than 30 subunits (Figure 4). The proteasome subunits are highly conserved among eukaryotes: most proteasome subunits exhibit >40% similarity between yeast and humans, while certain crucial components, such as the proteasomal ATPases, display similarity as high as 70-80%. The subunits are also functionally conserved, and several yeast proteasome knockout phenotypes can be rescued by expressing their human counterparts (Glickman and Coux 2001). Functional proteasomal degradation is essential to virtually all cellular processes, and the ubiquitin-proteasome pathway may therefore be one of the most conserved regulatory pathway in eukaryotes.

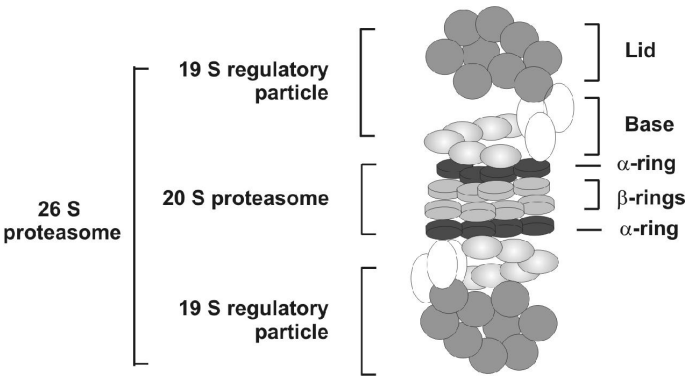

Figure 4. The 26S proteasome is a ~2.5 MDa holoenzyme consisting of the 20S core particle and one or two 19S regulatory particles. The 20S core includes two β-rings surrounded by two α-rings, forming a proteolytic cylinder. The 19S regulatory particle, in turn, consists of the lid and the base subcomplexes, which help recognize and unfold ubiquitylated substrates. The lid and the base are held together by another subunit called Rpn10. Adapted from Murata et al. (2009).

The 26S proteasome consists of two major subcomplexes, the 20S core particle and the 19S regulatory particle. The 20S core is barrel-shaped, containing four rings of seven subunits each. The β-rings in the middle form a proteolytic chamber in which the chymotrypsin, trypsin, and peptidoglutamyl transferase active sites are directed inwards (Groll et al. 1997, reviewed by Glickman and Ciechanover 2002). The α-rings at the outer ends of the β-rings, in turn, form narrow chambers by their N-terminal tails and control the

passage of substrates into the proteasome core and the release of peptides from the other end (Groll et al. 2000). One or both ends of the 20S core are capped with 19S regulatory particles. The 19S subunit contains at least 19 subunits and it can be further divided into two subcomplexes, the base and the lid (Glickman et al. 1998). The interaction between the lid and the base is stabilized by the Rpn10 subunit (reviewed by Soboleva and Baker 2004). The selectivity of proteasomal degradation is established by the sequestration of the 20S core particle, as well as the limited access of the substrates, which is governed by the α-rings and the 19S regulatory particle.

The base consists of nine subunits, the Rpt1-6 ATPases and the Rpn1, -2, and -10 non-ATPases, all of which directly interact with the α-subunits of the core particle (Davy et al. 2001, Fu et al. 2001). The ATPases are responsible for unfolding the substrates, thereby enabling the transportation of the peptides to the core. The lid is located on top of the base, consisting of eight non-ATPase subunits, Rpn3, 5-9 and 10-12. The lid subunits are capable of detaching and rebinding to the proteasome, indicating that the lid is a flexible structure that allows the proteasome to react to changing conditions. The lid is necessary for efficient degradation of polyubiquitylated proteins, and it is thought to be involved in recognizing and processing ubiquitin chains (Glickman et al. 1998).

3.6.2 Mechanisms of proteasomal degradation

The mechanism by which the proteasome identifies its ubiquitylated substrates is not yet fully understood. Efficient proteasomal degradation is mediated by K48-linked polyubiquitylation, and some E3 ligases, such as Parkin and Hul5, directly bind and cooperate with the proteasome (Leggett et al. 2002, Sakata et al. 2003). Rpn10 recognizes the hydrophobic patches of ubiquitin molecules via its UIM (Young et al. 1998, Fu et al. 2001). However, where substrate preparation for degradation ends and processing by the proteasome begins is still somewhat obscure. Although Rpn10 efficiently binds K48-linked tetraubiquitin chains, K6- or K11- linked multiubiquitin chains have been reported to bind Rpn10 with similar affinity (Baboshina and Haas 1996). This indicates that recognition by the proteasome does not guarantee the degradation of the substrate. It has been suggested that the UBD-containing proteins Rad23 and Dsk2 assist the proteasome in the recognition of ubiquitylated substrates (reviewed in Hartmann-Petersen and Gordon 2004).

After the substrate has reached the proteasome, the regulatory particle unfolds the substrate and positions it for the entry into the proteolytic core of the proteasome. The proteasome lid keeps the ubiquitin chain from blocking the channel while the base efficiently recognizes non-ubiquitylated, unfolded proteins and aids their refolding (Braun et al. 1999, Strickland et al. 2000). Proteolysis within the proteasome depends on ATP, and the conformational changes of the proteasome are accompanied by cycles of ATP hydrolysis (Hershko et al. 1984, DeMartino et al. 1994). The ATPase cycle is coupled to gating of the core by the N termini of the α-rings, unfolding of the substrate, and threading the unfolded polypeptide through the channel into the cylinder of the core particle. These cycles cause the regulatory particle to sequentially bind and release the substrate.

Proteasomal ATPases of the regulatory subunit prepare the substrates for degradation (Glickman et al. 1998). The regulatory particle forms interactions that compete with the amino-terminal tails of the α-subunits, enabling the proteolytic core to control the channel opening (Groll et al. 2000). Once the substrate has entered the core particle, the proteasome is able to cleave all peptide bonds in the substrate, albeit the active site-containing β-subunits prefer cleaving after certain amino acids. β1 prefers cutting after acidic or small hydrophobic amino acids and β2 after basic or small hydrophobic amino acids, whereas β5 prefers hydrophobic residues regardless of their size (Dick et al. 1998). The proteasome only processes one substrate at a time. Proteolysis in the core channel

produces small peptides of 3-23 amino acids, the average length being seven to nine amino acids (Nussbaum et al. 1998, Kisselev et al. 1999). Once released from the proteasome, the small peptides are hydrolyzed by downstream proteases and aminopeptidases.

4 Multiform regulation of programmed cell death

As tens of millions of cells die daily in the human body, efficient and safe mechanisms are called for to execute cell death and to dispose of the cell remnants (Reed 1999). Multicellular organisms utilize programmed cell death to eliminate excess, damaged, or potentially dangerous cells. The term “programmed cell death” is used to distinguish between expedient, regulated forms of cellular destruction and accidental, violent cell death. By programmed cell death, an organism is able to avoid inflammation caused by leakage of the cell contents to the surrounding tissue. Apoptosis, the classical form of programmed cell death, was first described in the 18th century and identified as a dynamic process in the 1970s in a founding work by John Kerr, Andrew Wylie and Sir Alastair Currie (Kerr et al. 1972). This study introduced the term apoptosis and described the morphology of an apoptotic cell. A typical apoptotic cell rounds up and retracts its pseudopodes. In addition, the cellular volume is reduced, the chromatin condensates, the nucleus becomes fragmented, and the plasma membrane blebs. Finally, the apoptotic cell is decomposed into apoptotic bodies, which are then engulfed by phagocytes (Kerr et al. 1972).

In addition to apoptosis, other modes of cell death have been described, displaying a variable degree of regulation. Necrosis has traditionally been defined as accidental cell death characterized by plasma membrane rupture and subsequent loss of intracellular contents, but necrosis has recently been found to be more regulated than originally thought (Fiers et al. 1999). However, many of the causative elements and bystander effects of necrosis are still unclear. Autophagy, in turn, is primarily a mechanism for keeping the cell operative during nutrient deprivation. However, autophagic cell death characterized by massive autophagic cytoplasmic vacuolization occurs in response to prolonged lack of nutrients (reviewed by Baehrecke 2005, Kroemer and Jäättelä 2005). Different types of cell death form a continuum rather than represent totally separate physiological processes (reviewed by Kroemer et al. 2009). Although apoptosis relies on caspases in the execution of the death program, it is possible to re-route death signals towards necrosis or other modes of cell death, if the caspases are inhibited. In concert, the presence of limited caspase activity does not usually constitute apoptosis, as caspases perform specific tasks during diverse physiological processes, including differentiation and proliferation. The following section is dedicated solely to apoptosis.

4.1 Execution of apoptosis through the intrinsic and extrinsic pathways

The apoptotic program can be carried out by two main biochemical routes (Figure 5). The extrinsic apoptotic pathway operates during development, in the immune system and in immunosurveillance for tumor removal, whereas the intrinsic pathway eliminates cells in response to ionizing radiation, chemotherapeutic drugs, and mitochondrial damage. Both pathways mediate the activation of caspases, a family of cysteine proteases that execute the organized destruction during apoptosis in a stepwise fashion. Without apoptotic stimuli, caspases reside in the cytosol as inert proforms. While the extrinsic pathway is triggered by death receptor ligation, the intrinsic pathway activates caspases in response to mitochondrial membrane permeabilization, which leads to the release of apoptotic molecules into the cytosol. Both pathways transmit the death signal through a series of

proteolytic events called the caspase cascade. Initiator caspases are first activated in multiprotein complexes, called the death inducing signaling complex (DISC) in the extrinsic pathway and the apoptosome in the intrinsic pathway. Downstream caspases are then activated by mature initiator caspases, and the proteolytic signal is thereby extended. Executioner caspases are responsible for cleaving hundreds of cellular substrates, evoking the morphological features characteristic to apoptosis.

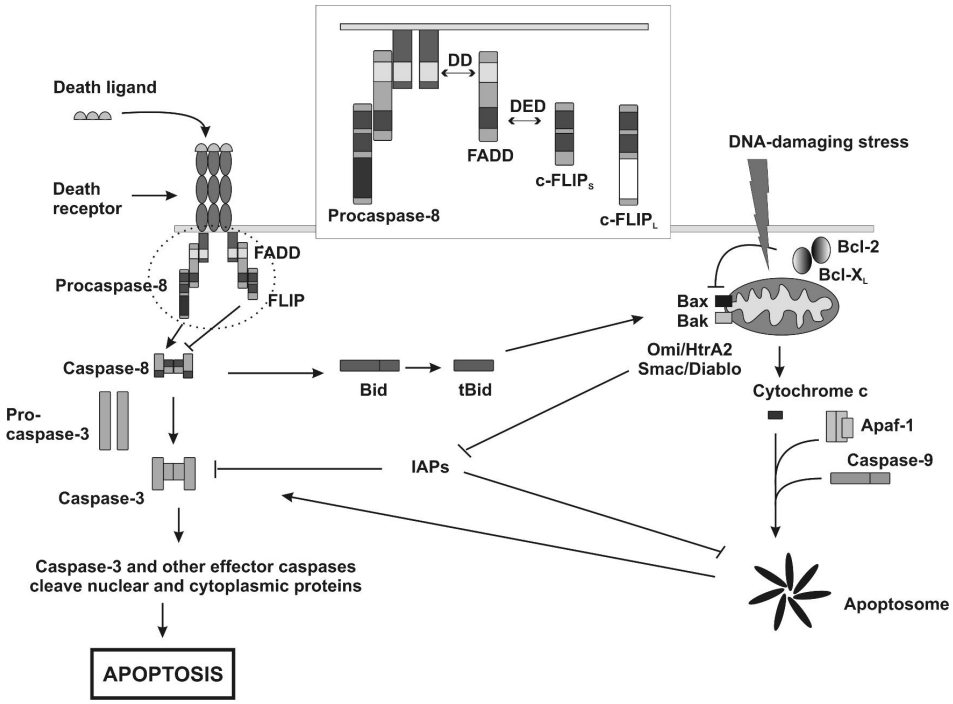

Figure 5. Schematic presentation of the major apoptotic pathways. In the intrinsic apoptotic pathway, the apoptotic program is initiated in response to severe stress stimuli, for example genotoxic damage. As a result, the mitochondrial membrane potential is lost, cytochrome *c* gets released from the intermembrane space, and the apoptosome is formed in the cytosol, enabling caspase-9 activation. Active caspase-9 initiates the caspase cascade leading to cleavage of several cytoplasmic and nuclear substrates. The extrinsic pathway is initiated by death receptor ligation, which leads to the formation of the death-inducing signaling complex (DISC). DISC brings together caspase-8 molecules, which then dimerize and activate each other, resulting in downstream caspase activation. The extrinsic pathway can be amplified through the mitochondrial loop by caspase-8-mediated cleavage of Bid.

4.2 Bcl-2 proteins regulate the intrinsic pathway and mitochondrial apoptotic signaling

The initiation and progression of the mitochondrial apoptosis is controlled at multiple levels by the B-cell lymphoma (Bcl) proteins, named after the human Bcl-2 oncogene (Tsujimoto et al. 1984). The Bcl-2 family includes both pro- and anti-apoptotic members, which activate or neutralize each other by homo- and heterodimerization. The pro-apoptotic Bcl-2 proteins

sense the apoptotic stimuli and transmit it to the mitochondria, while the anti-apoptotic Bcl-2 proteins act as to prevent them. Therefore, the Bcl-2 proteins are considered the gatekeepers of the mitochondrial membrane integrity.

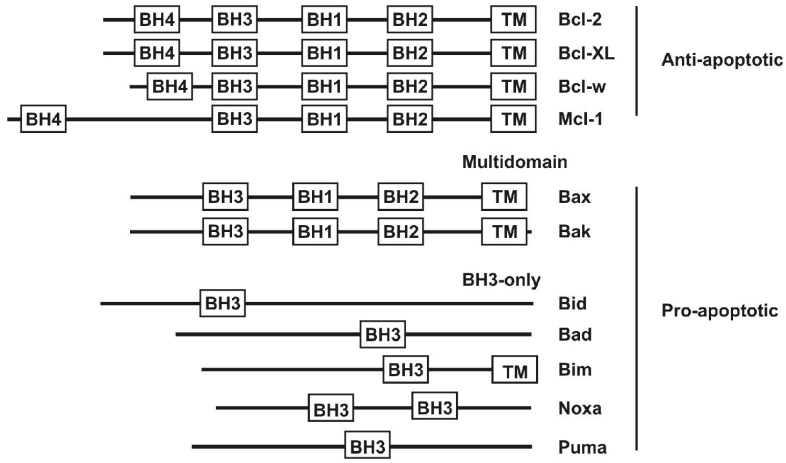

Figure 6. The anti-apoptotic Bcl-2 proteins protect the cell from apoptosis by inhibiting the pro-apoptotic Bcl-2 proteins. The pro-apoptotic multidomain proteins induce mitochondrial outer membrane permeabilization. The BH3 domain-only proteins act as sensors of variable apoptotic signals and transfer them further to the multidomain pro-apoptotic Bcl-2 proteins. Bcl-2 homology (BH) domains and putative transmembrane (TM) domains are indicated. Adapted from Adams and Cory (2007). The figure is not drawn to scale.

Based on their structure, the Bcl-2 proteins can be divided into three groups, the anti-apoptotic, the pro-apoptotic multidomain and the pro-apoptotic BH3-only Bcl-2 proteins (Figure 6; reviewed by Adams and Cory 2007). All Bcl-2 proteins are characterized by one or more Bcl-2 homology domains (BH), and the number and location of the BH domains dictates the attributes of an individual Bcl-2 protein. The pro-apoptotic Bcl-2 proteins are guarded by the pro-survival Bcl-2 proteins, which include Bcl-2, Mcl-1, and the Bcl-XL isoform of the Bcl-X gene. The anti-apoptotic Bcl-2 proteins have been shown to bind to the BH3-only proteins in order to keep them from activating Bax and Bak, but it has also been suggested that the Bcl-2 proteins directly bind and inhibit Bax and Bak. Nevertheless, correct execution of the anti-apoptotic function necessitates that Bcl-2 and Bcl-XL are correctly targeted to the outer mitochondrial membrane. Since the anti-apoptotic Bcl-2 proteins set the balance for apoptotic sensitivity, their levels and activity are carefully regulated. Bcl-2, for instance, is marked for ubiquitylation and degradation by phosphorylation (Breitschopf et al. 2000). The levels of anti-apoptotic Bcl-2 proteins are often found to be upregulated in malignant cells, partly accounting for their abnormal resistance towards apoptosis (Adams and Cory 2007). In addition to ubiquitylation, other post-translational mechanisms also regulate the pro-survival Bcl-2 proteins. Upon DNA damage, Bcl-XL becomes deaminated and is thereby inhibited from binding to BH3-only proteins (Deverman et al. 2002). The activity of Bcl-2, in turn, is positively modulated by multiple phosphorylations (Ruvolo 1999, Ruvolo et al. 2001).

The BH3 domain only-proteins sense the signals that initiate mitochondrial apoptosis. There are currently 11 known BH3 domain-only proteins, including BH3-interacting domain death agonist (Bid), BH3-interacting mediator of cell death (Bim), Bcl-2-associated death protein

(Bad), Noxa, and p53-upregulated modulator of apoptosis (Puma). During normal circumstances, the BH3 only-proteins are present in an inactive form, but they are post-translationally activated in response to stress. It seems that different types of stress stimuli are sensed by distinct BH3 only-proteins. For example, Bid is cleaved by active caspase-8 into its active truncated conformation (tBid; Li et al. 1998, Luo et al. 1998), although other proteases, such as caspase-3, are also capable of cleaving Bid *in vitro* (Desagher et al. 2001, Degli Esposti et al. 2003). Full tBid activation requires further cleavage, ubiquitylation and degradation of the N-terminal fragment (Tait et al. 2007). Bad, in turn, is normally kept inactive by Akt-mediated phosphorylation, but is released from the 14-3-3 scaffold by dephosphorylation (Zha et al. 1996, Wang et al. 1999). Bim is activated and freed from the filament network in response to increased levels of calcium or cytokine deprivation (Puthalakath et al. 1999), but targeted to ubiquitylation and proteasomal degradation by phosphorylation (Ley et al. 2003, Ley et al. 2004). Finally, Puma and Noxa are targets of p53, and their transcription is upregulated upon DNA damage (Nakano and Vousden 2001, Yu et al. 2001, reviewed by Puthalakath and Strasser 2002). The BH3 only-proteins harbor some specificity towards the anti-apoptotic Bcl-2 proteins. Bim, Puma and tBid bind the anti-apoptotic Bcl-2 proteins with limited selectivity, whereas Noxa is selective towards Mcl-1 and A1, and Bad towards Bcl-2, Bcl-XL, and Bcl-w (Chen et al. 2005, Kuwana et al. 2005). Although the anti-apoptotic proteins need to be neutralized in order for apoptosis to proceed, it is currently not known to which extent the neutralization needs to progress to allow apoptosis.

After activation, the BH3-only proteins transmit the signal by activating the pro-apoptotic multidomain proteins, such as Bcl-2-associated X (Bax) protein and Bcl-2 homologous antagonist/killer (Bak). Bax and Bak are essential executioners of mitochondrial apoptosis, since the BH3-only proteins are not capable of initiating apoptosis in cells lacking both Bax and Bak (Cheng et al. 2001, Zong et al. 2001). However, Bax and Bak seem to be redundant, as either one alone is sufficient to transmit the death signal from the BH3 only-proteins. Bax and Bak are constitutively expressed and sequestered to the cytosol and to the mitochondria, respectively. Upon activation, Bax is translocated from the cytoplasm to the mitochondrial outer membrane, where Bak already resides. Both Bax and Bak have to undergo conformational changes and oligomerize to be able to penetrate the outer mitochondrial membrane.

There are currently two models for Bax and Bak activation by the BH3-only proteins. In the direct activation model (reviewed by Willis and Adams 2005), Bim and tBid directly bind Bax and Bak, thereby promoting their activation (Letai et al. 2002, Kuwana et al. 2005, Certo et al. 2006, Oh et al. 2006, Walensky et al. 2006), while the remaining BH3-only proteins bind the anti-apoptotic Bcl-2 proteins. According to this model, Bim and tBid are released from the anti-apoptotic Bcl-2 proteins because they are displaced by the other BH3-only proteins, allowing Bim and tBid to bind Bax and Bak. In the indirect activation model, in turn, the BH3-only proteins do not directly interact with Bax or Bak, but with the anti-apoptotic Bcl-2 proteins (Chen et al. 2005, Willis et al. 2005, Willis et al. 2007). Here, the efficiency of tBid and Bim as apoptosis inducers is measured by their ability to bind the anti-apoptotic Bcl-2 family members. Later studies have questioned the direct activation model, but it has also been stated that Bax and Bak need an additional activating signal in order to promote apoptosis (reviewed by Green 2005, Adams and Cory 2007). Therefore, the indirect model at its simplest may also be insufficient.

The depletion of Bax and Bak results in the lack of mitochondrial outer membrane permeabilization (MOMP), as well as resistance to a wide range of apoptotic stimuli in the double knock-out mice (Wei et al. 2001). It is not known, however, how Bax and Bak are able to induce the release of cytochrome *c*. It has been suggested that Bax and Bak form channels on their own (Wei et al. 2000), interact with other channel-forming proteins to create permeability transition pores (Tsujimoto and Shimizu 2000, Zamzami and Kroemer

2001), or form supramolecular openings to the membrane (Kuwana et al. 2002). Interestingly, a recent report proposes that the mitochondrial fission induced by Bax and Bak may in fact be separate from the cytochrome *c* release, as the anti-apoptotic Bcl-2 family members were capable of rescuing the cell from cytochrome *c* release, but not from the fragmentation of the mitochondrial network (Sheridan et al. 2008).

MOMP is considered the point of no return for the intrinsic apoptotic pathway. MOMP is induced by numerous pro-apoptotic and pathological stimuli which converge in the mitochondria, and it is required for caspase activation in the intrinsic apoptotic pathway. Biochemical mechanisms for MOMP induction have remained controversial, although several proteins have been assigned MOMP-inhibiting or -promoting functions (Letai et al. 2002, Chen et al. 2005, Kuwana et al. 2005, Willis et al. 2005). MOMP results in the release of apoptotic factors such as cytochrome *c*, SMAC/DIABLO, and OMI/HtrA2 to the cytoplasm. In addition, the loss of mitochondrial membrane potential leads to mitochondrial metabolic failure.

Two basic models for MOMP have been proposed (reviewed by Green and Kroemer 2004). According to the first model, small permeability transition pores are formed on the inner mitochondrial membrane, allowing water and molecules of 1.5 kDa to pass through. The opening of these pores results in loss of the mitochondrial membrane potential and swelling of the matrix, which disrupts the outer mitochondrial membrane and releases the contents, including the caspase-9 activation co-factor cytochrome *c*. In the second, permeability transition pore-independent model, the MOMP does not depend on permeability transition pores, but instead the pro- and anti-apoptotic Bcl-2 proteins that reside on the outer mitochondrial membrane (Green and Kroemer 2004). The apoptotic Bcl-2 proteins are perhaps accompanied by other proteins, and together they permeabilize the outer mitochondrial membrane.

The mitochondrial pathway is sometimes involved in amplifying the signal that stems from death receptor ligation. According to this type 1 - type 2 hypothesis (Scaffidi et al. 1999), death receptor activation in type 1 cells results in caspase-8 activation that is strong enough to initiate the caspase cascade and apoptosis on its own. In contrast, caspase-8 activation in type 2 cells is too weak to sufficiently activate the downstream caspases, and the cell requires a mitochondrial amplification loop to intensify the signal. This is achieved through caspase-8-mediated proteolysis of Bid, producing an active truncated Bid (tBid). tBid then translocates to the mitochondria, where it triggers the release of cytochrome *c* from the intermembrane space to the cytosol, thereby enabling the activation of caspase-9. However, although it is widely accepted that caspase-8 mediates Bid cleavage in type 2 cells during extrinsic apoptotic cell death, some aspects of the type 1 - type 2 model have remained controversial. Caspase-8 is not the only protease that is capable of cleaving Bid, but also caspase-2 and caspase-3 have been reported to process Bid (Guo et al. 2002, Bonzon et al. 2006, Bossy-Wetzel and Green, 1999, Slee et al. 2000). In addition, cathepsins and calpains have been found to activate Bid (Stoka et al. 2001, Mandic et al. 2002, Werner et al. 2004, Droga-Mazovec et al. 2008). Therefore, Bid cleavage is not always a corollary of caspase-8 activity, although active caspase-8 would simultaneously be present.

4.3 Apoptotic initiator and effector caspases

According to the current view, a cell is considered dead when its plasma membrane integrity is lost, it has been fragmented into apoptotic bodies, and these fragments are eventually engulfed by adjacent cells (Kroemer et al. 2009). The majority of the apoptotic morphology is due to substrate cleavage by the caspases. Caspases are cysteine aspartate

proteases that are responsible for organized destruction of proteins and DNA during apoptosis. The pivotal role of caspases was originally discovered in *Caenorhabditis elegans* studies. The product of the *C. elegans CED-3* (cell death abnormality) gene was similar to the human caspase-1 or interleukin-1β-converting enzyme, the first human caspase to be identified (Yuan et al. 1993). A total of four caspases have since been discovered in *C. elegans*, while CED-3 remains its only apoptotic caspase.

4.3.1 The classification and structure of caspases

Caspase enzymes have been classified into two subfamilies based on phylogenetic analyses. The first subclass is the interleukin-1β converting enzyme (ICE)-like caspases, whereas the second subclass resembles the *C. elegans* apoptotic caspase, CED-3. To date, 13 caspases have been cloned from mammals. Caspases fall into two major functional groups, the apoptotic and the inflammatory caspases. Of these, the apoptotic caspases (-2, -8, -9, -10, -3, -6 and -7) are involved in initiating and executing cell death in an orderly manner (Figure 7), whereas the inflammatory caspases (-1, -4, -5, -11, -12 and -13) function in response to a variety of immunogenic stimuli by forming and regulating a signaling complex called the inflammasome (reviewed by Martinon et al. 2009).

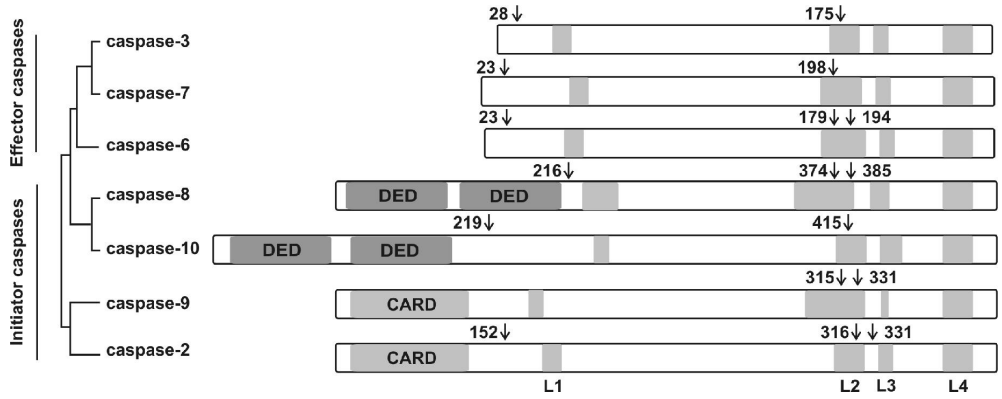

Figure 7. Structure, classification and conservation of the mammalian apoptotic caspases. Caspases -8 and -10 are the initiator caspases of the extrinsic pathway, both of them containing two N-terminal death effector domains (DED) and a C-terminal caspase domain. In contrast, the remaining initiator caspases -2 and -9 oligomerize via their caspase recruitment domains (CARD). During autoprocessing, all initiator caspases are cleaved twice to produce the large p20 and small p10 subunits. The effector caspases include caspase-3, -6 and -7, and they do not contain large prodomains. Instead, they are proteolytically activated by initiator caspases to produce the active conformation and a mature caspase. Adapted from Riedl and Shi 2004. The figure is not drawn to scale.

Caspases are produced as inactive zymogens that need targeted proteolysis for activation. Since inappropriate caspase activation would be detrimental to the cell, caspase zymogen processing is carefully regulated. The broad-scale activation of caspases is guarded by activation of the initiator caspases. Initiator caspases -8, -9, -2 and -10 mediate the cleavage of effector caspases -6, -7 and -3, which amplify the signal and cleave a large number of substrates. In addition to homology, caspases are categorized based on their

favored substrate motifs: while effector caspases prefer to cleave substrates after DEVD or VEVD, caspase-8 favors LETD and caspase-9 LEHD (Thornberry et al. 1997).

Unlike effector caspases, initiator caspases include conserved interaction domains (Figure 7). Hydrophilic interactions between the N-terminal death effector domains (DEDs) of caspases and the adaptor Fas-associated death domain protein (FADD) mediate the binding of caspase-8 and -10 to activated death receptors. In addition, the homophilic dimerization, which is crucial to caspase activation, relies on their second DED. Caspase-2 and -9, in turn, have single N-terminal caspase-associated recruitment domains (CARDs), which mediate homophilic, electrostatic interactions. Both DED and CARD are members of the death-fold domain family (reviewed by Liang and Fesik 1997), the two other members being the death domain (DD) and the pyrin domain (reviewed by Lahm et al. 2003).

4.3.2 Caspase-9 is activated in the apoptosome holoenzyme

The initiator caspases are activated in protein complexes. While caspase-8 and caspase-10 dimerize at the plasma membrane in a death receptor-based complex, caspase-9 is activated in the apoptosome holoenzyme following MOMP. MOMP releases proteins from the mitochondrial intermembrane space, some of which contribute to the formation of the apoptosome. The apoptosome signaling complex forms in the cytoplasm after some of its crucial subunits have been released from the mitochondria. One of these pivotal factors is cytochrome *c*, a water-soluble 13 kDa heme-containing protein, which under normal conditions is sequestered by cristae junctions in the spaces between cristae in the inner mitochondrial membrane and participates in the mitochondrial electron-transport chain (reviewed by Pellegrini and Scorrano 2007, Ow et al. 2008). After being released from the mitochondria, cytochrome *c* binds to the conserved WD40 domains of the adaptor molecule Apaf-1, which mediates the oligomerization of seven Apaf-1 molecules. Apaf-1-cytochrome *c* binding also allows ATP-binding by the CED-4-like domain of Apaf-1, leading to an elongated conformation. In addition, upon cytochrome *c* binding, the CARD domain of Apaf-1 is exposed, enabling the homotypic interaction between Apaf-1 and caspase-9 (Hao et al. 2005, Riedl et al. 2005). Together these molecules form the apoptosome, a complex with a wheel-like structure (Yu et al. 2005). The apoptosome provides a platform for caspase-9 dimerization, which is required for its activation. It has been suggested that the apoptosome operates like a molecular timer: the concentration of unprocessed procaspase-9 determines the duration of the complex, while the autoprocessing of procaspase-9 activates the timer, and the dissociation rate of mature caspase-9 from the apoptosome determines how long it is capable of activating procaspase-3 (Malladi et al. 2009).

Preceding MOMP and cytochrome *c* release, caspase-9 is an inactive monomer. In the cytosol, cytochrome *c* forms the apoptosome complex around the adaptor protein apoptotic protease activating factor 1 (Apaf-1; Zou et al. 1999). The interaction between Apaf-1 and cytochrome *c* alters the conformation of Apaf-1 into an ATP-binding, elongated state, enabling the assembly of the 1.4 MDa apoptosome complex structured like a seven-spoked wheel. The N-terminal CARD domains of Apaf-1 recruit caspase-9 molecules to the central hub of the apoptosome, where the induced proximity of these caspases promotes their dimerization and activation. Similarly to caspase-8, further cleavage is unnecessary for the enzymatic activity of caspase-9. A fully active caspase-9 dimer harbors only one active domain, and it is formed by small rearrangements of the surface loops defining the substrate cleft and catalytic residues (Hao et al. 2005, Riedl et al. 2005). The apoptosome has been considered the only activation platform for caspase-9, but lately this view has challenged. Caspase-9 was found to be in a complex with downregulated in rhabdomyosarcoma LIM-domain protein (DRAL), when the dependence receptor Patched is not occupied by its ligand, Sonic hedgehog (Mille et al. 2009). In addition, caspase-9 has been reported to be cleaved and activated by caspase-8 during TNF-mediated lysosomal

cell death (Gyrd-Hansen et al. 2006), illustrating the overlap between both different cell death programs and the extrinsic and intrinsic pathways.

4.3.3 Activation of the effector caspases

Following apoptotic stimuli, initiator caspases cleave and activate the effector caspases. The mechanism for activation of effecetor caspases -3, -6 and -7 differs markedly from that of initiator caspases. In contrast to initiator caspases, effector caspases are present in the cytosol as inactive dimers. Effector caspases contain a short prodomain spanning 20-30 amino acids, active-site loops L1-4, and C-terminal caspase domains. Caspases -3, -6 and -7 are activated by limited proteolysis within the interdomain linker at L2 loops, and following cleavage, the active cysteine residues in the N-terminal segment of loop L2 and the C-terminal L2' loop translocate to opposite directions to stabilize the active conformation (Figure 8). After the first cleavage by the initiators, effector caspases cleave the remaining loops in autoproteolytic fashion. The model of effector caspase activation is largely based on the crystal structure data obtained from the inactive and active conformations of caspase-7 (Chai et al. 2001, Riedl et al. 2001).

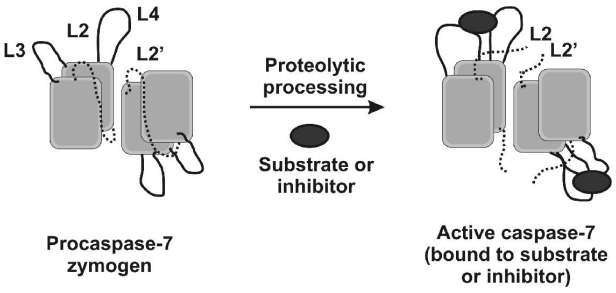

Figure 8. Model for effector caspase activation based on the crystal structure of caspase-7. The procaspase-7 dimer (left) and its active site loops are shown (L1 and L1' excluded). Proteolytic processing of the L2 loop from its interdomain linker (dotted line) between the small and large subunits produces a mature, stabilized structure, where the active cysteine is differentially located compared to the unprocessed procaspase-7 zymogen. Adapted from Riedl and Shi (2004).

4.3.4 Regulation of caspase activity

The activity of caspases is regulated on multiple levels to ensure safe and precise execution of apoptosis and to maintain appropriate caspase activity during development, differentiation and proliferation. The activation of caspase-8 is inhibited by cellular FLICE-inhibitory proteins (c-FLIPs). Active caspases, on the other hand, are specifically regulated by the members of the IAP (inhibitor of apoptosis) protein family (reviewed by Srinivasula and Ashwell 2008). After the viral IAPs were originally cloned from baculovirus-infected insect cells, their cellular orthologs have been found in organisms including yeast, nematodes, flies, mammals and even plants (Higashi et al. 2005). To date, 8 mammalian IAPs have been identified (Figure 9), among which cellular inhibitor of apoptosis proteins 1 (c-IAP1 or MIHB, hiap2, BIRC2) and 2 (c-IAP2 or MIHC, hiap2, BIRC3), as well as X-linked IAP (XIAP, hILP, MIHA, BIC4) are the most thoroughly characterized. The remaining five, BRUCE, ILP2, ML-IAP, NAIP and survivin, regulate variable physiologial processes, including innate immunity (Srinivasula and Ashwell 2008). Surprisingly, IAP gene deletion studies show that BRUCE, a 528 kDa IAP with an N-terminal BIR and a C-terminal UBC domain, is absolutely required for development, as *BRUCE* deficiency causes embryonic or neonatal lethality (Hao et al. 2004). In contrast, XIAP or c-IAPs are not required for survival

or normal development in mice, perhaps due to functional redundancy between the RING-containing IAPs (Harlin et al. 2001, Conze et al. 2005, Conte et al. 2006).

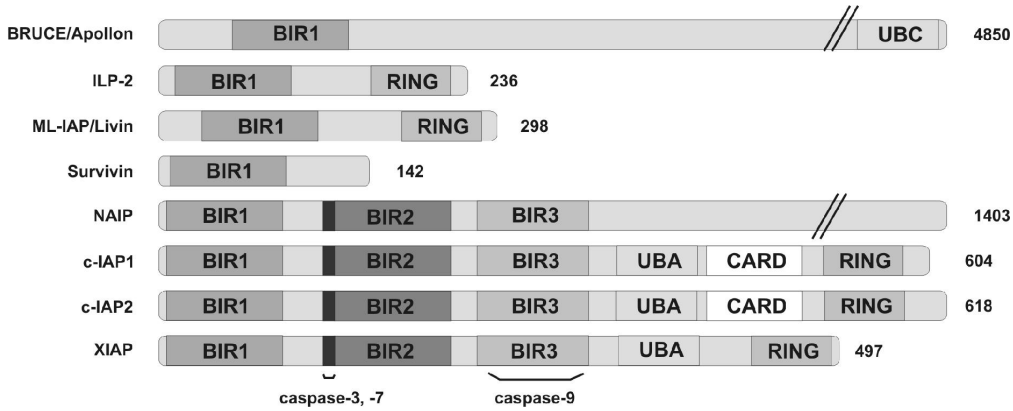

Figure 9. Schematic presentation of the mammalian IAPs. IAPs contain several conserved domains, of which the baculoviral IAP repeat (BIR) domain is common to all IAPs. In addition, c-IAPs contain CARDs, which enable them to interact with caspases, and IAPs containing RING domains can act as E3 ubiquitin ligases. The c-IAPs and XIAP also contain ubiquitin-associated domains that enable them to bind K63-linked ubiquitin in a non-covalent manner (Gyrd-Hansen et al. 2009). In addition, BRUCE is a large ubiquitin conjugating enzyme. The IAPs are known to inhibit different caspases: c-IAPs, XIAP and NAIP are capable of inhibiting caspase-3 and caspase-7 by the linker region N-terminal to BIR2, while XIAP also inhibits caspase-9 by its BIR3 domain. Adapted from Riedl and Shi (2004). The figure is not drawn to scale.

The IAPs are characterized by a variable number of the baculovirus IAP repeat (BIR) domains, which mediate direct binding and inhibition of proteolytically active caspases (reviewed by Eckelman et al. 2006). The BIR domains encompass about 70 amino acids, including a signature sequence C-X^2-CX^{16}-H-X^6-C (where X denotes any amino acid), which forms a hydrophobic core with a zinc atom in the center. In addition to the BIR domains, a subset of IAPs contains RING domains, thereby equipping these proteins with ubiquitin ligase E3 activity. c-IAPs also contain CARD domains, although their biological function has remained obscure.

An acidic surface groove of the BIR domain operates as an anchoring motif for caspases. Different BIR domains, however, display some specificity for caspase binding. For example, the surface groove of BIR2 strongly binds caspase-3 and -7, but not caspase-9. The same motif, when surrounded by different residues in BIR3, only binds caspase-9, but not caspase-3 or -7 (Eckelman et al. 2006). Binding alone, however, does not effectively inhibit caspase activity, and XIAP seems to be a more potent inhibitor of caspases than the c-IAPs (Eckelman and Salvesen 2006). Instead, XIAP blocks the substrate binding site of caspase-3 and -7 by its flexible linker region (Chai et al. 2001, Huang et al. 2001, Riedl et al 2001). Caspase-9, in turn, is inhibited by XIAP by a distinct mechanism: after caspase-9 has been processed in the apoptosome holoenzyme, the XIAP BIR3 domain binds to the exposed linker peptide of the small caspase-9 subunit (Srinivasula et al. 2001).

The IAPs are regulated by several pro-apoptotic proteins, including second mitochondrial-derived activator of caspases/direct inhibitor of apoptosis-binding protein with low pI

(SMAC/DIABLO; Du et al. 2000, Verhagen et al. 2000) and Omi/high temperature-requirement protein A2 (HtrA2; Suzuki et al. 2001, Hegde et al. 2002). The IAP inhibitors do not share any obvious sequence homology, apart from the IAP-binding motif (IBM) characterized by an N-terminal tetrapeptide (AVPI/AVSI) that is revealed by cleavage of the mitochondrial targeting sequence. During apoptosis, the IAP inhibitors move from the mitochondrial intermembrane space to the cytosol and bind to BIR2 and BIR3, thereby preventing IAPs from binding to caspases (Srinivasula and Ashwell 2008). In addition, SMAC triggers the autoubiquitylation and proteasomal degradation of c-IAPs. The recent development of SMAC mimetics has provided important tools for dissecting the role of IAPs in diverse physiological contexts. As described by several independent studies, SMAC mimetics induce the autoubiquitylation and degradation of c-IAP1 and c-IAP2 (Petersen et al. 2007, Varfolomeev et al. 2007, Vince et al. 2007). Intriguingly, SMAC mimetics seem capable of inducing both NF-κB and subsequent apoptosis. Following c-IAP depletion, RIP becomes deubiquitylated by CYLD, which leads to the release of RIP form the membrane-associated complex I into the cytosol. There RIP interacts with FADD and caspase-8 to form a death-inducing complex (Wang et al. 2008). How caspase-8 activation is regulated in this context is not entirely known.

Some IAPs possess a RING domain providing them with ubiquitin ligase activity, which partly explains the versatile roles of IAPs in death and survival signaling. The RING-containing IAPs include XIAP, ILP2, ML-IAP, c-IAP1 and c-IAP2 (Figure 9). At least XIAP and c-IAPs have been shown to be able to ubiquitylate themselves to mediate their own degradation. In addition, controversing reports exist on the effect of SMAC to the ligase activity of the IAPs (Creagh et al. 2004, Yang et al. 2004).

4.3.5 Processing of caspase substrates

The name 'caspase' derives from cysteine-dependent aspartate specific protease. As many other proteases, caspases use the cysteine side chain as a nucleophile during peptide bond hydrolysis. However, unlike most other proteases, caspases have strict specificity for cleavage after D residues (Sleath et al. 1990, Thornberry et al. 1992). The caspase consensus sequences are often used to indicate specificity, although the preferred caspase cleavage sites are far from exclusive. In general, caspases recognize a tetrapeptide (P4-P3-P2-P1) in which P1 is D. Charged or bulky residues are poorly tolerated at the P1' position after the scissile bond - G, A, T, S and N are suitable for caspase processing, whereas E, D, K, R and W do not favor catalysis. Several caspases prefer Q at P3, whereas the nature of the P2 seems to be less important, and the preferences for P4 vary among the caspases (Sleath et al. 1990, Talanian et al. 1997, Thornberry et al. 1997). Usually, the cleavage site of caspase substrates adapts a disordered and flexible conformation (Hubbard et al. 1991, reviewed by Timmer and Salvesen 2007). Although caspases favor slightly differing substrate sequences, the degree of overlap is still largely unknown. According to recent findings, caspase-3 and caspase-7 exhibit some differences in protease activities: caspase-3 appears more promiscuous than caspase-7, and therefore caspase-3 is likely to be the major executioner of apoptosis (Walsh et al. 2008).

Caspases were previously considered solely responsible for all the biochemical and morphological changes that occur during apoptosis. Yet it is difficult to distinguish the specific effects of active caspases from indirect secondary effects. Although hundreds of proteins have been identified as potential caspase substrates, only a subset has been confirmed to be *in vivo* substrates with a designated physiological role. Established caspase substrates include the executioner caspases, the pro-apoptotic Bcl-2 protein Bid, the tumor suppressor Rb, the nuclease regulator inhibitor of caspase-activated DNase (ICAD), and the poly(ADP-ribose) polymerase (PARP). Caspase cleavage typically results either in gain-of-function or in loss-of-function of the substrate. For example, the activation of

executioner caspases -3 and -7 is a gain-of-function event. The cleavage of ICAD, in turn, results in loss of enzyme inhibition, and as a result, the CAD is free to cleave double-stranded DNA (Liu et al. 1997, Enari et al. 1998). The degradation of DNA is a classical biochemical feature of apoptosis, along with the caspase-mediated cleavage and subsequent inhibition of the PARP enzyme, which routinely repairs breaks in double-stranded DNA (D'Amours et al. 2001). The ATP-dependent enzymes and processes are generally inhibited by apoptosis, because programmed cell death requires energy.

4.4 The death receptor systems and the extrinsic apoptotic pathway

4.4.1 The TNF superfamily of death ligands and receptors

Whereas the intrinsic pathway is induced from within the cell, the death signals initiating the extrinsic pathway may be expressed by the cell itself or by the neighboring cells. The extrinsic apoptotic pathway is initiated by binding of a death ligand to its cognate death receptor on the plasma membrane. The key mediators of receptor-mediated apoptosis are the ligands and receptors of the tumor necrosis factor (TNF) superfamily. The finding and characterization of the TNF cytokines and their respective receptors was a result of vigorous scientific work, the first discoveries dating back to the 19th century. Initial observations of TNF addressed tumor regression after bacterial infection (Bruns 1868), a phenomenon which has since been demonstrated in several models from guinea pigs to humans. TNFα and TNFβ were purified, sequenced and cloned in the 1980s, and TNF receptors (TNFR) 1 and 2 followed shortly thereafter (Aggarwal et al. 1984, Gray et al. 1984, Pennica et al. 1984, Aggarwal et al. 1985a, Aggarwal et al. 1985b). The 19 ligands and 32 receptors of the TNF superfamily are involved in a wide array of biological processes, including the regulation of cell death and survival, innate and adaptive immunity, and differentiation (reviewed by Aggarwal 2003). During the last decade, also non-apoptotic roles have begun to emerge for several death receptors.

TNF receptors and ligands are expressed in varying degrees in a wide range of tissues. Nearly all TNF ligands are expressed in immune cells, with the exception of the secreted vascular endothelial cell-growth inhibitor (VEGI), which is mainly expressed by the endothelial cells (Zhai et al. 1999). In contrast to TNF ligands, almost all cell types express some death and decoy receptors, thereby equipping them with the capacity to undergo receptor-mediated apoptosis. Receptors of the TNF superfamily are type 1 transmembrane proteins with an extracellular N terminus and an intracellular C terminus, and they reside on the membrane as preassembled homotrimers. The TNF receptors are characterized by one to six conserved cysteine-rich extracellular domains that mediate the receptor-ligand-interactions (reviewed by Ashkenazi and Dixit 1999, Chan 2007). In addition, some of the TNF receptors share a common cytoplasmic death domain (DD). These receptors are *bona fide* death receptors, as opposed to the decoy receptors which lack the cytoplasmic death domains and are therefore unable to transmit the death signals. However, decoy receptors are still potent modulators of death receptor signaling, because they bind death ligands. Many vertebrate death receptors contain DDs, but, surprisingly, the *Drosophila* TNFR homologue Wengen is able to transmit apoptotic signals although it lacks a functional death domain (Kanda et al. 2002, Kauppila et al. 2003). However, as other *Drosophila* proteins contain functional DDs, this is probably not an indication of the evolutionary development of the DD. In addition to the DDs, another homologous structure has been addressed to TNFR-1, CD95 and TRAILR-1, called the pre-ligand-binding assembly domain (PLAD). The PLADs mediate receptor homotrimerization, which is essential for ligand binding (Chan et al. 2000).

The TNF ligands are type 2 transmembrane proteins with an intracellular N terminus and an extracellular C terminus. The ligands reside at the plasma membrane as homotrimers (Chan et al. 2000). Similarly to the death receptors, death ligands contain two to four cysteine-rich repeats, some of which are required for binding to the receptor. In addition, the cysteine-rich repeats mediate the trimerization of CD95L (Orlinick et al. 1997a, Orlinick et al. 1997b). While some death ligands require solubilization to become activated, others are reportedly inhibited by shedding from the plasma membrane. For example, the membrane-bound CD95 ligand has been found to induce apoptosis, whereas the soluble CD95L antagonizes the membrane-bound form and suppresses apoptosis in human peripheral T lymphocytes (Suda et al. 1997). However, the mechanism for ligand activation or inactivation by shedding has remained elusive. A soluble ligand is produced from a membrane-bound ligand by matrix metalloproteinases (MMPs; Mitsiades et al. 2001, Ethell et al. 2002). To transmit apoptotic signals, the ligand must at least be in a hexameric state to bind multimerized death receptors (Holler et al. 2003). In addition, sensitivity to programmed cell death can also be adjusted through the shedding of death receptors from the cell surface (Tanaka et al. 1998, Ahonen et al. 2003, Strand et al. 2004). Interestingly, many of the TNF superfamily ligands have more than one cognate receptor, and this phenomenon has fundamental consequences: for example, the diverse functions of TNFα and the toxicity of its pharmacological derivatives are due to ubiquitous expression of the TNF receptors.

4.4.2 The TNF receptor system in cell death and survival signaling

The TNF cytokines TNFα and TNFβ (also called lymphotoxin α) transmit signals via TNFR-1 and TNFR-2. Since TNFR-2 lacks the intracellular death domain, TNF signaling is primarily mediated via TNFR-1 (Tartaglia et al. 1993). The TNF receptors are single transmembrane glycoproteins, which contain four extracellular cysteine-rich domains. It is interesting to note that these domains can be proteolytically shed, and the soluble receptor has neutralizing potential (Wallach et al. 1991). The TNF ligand is a type 2 transmembrane protein existing as stable trimers in a 26-kDa membrane-bound proform and a 17-kDa soluble form cleaved by matrix metalloproteinases, such as TACE (Smith and Baglioni 1987, Tang et al. 1996, Black et al. 1997). TNF signaling is extremely versatile and may promote either death or survival, depending on the context.

Ligation of TNFα to the TNFR-1 may initiate a number of signaling pathways through different TNFR complexes. The TNFR complexes located at the plasma membrane form around the adaptor protein TRADD (TNFR-associated death domain) in a fashion analogous to the CD95 or TRAIL DISCs. The death domains of TRADD mediate interactions between the intracellular parts of TNFR-1. TRADD recruits another adaptor protein, TNF receptor-associated factor 2 (TRAF2), and a serine/threonine kinase, receptor-interacting protein (RIP) to TNFR complex 1 at the plasma membrane (Figure 10). Complex 1 activates the canonical NF-κB pathway by activating the IκB kinase (IKK) complex, which then phosphorylates IκB, the inhibitor of NF-κB. Phosphorylation primes IκB for ubiquitylation and proteasomal degradation by recruiting the E3 ligase $SCF^{\beta\text{-}TrCP}$ (Yaron et al. 1998, reviewed by Karin and Ben-Neriah 2000). Degradation of IκB leads to the exposure of the nuclear localization signal of NF-κB, allowing it to translocate to the nucleus and act as a transcription factor.

While the formation of TNFR complex 1 relies on TRADD and RIP, TRADD can also mediate the formation of a FADD-dependent complex 2, which is cytosolic and signals cell death instead of survival. FADD is recruited to TNFR complex 2 by TRADD or ubiquitylated RIP1, after which FADD binds caspase-8 molecules to initiate apoptosis (Micheau and Tschopp 2003, Wang et al. 2008, reviewed by Wilson et al. 2009). Caspase activity in

complex 2 is regulated by c-FLIPs, c-IAPs, and the deubiquitylating enzyme CYLD (Figure 10; Micheau and Tschopp 2003, Wang et al. 2008).

Ubiquitin-mediated modifications have important regulatory roles in death receptor signaling, in particular in defining the outcomes of TNF receptor stimulation. TRAF2 is an adaptor protein that is recruited to the TNF complex 1 at the plasma membrane. TRAF2 is also a RING-finger ubiquitin E3 ligase, and it attracts yet more ligases, c-IAPs, to the same complex. The interaction between c-IAPs and TRAF2 is primarily mediated through the c-IAP BIR domain (Samuel et al. 2006), and it conveys c-IAP-mediated modification of RIP with K63-linked ubiquitin chains. The K63-linked ubiquitin chains of RIP serve as a binding platform for the IKK and TAK kinase complexes (Ea et al. 2006), and thereafter TAK phosphorylates and activates IKKβ of the IKK complex (Figure 10). After IKK-phosphorylated IkB is recognized and tagged with K48-linked ubiquitin chains by $SCF^{\beta\text{-}TrCP}$, NF-κB is free to translocate to the nucleus to drive the transcription of its target genes.

After forming at the plasma membrane, complex 1 is released to the cytosol, where the assembly of complex 2 begins. In the absence of the c-IAPs, RIP remains unubiquitylated and is free to recruit procaspase-8 molecules. The induced proximity of procaspase-8 proteins promotes their dimerization and activation, initiating apoptosis. Apoptosis is further promoted by A20, an enzyme harboring both ubiquitin ligase and deubiquitylating enzyme-like activities. A20 has been reported to deubiquitylate RIP, thereby contributing to apoptosis (Wertz et al. 2004). Another deubiquitylating enzyme, CYLD, has been demonstrated to regulate TNF responses by a similar mechanism (Wang et al. 2008). In the non-canonical NF-κB pathway, the c-IAPs have an unexpected negative role in ubiquitylating the NF-κB-inducing kinase (NIK), facilitating its proteasomal degradation.

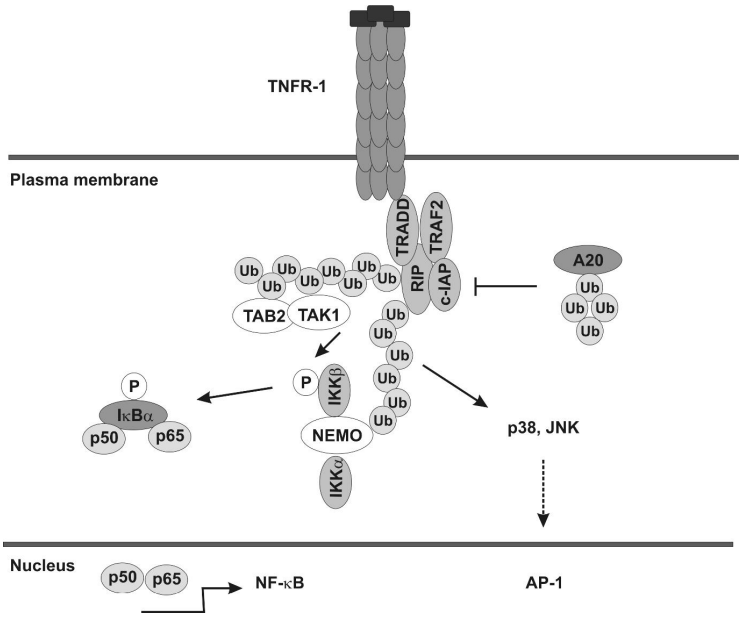

Figure 10. Schematic examples of ubiquitin signaling mediated by TNFR-1. Upon the stimulation of TNFR-1, complex 1 is formed at the plasma membrane. K63-linked ubiquitin chains are generated by E3 ligases within the complex, and used for recruiting and activating other proteins. Adapted from Broemer and Meier (2009) and Wilson et al. (2009).

The IKK complex has been shown to be regulated by ubiquitylation in numerous ways (Figure 10). In addition to phosphorylation, IKK is activated by TRAF6-mediated polyubiquitylation (Sun et al. 2004). Furthermore, the polyubiquitylation of NEMO has been shown to promote NF-κB signaling (Ea et al. 2006). NEMO ubiquitylation by linear ubiquitin

was recently found to be catalyzed by linear ubiquitin chain assembly complex (LUBAC; Tokunaga et al. 2009). However, it has also been reported that NEMO contains an ubiquitin binding in ABIN and NEMO (UBAN) motif, an interaction surface that mediates non-covalent ubiquitin binding, and that NEMO specifically recognizes linear diubiquitin (Rahighi et al. 2009). Previous reports, however, demonstrated that NEMO binds K63-linked ubiquitin chains (Wu et al. 2006).

In addition to ubiquitin signaling, phosphorylation has been shown to regulate TNF responses. However, no clear physiological function has been demonstrated. For instance, TNFR-1 is phosphorylated by MAPK on several residues, and the phosphorylation alters its subcellular localization, yet there are no documented effects on receptor function by phosphorylation during apoptosis (Cottin et al. 1999). The TNFR-1 receptor itself is phosphorylated primarily on T236 and S244, and secondarily to S240 and S244, by mitogen-activated protein kinase/extracellular-signal-regulated kinase 2 (MAPK/Erk2; Van Linden et al. 2000). Phosphorylation induces the relocalization of TNFR-1 from the Golgi to tubular ER structures to recruit Bcl-2 (Cottin et al. 1999, Cottin et al. 2001). TRADD, the adaptor protein of the TNFR-based signaling complexes, becomes phosphorylated upon the dissociation of the TNFR complex 1 from the receptor (Jiang et al. 1999). However, the exact functions of this modification are also currently unknown. Finally, recent studies suggest that programmed necrosis induced by TNFα depends on RIP3-mediated phosphorylation of RIP, and that this pathway is operates during viral inflammation and exposure to reactive oxygen species (Cho et al. 2009, He et al. 2009, Zhang et al. 2009). In summary, post-translational modifications are essential regulators of the known TNF pathways.

4.4.3 The CD95 and TRAIL receptor systems

CD95 is perhaps the best characterized of the TNF death receptor systems. The CD95 receptor (also known as Fas or APO-1) was originally discovered as the target of two mouse antibodies capable of inducing apoptosis in tumor cell lines and in xenotransplants planted in immunodeficient mice (Itoh et al. 1991, Oehm et al. 1992). Later, CD95L was cloned and identified based on its sequence homology to TNF (Suda et al. 1993). CD95 is expressed on the surface of a wide range of cell types, but it is especially pronounced in thymocytes and activated T cells, as well as in the heart, kidney and liver. However, the expression of CD95L is restricted to cells in immune privileged sites, and to cytotoxic T cells and natural killer cells which utilize CD95L to efficiently kill target cells (Suda et al. 1993).

The CD95 machinery is a central regulator of the immune system. The physiological roles of CD95 and CD95L were discovered in spontaneous mutant mouse strains, *lpr* and *gld*, which developed systemic lupus erythematosus (SLE) and lymphoadenopathy due to mutations in CD95 or CD95L, respectively (Watanabe-Fukunaga et al. 1992, Takahashi et al. 1994). In addition, mice completely deficient in CD95 or CD95L exhibit lymphocyte hyperproliferation, and substantial liver hyperplasia (Adachi et al. 1995). Moreover, lymphoadenopathy and splenomegaly are more pronounced and develop faster in *CD95*$^{-/-}$ mice compared to *lpr* mice, demonstrating the severity of the knock-out phenotype (Adachi et al. 1996). Correspondingly, complete loss of CD95L results in early death of the animal, alongside with a more severe phenotype of general lymphoproliferative disease than in the *gld* mice (Karray et al. 2004). The role of CD95 in maintaining homeostasis in the immune system was further demonstrated by the discovery of hereditary heterozygous mutations in the CD95 gene in a portion of autoimmune lympho-proliferative syndrome (ALPS) patients (Fisher et al. 1995, reviewed by Rieux-Laucat et al. 1995). Moreover, the CD95 and CD95L-deficient mice and ALPS patients have an increased risk for developing lymphoma, whereas excessive CD95 activity may lead to serious immunopathological conditions, such

as hepatitis (Ogasawara et al. 1993, Straus et al. 2001). Together, these reports highlight the importance of CD95 in preserving homeostasis in the immune system.

TNF-related apoptosis inducing ligand (TRAIL), the physiological ligand for the TRAIL receptors, was identified through its homology to other TNF family members (Wiley et al. 1995, Marsters et al. 1996). TRAIL is a type 2 transmembrane protein that can be proteolytically shed from the membrane to produce a soluble ligand. There are five TRAIL receptors, TRAILR-1-5, of which TRAILR-1 and TRAILR-2 (also called DR4 and DR5) are *bona fide* death receptors, whereas TRAIL receptors 3-5 are antagonistic decoy receptors. The expression of TRAIL receptor genes is regulated by p53 (reviewed by Kontny and Kovar 2005) and NF-κB (for example Ravi et al. 2001). The biological relevance of the TRAIL system has been studied by genetic depletion of genes encoding TRAIL receptors or ligand. In contrast to humans, mice only express one TRAIL receptor, which closely resembles human TRAILR-1 and -2 (Wu et al. 1999). The generation of TRAILR knockout mice showed that TRAIL receptor is not essential for embryonic development, but instead plays a role in innate immunity. Although the *TRAILR*$^{-/-}$ mice develop normal lymphocyte populations, they exhibit elevated levels of several cytokines and enhanced responses to the murine cytomegalovirus (Diehl et al. 2004). Late phase Toll-like receptor (TLR) signaling is also abnormal in the *TRAILR*$^{-/-}$ mice. In addition, the DNA damage response of the TRAILR-deficient mice appears to be compromised, as their spleen, thymus and brain tissues fail to undergo normal apoptosis after radiation (Finnberg et al. 2005).

Although the knockout phenotypes of CD95 and TRAIL ligands and receptors are dissimilar, it is interesting to speculate that they result form distinct expression patterns rather than distinct downstream signaling mechanisms. The differences in the way the CD95 and TRAIL systems operate, however, has had profound implications on therapeutic approaches. When the tumor toxicity of CD95 was discovered, anti-CD95 antibodies were seen as future drugs for cancer. However, early studies demonstrated that systemic administration of anti-CD95 antibodies *in vivo* was severely hepatotoxic (Ogasawara et al. 1993). In contrast to CD95, TRAIL has turned out to be non-toxic to normal tissues in animal models (Ashkenazi 2008, Ashkenazi and Herbst 2008), and it is also effective in inducing apoptosis in solid tumor cell lines (Ashkenazi et al. 1999, Walczak et al. 1999). Monoclonal antibodies against TRAIL and TRAIL receptors are in clinical trials for treating different types of cancer either alone or in combinations with traditional cancer therapies (reviewed by Melnikova and Golden 2004).

The TRAIL and CD95 receptor systems also transmit similar signals downstream from the receptor. The ligation of the CD95 or TRAIL receptor initiates the formation of the DISC. For the CD95 DISC assembly, a minimum of six oligomerized receptors is required (Holler et al. 2003). In addition, the preassociation of the receptors is essential for efficient signaling (Siegel et al. 2000a). The crystal structure of TRAIL bound to TRAILR-2 illustrated that the principle mechanisms of ligand and receptor binding are similar to the CD95 systems (Hymowitz et al. 1999). The cytoplasmic parts of individual CD95 receptors adapt a stable, closed conformation, which does not reveal the DDs or favor interaction (Scott et al. 2009). However, when ligands bind to the receptors, causing their oligomerization at the plasma membrane, the cytoplasmic parts of the receptors are reorganized into an extended conformation which is stabilized by interactions between the DDs. The extension of the cytoplasmic stem helices enables FADD to bind to the receptors. Both CD95 and TRAIL DISCs are formed around the adaptor protein FADD. FADD contains a death domain and a death effector domain, enabling homophilic interactions with the death receptor and the recruiting of additional DED-containing proteins to the DISC. These proteins include caspase-8 and caspase-10, as well as the caspase inhibitor c-FLIP. Depending on the strength of the death signal and the vicinity of DED-containing proteins, DISC formation results in substantial or little caspase-8 activity, leading either to apoptosis or alternative

signaling pathways (Figure 11). For instance, c-FLIP isoforms differentially transmit signals that preferably activate either the Erk or the NF-κB pathway (Kataoka et al. 2000).

Post-translational modifications affect death receptor signaling on several levels. Membrane trafficking can be adjusted to augment the activation of DISC. When the intracellular domain of CD95 is palmitoylated, it is translocated to the plasma membrane lipid rafts to favor receptor clustering and activation thereafter (Feig et al. 2007). *O*-glycosylation of TRAILR-1 and TRAILR-2 extracellular domains has been found to promote ligand binding-induced receptor clustering (Wagner et al. 2007). In contrast, proteolytic processing has profound implications on death receptors. For instance, the cleavage of CD95L between S126 and L127 by matrix metalloproteinases results in loss of cytotoxicity and pro-apoptotic potential (Schneider et al. 1998). The adaptor protein FADD, in turn, has been found to be phosphorylated on S194 by casein kinase 1α (CKIα; Scaffidi et al. 2000, Appalat et al. 2005). However, this modification seems to be irrelevant to apoptotic signaling, because S194 phosphorylation affects the subcellular localization of FADD and apparently regulates its non-apoptotic functions of FADD during mitosis (Appalat et al. 2005).

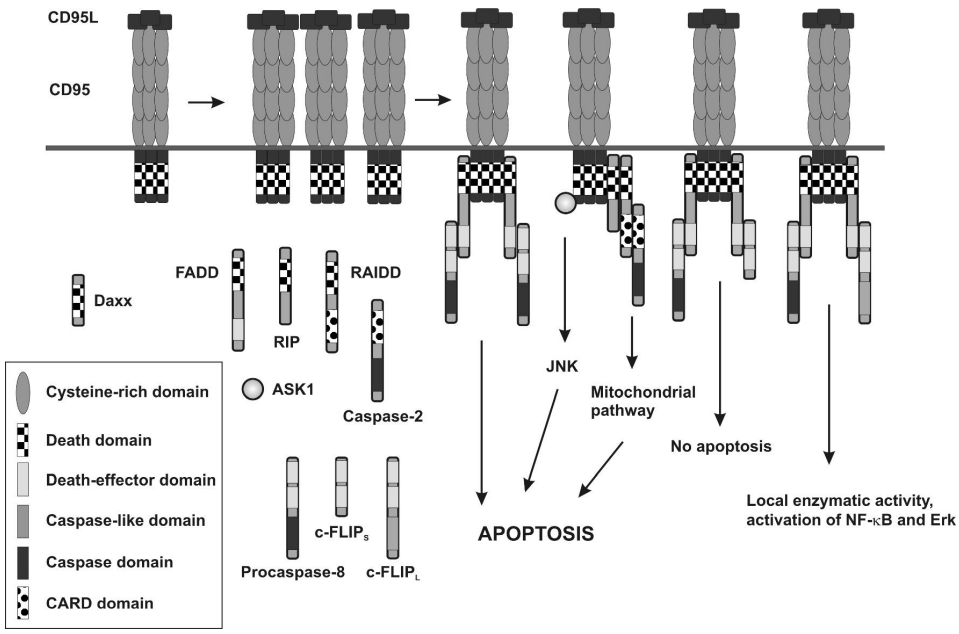

Figure 11. Examples of how the composition of the DISC dictates the outcome of death receptor stimulation. After CD95L binds to the CD95, the recruitment of FADD and procaspases-8 molecules results in caspase cascade and apoptosis. Some reports suggest that also caspase-2 can be recruited to the DISC via RAIDD to initiate apoptosis. In addition, death receptor stimulation may lead to stress or survival signaling. The short and long c-FLIP isoforms are differential regulators of DISC signaling. Adapted from Curtin and Cotter (2003).

4.4.4 Activation and biological roles of caspase-8 and caspase-10

Initiator caspases -8 and -10 are structurally similar, both containing two death effector domains and a caspase domain. Caspase-8 and -10 have evolved by genetic duplication, located at 2q33-q34, and they are both expressed in various products of alternative splicing (Rasper et al. 1998, Hadano et al. 2001). Caspase-8a/b of 55 and 53 kDa are widely expressed and well characterized. Another caspase-8 isoform, caspase-8L, has been cloned and proposed to have an anti-apoptotic function (Himeji et al. 2002). Caspase-10 is known to act similarly to caspase-8 during receptor-mediated apoptosis, but it is yet unclear whether caspase-8 and caspase-10 are functionally redundant. For example, caspase-10 appears not to be expressed in mice or lower organisms (Reed et al. 2003). Caspase-10a/d splice variants are the large isoforms of 55 and 59 kDa, respectively. Interestingly, the short caspase-10 isoform, caspase-10g, was found to inhibit receptor-mediated apoptosis and activate NF-κB, thereby operating in a dominant negative manner against the long caspase-10 isoforms (Wang et al. 2007).

The unprocessed initiator procaspases possess little, if any, catalytic activity. However, when a large number of initiator procaspases is brought together, the high concentration promotes homophilic interactions between inactive precursors, producing active caspase dimers. Initiator caspase activation is a result of limited proteolysis. During this process, the N-terminal activation domains are removed, and the proteolytic domain is cleaved to produce a fully mature caspase. This proximity-induced dimerization model for caspase activation was recently refined from the preceding induced proximity model (Boatright et al. 2003, reviewed by Riedl and Shi 2004). According to current understanding, the enhanced proximity of procaspases alone is enough to induce dimerization, which in turn suffices for enzymatic activity, contradicting the previous model according to which proteolytic cleavage precedes full initiator caspase activation.

To facilitate close proximity, dimerization and activation, initiator caspases are recruited to protein complexes whose formation depends on specific stimuli. Caspases -8 and -10 of the extrinsic apoptotic pathway are activated following death receptor activation in the DISC. An inactive initiator caspase-8 is a monomer, but upon death ligand ligation several caspase-8 monomers bind to the DISC via FADD and gather many low-activity enzymes to a close proximity to enable dimerization. The N-terminal DEDs allow for homophilic interactions among the procaspase-8 molecules, which is enough to produce an active enzyme (Boatright et al. 2003). Dimerization is followed by two-step proteolytic processing. The first cleavage of caspase-8 at D374 produces the p43/41 and p12 fragments, whereas the p18 and p10 subunits of the mature caspase-8 are formed upon the second cleavage at D216 and D384 (Chen et al. 2002). These proteolytic events have been thought to stabilize the active conformation of the enzyme (Boatright et al. 2003), but new structural studies have shown that complete processing is needed for caspase-8 to be fully active (Keller et al. 2009). After the proteolytic shedding of the N-terminal DEDs, a mature caspase-8 enzyme is released from the membrane into the cytosol. Recently, caspase-8 was appointed a new cleavage site at D210, which produces a cleavage fragment p30, an intermediate that is thought to amplify procaspase-8 activation (Hoffmann et al. 2009).

The functional differences between caspase-8 and -10 are still poorly understood. Despite high degree of homology, they do not seem to interact to generate caspase activity (Chen et al. 2002). However, caspase-8 and -10 coimmunoprecipitate with each other and seem to have some redundant functions due to partly overlapping substrate specificities (Fischer et al. 2006). However, caspase-10 has proven unable to entirely substitute for caspase-8 (Sprick et al. 2002).

Although the studies on caspase-8 have focused on death receptor signaling, it is becoming increasingly clear that caspase-8 is involved in a variety of non-apoptotic processes.

Targeted caspase-8 gene deletion has shed light on its role in development, differentiation and migration. Whereas mice deficient in death receptors are viable, deficiency in caspase-8, FADD or c-FLIP results in embryonic lethality (Varfolomeev et al. 1998, Yeh et al. 1998a, Yeh et al. 2000). Interestingly, the caspase-8 knock-out phenotype is strikingly similar to c-FLIP and FADD knock-out animals. Major developmental defects occur by day E11.5 of gestation, and the fetus displays impaired heart and neural tube development, hyperemia and liver erythrocytosis (Varfolomeev et al. 1998). These effects are probably due to abnormalities in the yolk sack of the caspase-8 null mouse (Varfolomeev et al. 1998, Sakamaki et al. 2002, Kang et al. 2004).

Caspase-8 also plays a major role in the immune system, and in humans caspase-8 deficiency leads to immune system defects. A homozygous R248W mutation in the caspase-8 gene, as well as mutations in CD95, CD95L and caspase-10 genes, have been linked to autoimmune lymphoproliferative syndrome-like symptoms, such as lymphoadenopathy, splenomegaly and autoimmunity (reviewed by Rieux-Laucat 2006). These symptoms are a consequence of defective apoptosis during negative selection of autoreactive CD4+/CD8+ T cells. Cleavage of caspase-8 has been reported to occur rapidly upon CD3/CD28-induced proliferation of T cells, although no apoptosis takes place (Alam et al. 1999, Kennedy et al. 1999), and caspase-8 deficient cells even fail to proliferate upon T cell receptor stimulation (Salmena et al. 2003). These data indicate that caspase-8 has a pivotal role in the proliferation of human T cells. Caspase-8 is also involved in modulating innate immunity, as it is found to bind to receptor signaling complexes initiated by the recognition of specific microbial components by the Toll-like receptors (TLRs; Su et al. 2005). In addition, caspase-8 deficiency has been shown to abolish NF-κB signaling in response to antigen receptor and Fc receptor activation in T, B and natural killer cells, supporting a major role for caspase-8 in immune regulation (Su et al. 2005). Some reports show a role for caspase-8 in NF-κB signaling in B cells, but conclude that caspase-8 is dispensible for B cell proliferation (Imtiyaz et al. 2006, Lemmers et al. 2007).

Substantial body of evidence suggests that the activity of caspases is modified by post-translational modifications, especially ubiquitylation. In *Drosophila*, both the initiator caspase Dronc and the effector caspase drICE are ubiquitylated by DIAP1 (Wilson et al. 2002, Ditzel et al. 2008). As drICE is sterically inhibited by DIAP1-mediated polyubiquitylation by K63-linked chains (Ditzel et al. 2008), and polyubiquitylation of Dronc by DIAP1 suppresses Dronc-mediated cell death, it seems evident that ubiquitylation of caspases generally inhibits their functions. However, the aggregation and subsequent activation of the mammalian caspase-8 was recently reported to be regulated in a positive manner by Cullin3-catalyzed polyubiquitylation upon TRAIL receptor stimulation (Jin et al. 2009). According to Jin and coworkers, polyubiquitylation on K461 enhances caspase-8 aggregation by enabling the sequestration of ubiquitylated caspase-8 on specific regions on the plasma membrane by the ubiquitin-binding protein p62/sequestosome-1. It is conceivable that ubiquitylation that occurs in different cellular compartments, under dissimilar physiological conditions, or is catalyzed by various E3 ligases on different target lysines, may have different implications on the function of the caspase. Future studies will show whether the current reports can be reconciled.

4.5 Regulation of death receptor signaling by c-FLIP

The FLICE-inhibitory proteins (FLIPs) are homologues of caspase-8 and -10 that fundamentally influence the outcome of DISC signaling. The FLIPs were originally cloned from viruses amidst a quest for new DED-containing proteins. The first discovered FLIP was bovine herpesvirus v-FLIP (Thome et al. 1997), and more viral FLIPs exist in the family of the γ herpes viruses (equine herpesvirus-2 v-FLIP E8, herpesvirus saimiri v-FLIP ORF71,

human herpesvirus-8 v-FLIP K13), as well as the human molluscipox viruses (v-$FLIP_S$ MC159 and MC160). The viral FLIPs are quite variable in their amino acid sequence, but also v-FLIPs inhibit caspase-8 activation in order to promote the transformation and survival of virally infected cells (Thome et al. 1997).

Shortly after the identification of the viral FLIP proteins, several groups independently cloned cellular FLIPs. For this reason, the cellular FLICE-inhibitory protein (c-FLIP; Irmler et al. 1997), is also called caspase homolog (CASH; Goltsev et al 1997), inhibitor of FLICE (I-FLICE; Hu et al. 1997), FADD-like anti-apoptotic molecule (FLAME-I; Srinivasula et al. 1997), caspase-like apoptosis-regulatory protein (CLARP; Inohara et al. 1997), MACH-related inducer of toxicity (MRIT; Han et al. 1997), caspase-eight-related protein (Casper; Shu et al. 1997) and usurpin (Rasper et al. 1998). The c-FLIP gene is located at 2q33-34 close to the caspase-8 and -10 genes, indicating that these homologues have arisen by gene duplication (Rasper et al. 1998, Hadano et al. 2001). Although 14 c-FLIP splice variants have been detected at the mRNA level, only two were originally identified at the protein level. c-FLIP short (c-$FLIP_S$) is a small, 26 kDa protein, whereas c-FLIP long (c-$FLIP_L$) is large, 55 kDa (Irmler et al. 1997, Shu et al. 1997). Another short isoform was later identified from Raji cells and hence named c-$FLIP_R$ (Djerbi et al. 2001, Golks et al. 2005). A recent study indicates that c-$FLIP_R$ is, in fact, of more ancient evolutionary origin than c-$FLIP_S$, as it is present in a wider array of organisms. However, c-FLIP proteins appear not to be present in non-mammalian eukaryotes. The production of the short isoforms is determined by a single nucleotide polymorphism in a 3' splicing site in the *CFLAR* gene (Ueffing et al. 2009).

All c-FLIP isoforms are identical in their 202 N-terminal amino acids (Figure 12). These residues include two death effector domains that mediate binding to the DISC and dimerization with caspase-8 (Irmler et al. 1997). In contrast, the remaining C termini are entirely different in all isoforms. The short isoforms have unique splicing tails of 11 and 19 amino acids. c-$FLIP_L$, in turn, contains a C-terminal caspase-like domain which lacks the active cysteine residue and hence the caspase-like catalytic activity. These structural differences provide basis for differential regulation and function of the c-FLIP isoforms.

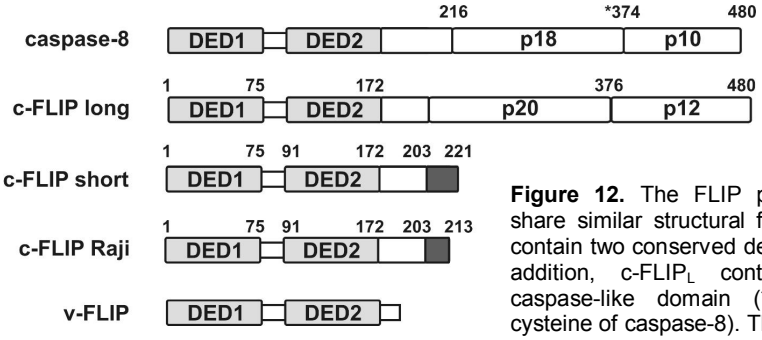

Figure 12. The FLIP proteins and caspase-8 share similar structural features. First, all FLIPs contain two conserved death effector domains. In addition, c-$FLIP_L$ contains a non-functional caspase-like domain (* denotes the active cysteine of caspase-8). The short c-FLIP proteins, c-$FLIP_S$ and c-$FLIP_R$, only have short splicing tails or 19 and 11 amino acids, respectively. C-terminal structures of the viral FLIP proteins differ in length and sequence.

Upon the cloning of c-FLIP, its biological functions were somewhat controversial. Some initial findings described c-FLIP as an inhibitor of apoptosis, whereas others stated it had a pro-apoptotic role. It was then noted that high levels of overexpressed c-$FLIP_L$, as well as

any other DED-containing protein, form toxic aggregates that activate caspase-8. The anti-apoptotic role of c-FLIP was established by the observation that the embryonic fibroblasts derived from c-FLIP deficient mice are highly sensitive to TNFα- and CD95-mediated apoptosis (Yeh et al. 2000). The generation of a knock-out animal also provided data on the physiological roles of c-FLIP. The phenotype of the knock-out animals was strikingly similar to those of the *CASP8*$^{-/-}$ and *FADD*$^{-/-}$ mice. The c-FLIP-deficient animals died at day E10.5 because of impaired heart development and cardiac failure (Varfolomeev et al. 1998a, Yeh et al. 2000). Interestingly, recent reports have addressed the role caspase-8 in development. This raises the possibility that also c-FLIP may regulate these less well characterized functions of caspase-8, and that some features of the *CFLAR*$^{-/-}$ phenotype can be derived from these tasks.

4.5.1 c-FLIP is recruited to diverse signaling complexes

c-FLIP is recruited to a number of death receptor signaling complexes, where it regulates receptor-mediated apoptosis by restraining caspase-8 (Figure 13). The c-FLIP splice variants have been extensively studied in the CD95 and TRAIL systems, where c-FLIP competes with procaspase-8 in DISC binding (reviewed by Krueger et al. 2001). It has been estimated that c-FLIP binds to the DISC with affinity that is 18 times higher than that of procaspase-8 (Chang et al. 2002), while the reported number of c-FLIP molecules in one cell is over 100 times smaller than the number of procaspase-8 molecules (Scaffidi et al. 1999). Moreover, the interaction between c-FLIP$_L$ and caspase-8 is stronger than within the caspase-8 homodimer (Chang et al. 2002), further underlining the capacity of c-FLIP to modulate death receptor responses. The short and long isoforms, however, affect caspase-8 differently. The short c-FLIP isoforms, c-FLIP$_S$ and c-FLIP$_R$, simply block the recruitment and the activation of caspase-8 (Krueger et al. 2001, Golks et al. 2005). The binding of murine c-FLIP$_R$ to the DISC depends on five critical residues of the DED2 (Ueffing et al. 2008). In contrast to the short c-FLIP proteins, the outcomes of the caspase-8:c-FLIP$_L$ interaction are more diverse. The dimerization allows the first cleavage of caspase-8 to p43/41 and p10 fragments, and c-FLIP$_L$ itself is also cleaved once. A recent piece of work by Yu and coworkers (2009) has provided structural insight into for the formation and processing of the procaspases-8:c-FLIP$_L$ heterodimer. Compared to full-length c-FLIP$_L$, p43 markedly promotes caspase-8 activation, explaining why caspase-8 is differently regulated by c-FLIP$_L$ compared to the short c-FLIP isoforms.

When procaspase-8 has processed itself and c-FLIP$_L$ once, the larger fragments remain in the DISC, while no active caspase-8 is formed or released to the cytosol. The remaining heterodimer binds additional proteins and is able to transmit signals. For example, the caspase-8:c-FLIP$_L$ heterodimer may bind TRAF2, a RING-containing E3 ligase, and adaptor protein, and the death domain-containing kinase RIP (Kataoka and Tschopp 2004). These recruitments have been shown to mediate the activation of the NF-κB pathway. In addition, c-FLIP$_S$ and c-FLIP$_L$ have been suggested to preferably promote NF-κB and MAPK/Erk pathways, respectively (Kataoka et al. 2000). This occurs presumably via the dissimilar C-terminal domains, but the detailed mechanisms are not currently known. In addition to DISC composition, the nature of death receptor signaling is governed by the strength of receptor activation. For example, the stimulation of the CD95R at sub-lethal levels activates the Erk pathway, but does not induce apoptosis (Lavrik et al. 2007).

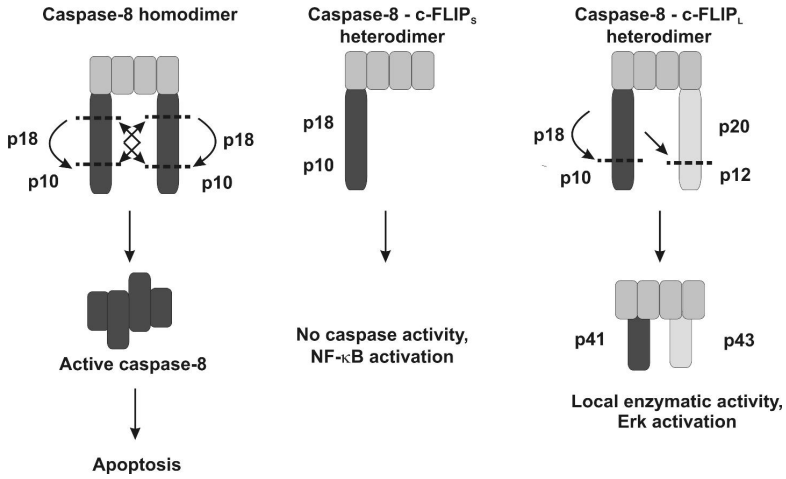

Figure 13. The c-FLIP isoforms differentially regulate caspase-8 processing in the DISC. While caspase-8 alone is autoproteolytically processed into a mature caspase-8 enzyme, no active caspase-8 is formed in a complex with c-FLIP$_S$. Sublethal death receptor stimulation may instead activate the NF-κB pathway. In contrast to c-FLIP$_S$, the caspase-8-c-FLIP$_L$ heterodimer is partially processed, but does not produce mature caspase-8. However, this heterodimer displays local enzymatic activity, which has been reported to promote Erk signaling. Adapted from Kataoka et al. (2000).

c-FLIP and caspase-8 have been found to contribute to NF-κB signaling also within the TNFR complex 2 and the T-cell receptor complex (reviewed in Budd et al. 2006). Upon the ligation of the T-cell receptor (TCR), an intracellular CD3 complex is formed in a PKCθ-dependent manner. Subsequently, a second complex is assembled, containing caspase-recruitment domain membrane-associated guanylate kinase (MAGUK) protein 1 (CARMA1), B-cell lymphoma 10 (Bcl-10), and the paracaspase mucosa-associated lymphoid tissue lymphoma-translocating gene 1 (MALT1). This Bcl complex regulates IκB degradation and NF-κB localization upstream of the IKK complex. Caspase-8 and c-FLIP are recruited to the Bcl complex via Bcl-10 and the death domain-containing MALT, and the enzymatic activity of caspase-8 is required for the Bcl-mediated NF-κB activation. In addition, caspase-8 is also involved in NF-κB signaling via the Fc receptor and Toll-like receptor 4, but not via TNFR complex 1 or CD40 (Su et al. 2005).

Strikingly, c-FLIP has been found to promote NF-κB activation in the absence of receptor stimulation in certain tumor cell types. Golks and colleagues (2006) found that in malignant non-apoptotic primary T and B cells and mature dendritic cells, the basal caspase-8 activity is sufficiently high to cleave a portion of c-FLIP at D196 to form an N-terminal fragment called c-FLIP-p22. c-FLIP-p22 was reported to directly interact with NEMO, the regulator of the IKK complex, to induce NF-κB. A recent report described the structure of KSHV-FLIP bound to NEMO (Bagneris et al. 2008), demonstrating how c-FLIP-p22 is able to induce NF-κB through conserved interaction surfaces. Nevertheless, the characterization of p22 still warrants further studies, and its biological significance remains to be determined.

In contrast to several reports described above, some studies conclude that c-FLIP$_L$ prevents NF-κB activation. In these studies, however, NF-κB activity was originated by stimulation of CD95 and TRAIL receptors (Imamura et al. 2004, Wachter et al. 2004). Although caspase inhibition has been shown to prevent caspase-8 and/or c-FLIP-mediated NF-κB activation (Chaudhary et al. 2000, Hu et al. 2000, Kataoka et al. 2000, Shikama et al.

2003, Kreuz et al. 2004), others have also reported that caspase-8 and c-FLIP are, in fact, dispensible for NF-κB activation. This was indicated by detection of normal NF-κB activity in c-FLIP deficient T lymphocytes (Zhang and He 2005), as well as in *CASP8*$^{-/-}$ and *CFLAR*$^{-/-}$ mouse embryonic fibroblasts (MEFs; Varfolomeev et al. 1998). These findings indicate that the roles of caspase-8 and c-FLIP in regulating NF-κB vary a lot depending on cell type and physiological context.

4.5.2 Transcriptional regulation of c-FLIP

c-FLIP is widely expressed in mammalian tissues, especially the heart, lymphoid tissue, skeletal muscle and kidney (Irmler et al. 1997, Rasper et al. 1998). The expression pattern includes neurons, endothelial cells, keratinocytes, dendritic cells, macrophages, hematopoietic cells, spermatocytes and pancreatic β cells. In most cell types, c-FLIP$_L$ is present in higher abundace than the short c-FLIP isoforms (Irmler et al. 1997, Rasper et al. 1998).

The expression of c-FLIP is administered by several pro-survival signaling pathways. The mitogen-activated Erk pathway positively regulates c-FLIP expression in stimulated T cells (Yeh et al. 1998), and inhibition of Erk downregulates c-FLIP in certain cancer cell lines (Panka et al. 2001). The growth-factor-induced PI3K/Akt pathway has been shown to promote c-FLIP expression in several tumor cell lines, including breast, prostate and melanoma cancer, emphasizing PI3K/Akt as a key regulator of c-FLIP transcription (Panka et al. 2001). In addition to tumor cells, the PI3K pathway regulates death receptor sensitivity in B cells by inducing the rapid up-regulation of c-FLIP expression in response to antigen stimulation (Moriyama and Yonehara 2007). Moreover, endothelial cells become sensitized to CD95-mediated apoptosis upon PI3K/Akt inhibition due to c-FLIP repression (Suhara et al. 2001).

Interestingly, isoform-specific regulation of c-FLIP proteins has been reported to occur in glioblastoma cells via the Akt/ mammalian target of rapamycin (mTOR) pathway (Panner et al. 2005). In human glioblastoma multiforme cells, the level of c-FLIP$_S$, but not c-FLIP$_L$, determines the sensitivity towards TRAIL. The overexpression of c-FLIP in these cells is mediated by mTOR, and rapamycin specifically downregulates the translation of c-FLIP$_S$. Although c-FLIP$_S$ and c-FLIP$_L$ originate from the same transcript, they differ in their 3' untranslated regions (Irmler et al. 1997, Ueffing et al. 2009), which may be occupied by a range of splicing factors recruited by mTOR in c-FLIP$_S$, but not in c-FLIP$_L$ (Panner et al. 2005). To date, little is known about the isoform-specific regulation of the stability of the c-FLIP messenger RNA.

c-FLIP not only promotes NF-κB signaling, but the *CFLAR* gene is also a transcriptional target of NF-κB. Kreuz and coworkers (2001) reported that several stimuli, including TNFα, IL-1, and agonistic CD40 antibodies, induce the expression of c-FLIP. The induction of the *CFLAR* gene by TNF required NF-κB activity and occurred in a variety of cell lines. Moreover, c-FLIP expression is rapidly induced via NF-κB in fibrosarcoma cells and T lymphocytes, in which c-FLIP determines the sensitivity against CD95L and TNFα (Micheau et al. 2001). Interestingly, the kinetics of induction vary in a cell type and isoform-specific manner (Micheau et al. 2001). Therefore, c-FLIP isoforms provide possibilities to fine-tune cellular responses.

The nuclear factor of activated T cells (NFAT) family of transcription factors has been shown to promote c-FLIP expression. NFATc2 is recruited directly to the *CFLAR* promoter in response to VEGF, providing a mechanism for regulating endothelial apoptosis by angiogenic factors (Volpert et al. 2002, Zaichuk et al. 2004). p53, in turn, is a tumor suppressor that mediates apoptosis via the intrinsic pathway upon DNA damage (reviewed

by Levine 1997). p53 regulates the expression of several key proteins that affect apoptosis, including c-FLIP (Bartke et al. 2001). Counter-intuitively, however, p53 promotes c-FLIP expression, shifting the focus of p53-mediated apoptosis from the extrinsic towards the intrinsic pathway (Bartke et al. 2001). Moreover, expression of c-$FLIP_L$ has been shown to inversely correlate with the accumulation of p53 in several ovarian carcinoma cell lines (Mezzanzanica et al. 2004).

In addition to positive transcriptional regulators, some transcription factors act as repressors of *CFLAR*. One of them, c-Myc, sensitizes a variety of tumor cells to TRAIL-mediated apoptosis (Ricci et al. 2004). c-Myc binds directly to the promoter of *CFLAR* and represses *CFLAR* messenger RNA levels (Ricci et al. 2004). In addition, transcription factor FOXO3 modulates endothelial cell viability by downregulating c-FLIP expression (Skurk et al. 2004). In addition to these transcription factors, c-FLIP expression is decreased in response to histone deacetylase (HDAC) inhibitors. HDAC inhibitor-mediated c-FLIP repression was sufficient to sensitize CD95-resistant osteosarcoma cell lines to apoptosis (Watanabe et al. 2005). HDAC inhibitors have also been shown to increase the apoptotic sensitivity of hepatocellular carcinoma cells, which normally are resistant towards chemotherapeutic drugs and other apoptotic stimuli (Pathil et al. 2006, Schuchmann et al. 2006). Notably, the mechanism for HDAC-mediated apoptosis in these cells was tumor-specific, raising the possibility of successful treatment of hepatocellular carcinoma by HDAC inhibitors (Pathil et al. 2006). In summary, the transcription of c-FLIP is affected by the central, well-established signaling pathways that regulate cell survival. However, the mechanisms that regulate the alternative splicing of *CFLAR* are still incompletely understood and require further efforts.

4.5.3 Post-translational regulation of c-FLIP

The c-FLIP isoforms are short-lived proteins whose activity can be regulated isoform-specifically by post-translational modifications. The modifications most commonly connected to c-FLIP are ubiquitylation and phosphorylation. Although c-FLIP is targeted by several modes of post-translational regulation, the interplay between different post-translational modifications is poorly characterized.

The proteasomal degradation of c-FLIP is determined by ubiquitylation. Initially, c-FLIP proteins were found to accumulate in response to proteasome inhibitors such as MG132, epoxomicin, lactacystin, and bortezomib/Velcade (Fukazawa et al. 2001, Kim et al. 2002a, Perez and White 2003, Chanvorachote et al. 2005, Zhang et al. 2005, Chang et al. 2006). Ubiquitylation of c-FLIP has been reported to occur during adenoviral infection (Perez and White 2003), p53 activation (Fukazawa et al. 2001), PPARγ modulators (Kim et al. 2002) and inhibition of cyclo-oxygenase-2 (Liu et al. 2006). In contrast, *S*-nitrosylation on C254 and C259 was demonstrated to protect c-$FLIP_L$ from ubiquitylation (Chanvorachote et al. 2005). These data suggests that diverse stimuli can affect c-FLIP ubiquitylation. Although some interaction partners of c-FLIP have conserved HECT or RING domains providing them with E3 ubiquitin ligase activity, the enzymes that regulate c-FLIP ubiquitylation are still largely unknown. The available data, however, supports the notion of isoform-specific regulation also in this respect. Chang and coworkers (2006) demonstrated that the HECT E3 ligase Itch specifically targets c-$FLIP_L$ for degradation in response to TNFα. The stability of the most recently identified isoform, c-$FLIP_R$, was reported to be similar to c-$FLIP_S$ (Golks et al. 2005). In addition, c-Cbl was recently shown to regulate the stability of the murine short c-FLIP isoform (Kundu et al. 2009).

In addition to ubiquitylation, c-FLIP proteins are regulated by phosphorylation. Earlier reports on c-FLIP phosphorylation indicate the involvement of several signaling pathways. Calcium-calmodulin-dependent protein kinase II (CaMKII) has been demonstrated to phosphorylate and stabilize c-$FLIP_L$ in the DISC, thereby providing protection against CD95

and TRAIL-mediated apoptosis (Yang et al. 2003, Xiao et al. 2005). In contrast, others have detected the loss of c-FLIP from the DISC in response to bile acid-induced, PKC-mediated phosphorylation, resulting in sensitization to TRAIL-mediated apoptosis (Higuchi et al. 2003). Recently, c-FLIP$_L$ S273 was identified as a PI3K/Akt-phosphorylation site (Shi et al. 2009). The authors reported that S273 phosphorylation is induced by TNFα-mediated macrophage activation, and that phosphorylation renders c-FLIP$_L$ susceptible for proteosomal degradation. This effect was not, however, carried out by the E3 ligase Itch. Murine short c-FLIP isoform was recently described to be phosphorylated on S4 and Y211 by p38 and c-Abl, respectively, enabling the recognition by the E3 ligase c-Cbl and subsequent ubiquitin-mediated proteasomal degradation (Kundu et al. 2009). Together, these reports show that c-FLIP proteins are likely to be phosphorylated on several residues by an array of kinases, and the consequences depend on cell type and physiological context.

4.5.4 Dynamic regulation of c-FLIP levels affects a multitude of physiological processes

The physiological roles of c-FLIP encompass a variety of biological processes from development to correct function of the immune system. In addition, c-FLIP is indicated in autoimmune diseases and cancer. In various cell types, c-FLIP expression directly correlates with death receptor sensitivity (Irisarri et al. 2000, reviewed by Krueger et al. 2001). Importantly, the *CFLAR* knock-out MEFs are extremely sensitive to CD95 and TNFα-induced apoptosis (Yeh et al. 2000). In addition, genetic knock-down of c-FLIP has been shown to increase the death receptor sensitivity of several cancer cell types, such as breast cancer and T cell lymphoma (Fulda et al. 2000, Siegmund et al. 2002, Ricci et al. 2004, Krueger et al. 2006, Palacios et al. 2006). The levels of c-FLIP are adjusted during physiological processes and stresses, such as erythroid differentiation or hyperthermia (Hietakangas et al. 2003, Tran et al. 2003).

Several studies have convincingly demonstrated the anti-apoptotic role of c-FLIP (Inohara et al. 1997, Irmler et al 1997, Shu et al. 1997, Fulda et al. 2000, Chang et al. 2002, Kim et al. 2002b, Davidson et al. 2003). While c-FLIP$_S$ is an unequivocal inhibitor of apoptosis (for example Bin et al. 2002), overexpression of c-FLIP$_L$ may lead to a different outcome. For example, Panner and colleagues were unable to detect any changes in death receptor sensitivity after overexpressing c-FLIP$_L$ (Panner et al. 2005). High overexpression of c-FLIP$_L$ has even led to increased apoptosis, either due to the formation of death effector filaments, or the spontaneous dimerization of c-FLIP$_L$ with caspase-8 (Inohara et al. 1997, Irmler et al. 1997, Shu et al. 1997, Fulda et al. 2000, Chang et al. 2002). However, several tumor cell types are resistant to death receptor-mediated apoptosis due to elevated c-FLIP expression, strongly supporting the anti-apoptotic role of c-FLIP$_L$. Therefore, it can be concluded that although extremely high levels of c-FLIP$_L$ are capable of promoting caspase-8 activation and sometimes even apoptosis, at physiological levels c-FLIP$_L$ is also an inhibitor of apoptosis.

The ability to resist apoptosis is a classical hallmark of cancer (Hanahan and Weinberg 2000). High expression levels of c-FLIP can provide selection benefits for cancer cells. In fact, it was discovered early on that c-FLIP is overexpressed in human metastatic melanoma tumors (Irmler et al. 1997). It was later demonstrated that overexpression of c-FLIP provides selection advantage to tumor cells compared to cells with lower c-FLIP levels (Medema et al. 1999). Experiments in murine tumor models showed that the overexpression of c-FLIP provided significant resistance towards cytotoxic T lymphocytes and CD95-mediated apoptosis. In addition to c-FLIP, v-FLIP proteins have been shown to promote tumorigenesis and mediate abnormal death receptor resistance (Djerbi et al. 1999). A tumor-promoting role has been described for the KSHV-FLIP, which is expressed

by HHV-8 and associated to certain lymphomas. In accordance with cellular FLIP, the v-FLIP protected cells from CD95-mediated apoptosis and permitted the clonal growth in the presence of death receptor stimulation by the cytotoxic T cells (Djerbi et al. 1999). These reports indicate that while death receptor triggering by cytotoxic T cells is indeed a crucial method for evading tumor cells, the expression of c-FLIP provides significant protection to tumor cells *in vivo*.

The dynamic regulation of c-FLIP has been described to play multiple roles in immune cells. The physiological roles of c-FLIP have been extensively studied in T cells. c-$FLIP_S$ was originally found to rescue primary human T cells from activation-induced cell death that occurs in activated and re-stimulated T cells (AICD; Kirchhoff et al. 2000). Later, c-$FLIP_R$ was reported to possess similar properties in primary human T cells (Golks et al. 2005). It seems that the isoform-specific roles of c-FLIP are relevant in physiological settings. Since c-FLIP is a major determinant for T lymphocyte persistence, imbalances in its regulation lead to harmful consequences. Abnormal c-FLIP levels and functions have also been indicated in several autoimmune diseases. The initial onset of autoimmune diseases is incompletely understood, but it is thought to include the failure of activated T lymphocytes to die via AICD, leading to overt production of autoantibodies by activated B cells. Decreased sensitivity to apoptosis due to increased c-FLIP levels has been detected in patients suffering from multiple sclerosis (MS). In healthy cells, c-FLIP levels decline towards the end of T cell activation, which makes the cells sensitive to AICD by CD95 receptor stimulation (Semra et al. 2001). In contrast, the T lymphocytes of an MS patient are unable to downregulate c-FLIP, thereby promoting the survival of the activated T cells and contributing to the clinical symptoms of MS.

In addition to regulating AICD, c-FLIP contributes to the differentiation of the naïve CD4+ T lymphocytes to Th1 and Th2 cells. In transgenic mice overexpressing c-$FLIP_L$, the Th2 differentiation is favored, and as a result, the mice are hypersensitive to allergens and produce abnormal quantities of immunoglobulins. In addition, the mice are sensitive to allergic airway inflammations and their T cells are abnormally resistant towards CD95-mediated apoptosis (Tseveleki et al. 2004, Wu et al. 2004). This phenotype is probably provoked by c-$FLIP_L$-mediated activity of caspase-8, leading to increased apoptosis of the sensitive Th1 cells (Wu et al. 2004). In accordance with these results, it has been shown that c-$FLIP_L$ transgenic mice are protected from experimental autoimmune encephalomyelitis (EAE), an autoimmune disease driven by overaccentuated Th1 responses (Tseveleki et al. 2004). The results are different, if c-$FLIP_L$ is introduced after the EAE onset: in this case, the overexpression of c-$FLIP_L$ prolongs the inflammatory response in the central nervous system by suppressing the death receptor sensitivity of activated T cells (Djerbi et al 2003).

Homeostasis in lymphoid cells is governed by fluctuations in c-FLIP levels. While death receptor sensitivity changes during T cell activation, cells respond differently to death receptor stimulation. Various reports have described roles for caspase-8, caspase-10 and c-FLIP in NF-κB activation in response to various stimuli (Kataoka et al. 2000, Golks et al. 2005, Su et al. 2005, Wang et al. 2007). As shown by Kataoka and colleagues, activated T cells expressing high levels of c-FLIP are protected from death signals and respond to CD95 receptor stimulation by growing and proliferating in an NF-κB and Erk/AP-1-dependent manner (Kataoka et al. 2000). In T cells, the activation of these pathways contributes to IL-2 production. Consistently, the primary T cells of the conditional c-FLIP knock-out mice display decreased NF-κB and Erk activity upon stimulation (Zhang and He 2005).

c-FLIP serves an important role in TCR-mediated NF-κB activation. Whereas the initial reports concluded that NF-κB could be induced by the DEDs of caspase-8, caspase-10 or c-FLIP (Shikama et al. 1998, Chaudhary et al. 2000, Hu et al. 2000, Kataoka et al. 2000),

later developments have shed some light on how the roles of different players change according to activating stimuli and experimental set-up. Su and coworkers (2005) demonstrated that the enzymatic activity of caspase-8 is required for TCR-induced NF-κB activity. The experiments were conducted on patient material from humans and mice with a disorder called caspase-8 deficiency, in which mutations in the caspase-8 gene cause defective apoptosis, and imperfect T, B, and natural killer cell activation leads to immunodeficiency (Chun et al. 2002). Caspase-8 was found essential for NF-κB signaling through the antigen receptor, the Fc receptor and Toll-like receptors 3 and 4 (Su et al. 2005). Although the potential role of c-FLIP was not addressed, the requirement for caspase-8 activity gives rise to two explanations: either the need for caspase-like activity implies that c-FLIP is a negative regulator of NF-κB, or then caspase-8 contributes to NF-κB by processing c-FLIP. The latter hypothesis is supported by a study conducted in c-$FLIP_L$ transgenic mice, where CD8+ T cell activation was regulated by c-$FLIP_L$ through caspase-8-dependent NF-κB activation (Dohrman et al. 2005).

Death receptor sensitivity of the B cells is also affected by c-FLIP levels. In unstimulated B cells the expression of c-FLIP is low, but upon CD40 or BCR stimulation, both c-FLIP isoforms are upregulated (Hennino et al. 2000). The upregulation of c-$FLIP_L$ seems to be stronger and more prolonged than that of c-$FLIP_S$, and the upregulation not only protects B cells from CD95-mediated apoptosis, but also contributes to the selection and maturation of B cells (Wang et al. 2000). In addition, the homeostasis of macrophages and dendritic cells are regulated by c-FLIP. During monocyte-macrophage differentiation, the levels of c-FLIP are increased, resulting in enhanced resistance towards CD95-mediated apoptosis (Perlman et al. 1999). A parallel phenomenon occurs upon the maturation of the dendritic cells, which further highlights the importance of c-FLIP as as essential regulator of homeostasis in the immune system.

4.6 PKC as a regulator of cell death

Many PKCs are known to be involved in regulating the sensitivity to apoptotic signals (reviewed by Gutcher et al. 2003). The diverse nature of PKC signaling explains why an individual kinase may act as a pro-apoptotic agent in one cell type, while inhibiting cell death in another context. Among the PKC family members, the anti-apoptotic PKCε and the pro-apoptotic PKCδ have been the focus of much research, of which a summary is provided below.

PKCε has been demonstrated to counteract apoptosis in several types of cells. Active PKCε enhances Akt activity and the subsequent, indirect upregulation of survival-related proteins. In addition, overexpression of PKCε has been shown to account for resistance to chemotherapeutic drugs (reviewed by Dempsey et al. 2000). PKCε is overexpressed in gliomas, and the ectopic expression of PKCε protects glioma cell lines from TRAIL-induced apoptosis. Conversely, silencing of the *PKCE* gene leads to loss of Akt expression and is sufficient to induce apoptosis (Okhrimenko et al. 2005). The relationship between PKCε and Akt is also evident in breast cancer cells, because PKCε cannot protect these cells from apoptosis in the absence of Akt (Lu et al. 2006). The role of PKCε in chemoresistance has been reported in several cell types. In non-small-cell lung carcinoma cells, the abnormally high expression of PKCε provides protection against etoposide and doxorubicin (Ding et al. 2002). In addition, increased PKCε expression is also indicated in cisplatin-resistant ovarian carcinoma cells (Basu and Weixel 1995). However, in contrast, PKCε has also been found to regulate Akt in a negative manner in mouse keratinocytes, thereby sensitizing them to UVC-induced apoptosis (Li et al. 2006).

PKCδ, in turn, is considered a pro-apoptotic kinase and a negative regulator of cell growth. Upon apoptotic stimulus, PKCδ is translocated to the mitochondria, where it promotes cytochrome *c* release. PKCδ is also capable of inducing the autocrine secretion of death ligands, resulting in the activation of the extrinsic apoptotic pathway (Gonzalez-Guerrico and Kazanietz 2005). Moreover, PKCδ is a substrate for caspase-3 and activated by this proteolytic processing (Emoto et al. 1995). The cleavage of PKCδ by caspase-3 is induced by UV radiation, etoposide and cisplatin, and if PKCδ is inhibited upon these treatments, apoptosis is notably dampened (Denning et al. 1998, Reyland et al. 1999, Persaud et al. 2005). The pivotal role of PKCδ in apoptosis was initially established by the observation that the *PKCD* null mice are defective in mitochondria-dependent apoptosis and caspase-3 activation (Humphries et al. 2006). PKCδ is most likely to act on many levels in apoptosis regulation; PKCδ is translocated to the mitochondria in response to diverse death stimuli, and once cytochrome *c* is released and caspase-3 activated, proteolytic processing of PKCδ serves as a positive feedback signal (Li et al. 1999, Majumder et al. 2000, Denning et al. 2002). In addition, PKCδ decreases the activity of Akt, thereby promoting UVC-induced apoptosis in mouse keratinocytes. Finally, the autocrine apoptotic loop, in which PKCδ is able to promote the secretion of TNFα and TRAIL, has been described in prostate cancer cells (Gonzalez-Guerrico and Kazanietz 2005). As far as other novel PKCs are concerned, the role of PKCθ has been mainly studied in the context of T cell apoptosis and AICD. The *PKCθ*$^{-/-}$ T cells are resistant to CD95L-induced apoptosis and AICD, indicating that PKCθ has a pro-apoptotic role in these processes (Manicassamy and Sun 2007).

Compared to PKCε and PKCδ, much less is known about the role of other PKCs in apoptotic regulation. PKCα predominantly promotes pro-survival pathways, such as Akt and Erk signaling, by phosphorylating Raf-1 (Ueda et al. 1996, Majewski et al. 1999, Li et al. 2006). Moreover, PKCα has been demonstrated to phosphorylate and stabilize the anti-apoptotic Bcl-2 protein (Ruvolo et al. 1998). In endometrial cancer cells treated with the DNA-damaging agent etoposide, inhibition of PKCα results in increased apoptosis, whereas whereas inhibition of PKCδ suppresses apoptosis (Haughian et al. 2006). However, contrasting reports suggest that PKCα may also play a pro-apoptotic role in some cell systems, such as prostate cancer (Powell et al. 1996). In addition to PKCα, another classical isoform, PKCβ, has been appointed anti-apoptotic functions. Similarly to PKCα, the PKCβII splice variant has been suggested to stabilize Bcl-2 (Whitman et al. 1997), and recent studies also indicated an essential role for PKCβ in NF-κB survival signaling in B lymphocytes (Su et al. 2002). Nevertheless, PKCβ was also reported to be necessary for JNK signaling and phorbol ester-induced apoptosis in monocytes (Ito et al. 2001). These examples depict the diversity of PKC signaling and describe how the PKC isoforms serve differing roles according to different stimuli.

OUTLINE OF THE STUDY

The aim of this PhD thesis was to study how death receptor signaling is affected by post-translational modifications of the anti-apoptotic c-FLIP proteins. In particular, I focused on phosphorylation and ubiquitylation, both of which have diverse and wide-ranging functions. Prior to this work, some reports indicated that c-FLIP proteins are subjected to phosphorylation and ubiquitylation. However, the underlying regulatory mechanisms and broader biological implications were largely unknown.

When this thesis work was initiated, our laboratory had discovered that while K562 erythroleukemia cells are generally resistant to TRAIL, they become sensitized upon hemin-induced differentiation due to downregulation of c-FLIP. Alongside with this observation, our group had shown that heat stress reduces c-FLIP levels, thereby sensitizing cells to CD95-mediated apoptosis. The goal of my study was to investigate how c-FLIP is regulated during these processes, as well as to determine whether the c-FLIP isoforms differ in their response to differentiating stimuli or heat stress. These experiments were conducted primarily in two cell models, the human erythroleukemia K562 cells and the human leukemic Jurkat T cells.

Furthermore, I aimed at finding and characterizing c-FLIP phosphorylation sites, as this had not been done before. We succeeded in finding two *in vivo* phosphorylation sites, one of which was present in all c-FLIP proteins and the other being c-FLIP$_L$-specific. A biological function and a regulatory pathway were assigned for the common phosphorylation site, and a potential role in survival signaling is suggested for c-FLIP$_L$-specific phosphorylation. During the course of this work, I discovered that c-FLIP phosphorylation and ubiquitylation are intertwined regulatory mechanisms that affect the two isoforms in distinct ways. While both c-FLIP proteins are protected from ubiquitylation by S193 phosphorylation, these modifications only affect the stability of the short c-FLIP isoforms. Therefore, although the phosphorylation of c-FLIP proteins is regulated by the PKC and it has similar effects on ubiquitylation, the biological outcomes are different, further highlighting the importance of isoform-specific functions of c-FLIP.

MATERIALS AND METHODS

1 Cell culture and treatments (I-III)

Human K562 erythroleukemia cells, Jurkat T lymphocytes and WM35 melanoma cells were cultured in a humidified 5% CO_2 atmosphere at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), antibiotics (penicillin and streptomycin), and 2 mM L-glutamine. For WM35 cells, the medium was supplemented with 5 µg/ml insulin. K562 cells stably overexpressing FLAG-tagged c-FLIP$_S$, c-FLIP$_R$ and c-FLIP$_L$ wild type isoforms (1E5 and 2E11, 3A6 and 2E10, respectively) or c-FLIP mutants (c-FLIP$_S$ S193A 2G7, S193D 2G10; c-FLIP$_L$ S193A 1G7, S193D 1F8; c-FLIP$_S$ Δ203-221/1-202 5F4) were maintained in RPMI 1640 medium containing G418 (500 µg/ml, Calbiochem). Jurkat T cell pools stably overexpressing FLAG-tagged c-FLIP$_S$ (c-FLIP$_S$ wild type and 1-202 deletion mutant) were maintained in RPMI 1640 medium containing G418 (1.5 mg/ml, Calbiochem).

Primary human peripheral T lymphocytes were collected from several healthy volunteers by venipuncture. The blood samples were diluted 1/2 in PBS, overlaid onto Ficoll-Paque PLUS (Amersham Biosciences), and centrifuged at 1500 rpm for 30 min. Mononuclear cells from the Ficoll-Paque separation were depleted by plastic adherence for 2 h. Nonadhering cells were further separated in a nylon wool column to exclude B lymphocytes. Resting T lymphocytes (day 0) were stimulated with 1 µg/ml phytohemagglutinin (PHA; Sigma-Aldrich) for 22 h. After 1 day of activation, cells were washed and T lymphocyte proliferation was supported by addition of 20 U/ml IL-2 (Sigma-Aldrich). The same dose of IL-2 was added at day 3 of activation. Human Jurkat T lymphoma cells (American Type Culture Collection) and primary human peripheral T lymphocytes were cultured in a humidified 5% CO_2 atmosphere at 37°C in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin, and 2 mM L-glutamine.

Apoptosis was induced with agonistic anti-CD95 Ab CH-11 (Biosite), 200 ng/ml for 2 h in Jurkat cells, if not otherwise indicated, and 1 µg/ml for 2 or 12 h in primary human T lymphocytes. To induce hyperthermia, heat shock treatments (HS) were performed in water bath at 40 or 42°C for 30 min if not otherwise indicated. After exposure to hyperthermia, cells were either harvested or returned to 37°C for recovery and/or additional treatments. The CD95L-blocking antibody NOK-1 (BD Pharmingen) was used at 1 µg/ml for 15 min before apoptosis induction.

The proteasome inhibitor epoxomicin (Calbiochem) was used at 200 nM for 14 h and the protein synthesis inhibitor cycloheximide (Sigma) was used at 5-50 µM for the indicated time periods. For K562 cells, hemin (Sigma) was added to a final concentration of 30 or 40 µM. For WM35 cells, cisplatin (Sigma) was added to a final concentration of 7 µg/ml. Apoptosis was induced by adding 100 ng/ml FLAG-tagged TRAIL (Alexis) together with 2 µg/ml cross-linking M2 anti-FLAG antibody (Sigma).

DISC formation was induced by adding 1 µg of human soluble recombinant FLAG-tagged TRAIL (Alexis, San Diego, California, USA) together with 2 µg/ml crosslinking M2 anti-FLAG antibody (Sigma) (DISC immunoprecipitation), or either human recombinant soluble Super*Killer* TRAIL (Alexis) or isoleucine zipper human recombinant TRAIL (kind gift from Dr. Henning Walczak, Imperial College, London, UK), neither of which requires a cross-linking enhancer, in the final concentration of 250 ng/ml for 1 h (detection of c-FLIP phosphorylation upon death receptor activation) or 90 ng/ml for indicated time points (detection of apoptosis markers after cycloheximide pretreatment).

For detecting S193 phosphorylation, K562 and HeLa cells were treated with 20 nM phorbol 12-myristate 13-acetate (TPA; Sigma) to activate the PKCs for indicated times. To inhibit type-1 and type-2A phosphatases, K562 and HeLa cells were treated with 20 nM calyculin A (Sigma) for 30 min before harvesting. To specifically inhibit PKCα and PKCβ, the cells were treated with 40 μM GÖ6976 (Sigma) for 4 h. To activate the TNF receptor, murine recombinant TNFα (R&D Systems) was added to K562 cells (10 ng/ml) for 1 h before calyculin A treatment.

2 Plasmid constructs (I-III)

The FLAG-tagged c-$FLIP_L$ and c-$FLIP_S$ were a kind gift from Dr. Jürg Tschopp (Institute of Biochemistry, University of Lausanne, Lausanne, Switzerland). c-$FLIP_S$, c-$FLIP_R$ and and c-$FLIP_L$ point mutations were made using the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. The FLAG-tagged c-$FLIP_S$ deletion mutant was constructed by PCR and cloned into the EcoRI and XhoI sites in-frame with the N-terminal FLAG tag in pCR3-Met-FLAG. FLAG-tagged c-$FLIP_R$ was constructed by PCR using FLAG-tagged c-$FLIP_L$ as a template with following primers:
Forward primer: 5'-ACAGTTGAATTCATGTCTGCTGAAGTC-3'
Reverse primer: 5'-
TCTAGACTCGAGTCATGCTGGGATTCCATATGTTTTCTCCAGACTCACCCTGAAGTTAT
TTGAAGG-3'
The PCR product was subcloned into the EcoRI and XhoI sites in-frame with the N-terminal FLAG tag in pCR3-Met-FLAG. The HA-tagged ubiquitin was a kind gift from Dr. Dirk Bohmann (University of Rochester, Rochester, NY, USA). The GFP-tagged kinase-dead PKCα and PKCβ constructs were kindly provided by Dr. Christer Larsson (University of Lund, Lund, Sweden).

3 Transient transfections and generation of stable cell lines (I-III)

For transient transfections, $5x10^6$ K562 cells were centrifuged and resuspended in 0.4 ml of Opti-MEM, and 20 or 30 μg of plasmid DNA was added. The expression levels of the mutants were titrated to be comparable because expression levels were noticed to affect ubiquitylation. Cells were subjected to a single electric pulse (220 V, 975 μF), in 0.4-cm gap electroporation cuvettes (BTX) using a Bio-Rad Gene Pulser electroporator followed by dilution to 5×10^5 cells/ml in RPMI 1640 medium with 10% FCS and antibiotics. Cells were incubated at 37°C for 24-36 h prior to the experimental treatments.

After transfections, the stable neomycin-resistant K562 cell lines overexpressing c-FLIP proteins were selected by G418 (500 μg/ml, Calbiochem) for 2 weeks, the resistant pool was serially diluted on a 96-well plate in the presence of G418, and the single cell clones were upscaled and screened for c-FLIP expression by Western blotting. The Jurkat T cell pools stably overexpressing c-FLIP proteins were transfected with the respective constructs and selected by G418 (1.5 mg/ml) for two weeks.

4 Ubiquitylation assay (I-III)

For immunoprecipitating ubiquitylated c-FLIP in K562 cells, the pellet from transiently transfected K562 cells was resuspended in 75 μl of boiling 1% SDS in PBS, and the resulting lysate was heated at 100°C for 5 min. The lysates were suspended 1:10 in 1% Triton X-100 in PBS. DNA was sheared by sonication, and the particulate material was centrifuged for 15 min at 15,000 x *g*. Samples were taken from the cleared lysates for input control. The lysates were further diluted 1:1 with 1% Triton X-100, 0.5% bovine serum albumin (BSA) in PBS and incubated with anti-HA (Santa Cruz Biotechnology) antibody and

15 µl of a 50% slurry of protein G-Sepharose under rotation for 2 h. After incubation, the Sepharose beads were washed four times with 1% Triton X-100 in PBS, and the immunoprecipitated proteins were separated on an 8 or 10% SDS-polyacrylamide gel and immunoblotted with anti-FLIP antibody.

For immunoprecipitating c-FLIP in Jurkat cells, the pellet from 3 x 10^7 c-FLIP stably overexpressing Jurkat cells was resuspended in 75 µl of boiling 1% SDS in PBS, and the resulting lysate was heated at 100°C for 5 min. The lysates were then suspended in 1 ml 0.5% Triton X-100 in PBS. DNA was sheared by sonication and the particulate material was centrifuged at 15,000 x *g* for 10 min. Samples were taken from the cleared lysates for input control. The lysates were precleared for 30 min without antibody at room temperature and then incubated with anti-FLIP antibody 1/10 (NF6 supernatant, a gift from Dr. Peter Krammer, German Cancer Research Center, Heidelberg, Germany) and 20 µl of a 50% slurry of protein A-Sepharose beads under rotation for 2 h at 4°C. After incubation, the Sepharose beads were washed four times with 0.5% Triton X-100 in PBS, and the immunoprecipitated proteins were run on SDS-PAGE, and immunoblotted with anti-ubiquitin antibody.

5 FLAG immunoprecipitation (III)

To immunoprecipitate FLAG-tagged c-FLIP, the cell pellets were lysed on ice in co-immunoprecipitation buffer (25 mM Hepes (pH 7.5), 150 mM sodium chloride, 5 mM EDTA, 0,5% Triton X-100, 20 mM sodium pyrophosphate, 0.5 mM PMSF, 0.1 mM sodium orthovanadate, 1 mM DTT, Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics GmbH)) and 10% of the lysates were used as input samples. The cleared cell lysate was immunoprecipitated with 15 µl of M2-agarose FLAG-beads (Sigma), at 4°C on a rotamix for 1-2 h. The beads were collected by mild centrifugation and washed five times with TEG-buffer (20 mM Tris-HCl, pH 7.5; 1 mM EDTA; 10% glycerol; 150 mM sodium chloride; 0.5% Triton X-100) and three times with FLAG-buffer (10 mM Tris-HCl, pH 7.5; 50 mM sodium chloride; 30 mM sodium pyrophosphate; 50 mM sodium fluoride; 5 µM zinc chloride; 10% glycerol; 0.5% Triton X-100). FLAG-tagged c-FLIP was eluted from the beads by adding 250 µg/ml of FLAG-peptide (Sigma) in FLAG-buffer and incubating in shaking overnight at 4°C. The supernatant was collected and mixed with 3xLaemmli sample buffer, boiled, and run on 12.5% SDS-PAGE, followed by autoradiography with an imaging plate using Fujifilm BAS-1800 Bioimaging analyzer.

6 CD95 and TRAIL DISC analysis (I-III)

A total of 5 x 10^7 Jurkat cells were resuspended in 1 ml of prewarmed RPMI 1640 medium. To ligate CD95 and induce DISC formation, 1 µg of Fc-CD95 ligand fusion protein (CD95L:Fc), a gift from Dr. Pascal Schneider (Institute of Biochemistry, University of Lausanne, Lausanne, Switzerland), was added to the cell suspension. The cells were incubated at 37°C for 12 min, and the reaction was stopped by adding 10 ml of ice-cold PBS. After washing, the cells were lysed in 1 ml of lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 0.2% Nonidet P-40, and complete protease inhibitor mixture (Roche)) for 20 min on ice. The cell debris was removed by centrifugation at 15,000 x *g* for 12 min at 4°C. 1 µg of CD95L:Fc was added to control samples without CD95 ligation. Samples were immunoprecipitated with 15 µl of protein G beads (Amersham Biosciences) for 2.5 h at 4°C. The beads were washed five times with lysis buffer, resuspended in Laemmli sample buffer, and finally boiled for 5 min. The immunoprecipitates and corresponding cell lysates were analyzed by Western blotting. A control sample without CD95L:Fc was used to exclude unspecific binding to the protein G beads (antibody control).

To stimulate TRAIL receptors, 4 x 10^7 K562 cells were pelleted (500 x *g* for 7 min) and resuspended in 1 ml of prewarmed RPMI 1640 medium. Thereafter 1 µg of FLAG-tagged TRAIL (Alexis) and 2 µg of anti-FLAG monoclonal M2 antibody (Sigma) were added to the cell suspension. The cells were incubated at 37°C for 20 min, and the reaction was stopped by adding 10 ml of ice-cold PBS. After washing, the cells were lysed in 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 0.2% Nonidet P40, and Complete protease inhibitor mixture (Roche Applied Science)) for 30 min on ice. The cell debris was removed by centrifugation at 15,000 x *g* for 15 min at 4°C. The amount of protein was determined by the Bradford assay, and an equal amount of protein from each sample was precleared with 50 µl of Sepharose CL-4B for 2 h at 4 °C. A total of 2.5 µg of monoclonal anti-DR5 and 2.5 µg of monoclonal anti-DR4 (Alexis) were added to samples and immunoprecipitated with 15 µl of protein G beads (Amersham Biosciences) for 2.5 h at 4°C. The beads were washed six times with 1 ml of lysis buffer, resuspended in 3x Laemmli sample buffer, and finally boiled for 3 min. The immunoprecipitated samples and corresponding cell lysates were analyzed by 11% SDS-PAGE and Western blotting.

7 SDS-PAGE and Western blotting (I-III)

For Western blot analysis, cells were harvested by centrifugation and washed once with PBS. Cells were lysed either in the Laemmli SDS sample buffer or in lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM PMSF, Complete miniprotease inhibitor mixture (Roche Applied Science)). Triton X-100 lysis buffer lysates were centrifuged to remove insoluble material, and the protein concentrations were determined by Bradford assay. Each lysate containing 30-50 µg of protein was loaded and resolved by SDS-PAGE and transferred to nitrocellulose membrane (Protran nitrocellulose, Schleicher & Schuell) by using a semidry transfer apparatus (Bio-Rad).

Western blotting was performed using antibodies against c-FLIP (NF6 FLIP antibody, kindly provided by Peter Krammer, German Cancer Research Center, Heidelberg, Germany; or NF6 and Dave-2, Alexis), Hsc70 (SPA-815; StressGen), DR5 (Alexis), FADD (BD Transduction Laboratories), GFP (JL-8), caspase-8 (C15, Alexis), CD95L (clone G247-4; BD Pharmingen), PARP (C-2-10, Sigma), ubiquitin (FK-1; Biomol), and c-FLIP phosphorylated on S193. HRP-conjugated secondary antibodies were purchased from Amersham and Southern Biotechnology. The bands were visualized using the enhanced chemiluminescence method (ECL; Amersham).

8 *In vivo* ^{32}P labeling, Edman sequencing and mass spectrometry (III)

K562 cells stably overexpressing FLAG-c-FLIP$_S$ or FLAG-c-FLIP$_L$ were grown in RPMI 1640 medium supplemented with 10% FCS, L-glutamine, and antibiotics. 2,5x10^6/ml cells were preincubated in 6 ml for 2.5 h with 0.3 mCi/ml ^{32}P-orthophosphate (ICN Pharmaceuticals) in phosphate-free minimum essential medium Eagle (MEME; Sigma-Aldrich) supplemented with 10% FCS. After treatment for 30 min with 50 nM of the phosphatase inhibitor calyculin A (Sigma), the cells were collected by centrifugation, washed with ice-cold PBS and subjected to the FLAG-immunoprecipitation. The resulting immunoprecipitates were separated by SDS-PAGE followed by autoradiography with Fujifilm BAS-1800 Bioimaging analyzer.

In-gel tryptic digestion of the ^{32}P-labeled FLAG-c-FLIP$_S$ and FLAG-c-FLIP$_L$ followed by 2D phosphopeptide mapping on a thin layer chromatography (TLC) sheet were carried out as previously described (Kochin et al. 2006). Labeled c-FLIP bands were excised from the dried gel and in-gel digested overnight, 37°C with 2 µg/ml sequencing grade trypsin

(Promega) in 50 mM ammonium bicarbonate solution (pH 8). The tryptic digest was applied on a cellulose TLC sheet (20x20 cm, Merck KgaA) and separated by 2D electrophoresis and TLC. The first dimension, electrophoresis, was performed in a pH 1.9 buffer (formic acid 2.3 %, acetic acid 2.9% v/v) at 750 V for 1.5 h using the Hunter Thin Layer Peptide Mapping System, model # HTLE-7000 (C.B.S. Scientific Co.). The sheet was dried and ascending TLC in the second dimension performed for 14 h in a chromatography tank saturated with a mobile phase containing 30% water, 37.5% n-butanol, 7.5% acetic acid, and 25% pyridine. The sheet was air-dried and the ^{32}P phosphopeptides visualized by autoradiography on a phosphorimager plate. For MALDI mass spectrometry and Edman sequencing corresponding ^{32}P peptides were extracted from cellulose sheets by scraping off the powder and then eluted twice with 30% ACN, 0.1% TFA solution. The resulting extract was vacuum-evaporated and MALDI MS and Edman sequencing were performed as described by Kochin and coworkers (2006). For Edman degradation, phosphopeptides were immobilized on arylamine membrane discs (Sequelon-AA membrane; Applied Biosystems) using water-soluble carboiimide. Individual c-FLIP phosphopeptides are immobilized on the Sequelon-AA discs via their C-terminal carboxyl groups. Therefore, the N termini of peptides are free, and amino acids are clipped off during Edman degradation cycles. The collected fractions were spotted on a Whatman filter paper and visualized by autoradiography on a phosphor imager plate to reveal the cycle at which the radiolabel is released, corresponding to the position of the phosphorylated amino acid counting from the N terminus.

9 Preparation and use of the anti-pS193 phosphopeptide antibody (III)

To generate an antibody that specifically recognizes phosphorylated human c-FLIP serine 193, the phosphopeptide (Ac-)CLQAAIQK(pS)LKDPSNN(-CONH2) was conjugated to keyhole lymphet haemocyanine and 150 µg of the conjugate was repeatedly injected into rabbits (NZ white, 5 kg). The antiserum against pS193-c-FLIP was collected on day 7 after injections and positively and consequently negatively affinity purified using (Ac-)CLQAAIQK(pS)LKDPSNN(-CONH2) and (Ac-)CLQAAIQKSLKDPSNN(-CONH2) conjugated to NHS-activated matrix columns (GE Bioscience), respectively. Whole-cell extracts, as well as lysis buffer lysates, were subjected to 10% SDS-PAGE and tested for their specificity. Immunoblotting with the anti-pS193-c-FLIP antibody was performed after SDS-PAGE in reducing conditions and subsequent electric transfer to nitrocellulose membranes. Membranes were briefly washed in MOPS buffer saline (MOPS 25 mM, NaCl 125 mM, pH=7.4, Tween 20 0.5%) and blocked in 5% BSA in the same buffer overnight. After three washes, the membranes were exposed to the primary antibodies (1:500) overnight. After antibody incubation, the membranes were washed three times, incubated with HRP-conjugated secondary antibody (Pierce), triple washed three times again, after which the membranes were developed with ACL chemiluminescent substrate (Amersham) and registered on X-ray films (Fuji).

10 Caspase-3 activity assay (I, II)

After treatments, the cells were washed once with ice-cold PBS, and the caspase-3 activity was analyzed with phycoerythrin-conjugated monoclonal active caspase-3 antibody apoptosis kit 1 (BD Pharmingen) according to the manufacturer's protocol.

11 Caspase-8 activity assay (III)

The activity of caspase-8 in the TRAIL DISC overpopulated with wild type or mutant c-FLIP was measured after down-scaled TRAIL DISC immunoprecipitation by Caspase-8 Glo® kit

(Promega) according to manufacturer's protocol. Luminescence was measured by Tecan Ultra luminometer at MediCity Turku.

12 Detection of apoptosis (II)

Apoptosis in Jurkat cells and primary peripheral blood T lymphocytes was detected by annexin V analysis. Cells were incubated for 10 min on ice with 4 µl/ml human recombinant FITC-conjugated annexin V (Sigma-Aldrich) in annexin V binding buffer (2.5 mM HEPES (pH 7.4), 35 mM NaCl, and 0.6 mM $CaCl_2$) and analyzed on a FACScan flow cytometer (BD Biosciences).

13 Surface expression analysis of CD95 (II)

Cells were washed twice with PBS and blocked for 30 min with 1% BSA in PBS. Cells were then incubated with CH-11 anti-CD95 antibody (Biosite), 1/500 in 1% BSA in PBS for 30 min followed by washing with PBS. Finally, cells were incubated with Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) for 30 min. After washes, cells were analyzed by flow cytometry on a FACScan flow cytometer (BD Biosciences). Samples without primary antibody were used as negative controls (second antibody control).

14 RNase protection assay (II)

Hyperthermia was induced in Jurkat cells at 42°C for indicated times and RNA was prepared with the Qiagen kit for RNA isolation according to the manufacturer's protocol. The RNase protection assay was done with the RiboQuant MultiProbe RNase Protection Assay System (BD Pharmingen) according to the manufacturer's standard RPA protocol using the hApo-3b probe (cat. no. 45611P). The results were visualized by autoradiography using BAS Imaging Plates (Fuji Film) and a BAS-1800 Imaging Plate Reader (Fuji Film).

15 Metabolic labeling by ^{35}S (III)

1-5x10^6 K562 cells stably overexpressing c-FLIP were washed once with methionine and cysteine-free RPMI 1640 supplemented with 10% dialysed FCS, antibiotics, and L-glutamine, and pulse labeled at 37°C for 3 h with 200 µCi (0.1mCi/ml) ^{35}S-methionine and cysteine (MP Biomedicals, Irvine, California, USA). The labeled cells were washed with supplemented RPMI 1640 containing unlabeled methionine and cysteine 100 times in excess (Sigma). The cells were chased for indicated times, washed with PBS and treated according to the FLAG-immunoprecipitation protocol. The relative amounts of ^{35}S-labeled c-FLIP were quantified by phosphorimager analyses, statistical significance of the differences between wild type and mutant c-$FLIP_S$ from three independent experiments were statistically analyzed.

16 Quantification, densitometry and statistical analysis of c-FLIP half-lives and caspase-8 activation (I-III)

The relative amounts of c-FLIP in the K562 CHX chases were analyzed by densitometry (ScionImage GlePlot 2 and Adobe Photoshop) and normalized against the Hsc70 loading control. The amounts of ^{35}S-labeled c-FLIP were quantified by phosphorimager analysis. The relative half-lives and the relative caspase-8 activation were illustrated using GraphPad Prism and statistical significance analyzed by Student's unpaired *t* test (n=3, *P=0.05, **P=0.005). The graphs show mean values and the standard errors of mean (n=3). Quantifications of c-FLIP half-lives in Jurkat cells were made using densitometry

(ScionImage GelPlot 2 and GraphPad Prism software. Student's unpaired *t* tests were performed on data from different individuals, i.e., primary T lymphocytes, whereas paired *t* tests were performed on data from Jurkat cells and Jurkat-based cell lines. Statistical significance is marked with asterisks (*P =0.05, **P=0.005, and ***P=0.001) and ns (P>0.05) stands for not statistically significant. The bar graphs represent mean values and standard deviation (n≥2). Caspase-8 activity induced in the K562 cells was given the value 1, and the activity readings from c-FLIP overexpressing cell lines were related against this value. Relative caspase-8 activation in different cell lines was illustrated using GraphPad Prism (version 5). The graphs show mean values and the standard errors of mean (n≥2).

17 Molecular modeling of c-FLIP$_S$ (I)

All models were built using the Modeler computer program. The sequence of c-FLIP$_S$ contains two conservative DEDs between S2 (numbering according to Irmler et al. 1997) and V79 and between Y93 and Q176. A model structure for the second DED in c-FLIP$_S$ (DED2) was built by using the NMR structural data for phosphoprotein enriched in astrocytes, 15 kDa (PEA-15; pdb code 1n3k) and the NMR structure of the FADD death effector domain (F25Y mutant; pdb code1a1w). The secondary structure of the c-FLIP$_S$ C-terminal segment (amino acids 176-221) was predicted using the following computer programs: PHD: Profile network prediction HeiDelberg (Rost and Sander 1993a, Rost and Sander 1993b, Rost and Sander 1994), SAM-T99 Secondary Structure Prediction (Karplus et al. 1997), SCRATCH (Sspro) (Pollastri et al. 2002), PROF-Secondary Structure Prediction System (Ouali and King 2000), and nnpredict (Kneller et al. 1990). The loop between the DED2 and the c-FLIP$_S$ C-terminal segment has been taken as seen in the structure of PEA-15, the only currently known representative structure covering this region (Hill et al. 2002). Surface construction and lipophilic potential calculations were done using SYBYL (Tripos Inc.). Figure 8 (I) was produced with MolScript v2.1 (Kraulis 1991) and Raster3D v2.4b (Merritt and Bacon 1997).

RESULTS AND DISCUSSION

1 Increased death receptor sensitivity during differentiation and hyperthermia is due to c-FLIP downregulation (I, II)

1.1 Death receptor sensitivity is adjusted by c-FLIP downregulation during differentiation and hyperthermia (I, II)

Death receptor sensitivity is dynamically regulated in response to environmental stimuli, and it may also change during differentiation. The human K562 cell line originates from a patient suffering from chronic myelogenous leukemia (CML). The CML cells express the Bcr-Abl fusion protein as a result of a mutation called the Philadelphia translocation (t9:22) (Lozzio and Lozzio 1975), producing a constitutively active tyrosine kinase that prolongs cell survival independently of growth factor signaling (Bedi et al. 1994). Hence, K562 cells are abnormally resistant to programmed cell death induced by a variety of stimuli (McGahon et al. 1994). The K562 cell line is a valuable research model because of its differentiating capacity. K562 cells are progenitors, and their non-terminal differentiation towards the erythroid-like lineage can be induced by hemin, the ferric chloride salt of heme (Andersson et al. 1979). In addition, the K562 cells can be stimulated to differentiate towards the megakaryocytic lineage by TPA (Tetteroo et al. 1984). In some cell types, including the K562 cells, differentiation causes changes in the sensitivity towards apoptotic stimuli. Earlier work from our group has shown that the induction of erythroid differentiation results in increased sensitivity to TRAIL in K562 cells (Hietakangas et al. 2003). Interestingly, the sensitization correlated with hemin-induced downregulation of c-FLIP. From this starting point, we aimed at determining how c-FLIP levels are decreased during K562 erythroid differentiation (I).

The ability to adjust death receptor sensitivity in response to environmental cues is a valuable asset to the cell and the organism. In addition to the K562 cells, also HEL and HL-60 cells were sensitized to TRAIL upon differentiation (Hietakangas et al. 2003). Therefore, differentiation-induced sensitivity is not a cell type-specific phenomenon. During differentiation of the primary erythroblasts, the expression of death receptors is dynamically regulated, but the expression of CD95L and TRAIL is restricted to mature erythroblasts (De Maria et al. 1999). This allows receptor-mediated apoptosis of immature erythroblasts to occur in locations where additional mature erythroblasts are not needed. c-FLIP has been demonstrated to regulate death receptor sensitivity also in other cell types. For example, the human CD34+ hematopoietic progenitor cells are protected from CD95-mediated apoptosis due to high expression levels of c-FLIP (Kim et al. 2002), but erythroid differentiation in response to Epo and kit ligand induces c-FLIP downregulation and sensitization to TNFα (Ratajczak et al. 2003).

In addition to differentiation, also cellular stresses, such as hyperthermia, have been shown to modulate death receptor sensitivity (reviewed by Nadeau and Landry 2007). Heat stress affects apoptotic sensitivity, but its impact largely depends on the cell type. Although heat shock protein expression has been shown to promote cell survival, stress may also change the expression and activity of the proteins that regulate apoptosis (reviewed by Beere 2004). Moreover, previous studies from our laboratory revealed that heat stress sensitizes Jurkat cells to CD95-mediated apoptosis (Tran et al. 2003). The Jurkat T lymphocyte cell line originates from the peripheral blood of a patient with acute T cell leukemia (Schneider

et al. 1977, Weiss et al. 1984). In Jurkat cells, changes in JNK or MAPK activities or Hsp70 expression did not contribute to the effect, although they have been shown to contribute to cell survival (reviewed by Jäättelä 1999; Kyriakis and Avruch 1996, Holmström et al. 1999, Tran et al. 2001). Instead, the sensitization was due to heat stress-induced downregulation of the c-FLIP proteins, rendering the cells susceptible for receptor-mediated apoptosis. This led us to examining how c-FLIP is regulated upon heat stress.

The downregulation of c-FLIP in response to heat shock and the following increase in death receptor sensitivity also apply to more than one cell model, as HeLa cervical adenocarcinoma cancer cells were sensitized to CD95 by hyperthermia treatment (Tran et al. 2003). Whereas the unstimulated primary T cells are not sensitive to TRAIL, the Jurkat cells are, and they are further sensitized by heat shock (Annika Meinander, PhD thesis). In addition to the malignant Jurkat leukemia cells, we confirmed our results using primary T cells. Indeed, there was a dramatic reduction in the levels of c-FLIP in response to hyperthermia in primary T cells, and the c-FLIP expression returned to normal after a 6-hour recovery period in normal cell culture conditions (Figure 3A and 3D, II). Furthermore, CD95 sensitivity was reduced to normal in correlation with the recovering c-FLIP levels (Figure 3B-C, II). Moreover, c-FLIP was absent from the CD95 DISC of the hyperthermia-treated Jurkat T cells (Figure 5, II). These results strongly suggest that c-FLIP acts as a switch dictating the sensitivity towards death receptor stimulation during hyperthermia in several cell models, including primary T cells.

Although heat shock proteins act to protect the cell, many reports still describe increased cell death sensitivity in response to heat stress. Human glioma cells, for instance, are sensitized to CD95-mediated apoptosis by hyperthermia (Hermisson et al. 2000). In addition, c-FLIP is not the only anti-apoptotic protein downregulated by hyperthermia. For example, the Bcl-2 family member Mcl1, another short-lived inhibitor of cell death, is destabilized by heat shock. The stabilizing interaction between Mcl1 and Noxa is lost upon hyperthermia, leaving Mcl1 available for a BH3-containing E3 ubiquitin ligase Mule. Mule catalyses the ubiquitylation and degradation of Mcl1, resulting in increased sensitivity to apoptosis after heat shock (Stankiewicz et al. 2009). We were interested in determining how c-FLIP downregulation occurs during hyperthermia, finding out if the ubiquitin-proteasome pathway is involved, and identifying the potential common denominators between hyperthermia- and differentiation-induced regulation of c-FLIP.

1.2 c-FLIP proteins are ubiquitylated and degraded in the proteasome following erythroid differentiation of K562 cells and upon fever-like hyperthermia of Jurkat T cells (I, II)

The c-FLIP isoforms are produced from one gene by alternative mRNA splicing (Shu et al. 1997). The messenger RNA levels of c-FLIP do not significantly change during hemin treatment (Hietakangas et al. 2003), suggesting that the downregulation of c-FLIP occurs primarily at the protein level. In addition, the kinetics of hemin-induced downregulation of the short and long isoforms are very different. These observations led us to explore the potential ubiquitylation and proteosomal degradation of the c-FLIP proteins. In addition, we wanted to assess whether the c-FLIP isoforms are differentially regulated. First, we used the proteasome inhibitor epoxomicin to investigate whether hemin-induced downregulation of c-FLIP upon erythroid differentiation was due to induced proteosomal degradation. Indeed, we observed that the hemin-induced downregulation of c-FLIP could be rescued by proteasome inhibition (Figure 1A, I). Notably, the effect was most prominent in Laemmli total cell lysates, as hemin treatment seemed to accumulate c-FLIP into the insoluble fraction. Moreover, treatment with epoxomicin led to the translocation of c-FLIP to the

insoluble fraction also in HeLa and Jurkat cell lines (data not shown). Similarly, the hyperthermia-induced downregulation of c-FLIP was rescued with epoxomicin (Figure 7A, II), suggesting a role for proteasomal activity in downregulation of c-FLIP.

The contribution of the proteasome in downregulating c-FLIP during differentiation and hyperthermia encouraged us to study if c-FLIP proteins were inducibly ubiquitylated during differentiation or heat shock. For this purpose, we transfected K562 cells with plasmids encoding FLAG-tagged c-FLIP and HA-tagged ubiquitin, and treated cells with hemin. Next, we prepared whole cell lysates, immunoprecipitated the overexpressed ubiquitin, and analyzed the ubiquitylation of c-FLIP by SDS-PAGE and Western blotting. We found that while exogenous wild type c-FLIP proteins were ubiquitylated during control conditions, hemin treatment clearly increased the ubiquitylation of both c-$FLIP_S$ and c-$FLIP_L$ (Figure 1C, I). After hemin and epoxomicin treatments, similar smears indicating polyubiquitylation could be seen in whole cell lysates prepared from K562 cell lines stably overexpressing c-$FLIP_S$ or c-$FLIP_L$ (Figure 1B, I).

To investigate c-FLIP ubiquitylation in response to hyperthermia, we overexpressed c-FLIP in Jurkat T cells, performed immunoprecipitation with antibodies against c-FLIP and immunoblotted the samples with antibodies recognizing endogenous ubiquitin. Importantly, we detected a clear increase in the ubiquitylation of both c-$FLIP_S$ and c-$FLIP_L$ in response to heat shock (Figure 7B, II). The high molecular weight ubiquitin smear was present on both endogenous and overexpressed c-FLIP, indicating that both c-$FLIP_S$ and c-$FLIP_L$ are inducibly ubiquitylated upon hyperthermia. As transcription and translation are generally repressed during hyperthermia, we investigated whether it affected the transcription of c-FLIP. However, according to the RNase protection assays, c-FLIP mRNA levels were unaffected by heat shock (Figure 6B, II). In addition, downregulation by the translation inhibitor cycloheximide was slower than the hyperthermia-induced degradation (Figure 6C, II), and since c-$FLIP_S$ and c-$FLIP_L$ declined rapidly upon heat stress (Figure 6A, II), this downregulation was more likely to occur via degradation of c-FLIP proteins than transcriptional repression of the *CFLAR* gene. Together, these results strongly support the idea of hyperthermia affecting death receptor sensitivity through destabilizing c-FLIP.

Prior to our work, the mechanisms of c-FLIP ubiquitylation had not been widely investigated. However, some reports indicated that c-FLIP is regulated by the ubiquitin-proteasome pathway, and these observations were later confirmed in a number of model systems. For example, Fukazawa and coworkers suggested that the stability of c-FLIP is under the transcriptional control of p53, as c-FLIP levels are low in p53 expressing cells, and the levels can be restored by proteasome inhibitors (Fukazawa et al. 2001). Consistently, cisplatin induces the ubiquitylation of c-FLIP in ovarian cancer cells by a p53-dependent mechanism (Abedini et al. 2008). In addition, the proteasomal degradation of c-FLIP can be induced by inhibition of PPARγ (Kim et al. 2002). Interestingly, also adenoviral E1A infection sensitizes tumor cells to apoptosis by specifically inducing the ubiquitylation and proteasomal degradation of c-$FLIP_S$, underlining the isoform-specific functions of the c-FLIP proteins (Perez and White 2003). Another example of isoform-specific regulation was recently shown in hepatoma cells, in which the flavonoid quercetin induces proteasomal degradation of c-$FLIP_S$ and sensitizes cells to TRAIL (Kim et al. 2008). In addition, nitric oxide was shown to counteract CD95-mediated apoptosis by inhibiting the proteosomal degradation of c-FLIP (Chanvorachote et al. 2005). A single report even shows that c-FLIP may, perhaps unexpectedly, regulate the proteasome, because in lung cancer cells, exogenous c-$FLIP_L$ forms aggregates which impair the proteasome function (Ishioka et al. 2007). However, whether this effect is c-FLIP-specific, or if the overexpression of any DED-containing protein would hamper the proteasome, remains to be elucidated.

2 Mechanisms of isoform-specific ubiquitylation and degradation of the c-FLIP splice variants (I, II)

2.1 c-FLIP isoforms display different half lives (I)

Despite the high degree of similarity between the c-FLIP proteins, the isoforms modulate apoptosis in disparate ways. It is therefore hardly surprising that the c-FLIP proteins are also regulated in an isoform-specific fashion. Hence, we were especially interested in examining the isoform-specificity of c-FLIP function and regulation. During this thesis work, Golks and coworkers (2005) reported the detection of the third c-FLIP isoform, c-FLIP$_R$, in the human Raji cell line. Prompted by this finding, we explored whether c-FLIP$_R$ was expressed also in other human cell lines. Surprisingly, the endogenous short c-FLIP isoform in K562 cells migrated faster compared to the short c-FLIP expressed in Jurkat or Hela cells, which indicates that the short isoform expressed by the K562 cells is more likely to be c-FLIP$_R$ than c-FLIP$_S$ (Supplementary Figure 2, III). It is therefore conceivable that we have determined the half-life of endogenous c-FLIP$_R$ and not c-FLIP$_S$ as indicated in article I. However, as most of the analyses of this thesis are based on overexpression experiments, conclusions on how c-FLIP$_S$ is regulated are likely to be correct and apply to c-FLIP$_R$ as well. This hypothesis is supported by our own data as well as the fact the short c-FLIP isoforms are yet to be assigned any differences as far as biological functions are concerned. To avoid confusion, I have chosen to refer to the respective isoforms by the names used in the original articles.

To determine the half-lives of the c-FLIP isoforms, we analyzed the c-FLIP protein levels in K562 and HeLa cells after a time course treatment with the translation inhibitor cycloheximide. Although these cell lines express differing amounts of the isoforms, c-FLIP$_L$ being more abundantly expressed than c-FLIP$_S$ in K562 cells whereas HeLa cells expressed similar amounts of both, we were able to see that the half-lives of the c-FLIP isoforms are different. c-FLIP$_S$ declined faster than c-FLIP$_L$ in both cell lines (Figure 2A-D, I), suggesting that although the c-FLIP isoforms are ubiquitylated and degraded in the proteasome, their stability is determined by distinct mechanisms. We have also determined the half-lives of c-FLIP proteins in Jurkat cells and found c-FLIP$_L$ more stable than c-FLIP$_S$ also in this cell model (data not shown).

Human c-FLIP$_S$	**MITPYAHCP-DLKILGNCSM**
Chimpanzee c-FLIP$_S$	**MIAPYAHCP-DLKILGNCSM**
Macaque c-FLIP$_S$	**MIAPYVHFP-DL--------**
Human c-FLIP$_R$	**VSLEKTYG-----IPA----**
Murine c-FLIP$_{S/R}$	**VSLEPVYG-----VPA----**
Rat c-FLIP$_{S/R}$	**VSLEPVCG-----ISA----**

Figure 14. Alignment of the splicing tails of c-FLIP$_S$ and c-FLIP$_R$ proteins in different species. c-FLIP$_R$ appears to be the predominant short c-FLIP isoform and evolutionarily older (Annika Meinander, PhD thesis; Ueffing et al. 2008, Ueffing et al. 2009). The predicted protein sequences of the chimpanzee and macaque c-FLIP$_S$ proteins, in turn, are homologous to human c-FLIP$_S$.

While c-FLIP$_L$ is a product of nine exons, c-FLIP$_S$ consists of five exons, exon 7 accounting for the unique C-terminal tail. The splicing tail of c-FLIP$_R$, however, is a result of run-through from exon 6 to intron 6 (Djerbi et al. 2001, Golks et al. 2005; Annika Meinander, PhD thesis). The C-terminal tail of c-FLIP$_S$ is 19 amino acids long and the tail of c-FLIP$_R$ is 11 amino acids, respectively (Figure 14). Notably, the sequences of the splicing tails are entirely different (Golks et al. 2005, Ueffing et al. 2008, Ueffing et al. 2009). In contrast to the short isoforms, c-FLIP$_L$ contains a C-terminal caspase-like domain, enabling it to dimerize with procaspase-8 (Scaffidi et al. 1999, Krueger et al. 2001, Micheau et al. 2002, Boatright et al. 2004).

The differential regulation of the c-FLIP isoforms can be explained by their differential functions in the DISC. Surprisingly, when we overexpressed c-FLIP$_S$ or c-FLIP$_L$ in Jurkat T cells and studied apoptosis after hyperthermia, we found that the heat stress-induced sensitivity was only counteracted by overexpression of c-FLIP$_S$, but not c-FLIP$_L$ (Figure 4, II). These results may be partially due to non-physiological expression levels of c-FLIP$_L$, which in other studies have been reported to induce cell death (Scaffidi et al. 1999). This is, however, unlikely, as overexpression of c-FLIP$_L$ did not significantly increase apoptosis on its own (Figure 4, II). Because c-FLIP proteins seem to play differential roles during hyperthermia, and yet both isoforms are downregulated in the process, it can be speculated that the underlying mechanisms are isoform-specific.

The differential stability of the c-FLIP isoforms has been addressed in a handful of studies. The overall data suggests that the short c-FLIP proteins are less stable than c-FLIP$_L$, and that half-lives of the c-FLIP proteins vary in a cell type-specific fashion. Cycloheximide experiments showed that in Boe^R cells, which express all three c-FLIP isoforms, the half-life of c-FLIP$_R$ was approximately similar to that of c-FLIP$_S$ (Golks et al. 2005, Ueffing et al. 2008). In contrast to K562 and HeLa cells, c-FLIP$_L$ is less stable than c-FLIP$_S$ in SHEP neuroblastoma cells (Fulda et al. 2000). In addition, the half-life of c-FLIP$_L$ in keratinocytes has been reported to be less than 90 minutes (Leverkus et al. 2000), whereas in SV80-CD40 cells both c-FLIP$_S$ and c-FLIP$_L$ were entirely degraded after four hours of cycloheximide treatment (Kreuz et al. 2001). In summary, our data and the studies of others demonstrate that the half-lives of the c-FLIP isoforms are modified in a cell type-specific fashion, and that in most cellular systems c-FLIP$_L$ displays a longer half-life than the short isoforms.

The specific regulation of the stability of splice variants provides variability in protein function and enables the cell to adapt to changing circumstances. In activated T lymphocytes, c-FLIP$_S$ expression was primarily responsible for resistance towards CD95-mediated apoptosis, while the role of c-FLIP$_L$ was slightly obscure. In contrast to c-FLIP$_S$, the biological functions of c-FLIP$_L$ extend beyond being a protector against apoptosis, and these features are likely to differ in compliance with the cell type and environmental cues. This is exemplified by an interesting study, according to which the lymphocytes of transgenic mice overexpressing c-FLIP$_L$ are not protected from AICD, but instead proliferate more than wild type lymphocytes in response to sub-optimal activation (Lens et al. 2002). Consequently, c-FLIP$_L$ could be acting as an amplifier for proliferatory signals. The divergent roles of the short and long c-FLIP isoforms in death receptor signaling may explain why the post-translational modification of these proteins is dissimilar.

A number of examples of differential stability of protein splice variants are described in the literature. For instance, the transcription factor suppressor of cytokine signaling 3 (SOCS3) is expressed in long and short isoforms, the latter of which is expressed under stress conditions, and the truncated SOCS3 is more stable than the full-length protein due to the absence of a major ubiquitylation site in the truncated SOCS3 isoform (Sasaki et al. 2003). The splice variants of the *S. cerevisiae* microtubules regulator, chromosome instability and karyogamy protein 1 (Cik1), in turn, are differentially expressed in response to mating

pheromones. Intriguingly, the short Cik1 protein, which is expressed upon exposure to mating pheromone α factor, is not a substrate for the APC/cyclosome complex, indicating that the short and long isoforms regulate different aspects of mitosis (Benanti et al. 2009). Moreover, the ubiquitin ligase Siah1 is expressed in two isoforms, Siah1 and Siah1 short (Siah1-S), of which Siah1-S, but not Siah1, is capable of autoubiquitylation and has a short half-life (Mei et al. 2007). Finally, an important cell cycle regulator, cyclin D1, is expressed in two isoforms, cyclin D1a and D1b, of which D1b is shorter and more stable, because it lacks the stabilizing C-terminal region (Leveque et al. 2007). These examples illustrate how several isoforms of a protein, possibly serving in disparate functions, are often targeted for degradation by distinct mechanisms.

2.2 K192 and K195 are the primary ubiquitylated lysines in c-FLIP$_S$ during K562 erythroid differentiation (I)

We and others have established that c-FLIP proteins are capable of modifying death receptor responses and that their protein levels are under precise regulation. While in most conditions all c-FLIP proteins counteract apoptosis, the short and long isoforms also play differential roles, prompting us to investigate the molecular mechanisms behind the induced downregulation of c-FLIP, and to examine the structural basis for isoform-specific modifications. We chose to perform most of these biochemical analyses in the K562 system for two reasons: firstly, the K562 cells could be easily transfected, allowing overexpression-based studies; and secondly, according to the current knowledge, c-FLIP operates similarly in the CD95 and the TRAIL DISCs. The fundamental findings regarding c-FLIP$_S$ have been confirmed in the Jurkat system.

Covalent modification of proteins by ubiquitylation occurs on lysine residues. The choice of a particular acceptor lysine may be primed by areas such as ubiquitin-interacting domains or other interaction motifs around the lysine which aid the docking of the ligase. However, ubiquitylation tends to be rather promiscuous, and it is often difficult to pinpoint one lysine residue that is entirely responsible for accepting ubiquitin. We were interested in determining which lysines of c-FLIP are subject to ubiquitylation in order to understand the mechanisms of isoform-specific regulation. To this end, we performed mutational analyses focusing on c-FLIP$_S$ because of its structural simplicity compared to c-FLIP$_L$. We mutated all 20 lysine residues of c-FLIP$_S$ in four clusters conservatively into arginines to preserve native protein structure. The cluster mutants were transiently transfected into K562 cells and their ubiquitylation was analyzed by immunoprecipitation. As seen in Figure 5C (I), mutation of three lysines (K192, K195 and K214) in the C-terminal part led to a marked loss of c-FLIP$_S$ ubiquitylation. To further dissect the role of the three lysines, we generated K214R, K192R, K195R and K192,195R mutants. Surprisingly, the only lysine present in the C-terminal splicing tail of c-FLIP$_S$, K214, did not appear to be the main target for ubiquitylation, but seemed to render c-FLIP$_S$ more susceptible for ubiquitylation (Figure 5A, I), probably due to conformational changes in the splicing tail of the mutant. In contrast, the mutation of K192 and K195 in the linker area between DED2 and the splicing tail significantly decreased c-FLIP$_S$ ubiquitylation, indicating that K192 and K195 are the primary ubiquitylated residues in c-FLIP$_S$ (Figure 5D, I). When K192 and K195 were individually mutated, c-FLIP$_S$ ubiquitylation was decreased, although not as efficiently as with the K192,195R mutant (Aura Kaunisto, unpublished data), signifying that the lysines are probably targeted by the same ligase. As K192 and K195 are conserved between species and present in other c-FLIP splice variants, we wanted to investigate their role in the ubiquitylation of c-FLIP$_L$. Surprisingly, mutating K192 and K195 did not affect the overall ubiquitylation of c-FLIP$_L$, underlining the isoform-specificity of the process. In addition, according to our results, K192 and K195 of c-FLIP$_S$ are not primarily ubiquitylated in Jurkat cells during hyperthermia (data

not shown), suggesting that in addition to isoform-specific regulation, selection of target residues is largely a cell type- and context-dependent event.

2.3 C-terminal regions determine the half-lives of c-FLIP proteins (I, II)

A single lysine residue seldom governs the ubiquitylation and stability of a protein. In fact, proteins often contain restricted motifs or larger domains that facilitate ubiquitylation and degradation by assisting the interaction with a ubiquitin ligase or a proteasome subunit. After we found that the c-FLIP isoforms display different half-lives, we sought to determine if this was due to structural differences, as the c-FLIP proteins share the 202 N-terminal amino acids, but differ in their C-terminal parts. To see whether the differential C-terminal regions explained the relative instability of c-FLIP$_S$, we deleted the splicing tail and studied the ubiquitylation of the remaining part encompassing only the amino acids common to all isoforms (called c-FLIP$_S$ Δ203-221 in I, c-FLIP1-202 in II and III). Our analysis revealed that deleting the splicing tail abolished most of c-FLIP$_S$ ubiquitylation, demonstrating that the C-terminal areas, unique to each individual c-FLIP, dictate their particular stability. In addition, we showed that these areas are not likely to contribute to anti-apoptotic functions and DISC recruitment, as the c-FLIP1-202 mutant was recruited to the TRAIL-induced DISC in K562 cells similarly to the wild type proteins, and its overexpression effectively protected cells from TRAIL-mediated apoptosis (Figure 4, I). Importantly, upon cycloheximide treatment, c-FLIP1-202 was remarkably more stable than wild type c-FLIP$_S$, as its half-life extended to approximately 12 hours compared to the 4 hours of the exogenous wild type c-FLIP$_S$ (Figure 6, I). These results strongly suggest that the C-terminal splicing tail of c-FLIP$_S$ is a destabilizing element. Although the half-lives of exogenous proteins do not correspond to those of endogenous protein, they do still provide an approximation on how certain regions affect protein stability. Our results show that the C-terminal areas of c-FLIP are crucial to their modification and processing by the ubiquitin-proteasome machinery.

To uncover the structural details of the interaction between the C-terminal part and lysines 192 and 195, we performed computational analysis to model these regions of c-FLIP$_S$. The analysis revealed that the C-terminal tail of c-FLIP$_S$ includes an α-helix encompassing amino acids Y182-D196 (Figure 7, I), but does not adapt a tightly folded state (Figure 8A, I). The loosely structured parts of the C terminus are likely to contribute to the unstable nature of c-FLIP$_S$. This idea is supported by the fact that although K192,195R double mutation clearly reduces the ubiquitylation of c-FLIP$_S$, it does not significantly impair c-FLIP$_S$ stability (data not shown). It is plausible that in the absence of K192 and K195, c-FLIP$_S$ would still be minutely ubiquitylated onto other lysine residues, and this would be enough to target c-FLIP$_S$ for degradation. Alternatively, the loose C terminus may be able to compensate for the loss of the primary target lysines by attracting the proteasome. Our model provides an indirect explanation on why K192 and K195 are especially important for the ubiquitylation of c-FLIP$_S$, but not c-FLIP$_L$. The computational programs did not predict α-helical structures for the corresponding area of c-FLIP$_L$ despite sequence similarity. It is possible that K192 and K195 are very differently positioned in c-FLIP$_L$ compared to c-FLIP$_S$, and therefore not available for ubiquitylation.

In addition to determining the structure adapted by the c-FLIP$_S$ C-terminal area, we analyzed its hydrophobic and hydrophilic properties. Our modeling showed that DED2 contains two lipophilic grooves, whereas the remaining C-terminal region has two lipophilic surfaces (Figure 8B-C, I). As these areas cannot reside freely or exposed in the cytosol, we suggested that they interact with each other or with an unknown protein by weak interactions. To test our hypothesis, we mutated Y182S, L186R and I190N, three residues of the α helix, to generate a triple point mutant called 3Xmut. In addition, we removed the α helix completely producing a deletion mutant called Δhelix. We then analyzed the

ubiquitylation and stability of these mutants comparing them to wild type c-FLIP$_S$. We found that both serial point mutations and the deletion of the helix significantly reduced the ubiquitylation of c-FLIP$_S$ (Figure 9A, I) and prolonged the half life of c-FLIP$_S$, supporting our hypothesis (Figure 9B-C, I). In terms of ubiquitylation, the α helix mutants resembled the K192,195R mutants, displaying only little ubiquitylation, yet more than the c-FLIP1-202 mutant (Figure 9A, I). However, although the K192,195R mutations did not destabilize c-FLIP$_S$, both α helix mutants exhibited a longer half-life compared to wild type c-FLIP$_S$ (Figure 9B-C, I). It therefore seems that disruption of the α-helical region crucially affects the conformation of the C terminus of c-FLIP$_S$, possibly rendering the primary target lysines less likely to be ubiquitylated, but probably also interfering with the overall structure of the C terminus. In summary, our model where weak interactions regulate the C-terminal structure of c-FLIP$_S$, thereby affecting stability, is likely to be correct.

To investigate whether the C-terminal region governed the ubiquitylation and stability of c-FLIP in our other experimental model, the Jurkat T lymphocytes, we compared the ubiquitylation status of wild type c-FLIP$_S$, the isoform primarily responsible for protecting T cells from receptor-mediated apoptosis, to the deletion mutant c-FLIP1-202, which only contains the amino acids common to all c-FLIP isoforms. Indeed, the c-FLIP1-202 mutant displayed less ubiquitylation than the wild type exogenous c-FLIP$_S$ in response to hyperthermia (Figure 7D, II). The faint remaining signal most likely originated from the endogenous ubiquitylation. Furthermore, when compared to the wild type c-FLIP$_S$, the c-FLIP1-202 mutant proved more resistant to heat stress, as it was not downregulated by 2-hour hyperthermia treatment, in contrast to wild type c-FLIP$_S$ (Figure 7C, II). What is more, the sensitizing effect of hyperthermia could be rescued by stable overexpression of the c-FLIP1-202 mutant (Figure 7E, II). In summary, these results firmly demonstrate that the sensitizing effect of hyperthermia in T lymphocytes is due to induced ubiquitylation and proteasomal degradation of the c-FLIP proteins, in particular c-FLIP$_S$.

Later studies have addressed how c-FLIP stability is affected by the characteristics of the C-terminal area. In the original article describing c-FLIP$_R$, the half-life of c-FLIP$_R$ was described to be very similar to that of c-FLIP$_S$ (Golks et al. 2005). Later, it was found by our group and recently reported by Ueffing and colleagues that the murine short c-FLIP isoform actually corresponds to c-FLIP$_R$, and not c-FLIP$_S$ as previously thought (Annika Meinander, PhD thesis; Ueffing et al. 2008). Interestingly, Ueffing and coworkers reported that lysines K196 and K200 of the murine c-FLIP$_R$, which correspond to K192 and K195 of the human c-FLIP$_S$, did not affect ubiquitylation, whereas the opposite was later demonstrated by another group (Kundu et al. 2009). Considering these results, it seems likely that similarly to human cell models, also murine cell models differ from each other with respect to target lysine selection.

In our experimental models, deletion of the C-terminal splicing tail or the caspase-like domain substantially stabilized c-FLIP. A possible explanation for this phenomenon is that these regions may provide a binding platform for E3 ubiquitin ligases. Although we have not addressed this question, our hypothesis was supported by the identification of Itch as the E3 ligase that specifically ubiquitylates c-FLIP$_L$ through interaction with the caspase-like domain (Chang et al. 2006). Recently, c-Cbl was reported to bind and ubiquitylate c-FLIP$_S$ upon mycobacteria infection-induced, TNFα-mediated apoptosis (Kundu et al. 2009). These results are in line with our observations, while other findings have indirectly challenged our idea. Golks and colleagues previously described c-FLIP-p22, a proteolytic product of caspase-8-mediated c-FLIP cleavage, which is generated in the absence of death receptor stimulation and activates NF-κB in malignant cells (Golks et al. 2006). The p22 fragment corresponds to amino acids 1-196 and should therefore be very stable in the light of our results, but the exogenous p22 was downregulated in Boe^R cells by cycloheximide within 4 hours. It is feasible that the malignant T and B cell lines expressing p22 have acquired additional mechanisms to regulate its turnover to be able to adjust the NF-κB activity. In

conclusion, C-terminal regions are essential for the regulation of c-FLIP stability, and they have been shown to attract ubiquitin ligases in an isoform-specific manner.

3 Regulation of TRAIL-mediated apoptosis by PKC-mediated c-FLIP phosphorylation (III)

3.1 Regulation of death receptor signaling by protein kinase C (III)

Post-translational modifications form complex networks. Phosphorylation is involved in almost every imaginable biological process, and it is perhaps the best characterized of all PTMs. It has been estimated that 30% of all proteins are targets for phosphorylation, and individual substrates are often targeted at several sites (reviewed by Pinna and Ruzzene 1996, Cohen 2000). Members of the protein kinase C family have been found to modulate death receptor-mediated apoptosis on multiple levels (reviewed by Tran et al. 2004). Unlike the classical family members PKCα and PKCβ, PKCε and PKCδ have not been found to act at receptor level, although they are often connected to receptor-mediated cell death. In Jurkat T cells, cPKC interferes with the formation of the CD95 DISC by blocking FADD recruitment and subsequent caspase-8 activation (Gomez-Angelats et al. 2000, Gomez-Angelats and Cidlowski 2001). PKC activation has also been observed to inhibit FADD recruitment to the TRAIL-DISC in HeLa cells (Harper et al. 2003). However, as these effects were not due to PKC-mediated FADD phosphorylation, their specific mechanism has remained unclear. The atypical PKCζ negatively regulates CD95-induced apoptosis in Jurkat cells by preventing FADD recruitment to the DISC (de Thonel et al. 2001, Leroy et al. 2005). However, it is not known to which extent these effects rely on the kinase activity of PKCζ. In addition, PKCα and PKCζ mediate opposing effects on cigarette smoke extract-induced apoptosis in human lung fibroblasts (Park et al. 2008). Collectively, these reports suggest that PKC modulates death receptor signaling both positively and negatively, but the underlying mechanisms of action are still largely unknown.

Recently, flavopiridol, an inhibitor of cyclin-dependent kinases currently undergoing clinical trials, was shown to sensitize breast tumor cells to TRAIL by inducing proteasome-dependent degradation of c-FLIP (Palacios et al. 2006). Also casein kinase inhibition has been shown to downregulate c-FLIP in a proteasome-dependent manner (Llobet et al. 2008). When this thesis was initiated, little was known about the mechanisms and biological implications of c-FLIP phosphorylation. We were especially interested in deciphering if there was a link between the modification of c-FLIP with ubiquitin and phosphorylation. Indeed, we found that the two modes of post-translational regulatory mechanisms cooperate to accommodate c-FLIP stability. We discovered a PKC-phosphorylated serine that specifically regulates the stability of the short c-FLIP isoforms, thereby describing a novel link between death receptor-mediated apoptosis and the classical PKC.

3.2 c-FLIP is phosphorylated on serine 193 (III)

c-FLIP proteins are targeted by several post-translational modifications including phosphorylation. The reports made hitherto indicate that many signaling pathways are involved in c-FLIP phosphorylation, and its physiological function varies according to the stimulus and the cell model. To resolve whether c-FLIP was regulated by phosphorylation in our model system, we treated K562 cell lines stably overexpressing c-FLIP$_S$ or c-FLIP$_L$ with the phosphatase inhibitor calyculin A and performed ^{32}P *in vivo* orthophosphate labeling.

Using electrophoresis and autoradiography, we detected clear signals of both isoforms, indicating that c-FLIP proteins are phosphorylated *in vivo* (Figure 1A, III). As no one had identified phosphorylated residues of c-FLIP when this study was initiated, we performed tryptic phosphopeptide mapping and mass spectrometric analyses to identify the sites. Indeed, serine 193 (S193) was revealed a novel phosphorylation site common to both c-$FLIP_S$ and c-$FLIP_L$ (Figure 1B-G, III). To continue the biochemical analyses on this site, we developed a polyclonal antibody against phosphorylated S193. Use of our antibody allowed the detection of overexpressed, immunoprecipitated c-FLIP in K562 cells treated with different phosphatase inhibitors (Figure 2A, III, and data not shown), but its titer was not high enough to detect endogenous phosphorylated proteins.

3.3 Serine 193 phosphorylation is mediated by PKCα and PKCβ (III)

S193 is conserved in all c-FLIP proteins and present also in the initiator caspase-10, but not in caspase-8. The amino acids surrounding S193 correspond to a PKC consensus sequence, which led us to detect the phosphorylation status of c-FLIP serine 193 after manipulating the activity of different PKCs. We treated cells with several PKC inhibitors and an activator, and found that S193 phosphorylation was differentially modulated in response to some of these agents. Importantly, we found that GÖ6976, an inhibitor of the classical PKC isoforms PKCα and PKCβ, considerably decreased S193 phosphorylation (Figure 2B, III). This effect was somewhat stronger in c-$FLIP_S$, which may either be due to c-$FLIP_L$ phosphorylation by additional kinases that are not inhibited by GÖ6976, or the differential affinity of the phosphopeptide antibody to the c-FLIP isoforms. In addition to GÖ6976, we also studied phosphorylation of S193 upon treatment with the pseudosubstrate of PKCα and PKCβ (data not shown), and obtained results similar to those with GÖ6976, supporting our hypothesis on PKC-mediated phosphorylation of c-FLIP S193. Remarkably, the phosphorylation of S193 was greatly enhanced by treatment with TPA, an activator of the classical and the novel PKCs (Figure 2C, III). A great increase in S193 phosphorylation was evident when the cells were treated both with TPA and calyculin A, but TPA also allowed the detection of S193 phosphorylation without phosphatase inhibition (Figure 2C, III). Similar results were obtained with c-$FLIP_R$, indicating that S193 phosphorylation of both short c-FLIP isoforms is regulated in a similar manner (Figure 2E, III). Furthermore, K562 cells stably overexpressing c-FLIP proteins were pretreated with GÖ6976 before treatment with TPA in order to see to what degree the TPA-mediated enhanced phosphorylation was due to PKCα and PKCβ, the kinases primarily inhibited by GÖ6976. Indeed, TPA-mediated phosphorylation of c-$FLIP_S$ was almost completely abolished by GÖ6976 pretreatment, indicating that the phosphorylation of c-$FLIP_S$ S193 is mainly mediated by PKCα and PKCβ (Figure 2D, III). The phosphorylation of c-$FLIP_L$ S193, in turn, was decreased by GÖ6976 pretreatment, but to a lesser degree than the phosphorylation of c-$FLIP_S$, implying that c-$FLIP_L$ is likely to be subject to phosphorylation by a wider array of kinases than c-$FLIP_S$ (Figure 2D, III).

We utilized genetic tools to ensure the specificity of our results obtained by the pharmacological PKC inhibitors. We transfected c-FLIP-expressing K562 cells with kinase-dead, GFP-tagged PKCα and PKCβ, treated cells with TPA and calyculin A, and monitored S193 phosphorylation. We expected the kinase-dead PKCs to act as dominant negative inhibitors of the endogenous PKCs upon TPA-induced activation and to thereby interfere with the induction of c-FLIP phosphorylation. Indeed, in line with our hypothesis, we detected a decrease in S193 phosphorylation levels in both c-$FLIP_S$ and c-$FLIP_L$ (Figure 2F, III), supporting our previous data. As the co-expression with PKC-GFP sometimes reduced the expression level of FLAG-c-FLIP, we normalized the signal from phospho-S193 to c-FLIP levels in the lysate in order to more accurately estimate the changes. Consequently,

as the co-expression of c-FLIP and PKC-GFP constructs was challenging, we were unable to express both PKCα and PKCβ together with c-FLIP in order to see how dual PKC inhibition would affect S193 phosphorylation.

Previous reports have suggested that c-FLIP is a substrate for CaMKII (Yang et al. 2003) and Akt/PI3K (Shi et al. 2009). However, we did not detect changes in S193 phosphorylation after treating cells with the CaMKII inhibitors KN-63 and KN-92 or the PI3K inhibitor LY29400, suggesting these pathways do not target S193. In addition, acknowledging that TPA is an activator of the novel PKCs and the MAP kinase family, the phosphorylation of S193 was monitored after treatment with Rottlerin, an inhibitor of PKCδ and PKCθ (Gschwendt et al. 1994) which is known to have some unspecific effects on MAPK and PKA (Davies et al. 2000), or with more specific MAPK inhibitors (data not shown). We could not observe any changes in S193 phosphorylation in response to Rottlerin or the MAPK inhibitors, indicating that the effect of TPA is likely to be mediated via the classical PKCs, possibly even by direct phosphorylation.

3.4 Serine 193 mutations have isoform-specific implications on c-FLIP ubiquitylation and stability (III)

In article I, we showed that the C-terminal regions are pivotal in regulating ubiquitylation and stability of the c-FLIP proteins. Specifically, we found that the primary ubiquitin acceptors in c-FLIP$_S$ during erythroid differentiation are K192 and K195, surrounding S193. We were therefore prompted to investigate whether S193 phosphorylation influenced c-FLIP ubiquitylation. We performed site-directed mutagenesis to mutate S193 into a non-phosphorylatable alanine or into aspartate to mimic constitutive phosphorylation, and assayed c-FLIP ubiquitylation by immunoprecipitation and Western blotting. Interestingly, the S193A mutation increased the ubiquitylation of all c-FLIP isoforms (Figure 3A-C, III). In contrast, the phospho-mimetic S193D mutation efficiently decreased the ubiquitylation of the short c-FLIP proteins (Figure 3A-B, III), while c-FLIP$_L$ S193D was ubiquitylated similarly to wild type c-FLIP$_L$ (Figure 3C, III).

Mutations of S193 may have an impact on the in the α-helical region of c-FLIP$_S$. It is possible that while S193A mutation probably does not significantly alter the properties of the α helix, S193D mutation creates a negative charge, and could thereby interfere with the potential interactions mediated by the α helix. While according to our modeling, K192 and K195 are susceptible for ubiquitylation in the native helix, they could be less favorably positioned in the S193D mutant, thereby inhibiting ubiquitylation. Therefore, it was important to detect c-FLIP ubiquitylation after GÖ6976 treatment to see if inhibition of S193 phosphorylation led to increased ubiquitylation. We found that c-FLIP$_L$ and c-FLIP$_R$ were, indeed, more ubiquitylated in response to GÖ6976 treatment. Moreover, the ubiquitylation of c-FLIP$_R$ S193D mutant was not affected by GÖ6976 treatment (Figure 2E, III), confirming that the change in ubiquitylation truly was due to changes in the phosphorylation state of S193. As c-FLIP$_L$ does not contain an α helix similar to c-FLIP$_S$, and the ubiquitylation of all isoforms was affected by S193 mutations as well as treatment with GÖ6976, it seems likely that changes in ubiquitylation are not mutation artifacts, but regulated by the phosphorylation status of S193. In addition, GÖ6976-induced ubiquitylation was more pronounced in c-FLIP$_L$ than in c-FLIP$_R$, indicating that phosphorylation is likely to diminish the ubiquitylation of all c-FLIP proteins, although its effects may differ among the short and the long isoforms. Given our previous results and the model on the C-terminal structure of c-FLIP$_S$, S193 phosphorylation probably modulates ubiquitylation sterically by inhibiting the exposure of K192 and K195. Nevertheless, how S193 affects the ubiquitylation of c-FLIP$_L$ is more difficult to hypothesize. It is conceivable that despite the lack of the α helix,

phosphorylation on S193 affects the folding of the caspase-like domain and thereby inhibits ligase binding, correct positioning of the target lysine(s), or both. The physical proximity of these binding sites or sites of modification is not necessarily required, since post-translational modifications have been described to affect interactions and other modifications also outside its instant vicinity.

After establishing that S193 phosphorylation indeed regulates c-FLIP ubiquitylation, we wanted to determine whether the C-terminal areas were required for S193-mediated effects. For this purpose, we performed site-directed mutagenesis using the deletion mutant c-FLIP1-202 as a template, producing the c-FLIP1-202 S193A mutant, which is prone to ubiquitylation, but lacks the crucial C-terminal structures. Our ubiquitylation assays showed that in the absence of the C-terminal part, c-FLIP cannot be ubiquitylated (Figure 2D, III). The C-terminal tail was not important for S193 phosphorylation, because c-FLIP1-202 was efficiently phosphorylated *in vivo* (data not shown). This indicates that while the C-terminal parts of c-FLIP are not needed for kinase recruitment, they are required for ubiquitylation. In addition, we wanted to examine the effect of S193 phosphorylation on ubiquitylation of c-FLIP$_S$ in the absence of the primary target K192 and K195. Surprisingly, when S193A mutation was introduced into the c-FLIP$_S$ K192,195R mutant, c-FLIP$_S$ was still more ubiquitylated than wild type (data not shown). This indicates that dephosphorylation is able to impose strong destabilizing effects on both c-FLIP proteins, even when the principal target lysines are unavailable. These data further underline the importance of S193 phosphorylation in determining c-FLIP ubiquitylation.

Targeting proteins to degradation is the most thoroughly described function of ubiquitylation. However, ubiquitin conjugation may also serve signaling purposes. To decipher if c-FLIP was a subject for K48-linked or other kind of ubiquitin conjugation, we determined the effect of S193 phosphorylation on the half-lives of c-FLIP isoforms. We therefore transfected K562 cells with low amounts of wild type and phosphomutant c-FLIP$_S$, c-FLIP$_R$ and c-FLIP$_L$ to mimic endogenous expression levels. This aim was met with c-FLIP$_L$, whose endogenous levels are higher, but we were unable to achieve such levels with c-FLIP$_S$ and c-FLIP$_R$, as their endogenous expression is very low. Nevertheless, we downregulated the translation of the c-FLIP constructs by cycloheximide to compare their half-lives (Figure 4A-C, III). Remarkably, mutations of S193 did not affect the half-live of c-FLIP$_L$ at all, suggesting that the function of S193 phosphorylation of c-FLIP$_L$ is unrelated to protein degradation (Figure 4C, III). In contrast, S193A mutation decreased the half-lives of both c-FLIP$_S$ and c-FLIP$_R$, whereas S193D mutation extended their half-life (Figure 4A-B, III). These results indicate that phosphorylation on S193 specifically affects the stability of the short c-FLIP proteins, while regulating the ubiquitylation of c-FLIP$_L$ in an unrelated fashion. To further solidify these findings, we performed ^{35}S metabolic labeling in K562 cell lines stably overexpressing c-FLIP$_S$ or c-FLIP$_L$. Again, due to the high levels of overexpression in the cell lines in question, the half-lives were longer than the endogenous ones, and we therefore detected c-FLIP levels at relatively long time points. Metabolic labelings yielded similar results as the cycloheximide chases: c-FLIP$_S$ S193A mutant was destabilized compared to wild type c-FLIP$_S$, whereas S193D displayed a longer half-life (Figure 4D, III). Furthermore, the stability of c-FLIP$_L$ was unaffected by S193 mutations also in this experimental setting (Figure 4E, III). Together, our results show that while phosphorylation on S193 affects the ubiquitylation of all c-FLIP isoforms, it specifically regulates the stability of the short c-FLIP proteins.

In addition to establishing a role for S193 phosphorylation in c-FLIP stability, we wanted to characterize its function in death receptor responses. We determined S193 phosphorylation upon TRAIL and TNF receptors stimulation, and found, intriguingly, that both TRAIL (Figure 5A, III) and TNFα (Figure 5B, III) increased S193 phosphorylation when combined with phosphatase inhibition, presumably leading to the stabilization of c-FLIP. The effect was more pronounced after TNFα treatment, possibly resulting from the stronger activation of

the cPKCs upon TNF receptor stimulation compared to TRAIL receptor stimulation. Death receptor stimulation may affect c-FLIP phosphorylation secondarily via signals transmitted from the receptor signaling complexes, resulting in increased PKC activity. These survival pathways are primarily mediated via the TNF receptor system, although low-level stimulation of the CD95 and the TRAIL receptor systems have also been reported to convey survival signals (reviewed by Siegel et al. 2000, Falschlehner et al. 2007). Alternatively, it is conceivable that the recruitment of c-FLIP to death receptor complexes augments direct physical interaction with active cPKCs, especially since cPKCs are recruited to cellular membranes during their activation process (reviewed by Greiner and Kazanietz 2007). The latter explanation is perhaps more likely in the case of TRAIL-mediated phosphorylation, because upon TNF receptor stimulation, c-FLIP is not recruited to the complex 1 at the membrane, but to the cytosolic complex 2 (Micheau and Tschopp 2003).

Since c-FLIP is a potent inhibitor of caspase-8 activation and a critical regulator of cell death, a crucial question is whether S193 phosphorylation affects these functions. Ueffing and coworkers reported that the C-terminal part (aa186-215) of murine c-FLIP$_R$ facilitated DISC binding, thereby raising the possibility that S193 was involved in regulating DISC assembly (Ueffing et al. 2008). To study this hypothesis, we analyzed the recruitment of wild type and phosphomutant c-FLIP proteins to the TRAIL DISC. According to our results, however, S193 phosphorylation does not affect DISC binding properties, as the c-FLIP mutants were recruited to the DISC at even efficiency, correlating with the relative expression levels of the stably overexpressing K562 cell lines (Figure 5C, III). This finding is logical, since S193 resides outside the DEDs responsible for DISC binding (Scaffidi et al. 1999). To ascertain whether S193 phosphorylation affects caspase-8 activation in the DISC, we analyzed the caspase-8 activity in the TRAIL DISC induced in cell lines stable overexpressing wild type, S193A or S193D c-FLIP$_S$ or c-FLIP$_L$. In accordance with our previous results and data published by others, we found that c-FLIP$_S$ is a potent inhibitor of caspase-8, whereas c-FLIP$_L$ allows or may even accentuate the activation of caspase-8 in the DISC (Figure 5D, III). S193 mutations did not, however, alter these features of the c-FLIP proteins. Statistically insignificant changes were detected among individual overexpressing clones, again correlating with their relative overexpression levels. These data suggest that S193 phosphorylation does not directly affect the ability of c-FLIP proteins to bind to the DISC or to regulate caspase-8 activation within the DISC.

Although S193 phosphorylation does not qualitatively affect the potential of c-FLIP to inhibit apoptosis, its isoform-specific stabilizing effects are likely to have profound implications on cell death sensitivity. To demonstrate that the prolonged half-life of c-FLIP$_S$ following S193 phosphorylation eventually results in delayed progression of apoptosis, we monitored apoptosis markers during cycloheximide-induced downregulation of wild type and S193A c-FLIP$_S$ (Figure 5E, III). Indeed, during the rapid degradation of S193A c-FLIP$_S$, we saw the cleavage of caspase-8 and PARP1 occurring earlier than in wild type c-FLIP$_S$ overexpressing cells. This result suggests that since S193 phosphorylation regulates the stability of the short c-FLIP isoforms, it is a pro-survival modification with physiologically relevant consequences. A simplified model of S193 phosporylation-directed events is depicted in Figure 15.

Our studies on S193 phosphorylation and c-FLIP stability add to understanding how malfunction in c-FLIP regulation may contribute to the pathogenesis of cancer. Abnormally high levels of c-FLIP have been reported to promote carcinogenesis and protect cancer cells from death receptor-mediated apoptosis (Djerbi et al. 1999, Medema et al. 1999). It can therefore be speculated that accumulating mutations that stabilize c-FLIP, either directly or indirectly, would offer malignant cells a selection advantage over cells that express lower amounts of wild type c-FLIP and are therefore more susceptible to apoptosis.

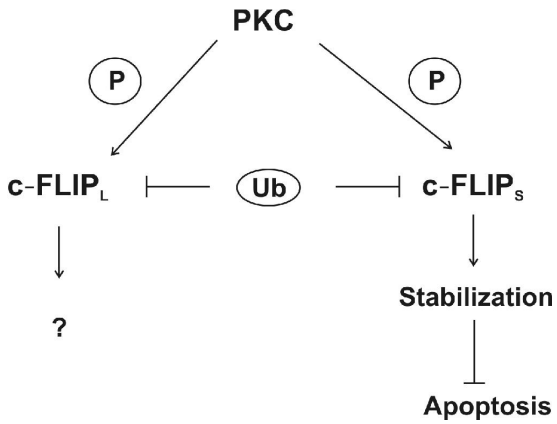

Figure 15. PKC-mediated phosphorylation of S193 counteracts c-FLIP ubiquitylation, but only prolongs the half-life of the short c-FLIP isoforms. Stabilization of c-FLIP$_S$ prevents caspase-8 activation and apoptosis. Although the phosphorylation of S193 also seems to protect c-FLIP$_L$ from ubiquitylation, it does not regulate the stability of c-FLIP$_L$. The biological function of c-FLIP$_L$ phosphorylation on S193 is currently unknown.

An interesting question, largely left unanswered by our studies, is the biological function of c-FLIP$_L$ S193 phosphorylation. Our investigations revealed that c-FLIP$_L$ is ubiquitylated in the absence of S193 phosphorylation, but the biological function of this modification is yet to be established. It is worth emphasizing that the ubiquitylation assay used in these studies does not disclose the type of ubiquitin chain c-FLIP is modified with. As the enhanced ubiquitylation of c-FLIP$_L$ S193A mutant did not affect the stability of the protein, it can be speculated that the mutation exposes c-FLIP$_L$ to regulatory ubiquitylation, conjugation of K63-linked ubiquitin chains for instance, instead of K48 chains favored by the proteasome. In this case, K63-linked chains could mediate binding to other proteins, perhaps even non-covalently via ubiquitin-interacting domains. Recent body of evidence has shown that the ubiquitin interacting motifs display an astounding specificity against ubiquitin chain linkage types, and that many players of the NF-κB pathway, for example, are modified by non-degradative ubiquitylation. Some of these proteins, RIP for instance, are established interaction partners of c-FLIP and might therefore be especially interesting in this respect. In our future studies, we will utilize mass spectrometric approaches to define the potential binding partners that specifically interact with non-S193-phosphorylated c-FLIP$_L$, and to elucidate which interactions are brought on or disrupted by physiological cues that affect S193 phosphorylation. Furthermore, antibodies that specifically recognize K48- or K63-linked ubiquitin chains have recently been developed (Newton et al. 2008), hopefully opening up new opportunities to study the ubiquitylation of c-FLIP.

While examining the protein sequences of c-FLIP, we found that the c-FLIP proteins contain a potential UIM (Figure 16). A functional UIM usually contains an acidic patch (..EDE..) N-terminal to sequence L-X^3-A-X^3-S-X^2-D. c-FLIP proteins contain a sequence that conforms to the UIM core, but lacks the acidic patch, raising the question whether the motif is functional. However, because S193 is one of the potential UIM residues, we wanted to find out whether the UIM was functional and if so, would phosphorylation of S193 regulate it. In preliminary pulldown analyses utilizing GST, single moieties of GST-ubiquitin, or ubiquitin agarose as bait, the putative UIM of c-FLIP$_{S/L}$ did not appear to bind ubiquitin noncovalently (Aura Kaunisto, unpublished data). In the light of our preliminary results and the fact that the acidic patch is missing from the c-FLIP sequence, it seems unlikely that the putative UIM of c-FLIP would be functional *in vivo*. Nevertheless, ubiquitin binding domains and motifs have preferences towards certain forms of ubiquitin, such as monoubiquitin, linear diubiquitin, K48-linked or K63-linked chains (reviewed by Ikeda and Dikic 2008). Therefore, further experiments could be conducted to determine whether c-FLIP is able to specifically bind linear, K48-linked or K63-linked ubiquitin chains.

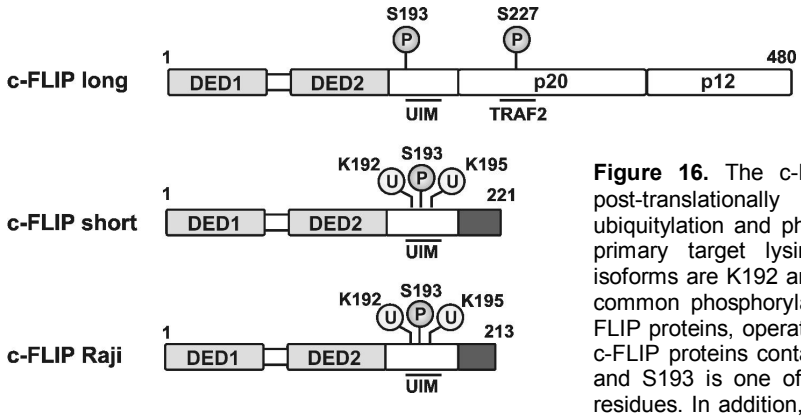

Figure 16. The c-FLIP isoforms are post-translationally modified by ubiquitylation and phosphorylation. The primary target lysines of the short isoforms are K192 and K195. S193 is a common phosphorylation site for all c-FLIP proteins, operated by the PKC. All c-FLIP proteins contain a putative UIM, and S193 is one of the potential UIM residues. In addition, we have identified another phosphorylation site, S227, in c-FLIP$_L$. S227 is a part of a putative TRAF2-binding domain. The figure is not drawn to scale.

As evidenced by the differential biological outcome of c-FLIP phosphorylation on S193, isoform-specific functions of c-FLIP can be adjusted by post-translational modifications. An open question is whether also other phosphorylation events contribute to isoform-specific functions of c-FLIP. While investigating the phosphorylation sites of c-FLIP proteins, we found one phosphorylated serine specific to c-FLIP$_L$ (Vitaly Kochin, PhD thesis). S227, located in the caspase-like domain (Figure 16), was found to be phosphorylated in our *in vivo* labeling experiments in K562 cells. According to computer-based prediction programs, the sequence surrounding S227 could potentially be recognized by PKC, CKII or GSK3. Intriguingly, the sequence S-I-Q-E also fills the minimal requirements for a TRAF2-binding domain. Interestingly, c-FLIP$_L$ has been reported to interact with TRAF2 to promote NF-κB (Kataoka et al. 2000). Similarly, the KSHV v-FLIP reportedly contains an interaction motif that is absent from c-FLIPs, but mediates the binding of v-FLIP to TRAF2 and TRAF3 to promote NF-κB and JNK signaling (Guasparri et al. 2006). It is therefore tempting to speculate that S227 phosphorylation may be involved in regulating c-FLIP$_L$-TRAF2 interaction. We are currently characterizing the role of S227 phosphorylation in the interaction between c-FLIP$_L$ and TRAF2, as well as its potential implications in NF-κB and JNK signalling. In addition, identifying the signaling pathways governing S227 phosphorylation warrants further studies.

Phosphorylation is known to regulate ubiquitylation and protein stability. In many cases, phosphorylation promotes ubiquitylation by providing a binding site for E3 ubiquitin ligases targeting phosphorylated substrates. For example, the tumor suppressor and transcription factor p53 is phosphorylated on S362 and S366 by IKKβ, which leads to ubiquitylation by the E3 ligase SCF$^{\beta\text{-TrCP}}$ and subsequent proteasomal degradation. Another example of a substrate that is recognized by an E3 ligase due to phosphorylation is cyclin E, whose multi-site phosphorylation promotes the binding of the SCF^{Fbw7} ubiquitin ligase (Ye et al. 2004, Xia et al. 2009).

Recent studies have begun to elucidate how the concerted phosphorylation and ubiquitylation jointly regulate the stability of c-FLIP. The c-FLIP$_L$-specific E3 ubiquitin ligase Itch is under the control of the JNK pathway, and the ligase is active upon mild JNK activity (Chang et al. 2006). The JNK pathway is also activated during hemin-induced differentiation of the K562 cells, although JNK activity is not required for TRAIL sensitization (Minna

Poukkula and Ville Hietakangas, unpublished results). Considering the specificity of Itch, it can be speculated that Itch would contribute to c-FLIP$_L$ downregulation during erythroid differentiation. It is also conceivable that c-FLIP$_L$ stability is adjusted by other phosphorylation events. During macrophage activation, the Akt1-mediated phosphorylation of c-FLIP on S273 has been reported to mediate proteasomal degradation of c-FLIP$_L$ (Shi et al. 2009). This is perhaps surprising considering that the PI3K/Akt pathway positively regulates the transcription of c-FLIP, but the destabilizing modification of c-FLIP by PI3K/Akt could also be viewed as a negative feedback loop. Finally, shortly after our study, two additional phosphorylation sites were identified in murine c-FLIP$_S$/c-FLIP$_R$. In this report, Kundu and coworkers (2009) described S4 and Y211 of murine c-FLIP$_S$ to be phosphorylated by p38 and c-Abl, respectively. Intriguingly, both of these phosphorylation events were required to facilitate the interaction between murine c-FLIP$_S$ and the E3 ligase c-Cbl. Y211 is located in the C-terminal splicing tail, and could therefore in part explain its importance for the rapid degradation of the human short c-FLIP isoforms, as the tyrosine residue is present in both. Whether S4 is phosphorylated in c-FLIP$_L$, or if it plays a role in regulating the stability of c-FLIP$_L$, requires further studies. In summary, these recent developments highlight the importance of correct regulation of c-FLIP levels and demonstrate that the stability of c-FLIP is, indeed, determined isoform-specifically by a complex interplay of post-translational modifications, especially phosphorylation and ubiquitylation.

The ability to resist apoptotic stimuli is a characteristic feature of a cancer cell, and the dynamic adjustment of death receptor signaling is essential for maintaining homeostasis in the immune system. Our results provide insights into how targeting of c-FLIP by phosphorylation and ubiquitylation affects c-FLIP levels and thereby determines death receptor sensitivity. These findings may have implications on the development of cancer therapies, as c-FLIP levels are upregulated in various malignancies that are resistant towards conventional treatments. In addition, our data may help understand the pathogenesis of various autoimmune diseases, since they are often characterized by abnormally low levels of c-FLIP. Furthermore, new aspects are presented how cPKC might contribute to the biological processes that function in an aberrant fashion in these conditions.

CONCLUDING REMARKS

Cells sense their environment by their surface receptors. The family of death receptors includes the TRAIL and CD95 receptors, which transmit signals engaging cellular suicide. c-FLIP proteins antagonize receptor-mediated cell death by regulating the activation of caspase-8. Since abnormal levels of c-FLIP are indicated in various autoimmune diseases and cancer, it is a potential therapeutic target. In this thesis, I have investigated how c-FLIP levels are adjusted through post-translational modifications, with a strong emphasis on phosphorylation and ubiquitylation.

Post-translational modifications provide means for rapid and exact modulation of crucial regulatory proteins and play an important role also in the molding of death receptor responses. When this thesis project was initiated, little was known about the regulation of c-FLIP via ubiquitylation or phosphorylation. During the work for this thesis, we established that while all c-FLIP proteins are ubiquitylated, the stability of the isoforms is governed by their unique C termini. These mechanisms are not restricted to only one cell model, since we were able to show that the differential regulation of c-FLIP isoforms determines death receptor sensitivity both in differentiating K562 erythroid leukemia cells as well as activated Jurkat and primary T lymphocytes. We were the first to identify phosphorylation sites of c-FLIP and develop an antibody that specifically recognizes S193-phosphorylated c-FLIP. Furthermore, we showed that the phosphorylation of S193 was mediated by classical PKC, and demonstrated that PKC-mediated S193 phosphorylation negatively regulates the ubiquitylation of all c-FLIP proteins, although it only affects the half-lives of the short c-FLIP isoforms. In the future, the post-translational modifications of c-FLIP and their effective biological significance should be studied in primary cells. These prospective endeavors would greatly benefit from the development of antibodies against individual post-translational modifications of c-FLIP, especially if they allowed the monitoring of endogenous c-FLIP. Our results underline the importance of c-FLIP stability in modifying death receptor sensitivity and especially in determining immune cell homeostasis.

During the course of these studies, other groups have discovered three additional phosphorylation sites, and two E3 ubiquitin ligases have been demonstrated to regulate c-FLIP turnover in an isoform-specific fashion, supporting our findings and hypotheses. In addition, independent studies have concluded that the stability of c-FLIP is adjusted by a cross-talk between phosphorylation and ubiquitylation. When the pathways and enzymes governing these events are characterized in more detail, c-FLIP will probably be even more closely linked to the key signaling complexes regulating cellular death and survival.

Due to its important role in determining sensitivity to apoptotic signals, c-FLIP has been considered a potential therapeutic target for treating cancer and diseases of the immune system. While TRAIL is perhaps one of the most promising candidates for inducing apoptosis in cancer cells, some tumors are resistant to TRAIL because of c-FLIP overexpression. The first steps in conferring this resistance by specific c-FLIP downregulation by RNAi, or by more general means such as protein synthesis inhibitors or existing chemotherapeutic drugs, have been successful and validated c-FLIP as a potential therapeutic target. Therefore, forthcoming studies addressing the regulation of c-FLIP stability and its roles in diverse signaling pathways are warranted.

ACKNOWLEDGEMENTS

This thesis work was performed during 2005-2009 at the Turku Centre for Biotechnology, University of Turku and Åbo Akademi University. I want to give thanks to Professor Riitta Lahesmaa for running such a fine research institute and for providing excellent facilities. I would like to thank the staff of CBT for maintaining all the vital functions from technical maintenance to computational assistance. I am grateful to Professor Mikko Nikinmaa and the Laboratory of Animal Physiology, University of Turku, for guiding and supporting me from my undergraduate years to this day. Professor Olli Lassila and the Turku Graduate School of Biomedical Sciences are acknowledged for their support - it has been a priviledge to be a part of this congenial community.

I am most indebted to Professor John Eriksson for welcoming me into his research group when I was a very young student and for keeping me employed all this time. John has never failed to support me in my efforts to develop as a scientist, or to offer encouragement in times of uncertainty. Professor Lea Sistonen is acknowledged for showing genuine interest in my work. I have also enjoyed the occasional chats stemming from our mutual interest in classical music. I especially appreciate the effortless way the Eriksson and Sistonen labs work together. It has had a unique impact on my life in many ways.

I want to express my gratitude to Professor Jannie Borst and Docent Päivi Ojala for reviewing this thesis, providing useful comments and ideas, and for formulating their critique in an utmost positive manner. I would also like to give many thanks to my superb supervisory committee, Professor Marja Jäättelä, Professor Dan Lindholm, and Docent Carina Holmberg-Still. I have been encouraged, uplifted and inspired by our annual meetings.

PhD is never an achievement of an individual, but of a group of people. I want to thank my co-authors Tomoko Asaoka, Tuire Colliander (née Katajamäki), Konstantin Denessiouk, John Eriksson, Ville Hietakangas, Mark Johnson, Vitaly Kochin, Annika Meinander, Andrey Mikhailov, Minna Poukkula, Lea Sistonen and Thomas Söderström for their contributions and support.

Long hours in the lab would have been much more difficult to endure, had I not been able to share them with such a fabulous crowd of people. Thanks to Johanna Ahlskog, Julius Anckar, Tomoko Asaoka, Johanna Björk, Henri Blomster, Marek Budzynski, Zhanna Chitikova, Aurelie de Thonel d'Orgeix, Alexandra Elsing, Saima Ferraris, Tao He, Eva Henriksson, Ville Hietakangas, Claire Hyder, Susumu Imanishi, Kimmo Isoniemi, Juha Kastu, Vitaly Kochin, Julia Lindqvist, Annika Meinander, Andrey Mikhailov, Mikko Nieminen, Hanna-Mari Pallari, Emilia Peuhu, Minna Poukkula, Mika Remes, Stephanie Robbins-Tran, Pia Roos-Mattjus, Cecilia Sahlgren, Anton Sandqvist, Heidi Stykki, Thomas Söderström, Elin Torvaldson, Anniina Vihervaara, Malin Åkerfelt and Päivi Östling. Special thanks to Helena Saarento for invaluable technical assistance.

I was very lucky to begin my thesis work under the guidance of Minna Poukkula, who has supported me throughout the project and everything that comes along. Not only has Minna been a wonderful mentor, but also a great friend, and I have been happy to find that this has not changed even when we reside in different continents. Special thanks for giving excellent comments and suggestions regarding this thesis! In addition, I want to give sincere thanks to Ville Hietakangas, who is also a friend and a mentor, and probably the only person who I can always count on to ease my better half's scientific anxiety when it hits him.

I want to thank Annika Meinander for joining forces with me more than once and for introducing me to many enjoyable scientists I probably would not have gotten to know otherwise. Thanks for standing up for your junior colleague in times of trouble, even during the small hours of the morning on the last day of a heavy conference! Annika is also acknowledged for providing important comments and suggestions for this thesis. During the past few years, I have been happy to see the new generation of us FLIP girls arise. Thanks to Tomoko Asaoka for your hard work, dedication, wonderful personality, and tolerance for my sense of humor. I am looking forward to our trip to Japan (as well as a few more sushi nights)! Special thanks to Emilia Peuhu for being my blonde twin and traveling companion in many meetings - it has been good fun! Thank you Johanna Ahlskog and Hanna-Mari Pallari for regularly arranging our Friday night specials filled with food, wine, girltalk and - first and foremost - top class Finnish entertainment that so few seem to appreciate these days.

Kiitos, rakkaat Tytöt, korvaamattomasta ystävyydestänne! Olen saanut siitä voimaa sekä hyvinä että huonoina päivinä. Töistä, velvollisuuksista ja välimatkasta huolimatta on näihin vuosiin mahtunut lukemattomia mahtavia yhteisiä kokemuksia, joilla ei (onneksi) ole mitään tekemistä solubiologian kanssa. Lisää niitä!

Tämä kirja on omistettu perheelleni sanan laajassa merkityksessä. Suurimmat kiitokset kuuluvat äidille ja isälle, jotka ovat kannustaneet minua tämänkin projektin ajan ja jaksaneet samalla muistuttaa, että vaikka työnteko olisi miten mielenkiintoista, ei se ole elämän tärkein asia. Kiitos Kallelle piristävästä asenteesta, hyvistä keskusteluista ja siitä, että tämän syyn varjolla saan sinut narrattua Turkuun toisen kerran puolen vuoden sisällä! Rakkaat kiitokseni Ritva-mummulle kullanarvoisesta tuesta ja suvun vanhimman näkökulmasta elämänmenoon. Jag vill också tacka Maja och Dag för att ha tagit mig in i familjen Anckar och för deras hjälp med all organisering som ingår i min disputation och doktorsmiddag.

Kiitos Julle, että tänäänkin olet elämänkumppanini, paras ystäväni ja Pohjantähteni.

Financial support from the Turku Graduate School of Biomedical Sciences, the Magnus Ehrnrooth Foundation, the Cancer Research Foundation of Southwestern Finland, the Medical Research Foundation “Liv och Hälsa”, the K. Albin Johansson Foundation, and the Cultural Foundation of Southwestern Finland are gratefully acknowledged.

Turku, October 2009

Aura Kaunisto

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