THE ROLE OF THE LINEAR UBIQUITIN CHAIN ASSEMBLY COMPLEX (LUBAC) IN DEATH RECEPTOR SIGNALLING

A DISSERTATION
SUBMITTED TO THE DEPARTMENT OF MEDICINE OF IMPERIAL COLLEGE LONDON
IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Stefanie Cordier

October 2011
Abstract

TNF is a cytokine with important functions in inflammation and immunity. Binding to its receptor TNF-Receptor-1 (TNFR1) induces the formation of a signalling platform which leads to TNF-target gene transcription and consequently the induction of cellular immune responses. To understand and possibly influence the effects observed upon TNF stimulation, it is important to define the molecular machinery responsible for signal transduction. In order to resolve the composition of the TNF-Receptor signalling complex (TNF-RSC) our group developed a highly sensitive modified tandem affinity purification method (moTAP). Using this technique, we recently reported on the recruitment of the two In-Between-RING (IBR) family members HOIL-1 (heme-oxidized IRP2 Ub ligase-1) and HOIP (HOIL-1 interacting protein) to the TNF-Receptor signalling complex (TNF-RSC) (reviewed in Haas et al, 2009). Together, HOIL-1 and HOIP form an E3 ligase complex that is capable of generating linear ubiquitin chains and therefore referred to as “linear ubiquitin chain assembly complex” (LUBAC) (Kirisako et al, 2006).

In addition to HOIL-1 and HOIP, we also identified Sharpin (SHANK-associated RH domain-interacting protein) as a further component that is recruited to the TNF-RSC in a stimulation-dependent manner. In this study I show that Sharpin forms an endogenous, stimulation-independent complex with HOIL-1 and HOIP. Although the mRNA levels of HOIL-1 and HOIP are unchanged in the absence of Sharpin, their respective protein levels are strongly diminished. These data indicate that Sharpin, HOIL-1 and HOIP form a tripartite complex. As previously shown for HOIL-1 and HOIP, overexpression of Sharpin together with HOIP also stabilises the TNF-RSC and leads to activation of NF-κB. Therefore, LUBAC does not only consist of HOIL-1 and HOIP, but also includes Sharpin.

A spontaneous point deletion in the Sharpin gene ablating its expression leads to the development of chronic proliferative dermatitis (cpdm) in mice (HogenEsch et al, 1993; HogenEsch et al, 1999; Seymour et al, 2007). Cpdm mice develop a multi-organ inflammatory disease with prominent skin lesions and display severe abnormalities in lymphoid architecture, including absence of Peyer’s patches, marginal zones, germinal centres, and follicular dendritic cells. Mouse embryonic fibroblasts (MEFs) and primary keratinocytes generated from these mice show impaired TNF-induced NF-κB and MAPK signalling. Although activation of these pathways is not completely dependent on LUBAC...
activity, its presence is required for full activation as demonstrated by proper induction of TNF-target genes. In line with these TNF-induced signalling defects, cpdm-derived cells are prone to TNF-mediated cell death, which is partially apoptotic and partially necroptotic. Thus, loss of Sharpin results in a cell death favouring dysregulation of TNF-induced signalling which is responsible for the inflammatory phenotype observed in cpdm mice.

Following the identification of a role for LUBAC in TNF signalling I determined whether it also played a role in signalling by the TNF related ligands TRAIL and CD95 ligand (CD95L/FasL). As compared to TNF, absence of a LUBAC component also sensitised cells to TRAIL- and CD95L- induced cell death, indicating that LUBAC plays a crucial role in cell survival. Accordingly, non-apoptotic TRAIL- and CD95L-induced signalling was also affected in the absence of LUBAC as determined by altered NF-κB and MAPK responses, respectively.

Taken together, the results obtained in this thesis determine LUBAC as a novel regulator of various death receptor-driven signalling pathways implicated in gene activation, cell death induction and, as a consequence, innate immunity and inflammation.
Declaration

I, Stefanie Cordier, declare that this PhD Thesis is my own work and has not been submitted in any form for another degree at any university or other institute of tertiary education. Information derived from the published and unpublished work of others has been acknowledged in the text and a list of references is given in the bibliography.

London, 25.10.2011

Stefanie Cordier
Acknowledgements

This thesis would not have been possible without the help of a variety of people whom I would like to thank at this point:

- My supervisor Prof. Henning Walczak who gave me the opportunity to work in his lab, take part in this fascinating project and who always supported me
- Prof. John Silke from the Walter and Elizabeth Institute in Melbourne who gave a lot of input to this project and with whom I worked in Australia for a couple of weeks
- My colleague Eva Rieser who did not only support my project and helped wherever possible, but also became a special friend who will always have a place in my heart
- My colleague, flat mate and friend Bjoern Gerlach without whom my PhD time would not have been the same
- My colleagues Chahrazade Kantari and Frank Reichenbach, who did not only help me with my project, especially with regard to cell death, but also became valuable friends
- The “Prieske family” for constantly generating a positive atmosphere in the lab and making me laugh even when I did not feel like
- My “LUBAC subgroup” colleagues Anna Schmukle and Christoph Emmerich for their scientific support and Tobias Haas, who identified SHARPIN in the TNF-RSC
- Pavanjit Rai, who became a special friend and whose constant questioning I will miss
- The entire former and present Walczak lab members, i.e. Torsten Hartwig, Mark Tuthill, Sebastian Kupka, Julia Zinngrebe and Johannes Lemke for helpful discussions
- Karen Chu for correcting my thesis, but moreover for becoming my friend
- My best friends Marie, Britta and Kerstin and her son Matti for their support, advice, but especially for their friendship
- My parents Uschi and Michael and my brother Matthias for their support and for always believing in me
- Lastly, but most importantly I want to thank Christian for always supporting but especially loving me the way I am.
# Table of Contents

Abstract ii
Declaration iv

Table of Contents ................................................................................................................ vi

1. Introduction ........................................................................................................................... 1
   1.1 The TNF and TNF-Receptor Superfamily ...................................................................... 1
   1.1.1 Biological function of the TNF/TNFR superfamily .............................................. 2
   1.2 The TNF/TNFR1 system .............................................................................................. 4
   1.2.1 Biological functions of TNF ................................................................................. 4
   1.2.2 The TNFR1 signalling complex ............................................................................ 5
   1.2.3 TNF-induced IKK and TAK1 activation ............................................................... 8
   1.3 TNF-induced NF-κB and MAPK signalling ................................................................. 10
       1.3.1 NF-κB activation ................................................................................................. 10
       1.3.1.1 Canonical NF-κB signalling .......................................................................... 11
       1.3.1.2 Non-canonical NF-κB signalling .................................................................. 12
       1.3.2 MAPK signalling ............................................................................................... 14
       1.3.2.1 MAP3Ks in TNFR1 signalling ....................................................................... 15
       1.3.2.2 The ERK1/2 module .................................................................................... 18
       1.3.2.3 The JNK module .......................................................................................... 18
       1.3.2.4 The p38 MAPK module ................................................................................. 19
   1.4 Cell death induction by death ligands of the TNF-Receptor superfamily .................. 20
       1.4.1 CD95L- and TRAIL-induced apoptosis ............................................................ 20
       1.4.1.1 The CD95L/CD95 and TRAIL/TRAIL-R1/2 systems .................................. 20
       1.4.1.2 The extrinsic apoptotic pathway .................................................................... 22
       1.4.1.3 The intrinsic apoptotic pathway .................................................................... 24
       1.4.1.4 Checkpoints in apoptotic responses ............................................................... 25
       1.4.1.5 Non-apoptotic TRAIL- and CD95L-induced signalling .................................. 29
       1.4.2 TNFR1-induced apoptosis .................................................................................. 30
       1.4.2.1 TNF-complex II formation .......................................................................... 30
       1.4.2.2 TNF-induced JNK mediated cell death .......................................................... 30
       1.4.2.3 Complex IIA versus Complex IIB ................................................................. 31
       1.4.3 TNF-induced necroptosis .................................................................................... 32
   1.5 The ubiquitin system ....................................................................................................... 36
       1.5.1 Ubiquitin chain formation .................................................................................... 37
       1.5.1.1 The HECT and RING family of E3 ligases .................................................... 37
       1.5.2 Physiological role of different ubiquitin chain linkages ...................................... 39
       1.5.3 Ubiquitin Binding domains .................................................................................. 42
       1.5.4 Deubiquitinases .................................................................................................. 45
   1.6 The Linear Ubiquitin Chain Assembly Complex (LUBAC) ........................................ 48
       1.6.1 HOIL-1, HOIP and SHARPIN .......................................................................... 48
       1.6.2 Chronic proliferative dermatitis (cpdm) ............................................................... 51
   1.7 Aims of the project ........................................................................................................ 54

2. Material and Methods ........................................................................................................ 55
   2.1 Materials ....................................................................................................................... 55
       2.1.1 Chemicals and reagents ....................................................................................... 55
       2.1.2 Specific inhibitors ................................................................................................. 55
# Table of Contents

2.1  Buffers and solutions .......................................................... 55  
2.2  Methods of Cell Biology ..................................................... 64  
2.2.1  HEK293T cells .................................................................. 64  
2.2.2  Cell culturing conditions ................................................. 65  
2.2.3  Freezing and thawing of eukaryotic cells ......................... 65  
2.2.4  Generation of primary keratinocytes ............................. 65  
2.2.5  Transfection of adherent cells .................................. 66  
2.2.6  Lentiviral infection of eukaryotic cells ....................... 66  
2.2.6.1  Production of recombinant viruses ....................... 66  
2.2.6.2  Generation of stable knockdown or overexpressing cells .......................................................................................... 66  
2.2.7  siRNA-mediated Knock-down ...................................... 67  
2.2.8  Inducible Protein Expression in MEF cell lines .......... 67  
2.2.9  cIAP1/2 degradation using SMAC mimetics ............... 67  
2.2.10  Quantification of Cell Death and Cell Viability ........ 67  
2.2.11  Determination of clonogenic survival ....................... 67  
2.2.12  Flow cytometry ......................................................... 68  
2.3  Methods of Molecular Biology ............................................ 68  
2.3.1  DNA digestion and restriction analysis ....................... 68  
2.3.2  Agarose gel electrophoresis of nucleic acids ................ 68  
2.3.3  Gel extraction of DNA fragments .................................. 69  
2.3.4  Ligation of DNA fragments ........................................ 69  
2.3.5  Transformation of competent _E. coli_ bacteria and Isolation of Plasmid DNA .. 69  
2.3.6  Polymerase Chain Reaction (PCR) .......................... 69  
2.3.7  Quantitative PCR ....................................................... 70  
2.3.7.1  RNA purification .................................................. 70  
2.3.7.2  cDNA preparation .................................................... 70  
2.3.7.3  Quantitative Real-time PCR .................................. 70  
2.4  Methods of Biochemistry .................................................... 71  
2.4.1  Determination of protein content ................................ 71  
2.4.2  Precipitation of Receptor Signalling Complexes .......... 71  
2.4.3  Protein interaction studies ......................................... 72  
2.4.4  Ubiquitin pull-down assay ............................................ 72  
2.4.5  SDS polyacrylamide gel electrophoresis (SDS-PAGE) .......... 72  
2.4.6  Western blotting ............................................................ 73  
2.4.7  Stripping of Western blot membranes ........................ 73  
2.4.8  Enzyme-Linked Immuno Sorbent Assay (ELISA) ....... 73  
2.5  Animal work .................................................................... 74
2.5.1 Chronic proliferative dermatitis mice ............................................................. 74
2.5.2 Conditional HOIL-1 mice ................................................................................ 74
2.5.3 Enbrel® administration .................................................................................. 74

3. Results .................................................................................................................. 75

3.1 Functional analysis of SHARPIN in TNFR1 signalling ..................................... 75
  3.1.1 SHARPIN is a novel constituent of the TNF-RSC and forms a stimulation-
       independent complex with HOIL-1 and HOIP ..................................................... 75
  3.1.2 SHARPIN is recruited to the TNF-RSC in a cIAP1/2-dependent manner and
       stabilises the receptor signalling complex .......................................................... 79
  3.1.3 SHARPIN stabilises the tripartite LUBAC complex ........................................ 82
  3.1.4 HOIP allows for the recruitment of LUBAC to the TNF-RSC .......................... 84
  3.1.5 SHARPIN is required for proper TNF-induced NF-κB and MAPK activation. 87
  3.1.6 Loss of SHARPIN sensitises to TNF-induced cell death .................................. 92
  3.1.7 Loss of SHARPIN promotes complex II formation ......................................... 97

3.2 SHARPIN regulates TRAIL- and CD95L-induced signalling ........................... 101
  3.2.1 TRAIL- and CD95L-induced cell death responses ........................................... 101
    3.2.1.1 Loss of SHARPIN sensitises cells to TRAIL-induced death ......................... 101
    3.2.1.2 Loss of SHARPIN increases CD95L-induced cell death ............................. 102
    3.2.1.3 TRAIL- and CD95L-induced death in LUBAC-deficient cells is independent
            of TNF ........................................................................................................... 104
    3.2.1.4 LUBAC is required in other death-inducing signalling pathways.............. 105
  3.2.2 SHARPIN and HOIL-1 regulate pro-survival signalling by TRAIL and CD95L 106
  3.2.3 LUBAC is recruited to the TRAIL and CD95 DISC ......................................... 107
    3.2.3.1 LUBAC is recruited to the CD95 and TRAIL DISC in a cIAP1/2-dependent
            manner ........................................................................................................ 110
    3.2.3.2 HOIL-1 absence alters the composition of the TRAIL-R1/R2 and CD95 DISC 111

3.3 Summary ............................................................................................................ 113

4. Discussion ............................................................................................................. 114
  4.1 SHARPIN is the third component of LUBAC and recruited to the TNF-RSC ...... 115
    4.1.1 SHARPIN, HOIL-1 and HOIP stabilise the native TNF-RSC ............................ 115
    4.1.2 LUBAC-a tripartite complex? ....................................................................... 119
  4.2 Identification of HOIL-1, HOIP and SHARPIN as novel components of the TRAIL-
      R1/R2 and CD95 DISC ...................................................................................... 121
  4.3 LUBAC balances TNF-, TRAIL- and CD95L-induced signalling outputs ........ 123
    4.3.1 LUBAC in TNFR1 signalling ....................................................................... 123
    4.3.2 LUBAC in TRAIL- and CD95L-induced cell death ...................................... 129
    4.3.3 Function of LUBAC in other signalling pathways ....................................... 132
  4.4 Deregulated LUBAC signalling ....................................................................... 133
    4.4.1 SHARPIN-deficient cpdm mice .................................................................. 133
    4.4.2 HOIL-1 and HOIP deficiency ..................................................................... 138
    4.4.3 LUBAC inhibition ....................................................................................... 140
  4.5 Summary and Outlook ....................................................................................... 141

5. Appendix ............................................................................................................... 142
  5.1 Abbreviations ................................................................................................... 142
  5.2 List of Figures .................................................................................................. 149
  5.3 List of Tables .................................................................................................... 151
  5.4 List of Publications ......................................................................................... 151

6. References ............................................................................................................ 152
1. Introduction

1.1 The TNF and TNF-Receptor Superfamily

In 1975, a bacterially-induced factor capable of initiating cell death in various tumours was discovered (Carswell et al, 1975). Designated as tumour necrosis factor (TNF), it was found to be the prototype of a family of molecules involved in immune regulation and inflammation. To date, a superfamily of TNF proteins consisting of 19 members that signal through 29 receptors has been reported. These families have been designated as the TNF and TNF-Receptor (TNFR) superfamilies. The receptors of this superfamily are type I transmembrane proteins containing one to six characteristic cysteine-rich domains (CRDs) in their extracellular amino-terminal domains that are required for ligand binding (Croft, 2003; Hymowitz et al, 1999). Their cognate ligands are type II transmembrane proteins with an extracellular, C-terminal TNF homology domain (THD). There is 20-30% amino acid identity between the THD of the different TNF superfamily members and the THDs are responsible for trimerisation and binding to the receptor (Locksley et al, 2001). The extracellular domain is frequently released as a soluble cytokine upon proteolytic processing (Bodmer et al, 2002). The 26 kDa transmembrane TNF molecule for instance is cleaved by the TNFα converting enzyme (TACE) into a 17 kDa soluble molecule (Black et al, 1997). Whereas some of the released ligands have agonistic activity, others are rather antagonistic by blocking binding of the transmembrane ligand to the receptor as reported for soluble CD95 ligand (CD95L) (Suda et al, 1997). However, a pre-dominant transmembrane expression of the TNF-superfamily ligands suggests that they are meant to act locally.

Members of the TNFR superfamily can be subdivided into three groups. The first group is characterised by the presence of an intracellular, 80-amino acid death domain (DD) that is required for cell death induction and forms six anti-parallel α-helixes with highly conserved topology. The DD was initially discovered in the cytoplasmic regions of CD95 and TNF-Receptor 1 (TNFR1) by two independent groups (Itoh & Nagata, 1993; Tartaglia et al, 1993). Subsequently, DDs have also been identified in TNF-related apoptosis inducing ligand receptor 1 and 2 (TRAIL-R1/R2), DR3, DR6 and EDAR.

The second group of TNFR superfamily members does not contain a death domain, but is rather distinguished by the presence of an intracellular TRAF-interacting motif (TIM). These
receptors are known to induce cell proliferation, survival and differentiation by delivering co-
stimulatory signals required for complete immune cell activation. Members of this second
family include 4-1BB, BAFF-Receptor, BCMA (B cell maturation antigen), CD27, CD30,
CD40, fibroblast growth factor 14 (Fn14), Glucocorticoid-induced TNFR family receptor
(GITR), Herpes virus entry mediator (HVEM), lymphotoxin β Receptor (LTβR), OX40,
receptor activator of NF-κB (RANK), transmembrane activator and calcium signal
modulating cyclophilin ligand interactor (TACI), TNFR2, TROY and X-linked EDA-A2
receptor (XEDAR).

The last sub-group neither contains a functional death domain nor a TIM but functions as
decoy receptors by binding their respective ligand without inducing subsequent signal
transduction. TRAIL-R3, TRAIL-R4 and osteoprotegerin (OPG) constitute this third class.

Upon receptor-ligand interaction, receptors of the TNFR superfamily associate in trimers and
become capable of transmitting distinct signals internally, leading to cell proliferation,
survival, differentiation or cell death. Since none of the TNFR superfamily members
possesses any enzymatic activity, intracellular adaptors need to be recruited in order to
facilitate downstream signalling events. A variety of yeast-two-hybrid approaches revealed
the interaction of TNFR-associated factors (TRAFs) with most TNFR superfamily members
following stimulation with the cognate ligand (Arch et al, 1998). So far, seven TRAF proteins
have been identified, designated as TRAF1-TRAF7 (Xu et al, 2004). Except for TRAF1, all
TRAF proteins contain five to seven amino-terminal zinc binding motifs, one of which
costimulatory signals required for complete immune cell activation. Members of this second
family include 4-1BB, BAFF-Receptor, BCMA (B cell maturation antigen), CD27, CD30,
CD40, fibroblast growth factor 14 (Fn14), Glucocorticoid-induced TNFR family receptor
(GITR), Herpes virus entry mediator (HVEM), lymphotoxin β Receptor (LTβR), OX40,
receptor activator of NF-κB (RANK), transmembrane activator and calcium signal
modulating cyclophilin ligand interactor (TACI), TNFR2, TROY and X-linked EDA-A2
receptor (XEDAR).

The last sub-group neither contains a functional death domain nor a TIM but functions as
decoy receptors by binding their respective ligand without inducing subsequent signal
transduction. TRAIL-R3, TRAIL-R4 and osteoprotegerin (OPG) constitute this third class.

Upon receptor-ligand interaction, receptors of the TNFR superfamily associate in trimers and
become capable of transmitting distinct signals internally, leading to cell proliferation,
survival, differentiation or cell death. Since none of the TNFR superfamily members
possesses any enzymatic activity, intracellular adaptors need to be recruited in order to
facilitate downstream signalling events. A variety of yeast-two-hybrid approaches revealed
the interaction of TNFR-associated factors (TRAFs) with most TNFR superfamily members
following stimulation with the cognate ligand (Arch et al, 1998). So far, seven TRAF proteins
have been identified, designated as TRAF1-TRAF7 (Xu et al, 2004). Except for TRAF1, all
TRAF proteins contain five to seven amino-terminal zinc binding motifs, one of which
constitutes a really interesting new gene (RING) finger and a TRAF domain that can be sub-
divided into a carboxy-terminal (TRAF-C) and amino-terminal (TRAF-N) TRAF domain
(Bishop et al, 2007; Pineda et al, 2007). The TRAF-C domain is highly conserved in the
TRAF family and facilitates homo- and hetero-dimerisation of the TRAF proteins as well as
binding to the intracellular TIM of TNFR superfamily members (reviewed in Ha et al, 2009);
in contrast, TRAF-N forms a coiled-coiled structure that is less conserved among the TRAF
family. As described in chapter 1.2.2, recruitment of TRAF proteins to the receptor signalling
platforms induces nuclear factor kappa B (NF-κB) and mitogen activated protein kinase
(MAPK) activation, resulting in survival, proliferation and induction of immune responses.

1.1.1 Biological function of the TNF/TNFR superfamily

Although TNF and other TNF superfamily members fulfil important physiological functions
in immune cell homeostasis and removal of transformed or infected cells, their expression and
activities need to be tightly regulated to prevent harmful reactions such as auto-inflammatory
diseases. Several reports showed the importance of this family of molecules in the regulation
and development of the immune system and revealed the generation of immune diseases if
one of the systems is genetically ablated or mutated (Basak & Hoffmann, 2008; Xie et al,
2008).

In particular, the immune system relies on signals exerted by TNF/TNFR superfamily
members to confer innate immunity, lymphoid organ development, co-stimulatory
lymphocyte activation, cytokine production, immunoglobulin switching and removal of virus-
infected cells (Chang et al, 2006; Gallagher et al, 2007; Sakurai et al, 2006). A good example
of deregulated TNFR superfamily signalling is seen in mice with mutated CD95 (lpr,
lymphoproliferation) or CD95L (gld, generalised lymphoproliferative disease). Under
physiological conditions, CD95L is up-regulated on activated T cells to induce activation-
induced cell death (AICD) of CD95-sensitive lymphocytes once an infection has been cleared
(Dhein et al, 1995). However, mutations in the CD95 ligand or the receptor prevent deletion
of mature T cells following activation and subsequent accumulation of activated T cells
results in the development of auto-inflammatory syndromes (Nagata, 1999).

A variety of TNF superfamily members has important functions in the co-stimulation and
subsequent activation and maturation of T cells and B cells. Accordingly, mice lacking the
expression of one of the TNF/TNFR superfamily members show defects in the development
of particular types of immune cells and/or in the induction of an efficient immune response. In
particular, BAFF, TNF, LT and CD40L were shown to be essential cytokines for the
development of B cells, OX40L, 4-1BBL and CD27L for T cells and CD40L and RANKL for
dendritic cells (reviewed in Aggarwal, 2003).

A role for TNF superfamily members in anti-tumour responses has been suggested. In
particular, TNF was initially discovered as a cytokine possessing anti-cancer activity
(Carswell et al, 1975). The oncologist William Coley was the first to treat cancer with
bacterial extracts (so-called Coley’s toxins) with some success (Coley, 1891). Ongoing
studies revealed the presence of TNF, as a result of lipopolysaccharide (LPS) signalling, in
these extracts and showed that TNF was able to induce necrotic cell death in a variety of
tumour samples (Carswell et al, 1975; O’Malley et al, 1963). However, the effectiveness of
TNF as an anti-cancer drug has been proven rather limited due to its systemic toxicity.
Furthermore, TNF was shown to even enhance tumour growth by promoting NF-κB
activation, therefore contributing to tumour invasion, proliferation and metastasis formation
(Szlosarek et al, 2006). Skin-specific TNF knockout mice were resistant to skin carcinogenesis, indicating a pro-tumoural activity of TNF (Moore et al, 1999). In contrast, TRAIL was shown to specifically induce cell death in transformed cells without affecting normal cells. Although most primary tumour samples were shown to be TRAIL-resistant, a variety of reagents were able to sensitise tumour cells to TRAIL-induced apoptosis (reviewed in Cordier et al, 2009b).

1.2 The TNF/TNFR1 system

1.2.1 Biological functions of TNF

As described in the previous chapter, TNF was the first cytokine of the TNF superfamily identified in the late 70s’ (Carswell et al, 1975) and is probably the best described member of the superfamily with more than 50,000 publications since its first isolation and characterisation in 1985 (Aggarwal et al, 1985). TNF can bind to two transmembrane receptors, TNFR1 and TNFR2 to execute its pleiotropic biological functions. Since TNFR2 lacks a death domain, this receptor has exclusively pro-survival activity, whereas TNFR1 can also induce cell death under certain circumstances. While the expression of TNFR2 is restricted pre-dominantly to immune cells and the endothelium, TNFR1 is ubiquitously expressed with the exception of erythrocytes (Wajant et al, 2003). In contrast to TNFR1, TNFR2 has very low affinity for soluble TNF and cannot be efficiently activated by this form of TNF; however, membrane-bound TNF induces strong cross-linking and activation of both, TNFR1, and TNFR2 (Grell et al, 1995; Grell et al, 1998; Krippner-Heidenreich et al, 2002).

TNF is the key cytokine involved in the initiation of innate immune responses against invading pathogens that is produced by a variety of cells including macrophages, dendritic cells (DCs), monocytes, lymphocytes and keratinocytes. Following bacterial infection, LPS or other pathogen-associated molecular patterns (PAMPs) derived from invading microbes are recognised by their specialised pattern recognition receptors (PPRs) on and in host cells to induce the expression and secretion of TNF and other pro-inflammatory cytokines. Subsequent binding of TNF to TNFR1 results in the initiation of host defence mechanisms including the production of additional pro-inflammatory cytokines associated with increased phagocytic activity of surrounding macrophages (Bekker et al, 2001). Furthermore, enhanced TNF-induced production of chemokines and cell adhesion molecules on the vascular endothelium allows for the infiltration of a variety of immune cells to the place of
inflammation to clear pathogens (Roach et al, 2002). Accordingly, it is not surprising that host
defence mechanisms in mice lacking TNF were severely impaired (Aggarwal, 2003). TNF
and TNFR1 knockout mice showed increased susceptibility to *Listeria monocytogenes* and
*Mycobacterium tuberculosis*, rapid death from infection and resistance to LPS-induced septic
shock, further underscoring the great importance of the TNF/TNFR1 system in protection
against infection (Ehlers, 2003; Pfeffer et al, 1993).

A variety of auto-immune diseases is associated with deregulated TNFR1 signalling,
including rheumatoid arthritis, Crohn’s disease and asthma (McInnes & Liew, 2005; O'Shea
et al, 2002; van der Poll & Lowry, 1995). Application of TNF-neutralising antibodies
(Infliximab/Remicade® and Adalimumab/Humira®) or TNFR2-Fc fusion proteins
(Etanercept/Enbrel®) has been proven beneficial in the treatment of these auto-immune
diseases (Feldmann & Maini, 2001; Taylor & Feldmann, 2009; Van Deventer, 1997). Thus,
even though TNF constitutes one of the major cytokines required for the induction of
immunity, it can also play an eminent role in the pathophysiology of a number of diseases. It
is therefore of great importance to understand the molecular mechanism underlying TNF
signalling.

1.2.2 The TNFR1 signalling complex

Binding of trimeric TNF to TNFR1, which was initially isolated and cloned in 1990 (Gray et
al, 1990; Loetscher et al, 1990; Schall et al, 1990), results in receptor trimerisation and
subsequent recruitment of a variety of intracellular adaptor proteins (see Figure 1). TNF
Receptor associated death domain (TRADD) is one of the most important adaptors required
for stable TNF-Receptor Signalling Complex (TNF-RSC) formation and ablation of TRADD
completely inhibits TNF-induced signal transduction. As indicated by its name, TRADD
possesses a DD, which can be utilised to bind to the intracellular DD of TNFR1 (Hsu et al,
1996b; Hsu et al, 1995; Micheau & Tschopp, 2003). TRADD then serves as a recruitment
platform for TRAF2 (and possibly TRAF5) (Hsu et al, 1996b; Tsao et al, 2000), which in turn
interacts with cellular inhibitor of apoptosis 1 and 2 (cIAP1/2) (Rothe et al, 1995; Shu et al,
1996). TRAF2 was shown to directly interact with cIAP1 and cIAP2 via its cIAP-interacting
motif (CIM) (Vince et al, 2009). On the other hand, the baculovirus IAP repeat 1 (BIR1) of
cIAP1/2 is essential for binding to TRAF2 (Samuel et al, 2006; Varfolomeev et al, 2006).
Zheng et al. recently demonstrated by crystallography that one cIAP2 molecule interacts
with a trimeric TRAF2 structure (Zheng et al, 2010). Although TRADD was originally described
to recruit the receptor-interacting protein kinase 1 (RIP1) to the TNF-RSC (Hsu et al, 1996a),
RIP1 can also directly bind to the DD of TNFR1 via its own DD. Yet, it has now been demonstrated that in the absence of TRADD and hence TRAF2/5 and cIAP1/2, RIP1 does not get ubiquitinated and is not capable of activating NF-κB and MAPKs (Ermolaeva et al, 2008; Haas et al, 2009; Pobezinskaya et al, 2008). Therefore, recruitment of RIP1 alone to the TNF-RSC is insufficient to activate downstream signals but requires additional modifications. Besides the DD, RIP1 also contains a kinase domain at its N-terminus. However, several reports have proven this kinase domain to be dispensable for TNF-induced signalling since

Figure 1: TNFR1 signal transduction. Binding of TNF to TNFR1 induces receptor trimerisation and subsequent binding of TRADD to the receptor via its DD. TRADD then recruits RIP1 and TRAF2/5, which in turn interacts with cIAP1/2 via the CIM domain. LUBAC is recruited to cIAP1/2-generated chains and linearly ubiquitinates NEMO and RIP1. The TAK1/TAB complex and the IKK complex are recruited to polyubiquitin chains and become activated by not completely resolved mechanisms. TAK1 then phosphorylates IKKβ, which subsequently phosphorylates IκB proteins that are then K48-ubiquitinated by SCFβTrCP and degraded by the proteasome. Thereby, the NF-κB heterodimers p65/p50 are released and translocates to the nucleus to drive the transcription of a variety of target genes implicated in cell survival and proliferation. TAK1 also activates the p38 and JNK MAPKs pathways, leading to ATF and AP-1 activation guiding in transcription of pro-survival target genes. Amongst these targets, the DUBs A20 and CYLD are transcribed and subsequently remove ubiquitin chains at the TNF-RSC to on the one hand terminate NF-κB and MAPK signalling and on the other hand induce internalisation of the signalling complex to form complex II. NF-κB/MAPK-driven up-regulation of c-FLIP inhibits caspase-8 activation in complex II, whose activation otherwise leads to cell death.
reconstitution of RIP1 null cells with a kinase-dead RIP1 mutant fully restored NF-κB signalling (Lee et al, 2004; Ting et al, 1996). Thus, regarding gene activatory signalling, RIP1 is thought to act as an adaptor protein rather than to exert catalytic activity within the TNF-RSC.

Both cIAP and TRAF proteins possess a RING domain with potential E3 ubiquitin ligase activity which allows for the recruitment of E2 proteins (UBCs) to the complex and therefore the formation of ubiquitin chains (see chapter 1.5). Considering that TRAF2 forms K63-linked polyubiquitin chains \textit{in vitro} and that RIP1 remains unubiquitinated in the absence of TRAF2 (Lee et al, 2004; Wertz et al, 2004), it has long been assumed that TRAF2 itself functions as an E3 ubiquitin ligase for RIP1 \textit{in vivo} following TNFR1 activation. However, a direct \textit{in vitro} ubiquitination of RIP1 could not be detected for TRAF2, but only for cIAP1/2 (Bertrand et al, 2008; Park et al, 2004). Furthermore, deletion of the E2 Ubc13, which was shown to act in concert with TRAF proteins to generate K63-linked polyubiquitin chains, did not alter TNF-induced NF-κB activation (Yamamoto et al, 2006). These results indicate that either the E3 activity of TRAF2 is dispensable for TNF signalling or that TRAF2 utilises another E2 to enzyme to generate ubiquitin chains. More recent reports suggested that the major role of TRAF2 in TNF signalling is to rather serve as a recruitment platform for cIAP1/2, which then in turn facilitates RIP1 ubiquitination (Bertrand et al, 2008; Ea et al, 2006; Varfolomeev et al, 2008). Indeed, only recently Vince \textit{et al}. showed that TRAF2/5 knockout mouse embryonic fibroblasts (MEFs) reconstituted with cIAP-binding deficient TRAF2 were unable to form a functional TNF-RSC and to activate NF-κB and MAPK signalling. In contrast, reconstitution with a RING deficient mutant of TRAF2 allowed proper TNF-RSC formation and signal propagation (Vince et al, 2009). Taken together, a revised model of TNFR1 signalling proposes TRAF2 to function as an adaptor protein for the recruitment of cIAP1/2, thereby bringing them in close proximity to possible targets within the TNF-RSC which can then be (poly-)ubiquitinated. In line with this notion, cIAP1/2 were described to be capable of forming a variety of different ubiquitin chain types \textit{in vitro} (Bertrand et al, 2008; Blankenship et al, 2009).

Only recently, Dynek \textit{et al}. showed the cIAP/UbcH5-dependent generation of K11-linked ubiquitin chains on RIP1 under endogenous, physiological conditions within the TNF-RSC (Dynek et al, 2010). Furthermore, our group recently expanded the complexity of TNFR1 signalling by showing the presence of linear ubiquitin chains attached to RIP1 and NEMO in the native TNF-RSC (Gerlach et al, 2011; Haas et al, 2009). These reports showed for the first
time that TNFR1 signalling is not only restricted to K63- and K48-linked ubiquitin chains but indicate that signal transduction is far more complex. We are just beginning to understand the complex interplay between ubiquitin-conjugating enzymes, ubiquitin accepting molecules and de-ubiquitinating enzymes, which will be discussed later in this chapter.

1.2.3 TNF-induced IKK and TAK1 activation

Ubiquitin chains generated within the TNF-RSC following stimulation serve as binding sites for the recruitment of the TGF-β activated kinase 1/TAK1-binding protein (TAK1/TAB) complex and the IKK complex to the TNF-RSC (Ea et al, 2006; Wu et al, 2006). The TAK1/TAB complex consists of the kinase TAK1 and the two adaptor proteins TAB1 and TAB2 (or its homologue TAB3). TAB2 and TAB3 were shown to preferentially bind to K63-linked ubiquitin chains via their Npl4-type zinc-finger (NZF) and are recruited via poly-ubiquitinated RIP1 to the TNF-RSC (Cheung et al, 2004; Kanayama et al, 2004; Lee et al, 2003).

The IKK complex consisting of Inhibitor of κB kinase α (IKKα, also known as IKK1), IKKβ (IKK2) and NF-κB essential modulator (NEMO, also known as IKKγ) is recruited to the TNF-RSC via NEMO. NEMO does not possess any catalytic activity but rather serves as an adaptor protein allowing binding of the IKK complex to ubiquitin chains of different linkages (as discussed in chapter 1.5.3) within the complex, preferentially to linear ubiquitin chains (Lo et al, 2009).

Through as yet incompletely resolved mechanisms, TAK1 auto-phosphorylates at Thr-187 and thereby gets activated once the TAK1/TAB complex is recruited to the TNF-RSC (Kanayama et al, 2004). Only recently, Xia et al. suggested free, unanchored poly-ubiquitin chains to directly activate TAK1 in vitro (Xia et al, 2009). However, considering the high turnover rate of free ubiquitin chains, it remains controversial whether such a model could also hold true in vivo. Alternatively, a conformational change within TAK1 induced by the recruitment to the TNF-RSC or a direct modification (e.g. ubiquitination or phosphorylation) might be responsible for its activation. Simultaneous binding of the IKK complex to ubiquitin chains (possibly on RIP1, but also other TNF-RSC components) within the complex allows TAK1 to phosphorylate and activate IKKβ which in turn phosphorylates inhibitor of κB (IκB) proteins. IκB proteins are known to sequester NF-κB subunits in the cytoplasm of unstimulated cells by masking their nuclear localisation sequence, thereby inhibiting the translocation of NF-κB to the nucleus. Once phosphorylated, IκB proteins are modified by
K48-linked ubiquitin chains by a ubiquitin ligase complex consisting of Skp1, Cul1, Roc1 and the F-box protein β-TrCP (β-transducin repeat-containing protein; SCFβ-TrCP) (Maniatis, 1999). Subsequently, IκB proteins are degraded by the 26S proteasome (Ben-Neriah, 2002), thereby liberating NF-κB subunits which then enter the nucleus to drive transcription (Ghosh & Karin, 2002). Besides NF-κB, MAPKs are also stimulated following TNFR1 ligation and both their functions will be described in more depths in chapter 1.3.

Though RIP1 has long been regarded as essential for IKK activation following TNF stimulation and for conferring protection against TNF-cytotoxicity (Kelliher et al, 1998; Pimentel-Muinos & Seed, 1999), a recent report by Wong et al. challenged this concept by showing that NF-κB activation was normal in a variety of cells obtained from RIP1 knockout mice (Wong et al, 2010). In line with this, the IKK complex was still recruited to the TNF-RSC in RIP1 knockout MEFs, suggesting that other ubiquitinated target proteins exist which might serve as a recruitment platform for NEMO. Indeed, TRADD (Micheau & Tschopp, 2003), cIAP1/2 (Haas et al, 2009) as well as TRAF2 (Brown et al, 2002; Habelhah et al, 2004; Li et al, 2002) have been reported to be ubiquitinated in the TNF-RSC. Thus, ubiquitin chains on these molecules might compensate for the loss of RIP1 to allow IKK recruitment and activation. Indeed, already in 2000, Devin et al. proposed that the IKK complex binds to TRAF2 in the absence of RIP1 (Devin et al, 2000).

The termination of NF-κB signalling at a certain stage is critical in preventing chronic inflammation and tumour formation (Pikarsky et al, 2004). The action of E3 ligases is reverted by deubiquitinating enzymes (DUBs) which remove ubiquitin chains of particular linkage from target proteins (described in more detail in chapter 1.5.4). A20 and cylindromatosis (CYLD) constitute the major DUBs involved in TNFR1 signalling and were both shown to de-ubiquitinate TNF-RSC components, particularly RIP1, thereby terminating NF-κB and MAPK activation (Brummelkamp et al, 2003; Kovalenko et al, 2003; Trompouki et al, 2003; Wertz et al, 2004).

Taken together, TNFR1 signalling is regulated at a variety of levels. Besides phosphorylation, ubiquitination has proven to be a central mechanism conferring control of signal propagation and termination. Conjugation of ubiquitin chains (e.g. by cIAPs), recruitment to ubiquitin chains (e.g. NEMO, TAB2/3) and their removal (e.g. A20, CYLD) represent decisive check points for TNFR1 signalling. Mutations in TNF-RSC components unbalance this output, leading to pathophysiological conditions as observed in various human diseases. Thus, it
remains essential to dissect the complex network of TNFR1 signallling in order to understand and possibly interfere with the signalling output.

1.3 TNF-induced NF-κB and MAPK signalling

The activation of NF-κB and MAPKs, albeit to different extents and with different kinetics, is shared by all stimulated TNFR superfamily members. While TNF-induced signalling is a fast and strong process, peaking 5-15 min following stimulation, TRAIL and CD95L are rather weak inducers of relatively late NF-κB and MAPK activation (Varfolomeev et al, 2005). The mechanism leading to the activation of these two major signalling pathways will be described in the following sections.

1.3.1 NF-κB activation

NF-κB is a heterodimeric family of transcription factors that control the expression of genes involved in immunity, inflammation and cell survival (Hayden & Ghosh, 2008). The family is composed of the five members p65 (RelA), p50 (NF-κB1), c-Rel, RelB and p52 (NF-κB2). p50 and p52 are generated from their precursors p105 and p100, respectively, by partial proteasomal degradation of their C-terminal, IκB-like domain. NF-κB subunits are characterised by a 300 amino-acid N-terminal Rel homology domain (RHD) that is required for dimerisation, nuclear translocation and DNA binding (Chen & Greene, 2004). Furthermore, the RHD domain is responsible for binding to IκB proteins, an ankyrin-repeat containing family of proteins that binds to NF-κB subunits via these repeats. The IκB family consists of the typical IκB proteins IκBα, IκBβ and IκBε, and the atypical members IκBδ, IκBζ and Bcl-3 (Figure 2). IκBα, IκBβ and IκBε reside in the cytosol and bind to NF-κB subunits, thereby masking their nuclear localisation sequence and preventing their entry into the nucleus. In contrast, the other IκB proteins reside in the nucleus and function as co-activators once NF-κB heterodimers are liberated and enter the nucleus (Bours et al, 1993; Kitamura et al, 2000).
1. Introduction

Besides the RHD, p65, c-Rel and RelB additionally contain a transcriptional activator domain (TAD) required to induce gene transcription. Since p50 and p52 lack a TAD, they have to heterodimerise with p65, c-Rel or RelB to form a functional NF-κB dimer. Alternatively, they can repress transcription when bound to κB sites as homodimers. Fifteen potential NF-κB homo- and heterodimers can form of which 12 are capable of binding DNA and nine possess transcriptional activator functions, thereby conferring a high degree of complexity (Hoffmann & Baltimore, 2006). However, most publications do not distinguish between the different dimers and simply monitor IκB degradation and p100 processing, thereby simplifying the view of NF-κB activation. However, one clear distinction has been made: NF-κB signalling can be subdivided into canonical and non-canonical pathways, which serve distinct physiological functions and are also molecularly very different (Sun & Ley, 2008).

1.3.1.1 Canonical NF-κB signalling

Canonical NF-κB activation is a fast process mediated by IκB degradation and subsequent rapid translocation of NF-κB dimers to the nucleus. It can be induced by all TNFR superfamily members as well as a variety of pro-inflammatory cytokines and Toll-like receptor (TLR) agonists (Vallabhapurapu & Karin, 2009). Following formation of a signalling platform at the membrane (induced by TNFR superfamily ligation) or intracellularly (mediated by a variety of PAMPs), the IKK complex consisting of the catalytic subunits IKKα, IKKβ and the regulatory subunit NEMO is recruited and activated (Rothwarf et al, 1998; Yamaoka et al, 1998; Zandi et al, 1997). IKKβ and NEMO fulfil non-redundant functions and are both essential for the canonical NF-κB pathway, whereas IKKα is dispensable (Hu et al, 1999; Li et al, 1999b; Rudolph et al, 2000). The canonical pathway is

**Figure 2:** Members of the NF-κB and IκB family. NF-κB family members are characterised by the presence of a RHD domain. Since p52 and p50 do not contain a TAD, they have to heterodimerise with another NF-κB family member to activate transcription. RHD: Rel homology domain; TAD: transactivation domain; LZ: leucine zipper; GRR: glycine-rich region; ANK: ankyrin repeat; DD: death domain; PEST: proline-, glutamic acid-, serine- and threonine rich.
involved in a variety of physiological processes including innate and adaptive immunity as well as cell survival. Activation of the IKK complex by yet unknown mechanisms leads to IKKβ-mediated phosphorylation of IkB proteins at two N-terminal regulatory serine residues. \(\text{IkB}\alpha\), the prototypic IkB member whose degradation is usually monitored and regarded as the definitive sign of NF-κB activation, becomes phosphorylated at serine 32 and serine 36 (DiDonato et al, 1996). Subsequently, IkB is targeted for ubiquitination by the SCF\(^{\beta\text{-TrCP}}\) E3 ubiquitin ligase complex, which attaches K48-linked ubiquitin chains to lysine residues K21 and K22, thereby marking it for proteasomal degradation. Subsequently, NF-κB is liberated, enters the nucleus and transcribes stimulus-dependent gene programs (Brown et al, 1995). Canonical NF-κB signalling is classically characterised by nuclear translocation of p50/p65 and c-Rel/p50 heterodimers (Basak & Hoffmann, 2008). Though the mechanisms remain elusive, p65 has been reported to become phosphorylated and acetylated following liberation, thereby presumably changing its transcriptional activity (Chen & Greene, 2004).

It is essential to terminate NF-κB responses in order to prevent the generation of chronic inflammation and/or tumourigenesis. Thus, NF-κB signalling is tightly regulated and has evolved to include negative feedback loops, including the transcription of inhibitory proteins able to down-regulate NF-κB activation. In particular, IkB itself becomes up-regulated to sequester liberated NF-κB dimers, thereby shutting off gene transcription (Sun et al, 1993). Furthermore, the expression of DUBs including A20, CYLD and Cezanne is induced, which then serve to dissolve the signalling platform responsible for IKK activation.

1.3.1.2 Non-canonical NF-κB signalling

The observation that overexpression of TRAF proteins was sufficient to activate NF-κB was in sharp contrast to an observed degradation of TRAF2 and/or TRAF3 following ligation of CD30 (Csomos et al, 2009; Duckett & Thompson, 1997), TNFR2 (Li et al, 2002) and CD40 (Brown et al, 2001; Liao et al, 2004), all of which are also associated with NF-κB activation. The missing link explaining these diametrically opposing effects of TRAF protein levels on NF-κB signalling was revealed with the discovery of an alternative, non-canonical NF-κB activation pathway.

Non-canonical NF-κB signalling is a rather slow process requiring new protein synthesis of NF-κB inducing kinase (NIK) resulting in IKK\(\alpha\)-mediated p100 processing to p52 (Hacker & Karin, 2006). In contrast to canonical NF-κB activation, it can only be initiated by a distinct subset of TNF superfamily ligands, including TNF weak inducer of apoptosis (TWEAK), RANKL, CD40L, BAFF and LT\(\beta\) (Claudio et al, 2002; Coope et al, 2002; Dejardin et al,
NIK is the central kinase regulating non-canonical NF-kB signalling (Xiao et al, 2001). In unstimulated cells, NIK levels are maintained low by a ubiquitin-ligase complex consisting of TRAF2, TRAF3 and cIAP1/2 (Liao et al, 2004; Vallabhapurapu et al, 2008; Zarnegar et al, 2008). Overexpression of the TNFR superfamily members RANK, CD30 and CD40 caused increased generation of p52 (Hauer et al, 2005), indicative of constitutive non-canonical signalling. Since concomitant TRAF3 overexpression inhibited this effect, TRAF3 can be regarded as negative regulator of non-canonical NF-kB activation. In line with this finding, TRAF3 knockout MEFs showed increased NIK levels in unstimulated cells and therefore a higher p100 turnover rate, without further stimulation. Constitutive ablation of TRAF3 expression caused perinatal death in mice, a phenotype that was completely rescued by concomitant depletion of NIK (Vallabhapurapu et al, 2008) or p100 (He et al, 2006). Thus, global, constitutive activation of non-canonical NF-kB signalling results in lethality.

Accordingly, TRAF2 knockout mice also died shortly after birth (Yeh et al, 1997). Traf2−/− cells were hypersensitive to TNF-induced death and the lethality of TRAF2 ablation was rescued by concomitant TNF or TNFR1 deletion, demonstrating that the death of the mice was TNF/TNFR1-dependent (Nguyen et al, 1999). Surprisingly, TRAF2 knockout cells did not show major defects on TNF-induced IKK activation, but rather exhibited defects in JNK activation and present with elevated levels of non-canonical NF-kB activation in unstimulated cells (Lee et al, 1997; Yeh et al, 1997). Concomitant deletion of only one allele of NIK sufficed to rescue the lethality of TRAF2 knockout mice (Vallabhapurapu et al, 2008).

Other major players regulating non-canonical signalling are cIAP1 and cIAP2. Genetic ablation of cIAP1/2 or forced degradation by IAP antagonists, also referred to as Smac mimetics (SM), were also associated with stabilisation of NIK and enforced p100 processing (Vallabhapurapu et al, 2008; Vince et al, 2007; Zarnegar et al, 2008). SM treatment of Traf2−/− cells did not further potentiate p100 processing, suggesting that cIAP and TRAF proteins act in the same pathway (Vince et al, 2007). Accordingly, in 2008 two independent groups provided the missing link explaining the mechanism of TRAF2/TRAF3/cIAP-mediated inhibition of alternative NF-κB activation (Vallabhapurapu et al, 2008; Zarnegar et al, 2008). They showed that NIK, TRAF2, TRAF3 and cIAP1/2 are part of the same complex, in which TRAF3 directly interacts with NIK and TRAF2. Binding of cIAP1/2 to TRAF2 brings them
in close proximity to NIK, allowing cIAP1/2 to attach K48-linked ubiquitin to NIK, thereby
targeting it for proteasomal degradation. However, stimulation of a distinct set of TNFR
superfamily members leads to recruitment of TRAF and cIAP proteins to the respective
receptor signalling complexes followed by their own degradation. Depending on the stimulus,
TRAF3 (CD30), TRAF2 and TRAF3 (TNFR2, CD40) or TRAF2 and cIAP1/2 (Fn14) are
degraded. Despite utilising different mechanisms, CD40, CD30, TNFR2 and Fn14 induce
inactivation of the TRAF/cIAP destruction complex accompanied by stabilisation of NIK
protein levels. Once stabilised, NIK phosphorylates IKKα, which in turn phosphorylates
p100, thereby inducing its ubiquitination by the SCFβ−TrCP complex. Interestingly, only the C-
terminal, ankyrin repeat-containing part of p100 is degraded, leaving the N-terminus (p52)
intact. Although the precise mechanism that governs this partial degradation remains to be
determined, it might be due to the tightly folded conformation formed by a stable dimer
(Piwko & Jentsch, 2006). Since ablation of one of the components of the
TRAF2/TRAFC/cIAP complex is sufficient to stabilise NIK levels, these proteins possess
non-redundant functions and are equally important for the regulation of non-canonical NF-κB
signalling.

1.3.2 MAPK signalling

MAPK signalling converts a variety of extracellular signals into a wide range of cellular
responses. So far, 14 MAPKs have been described in mammals. These can be divided into
conventional MAPKs including extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-
Jun amino (N)-terminal kinases 1, 2 and 3 (JNK1/2/3), p38 isoforms (α, β, γ, δ) and ERK5
(reviewed in Chen et al, 2001) and atypical MAPKs comprising ERK3/4, ERK7 and Nemo-
like kinase (NLK) (Coulombe & Meloche, 2007). MAPKs are characterised by a
Serine/Threonine (Ser/Thr) kinase domain flanked by N- and C-terminal regions of different
lengths. Additionally, some MAPKs possess a TAD or nuclear localisation sequences.
Conventional MAPKs are composed of a set of three evolutionarily conserved, sequentially
activated kinases: MAP kinase kinase kinases (MAP3Ks) are initially activated at the receptor
level on the membrane by proximity-induced auto-phosphorylation or co-recruited kinases
(MAP4Ks). Subsequently, they phosphorylate MAP kinase kinases (MAP2Ks) at Ser and Thr
residues, which then activate MAPKs through dual phosphorylation on Thr and Tyr residues
within a conserved Thr-X-Tyr motif located in the activation loop of the kinase domain.
While some MAP3Ks possess specificity for a single pathway, others are rather promiscuous
phosphorylating a wide array of down-stream MAP2Ks. MAPKs are proline-directed kinases
that phosphorylate target substrates on Ser and Thr residues followed by prolines. Activation of atypical MAPKs involves neither a three-tiered kinase cascade nor phosphorylation of the conserved Thr-X-Tyr motif and will not be discussed to any further extent in this thesis. Reversion of MAPK activation is achieved by MAPK phosphatases (MKPs) which remove stimulatory phosphate residues from the kinases, thereby rendering them inactive and terminating the signal.

TNFR superfamily members activate MAPKs via TRAF proteins following receptor trimerisation, leading to activation of transcription factors including activator protein-1 (AP-1) composed of Fos (c-Fos, FosB, Fra-1 and Fra-2), and Jun (c-Jun, JunB and JunD) members. Depending on the extracellular stimulus, the seven TRAF proteins are of differential importance. CD40-induced p38 and JNK activation for instance primarily rely on TRAF2, whereas the contribution of TRAF6 is less, though still significant (Matsuzawa et al, 2008). By contrast, TRAF2 is the main TRAF protein implicated in TNFR1 signalling, which most potently activates p38 and JNK. JNK activation was severely impaired in Traf2−/− fibroblasts, whereas NF-κB responses were only mildly affected (Yeh et al, 1997). This might be due to partial redundancy with TRAF5 given that Traf2−/− Traf5−/− double knockout cells completely lacked IKK activation (Tada et al, 2001). However, these results indicate that JNK activation primarily depends on TRAF2, whereas TRAF5 can partially compensate for TRAF2 in NF-κB responses.

1.3.2.1 MAP3Ks in TNFR1 signalling

As described before, activation of MAPKs requires a tightly controlled three-tiered cascade, in which a kinase is activated by phosphorylation by its particular up-stream kinase (Figure 3). However, the mechanisms leading to activation of the “initiator” kinases, i.e. the MAP3Ks remain inconclusive. Genetic approaches ablating expression as well as genome-wide small interference RNA-mediated down-regulation approaches both aim at identifying potential MAP3Ks involved in p38 and JNK activation.

Among the diverse pathways, receptor tyrosine kinase (RTK) signalling is probably best characterised. Early work revealed that the adaptor protein GRB2 is recruited though its SH2 domain to phospho-tyrosine residues of activated growth receptors. Subsequently, GRB2 serves to recruit the nucleotide exchange factor son of sevenless (SOS), which then mediates recruitment of the small GTPase Ras and its activation at the cell membrane. In its guanosine-triphosphate (GTP) bound form, Ras subsequently performs direct protein-protein interaction with the MAP3K Raf-1, inducing its phosphorylation and hence activation.
For TNFR1 signalling, several MAP3Ks have been described with TAK1 being the best characterised. TAK1 was initially identified as MAP3K activated by TGFβ (Yamaguchi et al, 1995). TAK1-deficient mice die embryonically at day E10.5 and fibroblasts generated from these embryos showed defective IL-1- and TNF-induced NF-κB, JNK and p38 responses, indicating a crucial function for TAK1 in activation of these pathways (Sato et al, 2005). TAK1 is recruited to the TNF-RSC by its adaptor proteins TAB2/3. Tab1−/− fibroblasts exhibited normal NF-κB and MAPK responses, indicating that TAB1 is dispensable for TAK1 activation. Although no defects were monitored in cells devoid of TAB2, concomitant depletion of its homologue TAB3 severely impaired TAK1 and hence IKK and MAPK activation (Ishitani et al, 2003), indicating that TAB2 and TAB3 play redundant functions and that TAK1 activation depends on the presence of at least one of these proteins. In particular, TAB2 or TAB3 are required to recruit the TAK1/TAB complex to the TNF-RSC by binding to K63-linked poly-ubiquitin chains via their NZF domains (see chapter 1.5.3) (Kanayama et al, 2004). Although TAK1 is clearly required for NF-κB, JNK and p38 activation, the mechanism leading to TAK1 activation itself remain poorly defined. Possible mechanisms include proximity-induced and conformational change-induced trans-autophosphorylation.

Figure 3: MAPK signalling. Following receptor recruitment, the MAP3Ks TAK1, MEKK1 and ASK are activated and subsequently phosphorylate down-stream MAP2Ks. MKK3/6 then activate the MAPK p38, whereas MKK4/7 are associated with JNK1/2 activation. Together with concomitant NF-κB activation, MAPKs activate gene transcription of pro-survival genes. Amongst these, SOD scavenges ROS, thereby inhibiting ASK activation. Hence, MKPs are no longer inhibited and allow for the termination of MAPK signalling. Active JNK1 stimulates the E3 ligase Itch, which attaches K48-linked ubiquitin chains to c-FLIP, thereby inducing its degradation and consequently allowing cell death to occur via complex II-activated caspase-8.
Alternatively, Wang et al. proposed that TAK1 becomes activated by K63-linked ubiquitination through an undefined ligase (Wang et al, 2001).

MEKK1 was the second MAP3K identified and was shown to potently activate p38 and JNK upon overexpression (Minden et al, 1994). These results were confirmed in knockout cells, which showed impaired JNK and p38 activation upon various stimuli (Xia et al, 2000; Yujiri et al, 2000). Co-sedimentation experiments showed that MEKK1 associated with oligomerised TRAF2, thereby providing a possible link between MEKK1 function and TNF-RSC formation (Baud et al, 1999). Together with TRAF2, MEKK1 was shown to recruit Germinal Centre Kinases (GCKs) and Germinal Centre Related Kinases (GCRKs) to the TNF-RSC, thereby providing another layer of kinases (MAP4Ks) involved in TNFR1 signalling (reviewed in Kyriakis, 1999). However, the exact mechanism for GCK/GCRK-mediated MEKK1 activation remains poorly described. Besides its kinase domain, MEKK1 possesses a RING finger motif exhibiting ligase activity (Lu et al, 2002). However, a target for this domain implicated in TNFR1 signalling has not yet been identified.

MEKK3 was proposed as further MAP3K implicated in TNFR1-mediated p38 and JNK activation, given that its overexpression was sufficient to activate both pathways (Blank et al, 1996). However, knockout experiments revealed a rather modest effect on TNF-induced MAPK activation, but a strong influence on NF-κB signalling (Yang et al, 2001). Consistently, MEKK3 has been shown to be recruited to RIP1 and to phosphorylate IKKα and IKKβ, providing another possible mechanism of IKK activation. Given its minor influence on MAPK activation, MEKK3 seems to act up-stream of TAK1 specifically stimulating NF-κB signalling.

Apoptosis signal-regulating kinase-1 (ASK1) was also described as MAP3K to activate the JNK and p38 pathway. Prolonged JNK activation was inhibited in ASK1-ablated fibroblasts, indicating a function for ASK1 in JNK stimulation (Matsuzawa & Ichijo, 2008). Following TNFR1 stimulation, ASK1 associates with TRAF2 and becomes activated (Hoeflich et al, 1999). ASK1 function is endogenously inhibited by thioredoxin (Trx), which directly binds to the N-terminal non-catalytic domain of ASK1, thereby preventing its activation by TNF (Saitoh et al, 1998). However, TNFR1 stimulation leads to TRAF2-dependent reactive oxygen species (ROS) production (Liu et al, 2000), which was shown to inhibit Trx binding to ASK1. Released ASK1 is then activated by TRAF2 by so far uncharacterised mechanisms, most likely involving homo-oligomerisation of ASK1. Moreover, ROS was shown to oxidise critical cysteine residues in the active centres of MKPs, thereby inhibiting their activity.
1. Introduction

1.3.2.2 The ERK1/2 module

ERK1 and ERK2 show 83% amino acid identity and are expressed in all tissues, though to various extents. ERK1 was the first MAPK identified and cloned in the early 90’s and found to be activated by growth factor RTKs (Boulton et al, 1991; Boulton et al, 1990). Apart from growth factors, ERK1/2 is also activated by a variety of other stimuli, including cytokines, osmotic shock and insulin (Raman et al, 2007). While the MAP3Ks A-Raf, B-Raf, Raf-1 are the major activators of ERK1/2 in RTK signalling, MEKK1 and Tpl2 (tumour progression locus 2) function in a more cell-type and stimulus-restricted manner to activate the MAP2Ks MEK1 and MEK2.

Stimulation of the ERK1/2 pathways results in its re-localisation from the cytoplasm into the nucleus, in which it activates the transcription factor Elk-1. Elk-1 is known to induce the expression of early-intermediate genes including c-Fos, which is furthermore stabilised by direct ERK1/2 phosphorylation. Dimerisation of c-Fos with c-Jun induces the formation of active AP-1 complexes, which subsequently activate the transcription of a variety of target genes including cyclinD1, a protein critically linked to cell cycle progression. Thus, ERK1/2 regulate cell proliferation and efficient G1 to S-phase transition (Cargnello & Roux, 2011).

1.3.2.3 The JNK module

JNK (also known as stress-activated protein kinase (SAPK)) is expressed in three different isoforms sharing 85% identity, which were cloned in the mid 90s by two independent groups (Derijard et al, 1994; Kyriakis et al, 1994). While JNK1/2 are widely expressed, JNK3 is primarily detected in neuronal tissues, testis and cardiac myocytes (Bode & Dong, 2007). The three-tiered kinase cascade leading to JNK1/2/3 activation involves the MAP2Ks MKK4 and MKK7 and the MAP3Ks MEKK1-4, MLK1-3, Tpl2, DLK, TAK1 and ASK1/2.

Both Mkk4 (Ganiatsas et al, 1998; Nishina et al, 1999; Yang et al, 1997) and Mkk7 genes (Dong et al, 2000) are required for embryonic viability, indicating non-redundant functions of these proteins. Indeed, JNK is preferentially phosphorylated on Tyr by MKK4 and on Thr by MKK7 (Lawler et al, 1998), suggesting that dual phosphorylation by both kinases is required for full JNK activation. Consistently, Tournier et al. demonstrated that M KK7-induced JNK phosphorylation on Thr residues activated JNK. However, maximal in vitro JNK activation required dual phosphorylation mediated by MKK4 and MKK7. Furthermore, intensive studies using M KK4 and M KK7 deficient fibroblasts showed an essential role for M KK7 in JNK activation induced by pro-inflammatory cytokines (IL-1, TNF) as well as exposure to extracellular stress. In contrast, though M KK4 was required for UV radiation-induced JNK
1. Introduction

activation, cytokine-induced signalling was only marginally affected, consistently with a lack of TNF- and IL-1-induced MKK4 activation (Tournier et al, 2001). Taken together, these data indicate that MKK4 is required for full JNK activation and that basal MKK4 activity in the presence of MKK7 is sufficient to maximally stimulate JNK activity.

Once activated, JNK phosphorylates c-Jun on Ser63/73, thereby increasing c-Jun-mediated transcription of target genes involved in cell proliferation, including cyclin D1 (Sabapathy et al, 2004). In contrast, Jnk1/2−/− double knockout fibroblasts were resistant to DNA damage- and UV-irradiation-induced cell death due to impaired cytochrome c release, indicating a function of JNK in intrinsic apoptotic responses (Tournier et al, 2000). Liu et al. showed that JNK1 had a more prominent function in TNF-induced cell death induction as compared with JNK2, indicating that although these proteins share high sequence homology, their functions are not completely redundant (Liu et al, 2004).

JNK signalling involves two phases (Ventura et al, 2006), with the first one being transiently activated early after receptor stimulation leading to the induction of pro-survival genes (Lamb et al, 2003). In contrast, the second, sustained phase is involved in cell death responses and tightly controlled by a crosstalk to NF-κB as well as activation by ROS-mediated ASK1 activation.

1.3.2.4 The p38 MAPK module

The p38 family of MAPKs consists of the four members p38α, p38β, p38γ and p38δ. The p38α isoform, which was identified in 1994, is the prototypic member of this family and is generally more responsive to stress stimuli as compared to ERK1/2 (Han et al, 1994; Lee et al, 1994; Rouse et al, 1994). Like p38β, p38α is ubiquitously expressed (Jiang et al, 1996). However, due to its higher expression in most tissues, most literature about p38 is restricted to the α isoform. MKK3 and MKK6 are the major MAP2Ks implicated in p38 activation, whereas MKK4 has a lower activity and is most prominently implicated in JNK activation. However, most stimuli do not exclusively activate one MAPK pathway, but p38 and JNK are generally activated at the same time by the same agents. While MKK6 activates all isoforms, MKK3 is unable to stimulate p38β. Various MAP3Ks have been implicated in the three-tiered p38 kinase cascade, including MEKK1-3, ASK1, Tpl2 and TAK1. The major function of p38 MAPKs is the production of pro-inflammatory cytokines, either by directly inducing their expression or by enhancing their mRNA stability and translation. Furthermore, p38 is implicated in cell proliferation and survival as it negatively regulates cell cycle transition at the G1-S and G2-M phase levels by down-regulating cyclins.
1.4 Cell death induction by death ligands of the TNF-Receptor superfamily

Most TNFR-superfamily members are involved in inflammation and immunity. However, based on the presence of a DD in the cytoplasmic part of the receptor, TNFR1, CD95 and TRAIL-R1/R2 are able to induce cell death. Although TNFR1 signalling is primarily involved in survival and proliferation, imbalances in NF-κB and MAPK activation lead to cell death. In contrast, the main function of CD95 and TRAIL-R1/R2 in immunity is to induce cell death of activated lymphocytes to prevent them from responding excessively against healthy tissue, thereby conferring tissue homeostasis. While CD95L- and TRAIL-induced cell death originates at the plasma membrane immediately following receptor engagement, TNF-induced death responses are rather slow and mediated via an intracellular complex derived from the membrane-associated complex. Initially, death ligand-induced cell death was solely attributed to be of apoptotic nature involving a tightly controlled cascade of caspase-cleavages resulting in the death of individual cells without affecting neighbouring tissue. However, intense research in the past decade revealed that death ligands can also induce a caspase-independent form of cell death (Laster et al, 1988) which has been referred to as necroptosis (Degterev et al, 2005). Unlike necrosis, in which cells die “accidentally”, necroptosis is, similar to apoptosis, a tightly controlled process regulated by the two closely related kinases RIP1 and RIP3 (Vandenabeele et al, 2010).

1.4.1 CD95L- and TRAIL-induced apoptosis

1.4.1.1 The CD95L/CD95 and TRAIL/TRAIL-R1/2 systems

Cell death mediated by TNFR superfamily members is primarily associated with the CD95L/CD95 as well as TRAIL/TRAIL-R1/R2 systems. The major function of CD95L (APO-1L, FasL) is mediated by its binding to CD95 (APO-1, Fas). Although binding to Decoy-Receptor3 (DcR3) has been suggested to neutralise its apoptosis-inducing function, the physiological relevance of this interaction is less clear (Wroblewski et al, 2003).

CD95 is ubiquitously expressed, though predominantly in the thymus, liver, heart, kidney and in virus-transformed lymphocytes. In contrast, CD95L expression is very restricted and tightly regulated. Following activation, T cells up-regulate CD95L and subsequently die by AICD (Dhein et al, 1995). Accordingly, deregulation of the CD95 system, as in ALPS (autoimmune lymphoproliferative syndrome) patients carrying a heterozygous mutation in the CD95 gene, is hallmarked by the inability to shut down immune responses. Activated T cells are not eliminated but remain active, thereby creating autoimmune reactions against self-antigens.
1. Introduction

(Rieux-Laucat et al, 1995). Another important function of the CD95L/CD95 system is to kill virus-infected and oncogene transformed cells.

CD95L can be found as membrane-bound ligand or soluble protein generated by the metalloprotease ADAM10 (Schulte et al, 2007). Aggregation of pre-assembled CD95L trimers is required for CD95L-induced cell death. Yet, the contribution of membrane-bound versus soluble CD95L remained obscure. To answer this question, O’Reilly et al. recently generated mice which either lacked soluble CD95L (lacking the ADAM10-cleavage site; FasLΔs/Δs) or membrane bound CD95L (lacking the transmembrane domain; FasLΔm/Δm). This study showed that only T cells obtained from FasLΔs/Δs, not FasLΔm/Δm mice were capable of killing target cells (O’Reilly et al, 2009). Furthermore, FasLΔm/Δm mice developed lymphadenopathy and hyper-gammaglobulinaemia, similar to FasLgld/gld animals, which exhibit mutations in the CD95L sequence abolishing its binding to CD95. However, FasLΔm/Δm additionally developed systemic lupus erythematosus (SLE)-like auto-immune kidney destruction and histocytic sarcomas, alterations only rarely seen in FasLgld/gld mice. Taken together, these results implicate that membrane-bound CD95L confers cytotoxicity and prevents autoimmunity and cancer development. In contrast, excessive accumulation of soluble CD95L amplifies non-apoptotic CD95 signalling, thereby promoting tumourigenesis and manifestation of autoimmune disorders.

TRAIL was found to bind to five receptors in the human system, i.e. TRAIL-R1 (DR4), TRAIL-R2 (DR5, APO-2, KILLER, TRICK2), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and OPG. However, only TRAIL-R1 and TRAIL-R2 are capable of inducing apoptosis due to the presence of an intracellular DD. It is still a matter of debate why cells express two receptors with apparently similar functions, although expression of only one is sufficient to induce cell death in a variety of tumour cells (Sprick et al, 2002). However, non-apoptotic functions including cell migration and proliferation might differentially require TRAIL-R1 and TRAIL-R2, respectively.

Although the extracellular domains of TRAIL-R3 (Degli-Esposti et al, 1997a; Degli-Esposti et al, 1997b) and TRAIL-R4 (Degli-Esposti et al, 1997a) are highly homologous to their apoptosis-inducing counterparts, both receptors completely or partially lack intracellular DDs and are therefore referred to as decoy receptors. Merino et al. proposed that both decoy receptors use diverse mechanisms to inhibit TRAIL-induced apoptosis. On the one hand, TRAIL-R3 was shown to retain TRAIL within lipid rafts, thereby competitively inhibiting its binding to TRAIL-R1/2. On the other hand, TRAIL-R4 formed hetero-dimers with
1. Introduction

TRAIL-R2 following TRAIL binding, which prevented the formation of active signalling platforms transmitting apoptotic signals (Merino et al, 2006). However, this study was based on overexpression experiments and a regulatory function of TRAIL-R3/4 under physiological conditions has not been demonstrated yet. Moreover, expression of the decoy receptors does not correlate with resistance of a given cell towards TRAIL-induced cell death, challenging a decoy function of these receptors.

OPG was described as the fifth, rather low-affinity receptor for TRAIL (Truneh et al, 2000). Initially, OPG was shown to competitively inhibit interactions between RANKL and RANK, thereby repressing osteoclast formation. However, given that $\text{Trail}^{-/-}$ and $\text{Trail-R}^{-/-}$ mice do not show any obvious defects in bone architecture, it is rather unlikely that TRAIL-OPG interactions are involved in bone remodelling (Cretney et al, 2002; Diehl et al, 2004).

TRAIL and TRAIL-R1/2 are ubiquitously expressed through human tissues, including spleen, thymus, peripheral blood lymphocytes, prostate, testis, ovary, uterus and multiple tissues along the gastrointestinal tract (Walczak et al, 1997; Wiley et al, 1995). Thus, in contrast to CD95 signalling, which is tightly controlled by regulated CD95L expression, other regulatory mechanisms except from mRNA expression have to account for the TRAIL system.

1.4.1.2 The extrinsic apoptotic pathway

Cell death initiated by TRAIL or CD95L is a tightly controlled process characterised by a caspase-activation cascade, which can be interrupted or promoted at various check-points by intracellular pro- and anti-apoptotic proteins. Pre-formed TRAIL or CD95L trimers bind to their respective receptors, which can form pre-assembled receptors connected via a pre-ligand assembly domain (PLAD). This domain allows for di- or trimerisation of the receptor without binding of the respective ligand. Although the PLAD domain differs from the interaction surface required for ligand binding and is not capable of transmitting a death signal, it might depict a pre-requisite for efficient higher-order oligomerisation of the receptor following binding of the respective ligand to ensure efficient signal transduction (Chan et al, 2000). Ligand binding to this pre-assembled receptor complex results in juxtaposition of the DDs, allowing for the recruitment of Fas-associated death domain (FADD), which binds via its DD to the DD of the receptor. Subsequently, pro-caspase-8 and -10, as well as the cellular FLICE-like inhibitory protein (c-FLIP) are recruited by interaction of their death-effector domains (DEDs) with the DEDs of FADD (Figure 4). Together, these proteins form the death-inducing signalling complex (DISC).
1. Introduction

Caspases are cysteinyl-aspartate specific proteases possessing cysteine residues in their active centres which are crucial for cleaving their target proteins after aspartic acid residues. They are synthesised as pro-enzymes consisting of a large and a small catalytic subunit and an amino-terminal pro-domain. Initiator caspases including caspase-2, -8, -9 and -10 activate caspase-cascades, while the effector/executioner caspases-3, -6 and -7 translate apoptotic signals through cleavage of non-caspase target proteins.

Recruitment of pro-caspase-8 and -10 to the DISC results in auto-catalytic processing through not completely defined mechanism, most likely involving proximity-induced activation. Once caspase-8 is processed to its active form, it cleaves down-stream caspases including caspase-3 by separating the large from the small subunit. However, this step is not sufficient to activate...
1. Introduction

Caspase-3 which requires a further auto-catalytic maturation step involving the removal of its pro-domain (Figure 5). Once activated, caspase-3 cleaves a variety of cellular proteins, including poly(ADP)-Ribose-Polymerase (PARP), lamins and cytokeratins. Furthermore, it inactivates ICAD/DFF45, the inhibitor of Caspase Activated DNase (CAD), thereby liberating CAD, which subsequently enters the nucleus to fragment DNA, thereby producing the “DNA ladder” characteristic for apoptotic cells (Inohara et al, 1999; Liu et al, 1998).

1.4.1.3 The intrinsic apoptotic pathway

Apart from death receptor-induced activation of the above described extrinsic pathway, apoptosis can also be initiated autonomously by intracellular caspase activation following irradiation- or chemotherapeutic agent-induced DNA damage. This so-called intrinsic apoptotic pathway primarily depends on mitochondria. Mitochondrial integrity is tightly regulated by a functionally diverse class of proteins that belong to the B cell lymphoma 2 (Bcl-2) family. Due to their domain structure, members of the Bcl-2 family can be subdivided.
into three groups (Borner, 2003). Anti-apoptotic proteins like Bcl-2, Bcl-extra large (Bcl-X₁) and myeloid leukaemia cell differentiation 1 (Mcl-1) are associated with the outer membrane of the mitochondria in order to maintain mitochondrial integrity, to prevent cytochrome c release and to promote cell survival. In contrast, members of the pro-apoptotic Bcl-2 family including Bcl-2-associated X protein (Bax), Bcl-2-antagonist/killer (Bak) and Bok destabilise mitochondrial integrity possibly by generating a transmembrane pore through the outer mitochondrial membrane during apoptosis, thereby inducing loss of the mitochondrial transmembrane potential and release of pro-apoptotic factors. Pro-apoptotic Bcl-2 family members are activated by molecules of the Bcl-2 homology 3 (BH3)-only family of proteins including BH3-interacting domain death agonist (Bid), Bcl-2 antagonist of cell death (Bad), p53 up-regulated modulator of apoptosis (PUMA), NOXA and Bcl-2-interacting mediator of cell death (Bim). It remains controversial whether these proteins directly activate the pro-apoptotic Bcl-2 proteins Bax and Bak or only indirectly via neutralisation of Bcl-2-like anti-apoptotic proteins (Strasser, 2005).

Genotoxic stress-activated BH3-only proteins increase the permeability of the outer mitochondrial membrane, resulting in the release of cytochrome c and other pro-apoptotic factors from the mitochondrial intermembrane space into the cytosol. Together with apoptotic protease-activating factor-1 (Apaf-1), dATP and pro-caspase-9, cytochrome c forms a complex referred to as apoptosome (Baliga & Kumar, 2003). Like caspase-8, apoptosome-activated caspase-9 is also able to activate pro-caspase-3. Activated caspase-3 not only cleaves down-stream targets involved in apoptosis execution, but also pro-caspase-9, thereby providing a positive feedback loop assuring apoptosis to be inevitably carried out.

Bid is not only activated by DNA damage and cellular stress but also cleaved by caspase-8. Truncated Bid (tBid) translocates to the mitochondria to allow the release of pro-apoptotic factors, which then facilitate full caspase-3 activation. Thus, Bid bridges the extrinsic and intrinsic apoptotic pathways.

1.4.1.4 Checkpoints in apoptotic responses

Depending on the need of the intrinsic apoptotic pathway to undergo death receptor-induced apoptosis, cells can be classified as type I and type II cells. Type I cells are characterised by strong DISC formation and therefore strong caspase-8 activation, which fully activates caspase-3. In contrast, DISC formation in type II cells is rather weak. Thus, these cells additionally require the mitochondrial amplification loop to efficiently activate effector caspases to undergo apoptosis (Ozoren & El-Deiry, 2002). However, this view was recently
challenged by Jost et al. who showed that the dependency on the intrinsic pathway is defined by intracellular X-chromosome linked inhibitor of apoptosis (XIAP) levels (Jost et al, 2009). CD95L induced caspase-dependent rapid loss of XIAP levels in thymocytes, which represent the prototype of type I cells. In contrast, XIAP levels increased in type II-classified hepatocytes in response to CD95 stimulation, whereas other anti-apoptotic proteins including Bcl-2, Bcl-XL and Mcl-1 or pro-apoptotic Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP binding protein with low PI) did not significantly change during CD95 engagement. Injection of CD95L or agonistic anti-CD95 antibodies into mice resulted in rapid death due to massive hepatocyte death and hence liver failure (Huang et al, 1999; Ogasawara et al, 1993). Given that type II cells depend on the mitochondrial amplification loop to execute cell death, it is not surprising that Bid−/− mice are resistant towards this treatment (Kaufmann et al, 2009). However, Bid−/− Xiap−/− double knockout mice succumbed to CD95L-induced death, indicating that XIAP is the crucial factor discriminating between type I and type II cells.

XIAP inhibits cell death by directly interacting via the BIR2, the preceding linker region and the BIR3 domain with caspase-3, -7 and -9 (Riedl et al, 2001). XIAP blocks the removal of the inhibitory pro-domain of caspase-3, thereby inhibiting the above-mentioned maturation step. Moreover, XIAP has been shown to catalyse the ubiquitination of caspase-3, leading to its proteasomal degradation (Vaux & Silke, 2005). Consistently, murine cells expressing an E3 ligase-deficient version of XIAP exhibited elevated levels of active caspase-3 and increased sensitivity towards apoptotic stimuli (Schile et al, 2008).

The activity of XIAP is in turn controlled by another set of proteins that antagonises their function. Once released from the mitochondrial intermembrane space, pro-apoptotic Smac/DIABLO interacts with the BIR domains of XIAP, cIAP1 and cIAP2 via its N-terminal four amino acid AVPI sequence (Liu et al, 2000; Wu et al, 2000). Binding of Smac/DIABLO or its mimetics to XIAP directly relieves its interaction with caspase-3, -7 and -9 (Chai et al, 2000; Li et al, 2004). Liberation of caspase-3 from XIAP subsequently induces its auto-catalytic cleavage and hence full activation, thereby allowing apoptosis to proceed. Furthermore, Smac/DIABLO functions as an allosteric activator of the E3 ligase activity of cIAP1/2, thereby promoting cIAP1/2 auto-ubiquitination and subsequent proteasomal degradation (Du et al, 2000; Varfolomeev et al, 2007; Vince et al, 2007).

The family of c-FLIP constitute another major control point regulating death receptor-induced responses. Due to the presence of two DEDs, c-FLIP closely resembles caspase-8 in structure
1. Introduction

and hence competes with it for binding to FADD (Krueger et al, 2001). However, due to a lack of the critical cysteine residue within the active centre, c-FLIP does not possess proteolytic activity. Hence, displacement of caspase-8 from the DISC prevents the initiation of the caspase cascade responsible for apoptosis transmission. The amount of c-FLIP within a cell inversely correlates with the amount of caspases that are activated at the DISC and is hence a decisive factor for apoptosis induction.

c-FLIP exists in three splice variants giving rise to a long poly-peptide of 55 kDa (c-FLIP_L), a short one of 26 kDa (c-FLIP_S) and a third, 23 kDa form which has been initially described in the Raji B cell line (c-FLIP_R) (Golks et al, 2005). Overexpression of c-FLIP_L modulates DISC formation in a way that the entire pool of DISC-recruited caspase-8 constitutes of immature p41/p43 fragments. However, generation of the active p18 fragment is inhibited. In contrast, high levels of c-FLIP_S in the DISC reduce overall DISC-recruited caspase-8 levels and completely inhibit its processing (Kuvari et al, 2011). Comprising two DEDs and an additional C-terminal caspase domain, c-FLIP_L closely resembles caspase-8 in its overall structure. Recruitment of c-FLIP_L to the CD95 and TRAIL-R1/2 DISC induces its caspase-mediated cleavage to produce a 43 kDa isoform which still contains the DEDs and the pseudo-caspase-motif. This fragment remains bound to FADD and inhibits caspase-8 maturation. Whereas c-FLIP_S and c-FLIP_R completely inhibit pro-caspase-8 cleavage at the DISC, cFLIP_L rather interferes with full maturation of DISC-recruited pro-caspase-8.

Another layer of complexity regulating death receptor signalling is provided by ubiquitination. Although the function of the ubiquitin system for cell signalling has been best characterised for TNFR1, Jin et al. described that caspase-8 ubiquitination is required for its full activity in TRAIL-R signalling (Jin et al, 2009). The RING E3 ligase Cullin3 was reported to be recruited to the DISC to induce caspase-8 ubiquitination, followed by p62-mediated aggregation of caspase-8, leading to its stabilisation and thereby augmenting its activation and full processing. Thus, ubiquitination in death receptor signalling has recently emerged as a new mechanism positively controlling its outcome.

Several tumours overexpress anti-apoptotic IAPs, a condition generally associated with poor prognosis (Hunter et al, 2007). The function of cIAP1/2 is also crucially linked to the regulation of death receptor responses given that cIAP1 ubiquitinates caspase-3 and -7 (Choi et al, 2009) as well as Smac (Hu & Yang, 2003), thereby enhancing cell survival. Constitutive activation of the NF-κB pathway by translocation-induced generation of a cIAP2-MALT1 (mucosa-associated lymphoid tissue) fusion protein is associated with the development of
non-Hodgkin MALT lymphomas, characterised by enhanced pro-survival and pro-inflammatory signalling, tumour progression and resistance to anti-cancer drugs (Zhou et al, 2005). Targeting IAPs in tumours exhibiting enhanced expression or activity of these E3 ligases constitutes a promising strategy in cancer therapy. The most promising approach developed so far involves small molecule IAP antagonists mimicking the N-terminal AVPI sequence of active Smac/DIABLO. IAP antagonists efficiently bind to the BIR domains of IAP proteins and have been proven efficient in inhibiting tumour growth \textit{in vivo} (Ndubaku et al, 2009). Unexpectedly, IAP antagonists not only prevent the interaction of XIAP and cIAPs with caspases, but induce conformational changes in cIAP1/2, resulting in their activation, auto-ubiquitination and subsequent degradation (Sun et al, 2007). As described in chapter 1.3.1.2, depletion of cIAPs induces NIK stabilisation and activation of non-canonical signalling. Translocation of p52/RelB dimers to the nucleus drives transcription of various target genes involved in pro-inflammatory responses, including TNF. Secreted TNF subsequently binds to TNFR1 and, in the absence of cIAP1/2, induces cell death (see chapter 1.4.2). However, as shown \textit{in vitro} using a panel of tumour cell lines, TNF is only produced by a subset of cell lines, which are therefore referred to as Smac mimetic (SM) sensitive (Vince et al, 2007). With regard to therapeutic use, IAP antagonists might be especially useful for the treatment of SM-sensitive tumours.

Only recently, Geserick \textit{et al.} provided new mechanistic insights into how cIAP1/2 regulate TRAIL- and CD95L-induced cell death responses. SM-induced depletion of cIAP1/2 in keratinocyte cell lines strongly sensitised for CD95L- and TRAIL-induced cell death, which was accompanied by increased levels of RIP1 at the DISC (Geserick et al, 2009). RIP1 as well as caspase-8 activity was required for cell death in the absence of cIAP1/2, indicating that SM-induced cell death possesses apoptotic as well as necroptotic characteristics (see chapter 1.4.3). Furthermore, c-FLIP\textsubscript{L} but not c-FLIP\textsubscript{S} inhibited RIP1 recruitment to the DISC and rescued cell death in the absence of cIAP1/2. Thus, RIP1 has a fundamental role in TRAIL- and CD95L-induced signalling and provides an alternative, caspase-independent death pathway.

So far, no differences in DISC composition between the CD95 and TRAIL-R1/R2 have been identified. However, biological outcomes of TRAIL versus CD95L-stimulation are extremely diverse. While systemic CD95 stimulation kills normal tissues including hepatocytes, TRAIL can specifically eliminate malignantly transformed cells without damaging healthy tissue, proposing TRAIL as promising strategy in anti-cancer therapy. More studies analysing the
1. Introduction

DISC compositions in the two systems will be required to identify target proteins explaining the diverse cellular responses.

Taken together, cell death induction by TRAIL and CD95L is a highly controlled process that can be modified at different levels, including the DISC (c-FLIP, caspase-8 ubiquitination), mitochondria (Bcl-2 family members, Smac/DIABLO release) as well as intracellular E3s (XIAP, cIAPs). Imbalances in the signalling cascade are readily linked to the development of autoimmune disorders and tumourigenesis.

1.4.1.5 Non-apoptotic TRAIL- and CD95L-induced signalling

Apart from their abilities to induce cell death, CD95 and TRAIL-R1/2 can also activate NF-κB and MAPKs, albeit to a lesser extent and with delayed kinetics when compared to TNFR1 signalling (reviewed in Falschlehner et al, 2007). Death receptor-induced activation of JNK and p38 in HaCaT cells appeared rather late, peaking at around two hours (Kavuri et al, 2011). Inhibition of caspases or overexpression of any c-FLIP isoform suppressed TRAIL- and CD95L-induced MAPK activation. In contrast, NF-κB activation, which was also inhibited by c-FLIP overexpression, did not depend on caspase-8 activity but on its mere presence. Consistent with a missing function of c-FLIP in TNF-RSC formation, its overexpression did not interfere with TNF-induced NF-κB and MAPK activation. Thus, anti-apoptotic TRAIL- and CD95L-induced signalling depends on the presence (NF-κB) or activity (MAPKs) of caspase-8 and is negatively regulated by c-FLIP (Kavuri et al, 2011).

In line with a pro-survival function of TRAIL, Varfolomeev et al. proposed the generation of one or several secondary intracellular complexes following TRAIL stimulation which contain caspase-8, FADD, NEMO, TRAF2 and RIP1 (Varfolomeev et al, 2005). However, little is known about the function of the secondary complexes, whether they merely serve anti-apoptotic functions or also contribute to cell death induction. Furthermore, it remains to be determined whether the proteins mentioned above form part of the same complex or constitute various sub-complexes with different functions. However, unlike delayed TNF-induced complex II (see next chapter), TRAIL- and CD95L-induced secondary complexes seem to form simultaneously with the primary, membrane-bound signalling platforms. Thus, more in-depths analyses are required to understand the function and relevance of these secondary complexes.
1.4.2 TNFR1-induced apoptosis

1.4.2.1 TNF-complex II formation

In 2003, Micheau and Tschopp described the formation of a secondary, intracellular complex following TNFR1 activation, which they referred to as complex II (Micheau & Tschopp, 2003). In contrast to the transmembrane complex, also known as complex I, complex II was shown to be implicated in cell death induction. Complex II forms as a consequence of complex I and appears with delayed kinetics once the primary complex disassembles. RIP1, TRADD and possibly TRAF2 dissociate from the membrane to form a cytosolic complex with FADD and caspase-8. This intracellular platform is capable of activating caspase-8 by cleavage, resulting in subsequent caspase-3 and Bid activation and hence apoptosis execution. Although the precise mechanism remains elusive, Oshima et al. proposed an anti-apoptotic function of ABIN-1 in complex II formation by inhibiting caspase-8-FADD interactions and subsequent TNF-mediated death (Oshima et al, 2009). However, TNF does not induce cell death in most cell lines under physiological conditions. Currently, this is thought to be due to rapid, complex I-induced activation of NF-κB, which results in the transcription of anti-apoptotic genes including c-FLIP, Bcl-2 family members, TRAF proteins and cIAPs (Micheau et al, 2001; Wang et al, 1998). Although several anti-apoptotic factors were shown to be up-regulated following TNF-induced NF-κB activation, major attention has been attributed to c-FLIP regulation, given that Flip⁻/⁻ fibroblasts undergo TNF-induced cell death even without inhibition of NF-κB-dependent gene expression (Yeh et al, 2000). High c-FLIP levels in complex II inhibit activation of caspase-8, thereby limiting the cytotoxic potential of TNF. Thus, TNF-induced cell death is negatively regulated by NF-κB-dependent c-FLIP induction.

1.4.2.2 TNF-induced JNK mediated cell death

In contrast to a well-established role for NF-κB in cell survival under normal physiological conditions, prolonged JNK activation has been associated with TNF-induced cell death (De Smaele et al, 2001; Papa et al, 2004; Tang et al, 2001). Crosstalk between these two pathways decides about the fate of a particular cell, i.e. survival or cell death. In this context, NF-κB was shown to induce the expression of antioxidants, including superoxide dismutase 2 (SOD2) (Pham et al, 2004). These antioxidants subsequently prevent oxidation and hence suppression of MKPs by ROS, which is also produced upon TNF-stimulation (Kamata et al, 2005). Thus, MKP-induced dephosphorylation of JNK terminates its activity and hence prevents JNK-mediated cell death under physiological conditions. However, in cells in which
NF-κB activation is attenuated, antioxidants are not expressed, ROS is not scavenged and JNK phosphatases therefore inhibited, resulting in prolonged JNK activation and cell death induction. Although prolonged JNK1 activation is required for TNF-induced cell death, prolongation of its activity alone in the absence of concomitant TNF stimulation is not sufficient to kill cells, indicating that JNK1 targets specific components of TNFR1 signalling to allow TNF-induced cell death to occur.

JNK1-deficient hepatocytes are resistant to TNF plus cycloheximide (CHX)-induced cell death and the absence of JNK1 delayed lethality caused by removal of IKKβ by four days, underscoring the physiological relevance of an NF-κB (IKKβ-mediated) and JNK crosstalk (Chang et al, 2006). The mechanism of JNK-mediated cell death induction has remained an open question for several years since a JNK-related target gene has not been identified. However, the observation that c-FLIP levels were stabilised in JNK1 deficient cells following TNF stimulation combined with the finding that JNK1 (and not JNK2) was able to phosphorylate and activate the HECT E3 ligase Itch provided new mechanistic insight to this conundrum (Gao et al, 2004). JNK1-mediated Itch activation was shown to induce ubiquitination and subsequent proteasomal degradation of c-FLIP, in particular c-FLIPL. Reduced levels of c-FLIP were unable to prevent caspase-8/FADD complex formation and caspase-8 processing, which ultimately resulted in cell death (Figure 6). Consistently, Itch or JNK1 deficiency in mice protects them from TNF-induced acute liver failure due to c-FLIP-mediated protection. Given that Itch phosphorylation is a reversible process, prolonged JNK1 activation is required to maintain Itch in an active state allowing for complete c-FLIP degradation. Taken together, c-FLIP levels, which are controlled on the transcriptional (NF-κB induction) and post-translational (JNK1-mediated degradation) level, are critical determinants deciding about the output of TNF-induced signalling.

1.4.2.3 Complex IIA versus Complex IIB

Complex II-mediated cell death can be executed in cells lacking substantial NF-κB activity, due to the inability to up-regulate anti-apoptotic factors (Muppidi et al, 2004). Thus, the balance between TNF-induced survival and cell death can be shifted to the latter in cells displaying mutations in the NF-κB-activating arm. Based on the dependency of c-FLIP levels for TNF-induced cell death, complex II can be subdivided in complex IIA and complex IIB (Wang et al, 2008). As mentioned before, most cells are resistant to TNF-induced cell death and rather induce pro-survival signalling. However, IAP antagonist-mediated down-regulation of cIAPs and CHX-induced inhibition of protein translation enforced complex IIB and IIA
formation, respectively, thereby allowing TNF-induced cell death to occur. Internalisation of complex I from the membrane induced conformational changes in RIP1 and TRAF2, which allowed TRADD to dissociate and to form complex IIA with caspase-8 and FADD. The activity of complex IIA depends on c-FLIP levels, which are regulated by TNF-induced JNK and NF-κB signalling and are negatively modified by CHX.

In contrast, complex IIB formation depends on the presence of RIP1, which has been shown to possess, based on its ubiquitination state, pro- as well as anti-apoptotic properties (Hsu et al, 1996; Stanger et al, 1995). TNF-RSC-recruited RIP1 in IAP antagonist-treated cells was not modified with ubiquitin and hence allowed efficient dissociation from the membrane and association with caspase-8/FADD. Thus, cIAPs prevent the recruitment of RIP1 to complex IIB (Bertrand et al, 2008). TRADD and TRAF2 were not found in complex IIB and TRADD knockdown was shown to even enhance SM-induced complex IIB formation and hence cell death (Wang et al, 2008). Furthermore, c-FLIP levels did not alter complex IIB activity, indicating that the two secondary complexes apply diverse mechanisms and proteins for cell death execution. While the kinase domain of RIP1 is dispensable for pro-survival TNFR1 signalling, it is required for cell death responses. Accordingly, caspase-8 interaction with FADD was strongly diminished when RIP1’s kinase domain was chemically inhibited or mutated (Degterev et al, 2008; Wang et al, 2008).

Taken together, TNF can induce cell death via two distinct, caspase-8-involving pathways that are formed under diverse conditions. Complex IIA-induced cell death depends on the presence of TRADD and is controlled by c-FLIP levels, but independent of RIP1. In contrast, complex IIB is insensitive to inhibition by c-FLIP, is regulated by cIAPs and requires RIP1 kinase activity.

1.4.3 TNF-induced necroptosis

Although the molecular signature of necroptosis is less well defined than the signalling pathways that induce apoptosis, a prominent function for RIP1 in death receptor-induced, caspase-independent cell death execution was demonstrated by Holler et al. in 2000 (Holler et al, 2000). However, the downstream mechanisms and description of other relevant factors remained poorly described. Accordingly, it remained obscure why some cells preferentially die by necroptosis, whereas others die by apoptosis. Performing a genome-wide siRNA screen, He et al. solved this mystery by identifying RIP3 as the determinant for cell death responses (He et al, 2009). They showed that cells expressing RIP3 were sensitive to TNF-induced caspase-independent necroptosis, whereas those lacking RIP3 expression purely died
1. Introduction

by apoptosis. Consistently, ectopic expression of RIP3 in RIP3-deficient cell lines rendered them susceptible to necroptosis, underscoring the importance of RIP3 in necroptotic responses. In contrast, cells expressing a RIP3 mutant lacking the kinase domain remained insensitive to necroptosis. However, necroptosis can only occur in RIP3-expressing cells if caspases are inhibited at the same time, indicating that caspase-8 blocks necroptosis (Figure 6). In line with this observation, it was shown that a heterodimer of caspase-8 and c-FLIP_L possessed stronger caspase-8 activity than a caspase-8 homodimer in a cell free system (Micheau et al, 2002). Moreover, Oberst et al. recently described the inhibition of RIP1/RIP3-mediated necroptosis by caspase-8/c-FLIP_L heterodimers, therefore providing the missing link explaining why caspase-8 deficient mice are not viable (Oberst et al, 2011). Backcrossing Caspase-8−/− or Fadd−/− mice to Rip1−/− (Kaiser et al, 2011; Zhang et al, 2011) or Rip3−/− (Kaiser

**Figure 6: TNF-induced necrosome formation.** Following TNF-RSC formation at the membrane, NF-κB and MAPKs are activated leading to the transcription of pro-inflammatory genes. In a negative feedback loop, NF-κB and MAPKs up-regulate the expression of CYLD and A20, which deubiquitinate RIP1 at the TNF-RSC, thereby allowing the formation of the cytosolic complex II and the termination of NF-κB/MAPK signalling. In TRADD-dependent secondary complexes, FADD and caspase-8 are recruited and activated, leading to apoptosis accompanied by RIP1 and RIP3 cleavage. Presence of IAP antagonists favours formation of a RIP1-dependent complex, whose function is independent of TRADD. The FADD-RIP scaffold induces activation of caspase-8 in a RIP1-dependent manner and leads to apoptosis. However, if caspase-8 activity is inhibited (indicated by the yellow star), RIP1 and RIP3 associate in a complex referred to as necrosome with FADD, caspase-8 and possibly TRADD, become activated and induce necroptosis.
et al, 2011, Oberst et al, 2011) mice rescued pre-natal liver failure-induced lethality of Caspase-8^-/- or Fadd^-/- mice. Besides its well characterised pro-apoptotic function required for immune homeostasis to prevent accumulation of abnormal T cell subsets, these studies demonstrate that the main function of caspase-8 during embryogenesis is to restrict RIP1/RIP3-dependent necrotic death rather than to induce apoptosis. However, Fadd^-/- Rip1^-/- double knockout mice died a few days after birth, consistently with the phenotype of Rip1^-/- single knockout mice, indicating that, unlike RIP3, RIP1 also possesses pro-survival functions independent of its necroptotic activity. Inactivation of RIP1 and RIP3 by caspase-8/c-FLIP heterodimers inactivates these proteins by an as yet unresolved mechanism, most likely involving their proteolytic cleavage. Consequently, caspase-8 inhibition switches the cellular response from apoptotic to necroptotic cell death.

RIP1 and RIP3 are quite similar in structure. Both contain a kinase domain, followed by a RIP homotypic interaction motif (RHIM), which is required for the interaction of both proteins following death receptor stimulation. However, unlike RIP3, RIP1 possesses a DD. TNF stimulation in the presence of caspase inhibitors and IAP antagonists induces complex IIB formation, which also includes RIP3. Closer examination revealed that the RIP3-pool interacting with RIP1 following TNFR1 activation was modified by phosphorylation. Although kinase-deficient RIP3 was still able to interact with RIP1 upon TNF stimulation, RIP3 phosphorylation was absent, indicating that RIP3 auto-phosphorylates following interaction with RIP1. Accordingly, inhibition of RIP1 kinase activity by its specific inhibitor necrostatin-1 (Degterev et al, 2005) prevented its interaction with RIP3 and hence necroptosis induction, indicating that RIP1 has to phosphorylate a target protein, most likely itself in order to interact with RIP3.

As described in chapter 1.5.4, the DUB CYLD has been shown to remove K63- and linear poly-ubiquitin chains in vitro (Komander et al, 2009), and to be recruited to the TNF-RSC where it removes polyubiquitin chains from target proteins, including RIP1. As the ubiquitination state of RIP1 determines whether its function induces survival or cell death, it is not surprising that necroptosis is inhibited in CYLD-depleted cells (Hitomi et al, 2008; Wang et al, 2008). These findings define CYLD as an inhibitor of necroptosis.

Although most work on necroptosis focused on TNF-induced cell death, TRAIL and CD95L were also shown to induce necroptosis in the absence of caspase-8 activity (Holler et al, 2000). While FADD was completely required for caspase-dependent and -independent TRAIL- and CD95L-mediated cell death in Jurkat T cells, Fadd^-/- T cells were even more
sensitive to TNF-induced cell death, indicating an anti-necroptotic function of FADD in TNFRI signalling. However controversially, TNF-induced necroptosis was blocked in Fadd\textsuperscript{-/-} fibroblasts (Lin et al, 2004), suggesting the presence of different necroptosis-inducing complexes (so-called necrosomes) in different cell types. Accordingly, the strict requirement of TRADD for TNF-induced necroptosis is under debate. Some studies showed the absence of apoptosis and necroptosis in Tradd\textsuperscript{-/-} cells, supporting an indispensable role for TRADD in both types of cell death (Ermolaeva et al, 2008; Micheau & Tschopp, 2003). In contrast, TRADD was not detected in IAP antagonist-induced, TNF-dependent complex II and siRNA-mediated TRADD knockout even enhanced complex II formation in some cell types, arguing against a crucial role for TRADD in complex II formation and activity (Declercq et al, 2009; Wang et al, 2008). Thus, it currently appears that necroptosis is a highly stimulus- (presence of IAP antagonists, CHX) and cell type-dependent process.

The signalling events that occur down-stream of RIP1-RIP3 interaction and are implicated in necroptosis execution are still poorly defined. RIP3 has been described to interact with glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1), thereby increasing their enzymatic activities and promoting a considerable metabolic burst (Zhang et al, 2009). Additional respiratory substrates are generated due to increased glycogenolysis and glutaminolysis, resulting in over-generation of ROS, which subsequently triggers mitochondrial membrane permeabilisation (MMP) and hence necroptosis. However, scavenging of ROS failed to prevent necroptosis in some cell lines (including HT-29), suggesting ROS as a mediator of necroptosis in some, but certainly not all cell types (He et al, 2009).

Several viruses were shown to produce proteins capable of inhibiting caspase-8, thereby preventing the host cells from undergoing apoptosis and clearing the viral infection. In scenarios where apoptotic responses are inhibited, RIP3-mediated necroptosis is believed to act as a back-up mechanism to eliminate virus-infected cells. Cells dying by necroptosis release danger-associated molecular patterns which then, together with the viral antigens, function as endogenous adjuvant to boost an efficient immune response. Consistently, although Rip3\textsuperscript{-/-} mice are viable and develop normally, they fail to control viral infection, most likely due to impaired necroptosis-mediated activation of innate, and consequently, adaptive immunity (Cho et al, 2009; He et al, 2009; Zhang et al, 2009). These results demonstrate that necroptosis possesses \textit{in-vivo} relevance.
1. Introduction

Taken together, although the signalling molecules used in the three death receptor systems are redundant, the outcomes of TNF versus TRAIL/CD95L signalling are very diverse and strongly depend on the cellular context. cIAPs have been described as critical determinants of cellular fate and their absence has been shown to shift the balance towards cell death in all three systems. However, more in-depth analyses are required to resolve the pathways in order to target them in various human diseases.

1.5 The ubiquitin system

Post-translational modifications of proteins constitute a major mechanism by which cells regulate and fine-tune signalling outputs initiated by various stimuli. In the 1950s’, phosphorylation was the first mechanism identified to regulate enzymatic activities (Burnett & Kennedy, 1954). Kinase-mediated phosphorylation describes the covalent attachment of a phosphate group to serine, threonine or tyrosine residues of target proteins, thereby ultimately altering their electrostatic properties. By changing the allosteric conformation of proteins, phosphorylation allows for the recruitment of downstream proteins and/or activation or inactivation of target proteins, thereby contributing to signal propagation. Phosphorylation is a complex, reversible process that involves more than 500 protein kinases, whose activities are counteracted by approximately 120 phosphatases which remove phosphate groups (Manning et al, 2002).

Ubiquitination was discovered in the late 1970’s as part of an energy-dependent protein-degradation system (Ciechanover et al, 1978), in which covalent conjugation of ubiquitin, at that time referred to as ATP-dependent proteolysis factor (APF-1), to lysine residues of target proteins was shown to be essential for their proteasomal degradation (Ciechanover et al, 1980; Hershko et al, 1980; Wilkinson et al, 1980). Shortly afterwards, the concept of ubiquitin-mediated degradation was confirmed in vivo (Ciechanover et al, 1984; Finley et al, 1984). It was well accepted for nearly two decades that ubiquitination was solely associated with degradation. However, in 1995 Spence et al. were the first to report a protein turnover-independent function of ubiquitin (Spence et al, 1995), suggesting non-proteolytic functions of ubiquitin. This was the starting point to extend ubiquitin research and led to the identification of numerous alternative functions including trafficking, DNA damage repair and kinase activation (Yang et al, 2010).
1.5.1 Ubiquitin chain formation

Ubiquitin is a highly conserved protein of about 8.5 kDa expressed in all eukaryotes from yeast to mammals (Makarova & Koonin, 2010). It is encoded by four individual genes, either as linear polyubiquitin chain, or as fusion protein with a ribosomal subunit (Finley et al, 1987). Processing of these pre-cursors generates a 76 amino-acid mature globular protein, which can be attached to lysine residues of target proteins. Ubiquitin possesses seven internal lysine residues (K6, K11, K27, K29, K33, K48 and K63) and all of them can be used for ubiquitin chain assembly (Ikeda & Dikic, 2008). Additionally, the amino-terminal methionine (M1) of ubiquitin can be connected to its C-terminal Glycine (G76), thereby generating so-called head-to-tail linear ubiquitin chains. Most interactions between ubiquitin and target proteins are due to a prominent hydrophobic surface patch centred on isoleucine 44 (Ile44) of ubiquitin. Given that all lysine residues reside on different surfaces pointing in different directions (except of K63 which is spatially close to the N-terminal amino group of M1), ubiquitin-binding proteins exhibit different affinities for the differentially linked ubiquitin chains. This chain-type selectivity of ubiquitin receptors hence explains the physiological differences induced by the different types of ubiquitin chain linkages.

**Figure 7: The ubiquitin system.** a) Ubiquitin initially gets activated in an ATP-consuming manner by an E1 and is then passed onto the reactive cysteine residue of an E2 conjugating enzyme. Subsequently, ubiquitin gets either transferred to the reactive cysteine of a HECT E3 enzyme, which then transfers it to a lysine residue of a target substrate or the E2 cooperates with a RING-box E3 which confers target selectivity and the E2 directly attaches ubiquitin to the target. b) Target proteins can be modified by mono-ubiquitination, multi-mono-ubiquitination or polyubiquitination. Polyubiquitin chains can be of homotypic nature, i.e. the ubiquitin chains contain only one linkage type, or of heterotypic nature, in which the polyubiquitin chain has alternating linkage types or is branched, i.e. two or more lysine residues of the same ubiquitin molecule are elongated.
The HECT and RING family of E3 ligases

Generation of mono- or polyubiquitin chains is mediated by a concerted action of three enzymes referred to as E1, E2 and E3 (Figure 7a). Initially, ubiquitin is activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent manner creating a thioester bond between its conserved cysteine residue and the C-terminal carboxy group of monomeric ubiquitin. Subsequently, ubiquitin is transferred to the active site of a ubiquitin-conjugating enzyme (E2), which is recruited by the C-terminal ubiquitin-fold domain of E1. Finally, an isopeptide bond between the C-terminus of ubiquitin and the ε-amino group of a lysine residue within a target protein is catalysed by a ubiquitin ligase (E3) (Kerscher et al, 2006). Up to date, two E1s, about 50 E2s and more than 600 E3s have been described in the human genome (Bhoj & Chen, 2009).

Classically, E2s are thought to determine linkage specificity whereas E3s confer substrate specificity bringing E2s in close proximity to target proteins. E2s contain a core UBC (ubiquitin conjugating) domain of approximately 140 amino acids including an invariant cysteine residue at the active site. Some E2s have been described to possess unique linkage specificity, including CDC34 and Ubc13/Uev1a which exclusively generate K48- and K63-linked ubiquitin chains, respectively (Hofmann & Pickart, 1999; Petroski & Deshaies, 2005). Preferential interactions between these particular E2s and ubiquitin which position the lysine residue of the ubiquitin to be conjugated in a distinct orientation most likely account for this specificity (Eddins et al, 2006; Petroski & Deshaies, 2005; VanDemark et al, 2001). On the contrary, E2s including those of the UbcH5 family are promiscuous and are capable of generating ubiquitin chains of different linkage types (Kim et al, 2007).

E3 ubiquitin ligases can be categorised in three classes based on their structure and function: homology to E6AP C-terminus (HECT), really interesting new gene (RING) and closely resembling U-Box E3s. HECT E3s possess highly conserved cysteine residues that initially accept ubiquitin molecules from E2s and subsequently transfer them to lysine acceptors in target proteins or to lysine residues of growing ubiquitin chains. In contrast, RING E3s lack enzymatic activity and rather function as adaptors bridging between E2s and target proteins facilitating the transfer of ubiquitin from E2s directly to targets. Some RING E3s including cIAPs and TRAFs contain substrate modules to directly function as E3, whereas others form part of multi-protein complexes (e.g. the SCFβTrCP complex).

Rachel Klevit and colleagues recently described a novel RING-HECT hybrid mechanism which is utilised by a sub-group of RING-type E3s referred to as RING-in-between-RING
1. Introduction

(RBR) E3s (Wenzel et al, 2011). RBR E3s are characterised by a canonical Cys$_3$HisCys$_4$-type RING domain (RING1), followed by two conserved Cys/His-rich Zn$^{2+}$-binding domains, an in-between RING (IBR) and a RING2 domain (Marin et al, 2004). While the RING1 domain is responsible for binding to an E2 enzyme, a conserved cysteine residue in the RING2 motif possesses catalytic activity. Similar to HECT E3s, this cysteine residue accepts ubiquitin from the E2 and forms a stable thioester bond before ubiquitin is transferred to the target. Thus, RBRs combine characteristics of both, RING and HECT E3s.

1.5.2 Physiological role of different ubiquitin chain linkages

As mentioned above, all seven lysine residues as well as the amino-terminus can be employed in ubiquitin chain formation as shown by mass-spectrometric analysis of total cell extracts (Peng et al, 2003). Only recently, quantitative analyses in budding yeast revealed that K48-linked chains are the most abundant, accounting for 29% of all ubiquitin linkages in the cell. Interestingly, presence of K11-linked chains was nearly as frequently detected as K48 linkages (28%). 17% of all linkages were conjugated via K63, whereas K6 (11%), K27 (9%), K29 (3%) and K33 (3%) linkages were present in relatively low abundance (Xu et al, 2009b). Since His-tagged ubiquitin was employed in this study, linearly linked ubiquitin chains could not be detected. Though these results indicate that all chain types serve physiological functions in vivo, more systematic analyses are required to understand the significance of each chain type during development or stress-induced signalling.

Though hundreds of papers have been published on ubiquitin during the past decade, most cover the functions, structures and physiological roles of K48-, K63- and mot recently also M1-linked ubiquitin chains. In contrast, recent studies just started to explore the character of other, “non-conventional” chains. These analyses revealed that not only the topology, but also the length of a given ubiquitin chain is able to regulate the signalling output, therefore placing ubiquitination as a central mechanism in signal regulation.

According to its position within the ubiquitin molecule, ubiquitin chains linked via the various internal lysine residues adopt different conformations. K48-linked ubiquitin chains form rather rigid, compact structures, in which the single ubiquitin monomers are tightly packed against each other and the Ile44 patches not exposed. In contrast, K63- and M1-linked ubiquitin chains adopt a rather flexible, extended, open conformation, forming no contacts between the individual ubiquitin molecules apart from the linkage itself. The Ile44 patches are exposed and capable of adopting different positions due to a relatively high flexibility in the ubiquitin chain. Hence, the only characteristic structural difference between these two chains
is constituted in the linkage itself. The peptide bond in linear chains with a methionine branching off is slightly more constrained as compared to more flexible isopeptide chains linked through lysine residues (Komander et al, 2009). Recently Virdee et al. solved the structure of K6-ubiquitin chains, which form an asymmetric, compact structure distinct from all other ubiquitin chains (Virdee et al, 2010). Additionally, the conformation of K11-chains is also unique among the ubiquitin linkages since the contacts between isopeptide-linked ubiquitin moieties are entirely polar and do not involve the hydrophobic ubiquitin surface patch around Ile44 (Bremm et al, 2010).

Apart from poly-ubiquitination, proteins can be modified with a single ubiquitin molecule on a single lysine residue (mono-ubiquitination) or on several lysine residues (multi-mono-ubiquitination; Figure 7b). (Multi)-mono-ubiquitination of surface receptors induces their internalisation and lysosomal degradation or recycling to the cell surface (Haglund et al, 2003). Furthermore, mono-ubiquitination of histones or the DNA sliding clamp PCNA (proliferating-cell nuclear antigen) was described to serve important functions during the DNA damage response (Alpi et al, 2008; Sigismund et al, 2004). Since mono-ubiquitination of certain substrates is sufficient to induce their proteasomal degradation (Shabek et al, 2009), the substrate’s affinity for the proteasome must be just high enough to allow the degradation process to initiate. It has also been suggested that distinct E2s might be involved in generation of mono- and poly-ubiquitination, respectively (Windheim et al, 2008).

As pointed out earlier, classical ubiquitin chains linked via K48 are solely associated with proteasomal degradation (Hershko & Ciechanover, 1998). Ubiquitin Binding Domains (UBDs; discussed in the next chapter) play important functions for the delivery and recognition of K48-modified substrates to the proteasome and its subsequent hydrolysis, which occurs from the distal end of the ubiquitin chain (Lee et al, 2010).

Besides K48, linkages via the K63 residue constitute the best studied and described form of ubiquitin chains. Groundbreaking work at the end of the 20th century by the groups of Pickart and Chen showed that the E2 complex consisting of Ubc13 and Uev1A specifically utilised K63 to elongate ubiquitin chains (Deng et al, 2000; Hofmann & Pickart, 1999). This type of chain serves non-degradative functions and has been implicated in NF-κB and JNK activation. In the IL-1 and TLR pathways, TRAF6 serves as major E3 that, in combination with Ubc13/Uev1a specifically and uniquely conjugates K63-linked ubiquitin chains to target proteins, including IRAKs, NEMO but also TRAF6 itself. Knockout of either TRAF6 (in particular its RING domain) or Ubc13 abolished IL-1- and TLR-induced IKK and MAPKs
1. Introduction

activation (Conze et al, 2008; Lamothe et al, 2007; Walsh et al, 2008; Yamazaki et al, 2009; Yin et al, 2009). Furthermore, using an elegant ubiquitin replacement strategy in which endogenous ubiquitin was knocked out and replaced by a mutant lacking K63, Xu et al. demonstrated the necessity of K63-linked ubiquitin chains generated by Ubc13/Uev1a in IL-1 signalling (Xu et al, 2009a). However surprisingly, formation of K63-linked ubiquitin chains was dispensable for TNF-induced NF-κB activation, which relied on the activity of UbcH5 family members rather than Ubc13. This study demonstrated for the first time that different pathways, though utilising similar components, differentially rely on the formation of distinct ubiquitin chain types. Consequently, the old model in which long K63-linked poly-ubiquitin chains function as recruitment and activation platform for the TAK1/TAB and IKK complexes has to be revised, at least with respect to TNF signalling.

Intriguingly, the linear ubiquitin chain linkage has recently been described to be involved in TNF-induced NF-κB and MAPK activation, i.e. linear ubiquitin chains (Gerlach et al, 2011; Haas, 2008; Ikeda et al, 2011; Tokunaga et al, 2011; Tokunaga et al, 2009). Unlike all other linkage types, linear chains do not utilise the ε-amino group of an internal lysine residue for chain propagation, but rather fuse the α-amino group of the M1 residue of ubiquitin to the C-terminal carboxy-group on G76 of the incoming ubiquitin. Though linear and K63-linked ubiquitin chains adopt similar conformations, the chemistry of the bonds between individual ubiquitin monomers (peptide vs. isopeptide bond) and the topology of the area surrounding the respective bonds clearly distinguish these two linkage types from another. As described in the next chapter, ubiquitin binding domains specifically bind to K63- or linear ubiquitin chain linkages, respectively, thereby dictating down-stream signalling.

Artificial conjugation of tetra-ubiquitin to the N- or C-terminus of various proteins induced their degradation (Zhao & Ulrich, 2010), suggesting that the position and/or length of the linear chain within a target determines its functional outcome. Binding of specific UBDs (see chapter 1.5.3) to linear ubiquitin chains possibly mask them from binding to the proteasome and hence from proteasomal degradation. Thus, although linear chains might mark proteins for degradation under certain circumstances, they also function as signalling molecules that work by recruiting factors involved in signal transduction. Consistently, free linear ubiquitin chains are rapidly cleaved by isopeptidase T, which binds to the C-terminus of ubiquitin, thereby disassembling ubiquitin chains (Reyes-Turcu et al, 2006). However, attachment of linear chains to target proteins blocks the C-terminus, thereby preventing isopeptidase T from hydrolysing linear chains.
A variety of proteins involved in diverse cellular signalling processes are modified with K11-linked ubiquitin chains (Xu et al, 2009b), suggesting its physiological importance in various pathways. The major function of K11-chains is attributed to proteasomal degradation (Baboshina & Haas, 1996; Jin et al, 2008). Accordingly, attachment of K11-linked ubiquitin chains to cyclin B1 promoted by the E3 anaphase-promoting complex (APC/C) led to its proteasomal degradation, therefore suggesting a role for K11 in cell cycle regulation (Jin et al, 2008). A prominent function of K11-chains in proteasomal degradation is further supported by the finding that K11-chains associate with the proteasome (Baboshina & Haas, 1996) and accumulate upon chemical inhibition of the proteasome (Xu et al, 2009b).

Although a heterodimeric complex of BRCA1 and BARD1 was capable of forming K6-linked ubiquitin chains in vitro (Morris & Solomon, 2004; Wu-Baer et al, 2003), a physiological function for this chain type has not been identified yet. However, since BRCA1 is associated with DNA damage repair and since it is frequently mutated in breast cancer, K6-linked ubiquitin chains are likely involved in DNA repair.

Analysing the function of K27-, K29- and K33-linked ubiquitin chains proves a challenging task owing to their close proximity within the ubiquitin molecule and hence formation of small tryptic-digest fragments during mass-spectrometry. Albeit accounting for approximately 10% of all ubiquitination events in yeast, no physiological function has so far been reported for K27-linked ubiquitin chains. In contrast, at least three independent E3s were described to generate K29-linked ubiquitin chains and predominantly associated its function with lysosomal degradation of substrates (Chastagner et al, 2006; Johnson et al, 1995; You & Pickart, 2001). Furthermore, conjugation of K29- as well as K33-linked ubiquitin chains to members of the AMP-activated protein-kinase (AMPK)-related family of protein kinases has been reported to inhibit their activity (Al-Hakim et al, 2008). However, the exact mechanism of inhibition remains elusive and requires further research.

In vitro, ubiquitin chains of mixed linkages (so called heterotypic chains) as well as branched chains in which several lysine residues of the same ubiquitin molecule are elongated can be formed. However, their existence and relevance in vivo remains to be shown.

1.5.3 Ubiquitin Binding domains

Similar to SH2-domain-containing proteins that are recruited to phosphorylated sites, ubiquitin chains also serve as platform for the recruitment of UBD-containing proteins also referred to as ubiquitin receptors. Approximately 200 proteins containing one or several
1. Introduction

UBDs have been identified. Non-covalent interactions between ubiquitin and UBDs control a variety of physiological processes, including protein degradation, receptor trafficking, cell-cycle progression, DNA repair, autophagy and apoptosis (Kirkin et al, 2009; Raiborg & Stenmark, 2009; Wickliffe et al, 2009; Winget & Mayor). As yet, five structural folds with more than 20 UBDs were identified (Dikic et al, 2009). Although ubiquitin is mostly composed of polar surfaces, most UBDs bind to a hydrophobic patch surrounding Ile44 of ubiquitin. Since ubiquitin chains linked via the different lysine residues adopt distinct conformations, the hydrophobic Ile44-containing patch is either embedded or faces outward, conferring a certain layer of specificity.

UBDs are commonly α-helical structures, zinc fingers, are similar to the Ubc-domain present in E2s or assume pleckstrin homology (PH) folds (Chen & Sun, 2009). Given a rather low binding affinity in the micro- to millimolar range for UBDs to ubiquitin (Winget & Mayor), additional interactions between the ubiquitinated target protein and the ubiquitin receptor are required for sufficient specificity and affinity.

Some UBDs were shown to specifically interact with a certain type of ubiquitin linkage. For instance, Husnjak et al. demonstrated preferential binding of the proteasome receptor Rpn13 via its pleckstrin-like receptor for ubiquitin (pru) domain to K48-linked di-ubiquitin (Husnjak et al, 2008).

Accordingly, the Npl4 zinc finger (NZF) of TAB2 and TAB3 selectively binds to K63-linked ubiquitin chains (Kulathu et al, 2009; Sato et al, 2009), providing a possible mechanism of recruitment of the TAK1/TAB complex to various signalling platforms. Precisely, two distinct sites of the NZF domain bind adjoining ubiquitin molecules at the hydrophobic Ile44 patch without contacting the K63-linker region (Kulathu et al, 2009; Sato et al, 2009). Due to steric constraints in the linker region of linear chains preventing their wrapping around NZF domains, linkage specificity is warranted.

NEMO and ABIN1-3 (A20 binding inhibitor of NF-κB) are critically linked with NF-κB activation and termination, respectively. Together with optineurin, NEMO and ABINs possess a type of UBD referred to as UBAN (UBD present in ABINs and NEMO). All five proteins specifically bind to linear ubiquitin chains (Komander et al, 2009; Rahighi et al, 2009). Although NEMO was originally identified as a K63-interacting protein (Ea et al, 2006; Wu et al, 2006), recent reports have provided biophysical proof that its affinity for linear chains was about 100-fold higher. Furthermore, competition experiments revealed NEMO to solely bind
to linear chains when offered a mixture of K48-, K63- and M1-linked tetra-ubiquitin chains (Komander et al, 2009).

Crystallographic analyses showed that two UBAN domains of NEMO form a parallel symmetric dimer, in which each UBAN domain interacts with one linear di-ubiquitin, revealing a 2:2 complex (two UBANs interacting with two di-ubiquitins). The NEMO UBAN directly contacts the residues involved in the linkage between two ubiquitin molecules, thereby explaining why NEMO specifically interacts with linear linkages, despite the fact that K63- and linear ubiquitin chains adopt similar overall conformations (Rahighi et al, 2009).

Deletion or mutation of NEMO’s UBAN domain led to impaired IKK and TAK1 activation by a variety of stimuli (Rahighi et al, 2009). Intriguingly, mutations in the UBAN have been identified in humans suffering from X-linked ectodermal dysplasia and immunodeficiency (Vinolo et al, 2006), further underscoring a physiological importance of linear ubiquitin binding by NEMO. NEMO binding to linear chains alters its conformation, thereby providing a possible mechanism translating to auto-activation of IKKβ. While these data clearly show the necessity of NEMO’s binding to ubiquitin chains, its role as ubiquitin acceptor being modified with ubiquitin chains itself remains elusive. Although several studies reported on the ubiquitination of NEMO with different linkage types and several potential acceptor lysines have been mapped (e.g. K285, K277, K309, K399), the contribution of the distinct linkage types and ubiquitination sites for signal propagation have not yet been confirmed (Tokunaga et al, 2009). It has been assumed that NEMO ubiquitination induced a conformational change, leading to oligomerisation and subsequent auto-phosphorylation of IKKβ (Tang et al, 2003). Thus, although IKK activation is doubtlessly the central mechanisms leading to NF-κB activation, the precise mechanisms are yet to be determined.

NEMO also possesses a C-terminal NZF domain, which has been shown to bind equally well to K63-, K48- and M1-linked ubiquitin chains (Bloor et al, 2008; Cordier et al, 2009a; Wu et al, 2006). Although a fusion protein containing both the NZF and UBAN domain binds by an order of magnitude more tightly to K63-linked chains in vitro (Cordier et al, 2009a), it is not clear whether this also translates into differential binding in vivo. However, initial binding of NEMO to K63- and possibly K11-linked ubiquitin chains (Dynek et al, 2010) generated upon stimulation might allow for a transient, weak recruitment of the IKK complex to a signalling platform. Subsequent generation of linear chains on NEMO but also other possible targets might strengthen the interaction, allowing for conformation-induced IKK activation or
phosphorylation by simultaneously recruited and activated TAK1. Thus, a concerted action of ubiquitin chains of different linkage specificity allows for strong, persistent signalling.

ABIN-1, which was initially identified in a yeast-two-hybrid screen as A20-interactor (Heyninck et al, 1999) and was shown to specifically bind to linear ubiquitin chains via its UBAN domain (Rahighi et al, 2009), also serves critical roles in immune responses but, unlike NEMO, is rather required for their termination. Consistently, overexpression of ABIN-1 resulted in inhibition of TNF-induced NF-κB activation (Heyninck et al, 2003). In contrast, Abin1−/− cells did not show any severe abnormalities with regard to ex vivo NF-κB activation, possibly due to a certain degree of redundancy between ABIN-1, ABIN-2 and ABIN-3. However, ABIN proteins clearly show some non-redundant functions given that ABIN-1 knockout mice died during embryogenesis due to TNF-dependent fatal liver apoptosis (Oshima et al, 2009). Although the mechanism remains elusive, deletion of the UBAN domain from ABIN-1 sensitised cells to TNF-induced cell death through increased complex II formation (Oshima et al, 2009). Furthermore, replacing endogenous wild-type ABIN-1 by a mutant lacking the UBAN domain led to auto-immunity in mutant knock-in mice (Nanda et al, 2011). Thus, the ability of ABIN-1 to bind to linear ubiquitin chains prevents auto-immune pathologies by on the one hand terminating NF-κB and MAPKs signalling and on the other hand inhibiting pro-inflammatory cell death.

Generation of eight possible ubiquitin linkage types combined with the presence of a variety of UBDs with different specificity confers a high level of complexity, which tightly regulates the localisation and function of hundreds of proteins in diverse cellular processes. Depending on whether UBDs are located in proteins involved in proteasomal degradation, endocytosis, vesicle-trafficking or signal transduction, ubiquitination leads to proteasomal degradation, lysosomal destruction or non-proteolytic signalling outputs, respectively. Thus, the fate of a given cell and generation of down-stream signals are dictated by the kind of ubiquitin chain recognised by its specific UBDs. Units of ubiquitin-dimers are thought to confer UBD binding specificity. However, little is known about the regulation of the length of ubiquitin chains in vivo and its influence on UBD recruitment. It will be of great interest to decode the ubiquitin network and identify which chain types attached to which target proteins are decisive for the different signalling outputs.

1.5.4 Deubiquitinases

Opposing the effect of ubiquitin ligases, the human genome contains nearly 100 deubiquitinases (DUBs) that are capable of removing ubiquitin chains of different linkages
from target proteins (Skaug et al, 2009). DUBs can be subdivided in 5 classes based on their structural motifs: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumour proteases (OTUs) and Josephins that act as cysteine proteases as well as the zinc-dependent JAB1/MPN/MOV34 metalloproteases (JAMMs) (Reyes-Turcu et al, 2009). USPs constitute the largest class of DUBs with more than 50 members.

A20 belongs to the OTU family (Evans et al, 2004) and is one of the best characterised DUBs that was initially identified as a TNF-target gene in human umbilical vein endothelial cell (HUVEC) (Opipari et al, 1990). While its expression level is rather low in most unstimulated cells, it gets rapidly up-regulated in an NF-κB-dependent fashion by a variety of stimuli. Only thymocytes and peripheral T lymphocytes show high expression of A20 per se, which conversely becomes down-regulated following T cell activation (Tewari et al, 1995). A20 functions as a ubiquitin-editing enzyme in TNFR1 signalling by removing K63-linked, and possibly other types of ubiquitin chains and simultaneously adding K48-linked ubiquitin chains to RIP1, targeting it for degradation. Remarkably, A20 does not possess a RING or HECT domain but mediates ubiquitination via its fourth zinc finger domain (ZnF4), thereby presenting a new type of E3 ubiquitin ligase domain. Thus, A20 occupies a dual function as ubiquitin-editing enzyme capable of removing (DUB activity) and adding (E3 ligase activity) ubiquitin chains in a controlled, sequential manner. Since RIP1 does not play a major function in TLR and IL-1 signalling, other mechanisms have to exist explaining the negative regulatory function of A20 in these pathways. Only recently, Shembade et al. reported A20 to disrupt the association of E2-E3 enzymes including binding of the E3 TRAF6 to the E2s Ubc13 and UbcH5c (Shembade et al, 2010). Thus, A20 utilises a variety of mechanisms to terminate signalling initiated by distinct stimuli.

As a result of excessive, uncontrolled NF-κB signalling, A20−/− mice die pre-maturely due to multi-organ inflammation and cachexia (Lee et al, 2000). Cells derived from A20-deficient animals exhibited elevated, prolonged TNF- and IL-1-induced IKK activation and were hypersensitive to TNF-induced cytotoxicity, suggesting A20 as negative regulator of NF-κB signalling possessing anti-apoptotic functions (Heyninck et al, 1999; Jaattela et al, 1996; Opipari et al, 1992; Song et al, 1996). However, the function of A20 is not limited to TNF since back-crossing A20 knockout mice to Tnf−/− or Tnfr1−/− mice did not revert the spontaneous inflammatory phenotype (Boone et al, 2004). Moreover, Rag−/− A20−/− double knockout mice were also not rescued, indicating that the inflammation was independent of the adaptive immune system (Lee et al, 2000). However, concomitant loss of MyD88, an adaptor
1. Introduction

protein commonly used in TLR signalling, rescued \( A20^{+/} \) animals from lethality (Turer et al, 2008), indicating that TLRs are implicated in manifestation of inflammatory lesions.

A20 does not preferentially cleave K63-linked ubiquitin chains \textit{in vitro} (Lin et al, 2008), suggesting that (i) this might not be the preferred target linkage \textit{in vivo} or (ii) that it needs to cooperate with distinct proteins conferring selectivity for this type of chain. Indeed, A20’s activity depends on a variety of proteins including Tax 1 binding protein 1 (TAX1BP1), Itch and RING finger protein 11 (RNF11). TAX1BP1, Itch and RNF11 knockout cells all present with hyperactivated NF-\( \kappa \)B signalling and it was shown that the function of A20 depended on these proteins. TNF-induced interaction of RNF11, Itch and TAX1BP1 is a pre-requisite for A20 recruitment to RIP1 (Iha et al, 2008; Shembade et al, 2007; Shembade et al, 2008; Shembade et al, 2009). Thus, A20 forms a multi-protein complex involved in negative regulation of NF-\( \kappa \)B signalling induced by a variety of stimuli.

ABIN-1 is another molecule crucially linked to A20’s inhibitory function. Following TNFR1 activation and formation of linear ubiquitin chains on NEMO and RIP1 (Gerlach et al, 2011), ABIN-1 functions as adaptor to recruit A20 in close proximity to these molecules. Subsequently, A20 deubiquitinates NEMO and RIP1, thereby terminating TNF-induced signalling (Mauro et al, 2006). However, an A20-binding-deficient mutant of ABIN-1 was still capable of inhibiting NF-\( \kappa \)B activation, suggesting the existence of A20-independent mechanisms. Presumably, ABIN-1 competes with other proteins required for NF-\( \kappa \)B stimulation for binding to the complex, thereby dampening complex stability.

The USP family member CYLD was initially discovered as tumour suppressor given its frequent mutations in benign tumours of skin appendage (Bignell et al, 2000). Counteracting ubiquitination of TRAF2 and NEMO, CYLD negatively regulates NF-\( \kappa \)B, JNK and p38 signalling (Brummelkamp et al, 2003; Kovalenko et al, 2003; Trompouki et al, 2003). Loss of CYLD did not only increase NF-\( \kappa \)B signalling, but also enforced c-Jun amino-terminal kinase (JNK) and p38 activation, most likely due to maintained ubiquitination of TRAF2 and NEMO (Zhang et al, 2006). However, its activity is quite distinct from A20, since it is thought to rather inhibit spontaneous signalling and its activity needs to be overcome to allow TNF-induced signal propagation. IKK was suggested to phosphorylate CYLD, thereby inhibiting its DUB activity, allowing TNF-induced signals to proceed prior to their termination by A20 (Reiley et al, 2005). Conversely, A20 is part of a negative feedback loop whose expression initially needs to be induced prior to accomplishing its inhibitory function. Thus, CYLD and A20 serve precise, temporally distinct roles in inhibiting NF-\( \kappa \)B. Furthermore, CYLD and
A20 possess different ubiquitin linkage specificities; A20 preferentially hydrolyses K48-linked ubiquitin chains, whereas CYLD specifically cleaves linear chains and has some, albeit reduced activity towards K63-linked ubiquitin chains \textit{in vitro} (Komander et al, 2008; Komander et al, 2009; Lin et al, 2008). However, these activities might be different \textit{in vivo} when these proteins function together with adaptors, as in the case for A20.

USP21 and Cezanne (cellular Zn finger anti-NF-κB) were also described as negative regulators of TNF-induced signalling, though the exact mechanisms remain elusive (Enesa et al, 2008; Evans et al, 2001; Xu et al, 2010). However interestingly, Cezanne was implicated in hydrolysis of K11-linked ubiquitin chains. At the time when K48- and K63-linked ubiquitin chains were the only linkage types regarded important for cell signalling, the development of a complex system of at least four DUBs with non-redundant functions remained puzzling. However, considering recent reports describing the presence of at least four different ubiquitin chain types on RIP1 (Gerlach et al, 2011), evolution of such an intricate scheme is a lot more intuitive. Each ubiquitin chain type formed on RIP1 or other TNF-RSC components needs to be removed to terminate an inflammatory signal and to prevent the development of auto-immune pathologies. This demand is warranted by a highly controlled termination system, in which each component (DUB) fulfils its precise, temporally and spatially-controlled function.

Taken together, the ubiquitin network comprises a complex system that we are just starting to understand. Considering the association of frequent mutations of the ubiquitin-mediated signalling cascade with the occurrence of certain diseases, interfering with the perturbation of the ubiquitin network or with the consequences thereof might constitute a starting point for the development of novel therapeutic strategies to treat these diseases.

1.6 The Linear Ubiquitin Chain Assembly Complex (LUBAC)

1.6.1 HOIL-1, HOIP and SHARPIN

Following identification of ubiquitination in the early 80s’, generation of ubiquitin chains was thought to be solely mediated via one of the ε-amino groups of one of the seven internal lysine residues. However, in 2006, Kirisako \textit{et al.} described that the α-amino-group of ubiquitin can also be utilised for chain elongation by covalently attaching it to the carboxy-terminus of the following ubiquitin (Kirisako \textit{et al}, 2006). This activity was attributed to a
complex consisting of HOIL-1 (heme-oxidised iron-regulatory protein 2 ubiquitin ligase-1; also known as RBCK1) and HOIP (HOIL-1 interacting protein; also known as RNF31), which was therefore referred to as linear ubiquitin chain assembly complex, LUBAC. Only recently, we and others found that LUBAC does not only consist of HOIL-1 and HOIP, but includes SHARPIN (SHANK-associated RH-domain-interacting protein) as a third component, which will be discussed in this thesis (Gerlach et al, 2011; Ikeda et al, 2011; Tokunaga et al, 2011).

Since ubiquitin genes are encoded as linear precursors which are rapidly processed to monomers by DUBs, the existence of linear ubiquitin chains has been known for years. However, recent proteomic approaches revealed only very low steady-state levels of linear ubiquitin conjugates in cells (Dammer et al, 2011). Thus, identification of an E3 ligase complex capable of \textit{de novo} assembly of these chains from mono-ubiquitin moieties was somehow surprising. However, research efforts during the past years by our lab and others showed that linear ubiquitin chains generated by LUBAC are required for certain aspects of cell signalling and indeed play an important physiological role in the prevention of inflammation.

HOIL-1 and HOIP are both members of the RBR family, a subgroup of the RING finger protein family (Marin et al, 2004). HOIL-1 was initially discovered as ubiquitin ligase for heme-oxidized IRP2 (Yamanaka et al, 2003) that contains, besides its C-terminal RBR, an NZF domain responsible for binding to ubiquitin as well as an N-terminal Ubiquitin-like domain (UBL) (Tokunaga et al, 2009; Ikeda et al, 2011).

In addition to its C-terminal RBR domain, HOIP possesses a ZF domain, followed by two NZFs and a ubiquitin-associated (UBA) domain (Hicke et al, 2005). The UBA domain of HOIP and the UBL domain of HOIL-1 mediate stable interaction of both proteins. Surprisingly, although a mutant consisting only of the UBA of HOIP was able to directly bind to HOIL-1, no interaction of this mutant with ubiquitin could be observed (Kirisako et al, 2006). Thus, the UBA of HOIP does not seem to be essential for binding to ubiquitin, but is both sufficient and required to form a complex with HOIL-1. Instead, the second NZF motif confers binding capacity to ubiquitin.

SHARPIN does not possess any enzymatic activity, but sequence analyses revealed a similar structure to HOIL-1, i.e. presence of a C-terminal UBL domain followed by an NZF motif. Additionally, SHARPIN contains an N-terminal coiled-coil domain of so far unknown function. Initially, SHARPIN was identified as a protein interacting with SHANK, a
1. Introduction

component of postsynaptic density in dendritic spines, sites of neuronal connectivity in the hippocampus (Lim et al, 2001). SHARPIN forms homodimers that facilitate SHANK cross-linking, suggesting a possible function of SHARPIN in neuronal signalling.

Initially, Kirisako et al. demonstrated that HOIL-1 and HOIP form a high molecular weight complex of approximately 600 kDa that possesses ubiquitin ligase activity in vitro (Kirisako et al, 2006). The catalytic activity of HOIL-1 is dispensable for linear ubiquitin chain formation, which solely depends on a functional RBR domain of HOIP (Haas et al, 2009; Tokunaga et al, 2009). Here, the RING1 domain of HOIP is thought to bind to E2s, whereas the RING2 motif confers enzymatic activity. Although HOIL-1 does not confer linear ubiquitin ligase activity to LUBAC, it was shown to ubiquitinate a number of proteins with K48-linked ubiquitin chains, including IRP2, the transcription factors Bach1 and interferon regulatory factor-3 (IRF3) as well as TAB2 and TAB3 (Ishikawa et al, 2005; Tian et al, 2007; Yamanaka et al, 2003; Zenke-Kawasaki et al, 2007). Targeting TAB2/3 for proteasomal degradation, HOIL-1 might negatively interfere with NF-κB activation in a LUBAC-independent manner. However, given that this study was based on overexpression of HOIL-1 and taking into account that Hoil-1-/- mice showed defects in NF-κB activation (Tokunaga et al, 2009), it is unlikely that HOIL-1 has a negative regulatory function in vivo. HOIL-1 and HOIP were shown to bind to activated conventional protein kinases (PKCs) and ubiquitinate them in vitro. Since degradation of activated PKCs was decreased in HOIL-1-deficient cells, linear ubiquitination was associated with proteasomal degradation at that time (Nakamura et al, 2006).

We and others showed that SHARPIN is also part of this high molecular weight complex and that SHARPIN-HOIP or SHARPIN-HOIL-1-HOIP complexes are also capable of linear chain formation in vitro. Thus, HOIP is the central component of the LUBAC complex conferring catalytic activity. However, HOIP alone is unable to fulfil its activity, indicating that it requires at least one binding partner, i.e. HOIL-1 or SHARPIN to act as an E3 ubiquitin ligase. Furthermore, the stoichiometry of the 600 kDa complex needs to be resolved in order to understand whether only tripartite complexes exist in vivo, or whether HOIP-SHARPIN and HOIP-HOIL-1 sub-complexes form that might target diverse proteins for ubiquitination.

In contrast to the initial observation that linear ubiquitination triggers proteasomal degradation (Kirisako et al, 2006), recent work by our group and others conclusively showed that LUBAC-mediated linear chain formation is rather required for proper NF-κB and MAPK activation in a variety of signalling pathways (Gerlach et al, 2011; Ikeda et al, 2011;
Tokunaga et al., 2011). As demonstrated in this thesis, LUBAC regulates the balance of TNF-induced pro-survival signalling and cell death induction (see Results section).

LUBAC is capable of forming linear chains with a variety of E2s, challenging the classical view that the E2 is primarily responsible for conferring linkage specificity. Moreover, LUBAC even forces E2-25K, an E2 described to uniquely catalyse K48-ubiquitin chain formation (Chen & Pickart, 1990), to generate linear chains. As proposed by Wenzel et al., LUBAC and other RBR family proteins utilise a RING-HECT hybrid mechanism that allows the E3 to confer linkage specificity independently of the E2s (Wenzel et al., 2011). Thus, LUBAC overrides E2-encoded linkage preference and determines linkage specificity. Consistently, LUBAC is unable to generate poly-ubiquitin chains in vitro with versions of ubiquitin, in which the amino-terminus is masked by a tag (Gerlach et al., 2011, Kirisako et al., 2006). Thereby, it can be concluded that LUBAC exclusively forms linear (and not lysine-conjugated) ubiquitin chains.

1.6.2 Chronic proliferative dermatitis (cpdm)

Mice harbouring a loss of function mutation in the Sharpin gene spontaneously develop chronic proliferative dermatitis (cpdm), an autosomal recessive, systemic inflammatory disorder at about four to six weeks of age. Deletion of a single base pair in the 3’end of exon1 encoding the coiled-coiled region of SHARPIN leads to a frame shift in the open reading frame predicted to cause a premature stop codon at position 624 and hence generation of a non-functional protein (Seymour et al., 2007). Although the skin is the organ that is affected most obviously, cpdm mice present with multi-organ inflammation including other epithelia, e.g. in the mouth, oesophagus and forestomach, but also in liver, lungs and several joints. The skin of these mice displays signs of hyperplasia, hyperkeratosis and parakeratosis that are accompanied by infiltration of inflammatory cells including macrophages, mast cells and granulocytes (Gijbels et al., 1995a; Gijbels et al., 1996). Increased ICAM-1 and E-Selectin expression on basal keratinocytes and endothelial cells of dermal blood vessels might account for this increased infiltration. Besides increased keratinocyte proliferation resulting in thickening of the dermis, elevated levels of keratinocyte cell death have also been reported in cpdm mice. However, cpdm mice are not born with these lesions but develop them with age, with the first alterations being macroscopically visible three to four weeks after birth. Once severe pruritus and scaling manifest, the condition of the mice worsens as demonstrated by significant weight loss, growth retardation and formation of self-inflicted wounds, entailing the necessity to cull the animals ten to twelve weeks after birth. Only recently, Xia et al.
demonstrated a role of SHARPIN in bone metabolisms, explaining the observed growth retardation phenotype (Xia et al, 2010).

Transfer of haematopoietic cells from \textit{cpdm} mice to irradiated syngeneic mice did not induce the development of skin lesions, indicating that haematopoietic cells do not account for the skin pathogenesis observed in \textit{cpdm} mice. In contrast, skin grafts transferred from \textit{cpdm} or wild-type mice to \textit{cpdm}, nude or wild-type mice revealed maintenance of the donor phenotype (Gijbels et al, 1995b). These results suggest that the pathology of \textit{cpdm} mice is a result of a disorder in the dermis caused by local factors and not a cause of systemic defects or defects of the immune system.

Apart from inflammatory lesions, mice also show developmental abnormalities as assessed by defective organisation of lymphoid tissues. In particular, \textit{cpdm} mice are completely devoid of Peyer’s patches and lack well-formed follicles, germinal centres and follicular dendritic cells in the spleen, lymph nodes and nasal-associated lymphoid tissues (HogenEsch et al, 1999), resulting in impaired B cell development without affecting T cell responses. In spite of a defective B cell population, absolute numbers of B cells and CD4$^+$ and CD8$^+$ T cells are unchanged. In contrast, total numbers of white blood cells in peripheral blood are 7-fold increased with an even 40-fold rise in eosinophil counts (HogenEsch et al, 2001), causing splenomegaly and lymphadenopathy in \textit{cpdm} mice. This accumulation is mediated by increased T$_{H2}$ cytokine production observed in skin homogenates of mutant mice. In particular, levels of interleukin-4 (IL-4), -5, -13 and granulocyte macrophage colony-stimulating factor (GM-CSF) were shown to be up-regulated in \textit{cpdm} samples, whereas no differences in TNF and IL-10 could be observed. Furthermore, \textit{cpdm}-derived splenocytes activated with Concanavalin A (ConA) produced less interferon-$\gamma$ (IFN$\gamma$), but more IL-13 and IL-5, further underscoring a dysbalanced cytokine response in the absence of SHARPIN.

Steady-state serum levels of the immunoglobulins (Ig) IgG, IgA and IgE in \textit{cpdm} mice are significantly lower as compared to wild-type, whereas IgM levels remain normal. Furthermore, production of antigen-specific antibodies of all isotypes upon antigenic challenge is severely decreased, indicating a function of SHARPIN in humoral immune responses, particularly in Ig class switching.

Treatment of mutant mice with IL-12 completely abolished thickening of the epidermis and development of skin abnormalities (HogenEsch et al, 2001). Most likely, IL-12 induced T cells and natural killer (NK) cells to produce IFN$\gamma$ and to inhibit the production of T$_{H2}$ cytokines, as assessed by decreased IL-5 secretion. IL-5 is a cytokine critically linked to
eosinophil homeostasis as it promotes maturation of eosinophils in the bone marrow, sensitises them to chemokines and enhances their survival in tissues (Stern et al, 1992; Yamaguchi et al, 1988). Treatment of cpdm mice with neutralising anti-IL-5 antibodies or crossing to Il-5⁻/⁻ mice reduced eosinophil numbers to normal levels. However, the severity of dermatitis was not changed, indicating that eosinophils do not account for manifestation of inflammatory skin lesions and that their accumulation in cpdm mice is rather a consequence of the inflammation than causative for it (Renninger et al, 2009).
1.7 Aims of the project

We recently reported on the development of a highly sensitive modified tandem-affinity purification (moTAP) method which can be used to purify receptor signalling complexes with high purity and selectivity (Haas et al, 2009). Analysis of the TNF-RSC revealed a stimulation-dependent recruitment of two novel components, HOIL-1 and HOIP, which form a ubiquitin ligase complex capable of generating linear poly-ubiquitin chains (Kirisako et al, 2006). Additionally, this mass-spectrometric analysis determined SHARPIN as a third novel constituent of the TNF-RSC. This thesis aims at identifying the function of SHARPIN in TNFR1 signalling and at answering the following questions:

1. How is SHARPIN recruited to the TNF-RSC?
2. What is the functional consequence of SHARPIN deletion on TNF-induced NF-κB and MAPK signalling?
3. Is SHARPIN required to prevent TNF-induced cell death, and if so what is the mechanism of sensitisation? Is there a difference in signalling output between SHARPIN-deficient as compared to HOIL-1-deficient cells?

TNFR1, CD95 and TRAIL-R1/R2 are members of the TNFR superfamily that are able to induce proliferation as well as cell death following receptor crosslinking by their respective cognate ligands TNF, CD95L and TRAIL. Given that the signalling outputs of all three receptor-ligand systems rely on similar down-stream molecules, I also analysed and compared the functions of SHARPIN, HOIP and HOIL-1 in the CD95L/CD95 and TRAIL/TRAIL-R1/2 systems. Thereby I aimed at answering the following questions:

1. Are SHARPIN, HOIL-1 and HOIP required for CD95L- and TRAIL-induced pro-survival signalling?
2. Are SHARPIN, HOIL-1 and HOIP required for preventing CD95L- and TRAIL-induced cell death?
3. What is the mechanism of sensitisation to death ligand-mediated cell death in the absence of one of the LUBAC components?
2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

All reagents used in this study were purchased from the following companies unless stated otherwise: Amersham Biosciences, AppliChem, Becton Dickinson, Gerba, Merck (Calbiochem), Invitrogen, Pierce, Roche, Roth and Sigma/Aldrich (Fluka). All chemicals were purchased in pA quality unless indicated otherwise.

2.1.2 Specific inhibitors

- COMPLETE Protease Inhibitor Cocktail: Roche
- Phosphatase Inhibitor Cocktail: Sigma/Aldrich
- Necrostatin-1: Sigma/Aldrich
- Q-Val-Asp(non-O-methylated)-OPh (QVD): R&D systems
- TNFR2-Fc (Enbrel®): Pfizer

2.1.3 Buffers and solutions

- Blocking Buffer: 5 % milk powder
  0.05 % Tween-20 in PBS
- Carbonate Coating Buffer (pH = 9.5): 8.4 g NaHCO$_3$
  3.56 g Na$_2$CO$_3$
- Citrate Buffer: 0.025 M citric acid (pH = 4.5)
- ELISA Assay Buffer: 0.5 % BSA
  0.05 % Tween-20 in PBS
- ELISA Substrate Solution: 10 ml Citrate Buffer
  10 µl H$_2$O$_2$
2. Materials and Methods

10 mg OPD

FACS buffer
- 1 x PBS
- 5% FCS (v/v)
- 0.05% NaN₃ (w/v)

Freezing medium (for cells)
- 90% FCS (v/v)
- 10% DMSO (v/v)

HBS (2x, pH = 7.0)
- 50 mM HEPES
- 280 mM NaCl
- 1.5 mM Na₂HPO₄ x 2 H₂O

IP-lysis Buffer
- 30 mM Tris-Base (pH 7.4)
- 120 mM NaCl
- 2 mM EDTA
- 2 mM KCl
- 10% Glycerol (v/v)
- 1% Triton X-100 (v/v)
- COMPLETE protease-inhibitor cocktail

Luciferase Assay Buffer A (pH = 7.8)
- 20 mM HEPES
- 33.3 mM DTT
- 8.0 mM MgCl₂
- 130 μM EDTA
- 530 μM ATP
- 470 μM Luciferin
- 270 μM Coenzyme A

Luciferase Assay Buffer B (pH = 5.0)
- 15 mM Natriumpyrophosphate
- 7.5 mM NaOAc
- 400 mM NaSO₄
- 10 mM CDTA
- 25 μM APMBT
2. Materials and Methods

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Methanol (v/v)</td>
<td>1% Methanol (v/v)</td>
</tr>
<tr>
<td>2 μM Benzyl-Colenterazin</td>
<td>2 μM Benzyl-Colenterazin</td>
</tr>
</tbody>
</table>
| MOPS Running Buffer (pH = 7.7) | 50 mM MOPS  
|                           | 50 mM Tris-Base                                |
|                           | 3.5 mM SDS                                     |
|                           | 1.0 mM EDTA                                    |
| PBS (pH = 7.4)            | 137 mM NaCl                                    |
|                           | 8.1 mM Na₂HPO₄                                 |
|                           | 2.7 mM KCl                                     |
|                           | 1.5 mM KH₂PO₄                                 |
| PI buffer                 | 1 μg/mL Propidium Iodide in PBS                |
| Pull-down buffer          | 150 mM NaCl                                    |
|                           | 50 mM Trizma Base (pH 7.5)                     |
|                           | 5 mM DTT                                       |
|                           | 0.1% NP-40 (v/v)                               |
| Stripping Buffer (pH = 2.3) | 50 mM Glycin in H₂O    |
| TAE buffer (10x)          | 400 mM Tris/HCl                                |
|                           | 200 mM Acetic Acid                             |
|                           | 10 mM EDTA                                     |
| TB buffer                 | 10 mM Pipes                                    |
|                           | 55 mM MnCl₂                                    |
|                           | 15 mM CaCl₂                                    |
|                           | 250 mM KCl                                     |
| Transfer Buffer           | 192 mM Glycin                                  |
|                           | 25 mM Tris-Base                                |
2. Materials and Methods

0.01 % EDTA
20% Methanol (v/v)

Wash Buffer 0.05% Tween-20 in PBS (1x)

2.1.4 Antibodies

Table 2.1: Unconjugated antibodies
(WB: Western blotting; IP: immunoprecipitation)

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Source</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>A20 (59A426)</td>
<td>rabbit</td>
<td>Imgenex</td>
<td>WB</td>
</tr>
<tr>
<td>ABIN1</td>
<td>Sheep</td>
<td>Gift by Philipp Cohen</td>
<td>WB</td>
</tr>
<tr>
<td>Actin (A5441)</td>
<td>mIgG1</td>
<td>Sigma</td>
<td>WB</td>
</tr>
<tr>
<td>Bid (#2002)</td>
<td>rabbit</td>
<td>Cell signalling</td>
<td>WB</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>rabbit</td>
<td>Axxora</td>
<td>WB</td>
</tr>
<tr>
<td>Caspase-8 (ALX-804-429)</td>
<td>mIgG2b</td>
<td>Axxora Life Science</td>
<td>WB</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>ratIgG1</td>
<td>Axxora Life Science</td>
<td>WB, IP</td>
</tr>
<tr>
<td>Caspase-8 (C20)</td>
<td>goat</td>
<td>Santa Cruz</td>
<td>IP</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>mIgG1</td>
<td>MBL</td>
<td>WB</td>
</tr>
<tr>
<td>cIAP1/2 Pan (MAB3400)</td>
<td>mIgG2a</td>
<td>R&amp;D Systems</td>
<td>WB</td>
</tr>
<tr>
<td>FLAG (M2)</td>
<td>mIgG1</td>
<td>Sigma</td>
<td>WB, IP</td>
</tr>
<tr>
<td>IxBα (44D4)</td>
<td>rabbit</td>
<td>Cell signalling</td>
<td>WB</td>
</tr>
<tr>
<td>JNK (56G8)</td>
<td>rabbit</td>
<td>Cell Signalling</td>
<td>WB</td>
</tr>
<tr>
<td>Myc (9E10)</td>
<td>mIgG1</td>
<td>Abcam</td>
<td>WB</td>
</tr>
<tr>
<td>pIkBα (5A5)</td>
<td>mIgG1</td>
<td>Cell Signaling</td>
<td>WB</td>
</tr>
<tr>
<td>pJNK (98F2)</td>
<td>rabbit</td>
<td>Cell Signalling</td>
<td>WB</td>
</tr>
<tr>
<td>p38 (7D6)</td>
<td>rabbit</td>
<td>Cell signalling</td>
<td>WB</td>
</tr>
<tr>
<td>p65 (A)</td>
<td>rabbit</td>
<td>Santa Cruz</td>
<td>WB</td>
</tr>
<tr>
<td>PARP1 (C-2-10)</td>
<td>mIgG1</td>
<td>Biomlo</td>
<td>WB</td>
</tr>
<tr>
<td>pp38 (3D7)</td>
<td>rabbit</td>
<td>Cell signalling</td>
<td>WB</td>
</tr>
<tr>
<td>RIP1 (#610459)</td>
<td>mIgG2a</td>
<td>BD</td>
<td>WB</td>
</tr>
</tbody>
</table>
2. Materials and Methods

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIP3</td>
<td>rabbit</td>
<td>Imgenex</td>
</tr>
<tr>
<td>SHARPIN</td>
<td>rabbit</td>
<td>Gift by Ivan Dikic</td>
</tr>
<tr>
<td>TNFR1 (ab19139)</td>
<td>rabbit</td>
<td>Abcam</td>
</tr>
<tr>
<td>TRADD (H-278)</td>
<td>mIgG1</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>TRAF2 (C-20)</td>
<td>rabbit</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Ubiquitin (#07-375)</td>
<td>Rabbit</td>
<td>Millipore</td>
</tr>
<tr>
<td>V5 (sV5-10)</td>
<td>mIgG1</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Table 2.2: Conjugated antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat-anti-mIgG1-HRP</td>
<td>Southern Biotech</td>
<td>WB</td>
</tr>
<tr>
<td>Goat-anti-mIgG2a-HRP</td>
<td>Southern Biotech</td>
<td>WB</td>
</tr>
<tr>
<td>Goat-anti-mIgG2b-HRP</td>
<td>Southern Biotech</td>
<td>WB</td>
</tr>
<tr>
<td>Goat-anti-mlgM-HRP</td>
<td>Southern Biotech</td>
<td>WB</td>
</tr>
<tr>
<td>Goat-anti-rabbit-IgG-HRP</td>
<td>Southern Biotech</td>
<td>WB</td>
</tr>
<tr>
<td>Goat-anti-rat-HRP</td>
<td>Southern Biotech</td>
<td>WB</td>
</tr>
<tr>
<td>Rabbit-anti-goat-IgG-HRP</td>
<td>Santa Cruz</td>
<td>WB</td>
</tr>
</tbody>
</table>

2.1.4.1  HOIL-1, HOIP ans SHARPIN antibodies

Polyclonal anti-HOIP serum was obtained by immunising rabbits with two peptides (CEDFQNWKRMDPEYQAQ-CONH2 and CGDPEKQRQDKMREEG-CONH2) coupled to KLH. Monoclonal HOIL-1 antibodies (2D2) were generated from 8-week-old BALBc mice immunised with recombinant full length GST-tagged HOIL-1 protein. The monoclonal antibody recognising human SHARPIN was generated from 8-week-old Balb/c x C57BL6 mice immunised with KHL-coupled peptide (GPDAEAQLRLQLSADC).

2.1.5  Cell culture Media and Additives

Dulbecco’s Modified Eagle Medium Invitrogen, Karlsruhe; Germany
Dulbecco’s PBS Invitrogen, Karlsruhe; Germany
RPMI 1640 Invitrogen, Karlsruhe; Germany
2. Materials and Methods

Opti-MEM® Reduced Serum Medium  Invitrogen, Karlsruhe; Germany
Keratinocyte Serum Free Media (KSFM)  Invitrogen, Karlsruhe; Germany
Ampicillin  Roth, Karlsruhe; Germany
Penicillin/Streptomycin  Invitrogen, Auckland; New Zealand
Gentamycine  Cambrex Bio Whittaker, Verviers; Belgium
Hygromycin B  Boehringer Mannheim, Mannheim; Germany
G418 (Geneticin®)  Invitrogen, Karlsruhe; Germany
Zeocin  Invitrogen, Karlsruhe; Germany
Sodium Pyruvate  Invitrogen, Karlsruhe; Germany
Hypoxanthine Thymidine (HAT)  Invitrogen, Karlsruhe; Germany
Trypsin/EDTA solution  Invitrogen, Karlsruhe; Germany
β-mercaptoethanol  Invitrogen, Karlsruhe; Germany

2.1.6 Beads for protein precipitation

Anti-Myc agarose  Sigma-Aldrich, Munich; Germany
anti-V5 Agarose (clone V5-10)  Sigma-Aldrich, Munich; Germany
FLAG M2 affinity Gel  Sigma-Aldrich, Munich; Germany
Protein G Sepharose  GE Healthcare, Munich; Germany

2.1.7 Kits and Ready-to-Use Solutions

ABsolute QPCR ROX Mix  ABgene, Epsom; UK
BCA Protein assay  Perkin Elmer, Wellesley; United States
Chemiluminescent Substrate  Perkin Elmer, Wellesley; United States
IL-6 ELISA  eBioscience, Frankfurt; Germany
Perhydrol® 30 % H₂O₂  Merck, Darmstadt; Germany
QIAprep Maxi Kit  Qiagen, Hilden; Germany
QIAprep Spin Mini Kit  Qiagen, Hilden; Germany
QIAquick Gel Extraction Kit  Qiagen, Hilden; Germany
### 2. Materials and Methods

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>RevertAid™ H Minus strand cDNA synthesis kit</td>
<td>Fermentas Life Science, St. Leon Rot; Germany</td>
</tr>
<tr>
<td>Trizol® reagent</td>
<td>Invitrogen, Karlsruhe; Germany</td>
</tr>
<tr>
<td>TrypanBlue</td>
<td>Serva, Heidelberg; Germany</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2.1.8 Consumables</strong></td>
<td></td>
</tr>
<tr>
<td>Cell Culture Petri dishes</td>
<td>TPP, Trasadingen; Switzerland</td>
</tr>
<tr>
<td>Cell Culture Test Plates (6-, 12-, 24-well)</td>
<td>TPP, Trasadingen; Switzerland</td>
</tr>
<tr>
<td>Round and flat bottom 96-well test plates</td>
<td>TPP, Trasadingen; Switzerland</td>
</tr>
<tr>
<td>Tissue Culture flasks (25, 75, 150 cm²)</td>
<td>TPP, Trasadingen; Switzerland</td>
</tr>
<tr>
<td>Falcons (15 ml and 50 ml)</td>
<td>TPP, Trasadingen; Switzerland</td>
</tr>
<tr>
<td>Plastic pipettes (5 ml, 10 ml and 15 ml)</td>
<td>Becton Dickinson, Heidelberg; Germany</td>
</tr>
<tr>
<td>Pipette tips (0.1-10, 1-200, 101-1000 µl)</td>
<td>StarLab, Ahrensburg, Germany</td>
</tr>
<tr>
<td>Safe-Lock Reaction Tubes (1,5ml, 2 ml)</td>
<td>Eppendorf, Hamburg; Germany</td>
</tr>
<tr>
<td>Vivaspin Concentrator MWCO 10000</td>
<td>Sartorius, Goettingen; Germany</td>
</tr>
<tr>
<td>PCR Tubes</td>
<td>StarLab, Ahrensburg; Germany</td>
</tr>
<tr>
<td>96 well PCR plate</td>
<td>ABgene, Epsom; UK</td>
</tr>
<tr>
<td>384 well PCR plate</td>
<td>ABgene, Epsom, United Kingdom</td>
</tr>
<tr>
<td>Sealing foil</td>
<td>Roche, Mannheim; Germany</td>
</tr>
<tr>
<td>NuPAGE® 4-12% Bis-Tris Gels</td>
<td>Invitrogen, Karlsruhe; Germany</td>
</tr>
<tr>
<td>Hybond ECL Nitrocellulose Membrane</td>
<td>Amersham Bioscience, Buckinghamshire; UK</td>
</tr>
<tr>
<td>X-Ray film Hyperfilm™ ECL</td>
<td>Amersham, Freiburg; Germany</td>
</tr>
<tr>
<td>Whatman paper</td>
<td>Schleicher&amp;Schuell, Maidstone; United Kingdom</td>
</tr>
<tr>
<td>Polypropylene round bottom tube (5 ml)</td>
<td>Becton Dickinson, Heidelberg; Germany</td>
</tr>
<tr>
<td>Cuvette</td>
<td>Greiner Bio-One, Flacht; Germany</td>
</tr>
<tr>
<td>Dialysis Tube</td>
<td>Roth, Karlsruhe; Germany</td>
</tr>
<tr>
<td>PS- Test Tubes for FACS</td>
<td>Greiner Bio-One, Flacht; Germany</td>
</tr>
</tbody>
</table>
2. Materials and Methods

Cryogenic vials      Nunc, Wiesbaden; Germany
F96 MaxiSorp Plates for ELISA Nunc, Wiesbaden; Germany
Cell Sieve (40 µm pore size) Becton Dickinson, Heidelberg; Germany
Vacuum driven Bottle Top filter (0.22 µm) Millipore, Billerica; United States
50 ml Reagent Reservoir Corning Inc., Corning; United States
Single-Use Syringe (5 ml, 30 ml, 50 ml) Terumo, Eschborn; Germany
Single-Use Syringe (1 ml, 2 ml) Becton Dickinson, Heidelberg; Germany
Sterile filter (0.22 µm pore size) Millipore, Billerica; United States
Single-Use Scalpel Feather, Osaka; Japan
Single-Use Needles Becton Dickinson, Heidelberg; Germany
Glassware Schott, Mainz; Germany

2.1.9 Instruments
Flow Cytometer FACSCalibur Becton Dickinson, Heidelberg; Germany
ABI Prism® 7900HT Sequence Detection System Applied Biosystems, Foster City; United States
Multiskan Ascent Thermo Labsystems, Vantaa; Finland
Hyper Processor X-Ray film Developer Amersham Bioscience, Buckinghamshire; UK
COBRA Auto-gamma counter Packard, Ramsey; United States
Blotting equipment X cell II™ Novex, Bergisch Gladbach; Germany
NanoDrop Spectrophotometer ND-1000 NanoDrop Technologies, Wilmington; USA
Photometer Ultrospec 3100 pro Amersham, Freiburg; Germany
Äkta Prime Amersham Pharmacia Biotech, Vienna; Austria
Steri-Cult Cell culture bench HERA Safe, Berlin; Germany
Incubator Stericult 2000 Forma Scientific, Scotia; United States
Millipore Super-Q water installation Millipore, Billerica; United States
Ice machine Scotsman, Vernon Hills; United States
## 2. Materials and Methods

<table>
<thead>
<tr>
<th>Item</th>
<th>Manufacturer/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microwave</td>
<td>AEG, Nuremberg; Germany</td>
</tr>
<tr>
<td>Table Centrifuge Biofuge</td>
<td>Heraeus, Hanau, Germany</td>
</tr>
<tr>
<td>Varifuge 3O-R</td>
<td>Heraeus, Hanau, Germany</td>
</tr>
<tr>
<td>Multifuge 3S-R</td>
<td>Heraeus, Hanau, Germany</td>
</tr>
<tr>
<td>Biofuge Stratos</td>
<td>Heraeus, Hanau, Germany</td>
</tr>
<tr>
<td>Gene Amplification PCR system 9700</td>
<td>Applied Bioscience, Mumbai; India</td>
</tr>
<tr>
<td>Power Supply for agarose gels</td>
<td>BioRad, Hercules; United States</td>
</tr>
<tr>
<td>Light Microscope</td>
<td>Zeiss, Oberkochen; Germany</td>
</tr>
<tr>
<td>Vortex</td>
<td>Heidolph, Schwabach; Germany</td>
</tr>
<tr>
<td>Cryo 1°C Freezing container</td>
<td>Nalgene Labware, Neerijse; Belgium</td>
</tr>
<tr>
<td>pH Meter</td>
<td>Mettler, Giessen; Germany</td>
</tr>
<tr>
<td>Thermomixer compact</td>
<td>Eppendorf, Hamburg; Germany</td>
</tr>
<tr>
<td>Freezer -20°C</td>
<td>Liebherr, Biberach; Germany</td>
</tr>
<tr>
<td>Freezer -80°C</td>
<td>Forma Scientific, Marietta; United States</td>
</tr>
<tr>
<td>GelSystem Flexi 4040</td>
<td>Biostep, Jahnsdorf; Germany</td>
</tr>
<tr>
<td>Digital Camera</td>
<td>Olympus, Hamburg; Germany</td>
</tr>
<tr>
<td>Pipettes (10 µl, 100 µl, 200 µl, 1 ml)</td>
<td>Gilson, Bad Camber; Germany</td>
</tr>
<tr>
<td>Pipetboy</td>
<td>Integra Bioscience, Fernwald; Germany</td>
</tr>
<tr>
<td>Multichannel pipettes</td>
<td>Micronic Systems, McMurray; United States</td>
</tr>
</tbody>
</table>

### 2.1.10 Software

<table>
<thead>
<tr>
<th>Item</th>
<th>Manufacturer/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>CellQuest Pro™ version 4.02</td>
<td>Becton Dickinson, Heidelberg; Germany</td>
</tr>
<tr>
<td>Vector NTI</td>
<td>Invitrogen, Karlsruhe; Germany</td>
</tr>
<tr>
<td>Microsoft® Excel 2003</td>
<td>Microsoft, Redmont; United States</td>
</tr>
<tr>
<td>Microsoft® Powerpoint 2003</td>
<td>Microsoft, Redmont; United States</td>
</tr>
<tr>
<td>Microsoft® Word 2003</td>
<td>Microsoft, Redmont; United States</td>
</tr>
<tr>
<td>Universal Probe Library Assay Design</td>
<td></td>
</tr>
</tbody>
</table>
2. Materials and Methods

Centre Roche, Mannheim; Germany
Sequencing Imperial College London, London, UK
ND1000 V3.3.0 NanoDrop Technology; Wilmington; USA
Ascent Software Version 2.6 Thermo Labsystems, Vantaa; Finland
SDS 2.1 Applied Biosystems, Foster City; United States

2.2 Methods of Cell Biology

2.2.1 Cell lines

2.2.1.1 HEK293T cells

HEK293T cells were generated by transformation of human embryonic kidney (HEK) cell cultures with sheared adenovirus 5 DNA (Graham et al, 1977). HEK293T cells are highly transfectable. The expression of the SV40 large T-antigen facilitates the extra chromosomal replication of plasmids bearing the SV40 origin of replication.

2.2.1.2 HeLa cells

The HeLa cell line was isolated from an adenocarcinoma of the cervix of a patient named Henrietta Lacks in 1952. It is the first human epithelial cancer cell line established in long-term cell culture (Scherer et al, 1953). HeLa cells have a hypertriploid chromosome number (3n+), 20 clonally abnormal chromosomes and contain multiple copies of HPV type 18 (HPV18), integrated at specific sites (Chen, 1988; Popescu et al, 1987).

2.2.1.3 Jurkat cells

The Jurkat cell line was first established from the peripheral blood of a 14-year old boy suffering from T cell leukemia in the late 1970s (Schneider et al, 1977).

2.2.1.4 MCF-7 cells

The human breast carcinoma cell line MCF-7 was first described in 1973 by Brooks and colleagues (Brooks et al, 1973).

2.2.1.5 Mouse Embryonic Fibroblasts (MEFs)

MEFs were generated from E15 embryos in accordance with standard procedures and were infected with SV40 large T antigen-expressing lentivirus. For the generation of HOIL-1−/−
MEFs, cells were additionally infected with Cre-expressing lentivirus and selected by addition of 2 μg/mL puromycin.

2.2.1.6 U937

The human monocytic cell line U937 was derived from a patient with generalised histiocytic lymphoma and was first described in 1976 by Sundstrom and Nilsson (Sundstrom & Nilsson, 1976).

2.2.2 Cell culturing conditions

All adherent cell lines were cultured in a 10% CO₂-humidified atmosphere at 37 °C. HEK293T, HeLa and MEF cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% foetal bovine serum. MCF-7 cells were cultured in DMEM containing 5% foetal bovine serum, 1% pyruvate and 0.1% human insulin. Cells were detached with a 1 x PBS/trypsin (Invitrogen) solution before they reached confluence.

All suspension cell lines were maintained in RPMI supplemented with 10% FBS at 5% CO₂ (37 °C) at a density of ~ 1x10⁶ cells/mL. Cell density was determined with a Neubauer chamber slide.

2.2.3 Freezing and thawing of eukaryotic cells

For freezing, approximately 2.5 x 10⁶ cells were re-suspended in FCS containing 10% DMSO, transferred to cryogenic vials and frozen down in an isopropanol box to -80° C. For long-term storage, cells were transferred to liquid nitrogen at -196 °C.

Frozen cells were thawed in a water bath at 37 °C. To get rid of the DMSO, cells were spun down with a centrifuge and re-suspended in fresh medium.

2.2.4 Generation of primary keratinocytes

The skin of the tails from 10 day-old mice was incubated in keratinocyte serum free medium (KSFM) supplemented with bovine pituitary extract, epidermal growth factors (Invitrogen) and gentamicin (50 μg/mL) containing 2.1 U/mL dispase II (in 50 mM HEPES and 150 mM NaCl, pH 7.4) at 4 °C overnight. The following day, the epidermis was separated from the dermis and keratinocytes extracted from the epidermis using Tryp LE express Trypsin (Invitrogen) under constant agitation using a P1000 pipette at room temperature for 15 min. Subsequently, cells were centrifuged for 10 min, keratinocytes re-suspended in growth factor-supplemented KSFM and seeded on 12-well or 96-well plates, respectively. Medium was
2. Materials and Methods

changed the next day to remove cells that have not attached. Five days later, keratinocytes were used for the respective assays.

2.2.5 Transfection of adherent cells

MCF-7 and HeLa cells were transfected with FuGene 6 (Roche Applied Science) according to the manufacturer’s protocol.

HEK293T cells were transfected by the calcium phosphate method. HEK293T cells were split 1:3 the day before transfection to obtain ~60% confluence. For transfection of a 10 cm² dish, 20 µg plasmid DNA and 50 µl CaCl₂ (2M) were added to 450 µl H₂O. Subsequently, the DNA/CaCl₂ solution was gently mixed with 500 µL 2xHBS. Following 30 min incubation at room temperature, the transfection mixture was gently added drop-wise to the cell medium.

For production of recombinant proteins secreted into the cell culture supernatant, the supernatant was harvested 48 hrs and 96 hrs post-transfection.

2.2.6 Lentiviral infection of eukaryotic cells

2.2.6.1 Production of recombinant viruses

HEK293T cells were used for the production of recombinant, lentiviral particles using the calcium phosphate method (see chapter 2.2.5). The transfection mixture containing 28 µg of the packaging vector pCMV-dR8.91 (HIV_Gag, HIV_Rev), 12 µg of the envelope pMD2G (VSVG) and 40 µg of the shRNA plasmid (pLKO.1) to be packed was added drop wise to the cells. Following 48 and 96 hrs incubation at 37° C, the lentivirus-containing cell supernatant was collected.

Lentiviral particles were then used to generate cell lines in which the gene of interest was stably silenced. Briefly, target cells were spin-infected in the presence of 5 µg/mL polybrene (2000 x g, 4 hrs, RT) and infected cells selected with 2 µg/mL puromycin. Knockdown efficiency was controlled by Western Blotting and qPCR.

2.2.6.2 Generation of stable knockdown or overexpressing cells

HOIL-1-specific shRNAs were obtained from the RNAi consortium (TRC). The sequence for stable knockdown of HOIL-1 was 5′-CCACAACACTCATCTGTCAAA-3′ (TRCN0000007599), RHS408 was used as control shRNA. For stable HOIP knockdown, the shRNA coding sequence (5′-AGACAAGGTTGAAGATGATAT-3′) from the pGeneClip vector (SA Biosciences) was cloned into pTWEEN-GFP. To excise HOIL-1 from targeted
HOIL-1 MEFs, cells were infected with Cre-expressing virus. The respective cDNA sequences of HOIL-1 and SHARPIN were cloned into the pCIGW vector and the cDNA sequence of wild-type or mutant HOIP into the pCIGW vector in order to over-express the respective proteins.

2.2.7 siRNA-mediated Knock-down

Transient knock-down of target genes was achieved by siRNA obtained from ThermoScientific using Dharmafect as transfection agent. Briefly, per six-well, 1.5 µL Dharmafect were mixed with 200 µL DMEM not containing FCS for 10 min at RT. Subsequently, 2.2 µL siRNA (20 µM) were added and incubated for 30 min at RT. 2 x 10^6 cells were transfected with the transfection mixture.

2.2.8 Inducible Protein Expression in MEF cell lines

An inducible lentiviral system was used for the induction of cIAP1 in cIAP1/2 double knockout MEFs using 20 nM 4-hydroxy-tamoxifen (4-HT) for 20 hrs (Mace et al, 2008). The cell line was kindly provided by John Silke.

2.2.9 cIAP1/2 degradation using SMAC mimetics

SMAC-83 (SM-83) was synthesised and kindly provided by Pierfausto Seneci and Leonardo Manzoni (Cossu et al, 2009). To obtain efficient cIAP1/2 degradation, HeLa cells were pre-treated with 100 nM SM-83 for 2 hrs.

2.2.10 Quantification of Cell Death and Cell Viability

Cell death was assessed by propidium Iodide uptake. Briefly, 5 x 10^4 cells were seeded per 12-well and cells pre-treated the next day in the presence or absence of 10 µM Q-Val-Asp(non-O-methylated)-OPh (QVD) or 30 µM necrostatin-1 (NEC-1) for 1 hr before addition of the respective death ligand. 24 or 48 hrs later, the percentage of cell death was assessed by propidium Iodide up-take using flow cytometry (FACS Calibur). Alternatively, 1 x 10^4 cells were seeded per 96-well, cells treated the next day and cell viability measured by Cell Titer glo (Promega) 24 hrs later according to the manufacturer’s instructions.

2.2.11 Determination of clonogenic survival

1 x 10^4 cells were seeded per 12-well and stimulated with the respective death ligand the following day. Six days later, cells were washed with PBS and fixed in 3% formaldehyde in
PBS for 10 min at RT. Subsequently, cells were incubated with crystal-violet for 15 min at RT and washed with PBS.

2.2.12 Flow cytometry

For flow cytometry, up to $5 \times 10^5$ cells per staining were initially incubated for 10 min on ice with FACS buffer containing Fc-Receptor blocking antibody (CD16/32) to block unspecific binding. Depending on the efficiency of the antibodies, different dilutions of the antibody were prepared in FACS buffer. Typically, 5 µg/mL primary, 3 µg/mL secondary and 1 µg/mL tertiary antibody were used. If the primary antibody was not directly conjugated to a distinct fluorescent dye, cells were washed three times with FACS buffer before the next antibody was applied. To control for specific binding of the antibody, isotype controls were used. Surface expression of distinct proteins was then assessed by flow cytometry using a FACS Calibur.

2.3 Methods of Molecular Biology

2.3.1 DNA digestion and restriction analysis

For the sequence-specific cleavage of DNA molecules samples were incubated with restriction endonucleases (Fermentas FastDigest®) for 1 hr at 37 °C.

2.3.2 Agarose gel electrophoresis of nucleic acids

Agarose gels in TAE buffer were used to analyse Plasmid DNA and DNA fragments. DNA was mixed with 1 x DNA loading buffer, loaded onto 1-2 % (w/v) agarose gels and ran at 130 V for 40 min in 1 x TAE buffer. To determine the size of the loaded DNA fragments, a marker containing DNA fragments of defined molecular sizes (Smart Ladder) was also applied. DNA bands were visualized by incubation in a solution containing 0.5 µg/ml ethidium-bromide for 15 min. UV light with a wavelength of $\lambda = 254$ nm enabled visualization of DNA fragments.
2.3.3 Gel extraction of DNA fragments

Isolation of DNA fragments from agarose gels was achieved using the QIAquick Gel Extraction kit (Qiagen) according to manufacturer’s instructions. DNA was eluted 30-50 µL and used in further applications.

2.3.4 Ligation of DNA fragments

The optimal ratio for ligation between linearised vector and insert is in the range of 1:3 to 1:5. The concentrations of both components were estimated by intensity comparison of the bands in agarose gels. Appropriate volumes were mixed with ligase buffer, polyethyleneglycol (PEG) and 2 U T4-ligase (NEB) in a total volume of 20 µL. The reaction was carried out in a PCR machine using a cycle ligation program overnight; alternately 10 min at 10 °C, 30 min at 30 °C.

2.3.5 Transformation of competent E. coli bacteria and Isolation of Plasmid DNA

Competent bacteria E.coli TOP 10 F’ (Invitrogen) were thawed on ice and incubated with 1-10 ng plasmid DNA or the ligation product for 30 min on ice. Heating at 42° C for 90 sec and subsequent cooling on ice for 3 min permeabilised the bacterial membrane and allowed the DNA to enter the cell. 300 µL SOC medium were added, the bacteria shacked for 30 min at 37 °C and then plated on LB agar plates containing the respective antibiotic to select for positive clones. The next day, single clones were picked, grown in 5 mL LB Amp medium overnight at 37 °C and the plasmids isolated with the QIAprep Miniprep Kit (Qiagen).

2.3.6 Polymerase Chain Reaction (PCR)

All PCRs were done in a volume of 50 µl, containing 1-50 ng plasmid- or cDNA as template. The synthetic oligonucleotides were designed with 40-60% GC content, no internal structure or complementarities at the 3’-ends. Annealing temperatures were chosen ~5-10°C lower than the melting temperature Tm. A typical PCR reaction contained 200 µM dNTP, 2.5 U Polymerase, 1 x Polymerase Buffer and 0.4 µM Primer.
2. Materials and Methods

2.3.7 Quantitative PCR

2.3.7.1 RNA purification

Total RNA from cells was isolated using Trizol (Invitrogen) according to the manufacturer’s instructions. Trizol was removed by addition of 0.2 ml chloroform followed by isopropanol precipitation. The concentration and purity of RNA was determined with a ND-1000 Spectrophotometer (NanoDrop).

2.3.7.2 cDNA preparation

5 µg of total RNA were transcribed into cDNA using the RevertAid™ H Minus first strand cDNA synthesis kit (Fermentas) according to the manufacturer’s instructions.

2.3.7.3 Quantitative Real-time PCR

Quantitative Real-time PCR (qPCR) was performed with the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) using the ABsolute™ QPCR ROX Mix (ABgene). The ProbeFinder software (Roche) was used to design the optimal assay, comprising the respective labelled probe of the Universal ProbeLibrary (Roche) and gene-specific primers. A specific probe-primer mix was prepared for each gene investigated:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific Probe</td>
<td>0.44 µl</td>
</tr>
<tr>
<td>5’ and 3’ Primer mix (10 pmol/µl)</td>
<td>1.76 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>6.4 µl</td>
</tr>
<tr>
<td>cDNA (diluted 1:10)</td>
<td>4.4 µl</td>
</tr>
<tr>
<td>2 x qPCR ROX Mix</td>
<td>13 µl</td>
</tr>
</tbody>
</table>

The qPCR reaction was performed in double determination (each time 11 µl) according to the following thermal cycling program:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature/Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiation</td>
<td>50 °C for 2 min</td>
</tr>
<tr>
<td>Enzyme activation</td>
<td>95 °C for 15 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C for 15 sec</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60 °C for 60 sec</td>
</tr>
</tbody>
</table>

40 cycles

After normalisation on basis of the housekeeping genes, relative differences in mRNA levels were assessed based on the cycling threshold (C(t)) determined by the PE Biosystems ABI 7900 sequencer software and the relative quantification calculated.
2. Materials and Methods

The following Primers and Probes were used:

**Table 2.3: Oligonucleotide sequences and corresponding Probe numbers according to the Roche Universal Probe Library.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward Primer 5’-3’</th>
<th>Reverse Primer 5’-3’</th>
<th>Probe number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>aaattcaacggcacagtcaa</td>
<td>gtagcccaagatgccttc</td>
<td>38</td>
</tr>
<tr>
<td>A20</td>
<td>gctcaacctggttgctgtaaag</td>
<td>atgaggcatttcctgccctc</td>
<td>21</td>
</tr>
<tr>
<td>HOIL-1</td>
<td>tctcccaacacaggacatc</td>
<td>aaatgggtgaggtgtgcat</td>
<td>88</td>
</tr>
<tr>
<td>HOIP</td>
<td>cccaggtgcacacagcttc</td>
<td>cctcacaacttcgctcctg</td>
<td>52</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>cccagcgtacctctgcctc</td>
<td>gatggatacctgagcatcacc</td>
<td>81</td>
</tr>
<tr>
<td>IkBα</td>
<td>acgcagcaaatttggtaagggag</td>
<td>atgattgccaagtgcaagga</td>
<td>38</td>
</tr>
<tr>
<td>TNF</td>
<td>tcgctatgtctcctctccttc</td>
<td>gaggccatttgggaacttct</td>
<td>49</td>
</tr>
<tr>
<td>FLIP</td>
<td>cttcgctccccaaattgagt</td>
<td>tccaaatcttggagtcttact</td>
<td>50</td>
</tr>
</tbody>
</table>

2.4 Methods of Biochemistry

2.4.1 Determination of protein content

To determine the protein concentration of cell lysates, the bicinchoninic acid (BCA)-containing protein assay was applied according to the manufacture’s protocol (Pierce). 1-5 µl of the lysate were incubated in 100 µl BCA solution at 60 °C for 15 min, followed by measuring light absorption at 560 nm in a Multiskan Ascent (Thermo Labsystems).

2.4.2 Precipitation of Receptor Signalling Complexes

Cells were stimulated with the respective death ligand for different time periods. His-FLAG tagged TNF (HF-TNF, referred to as TNF in the subsequent chapters), moTAP-TRAIL (3xFLAG-Precision-Avi tag; referred to as TRAIL) and Fc-CD95L (referred to as CD95L) were used. TNF and TRAIL were produced in bacteria and subsequently purified based on standard procedures; CD95L was produced in HEK293T cells, the supernatant collected and the protein content quantified. Following stimulation, cells were washed twice with ice-cold PBS and lysed in 1 mL IP-lysis buffer/cell dish at 4 °C for 35 min. The detergent-insoluble fraction was then pelleted by centrifugation (13,000 rpm, 30 min) and the cell lysates
transferred to a new tube. Equal amounts of protein were subjected to precipitation with 10 µL of the respective antibody-coupled beads at 4 °C overnight. Briefly, anti-FLAG coupled M2 beads (Sigma-Aldrich) were used for the immune-precipitation of the TNF-RSC and TRAIL-DISC and Protein G beads for the CD95 DISC. Alternatively, to precipitate any other component from the lysates, 1 µg of the antibody of interest was coupled to 10 µL Protein G beads per sample. Beads were washed five times the next day in IP-lysis buffer. In the last step, beads were completely aspirated and dried, re-suspended in 20-40 µL 2 x LDS buffer and incubated at 75 °C for 10 min.

2.4.3 Protein interaction studies

For in vivo interaction studies, 7.5 x 10⁶ HEK293T cells were transfected with 5-10 µg of the respective DNA plasmids by standard calcium phosphate transfection (see chapter 2.2.5). Cells were harvested 24 hrs post transfection and lysed in 1 ml IP-lysis buffer supplemented with 1% Triton X-100 for 30 min at 4 °C. Subsequently, lysates were centrifuged at 13,000 rpm for 30 min. Immuno-precipitations with the respective antibody-coupled beads were performed at 4 °C overnight. Beads were washed five times with IP-lysis buffer and the proteins were eluted in 30 µl 2 x LDS sample buffer. Precipitated proteins were analysed by Western blotting.

2.4.4 Ubiquitin pull-down assay

10 µg of purified GST-tagged protein were incubated with glutathione-sepharose resin in 600 µl Pull-down buffer (PDB) at 4 °C for 6 hrs. Beads were washed three times with PDB and incubated at 4 °C overnight with 2 µg ubiquitin tetra-ubiquitin chains of different linkage in 450 µl PDB. Beads were washed five times with PDB the next day and binding of the respective ubiquitin chains types to the GST-tagged proteins analysed by Western blotting.

2.4.5 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of proteins was performed based on the method by Laemmli (Laemmli, 1970) using 4-12% Bis-Tris-NuPAGE gels from Invitrogen. Samples were mixed with SDS-Sample buffer and heated for 10 min at 75 °C. The SeeBlue™ Plus2 Pre-Stained marker (Novex) was used as a molecular weight standard. The electrophoretic separation was carried out at a constant voltage of 100 V for 10 min and subsequently 180 V for 60 min. Depending on the molecular sizes of the proteins of interest, MES (proteins < 40 kDa) or MOPS buffer (> 40 kDa) was used.
2.4.6 Western blotting

Western blotting was performed using the NOVEX gel-system based on the method of Towbin (Towbin et al, 1979). Proteins from SDS-PAGE gels were transferred onto nitrocellulose membranes (Amersham Pharmacia) by application of a current of maximum 160 mA (30 V) per gel for 2 hrs. Afterwards, membranes were incubated for at least 15 min with blocking buffer at room temperature to occupy non-specific protein binding sites. Subsequently, membranes were subjected to immune-probing with primary antibodies and secondary horseradish-peroxidase (HRP)-conjugated antibodies. Proteins were visualised using different ECL® detection systems (Amersham Biosciences).

2.4.7 Stripping of Western blot membranes

Nitrocellulose membranes containing transferred proteins were incubated with alternative antibodies if necessary. For that purpose, the already bound immunoglobulins were removed by incubating the nitrocellulose membranes with Stripping buffer at RT for 15 min. The acidic pH of the stripping solution leads to denaturation of the bound antibodies. Subsequently, the membranes were washed 4-6 times with PBST, followed by incubation in blocking solution and probing as described in 2.4.6.

2.4.8 Enzyme-Linked Immuno Sorbent Assay (ELISA)

Cells were stimulated for 24 hrs with the respective ligands and the cell supernatant collected afterwards. ELISA reactions were performed according to the manufacturer’s instruction. Briefly, 96-well plates were coated with anti-IL-6 or anti-TNF antibodies diluted in coating buffer at 4 °C overnight. Plates were washed three times and blocked with 200 µl/well Assay buffer at RT for 1 hr. Plates were then washed again and the supernatant applied at RT for 2 hrs. In addition, distinct concentrations of recombinant IL-6 or TNF were applied to prepare standard curves. Subsequently, the detection antibody was applied followed by Streptavidin-HRP solution. Cytokine production was the visualised by the addition of ELISA substrate solution. The reaction was stopped with 100 µl 2 N H₂SO₄ and the absorbance measured at 450 nm with a Multiskan Ascent.
2. Materials and Methods

2.5 Animal work

2.5.1 Chronic proliferative dermatitis mice

The *cpdm* mutation arose spontaneously in the breeding stock of inbred C57BL/KaLawRij mice in the specific pathogen-free-breeding facility of TNO Rijswijk, Netherlands. Mice with a mutation in the SHARPIN gene were obtained from the Jackson Laboratory and kept under conventional conditions. Mutation of the SHARPIN gene causes a chronic proliferative dermatitis (*cpdm*) phenotype which was initially described by HogenEsch et al. (HogenEsch et al, 1993). SHARPIN-deficient *cpdm* mice of both sexes were equally affected. As female *cpdm* mice do not breed and males become infertile with 6-8 weeks of age, heterozygous males and females were mated and mutant mice born with Mendalian ratio. Animals were housed in micro-isolator cages under pathogen-free conditions in the Burlington Danes barrier facility at Imperial College London. Animal experiments were done in accordance with the Animals (Scientific Procedures) Act 1986; all were approved by local ethical review.

2.5.2 Conditional HOIL-1 mice

Conditional HOIL-1 knockout mice were generated at the Walter and Eliza Hall Institute (WEHI) in Melbourne, Australia. These mice contain a targeted cassette in which exon 1 and exon 2 of HOIL-1 are flanked by loxP sites and HOIL-1 can hence be excised by crossing these animals to tissue-specific Cre-expressing mice (Figure 8). Alternatively, isolated cells can be infected with Cre-expressing lentivirus to generate *Hoil-1*−/− cells.

![Figure 8: Schematic representation of the targeted allele used to generate conditional Hoil-1*−* mice.](image-url)

2.5.3 Enbrel® administration

Ten day old, lesion free *cpdm* animals were injected twice weekly with 125 µg Enbrel® (Etanercept; Pfizer) in 100 µl saline solution and monitored for skin lesion development. If lesions were too severe, mice were culled according to standard procedures. This experiment was conducted together with Eva Rieser.
3. Results

3.1 Functional analysis of SHARPIN in TNFR1 signalling

3.1.1 SHARPIN is a novel constituent of the TNF-RSC and forms a stimulation-independent complex with HOIL-1 and HOIP

We recently reported on the mass-spectrometrical identification of HOIL-1 and HOIP as new components of the TNF-RSC (Haas et al, 2009). Apart from HOIL-1 and HOIP, SHARPIN was the only other previously unknown component for which peptides were detected by this approach. The data obtained by mass-spectrometry could be confirmed by a kinetic analysis using Western blotting, in which a stimulation-dependent recruitment of SHARPIN to the TNF-RSC peaking between 5-15 min following receptor engagement and declining after 30 min was observed in different cell lines of human and murine origin (Figure 9). As previously described, RIP1, cIAP1/2, TRAF2 and TRADD were rapidly recruited being detectable in the TNF-RSC as early as 2 min following TNF administration and declined over

![Figure 9: SHARPIN forms part of the native TNF-RSC.](image-url)

*Figure 9: SHARPIN forms part of the native TNF-RSC. a) Hela and b) MEF cells were stimulated with 1 µg/mL TNF for the indicated times and the receptor complex precipitated. Post-lysis, 0.5 µg TNF was added to the unstimulated samples to pull down non-stimulated receptors. The isolated RSCs were immunoblotted with the indicated antibodies. A representative experiment of three independent experiments is shown. * marks unspecific bands.*
time once complex II forms (Micheau & Tschopp, 2003). In contrast, A20 expression, which is low in unstimulated cells, had to be induced by NF-κB and MAPK signalling prior to its recruitment to the TNF-RSC (Opipari et al, 1990), hence peaking in the TNF-RSC 1-2 hrs following stimulation.

Interestingly, SHARPIN was recruited to the TNF-RSC with similar kinetics as compared to the previously identified HOIL-1 and HOIP. Although SHARPIN does not possess a RING domain or any other catalytic activity, its structure closely resembles HOIL-1 given the presence of a C-terminal NZF motif and a UBL domain (Figure 10a). It was therefore next analysed whether the three proteins interacted with each other. As demonstrated in Figure 10b, SHARPIN formed a stimulation-independent complex with HOIL-1 and HOIP whose composition did not change following TNF stimulation. Consistently, immuno-precipitation of any of the three components resulted in co-precipitation of the other two in a variety of cell lines including monocytic U937 (Figure 10c) and Jurkat T cells (Figure 10d). Stimulation of U937 cells with TNF or Jurkat cells with the ionophore A23187 and PKC activator PMA did not change the interaction of SHARPIN with HOIL-1 and HOIP, indicating that the three proteins associate as a pre-formed tripartite complex in the cytoplasm. Consistently, overexpression experiments showed that SHARPIN interacted with both HOIL-1 and HOIP but

Figure 10: SHARPIN forms a stimulation-independent tripartite complex with HOIL-1 and HOIP. a) Schematic domain structure of SHARPIN, HOIL-1 and HOIP. b), c) U937 cells were stimulated with 1 µg/mL TNF for indicated times and the lysates were subjected to precipitation with the indicated antibodies. d) Jurkat cells were stimulated with 200 ng/mL A23187 and 10 ng/mL PMA for the indicated times and lysates were subjected to SHARPIN pull-down.
that its interaction with HOIL-1 was increased in the presence of HOIP, indicating that HOIP mediates binding of HOIL-1 to SHARPIN (Figure 11a). This notion was further supported by the finding that an interaction of endogenous HOIL-1 with endogenous SHARPIN was barely visible in cell lysates which have been depleted for HOIP (Figure 11b), suggesting that HOIL-1 and SHARPIN were unable to directly interact but are part of the same complex due to concomitant interaction with HOIP.

NF-κB was only activated by a combination of HOIP with SHARPIN and/or HOIL-1, but not by overexpression of SHARPIN and HOIL-1 (Gerlach et al, 2011; Ikeda et al, 2011; Tokunaga et al, 2011), indicating that HOIP is the catalytic active component of the tripartite complex. Considering this result, the interaction between SHARPIN and HOIP was studied in this thesis by mapping the domains responsible for the interaction. Overexpression of a mutant form of SHARPIN lacking the UBL domain strongly attenuated its binding to HOIP, whereas a mutant lacking the NZF domain was still capable of interacting with HOIP. Thus, similar to HOIL-1, SHARPIN utilises its UBL domain to bind to HOIP (Figure 12a).

![Figure 11: HOIP facilitates the interaction between HOIL-1 and SHARPIN. a) HEK293T cells were transfected with the indicated plasmids and half of the lysates were subjected to V5 (HOIL-1) or myc (SHARPIN) immunoprecipitation, respectively. b) HeLa cells were silenced for HOIP expression and lysates were subsequently further depleted by HOIP precipitation. Depleted lysates were subjected to HOIL-1 pull-down and immunoblots were analysed as indicated. ctr = control; HOIP refers to samples in which HOIP has been silenced by HOIP-specific siRNA.]
3. Results

Next, the domain within HOIP necessary for binding to SHARPIN was determined. Different HOIP mutants were co-expressed in the presence of wild-type SHARPIN, which was pulled down via its N-terminal myc-tag. As shown in Figure 12b, a HOIP mutant lacking the RING domains was sufficient to interact with SHARPIN. Albeit showing slightly decreased binding affinity, a HOIP mutant lacking the UBA domain was still capable of interacting with SHARPIN, indicating that this domain was also not required for the interaction of HOIP and SHARPIN, but might contribute to the strength of the association of both proteins. In contrast, a HOIP mutant containing only the N-terminal part of HOIP comprising the ZF and NZF domains was sufficient to bind to SHARPIN, indicating that the interaction between SHARPIN and HOIP was primarily mediated via their UBL and zinc finger domains, respectively.

HOIL-1 and HOIP are capable of binding to ubiquitin chains of different linkage specificity using their NZF domains (Haas et al, 2009; Tokunaga et al, 2009). To analyse whether SHARPIN was also able to bind to ubiquitin, different SHARPIN mutants were overexpressed

Figure 12: SHARPIN and HOIP interact via their UBL and NZF domains, respectively. a) HEK293T cells were transfected with the indicated plasmids and lysates were subjected to V5-HOIP IP and the co-precipitation of SHARPIN mutants was analysed. b) Full length myc-tagged SHARPIN (wt) was co-expressed with the indicated HOIP mutants, cell lysates were subjected to myc-precipitation and co-precipitation of HOIP was analysed.

Figure 13: SHARPIN binds to ubiquitin via its NZF domain. HEK293T cells were transfected with the indicated SHARPIN mutants and the lysates incubated with ubiquitin-coupled agarose beads. Binding of SHARPIN to ubiquitin was assessed by Western blot analysis.
3. Results

in HEK293T cells and the lysates incubated with ubiquitin-coated beads. As shown in Figure 13, SHARPIN bound to ubiquitin via its NZF domain. Taken together, SHARPIN interacts via its UBL with HOIP and via its NZF domain with ubiquitin.

HOIP alone was unable to produce linear ubiquitin chains \textit{in vitro}. However, addition of either HOIL-1, SHARPIN or a combination thereof activated the ubiquitin ligase activity of HOIP (Gerlach et al, 2011). Therefore, the terminology linear ubiquitin chain assembly complex (LUBAC) should be expanded to the tripartite complex consisting of HOIP, HOIL-1 and SHARPIN.

3.1.2 SHARPIN is recruited to the TNF-RSC in a cIAP1/2-dependent manner and stabilises the receptor signalling complex

HOIL-1 and HOIP are recruited to the TNF-RSC in a TRADD/cIAP1/2/TRAF2-mediated manner, independently of NEMO and RIP1 (Haas et al, 2009). Given that SHARPIN forms a tripartite complex with HOIP and HOIL-1, it was not surprising that the recruitment of SHARPIN to the TNF-RSC was also diminished in cIAP1/2-depleted MEFs or HeLa cells in which cIAP1/2 were degraded by the addition of the IAP antagonist SM-83 (Figure 14).

![Image](image_url)

\textbf{Figure 14: SHARPIN is recruited to the TNF-RSC via cIAPs.} a) MEFs of the indicated genotype were stimulated with 1 µg/mL HF-TNF for 10 min or left untreated and the TNF-RSC precipitated. 0.5 µg was added post-lysis to unstimulated samples. b) HeLa cells were pre-treated with 100 nM SM-83 for 2 hrs before cells were stimulated with TNF. Immunoblots were analysed with the indicated antibodies.
Moreover, SHARPIN was not recruited to the TNF-RSC of cIAP1/2 double knockout MEFs reconstituted with a cIAP1 mutant unable to form RING homodimers (F610A) and hence lacking catalytic activity (Mace et al, 2008). In contrast, reconstitution of cIAP1/2 double knockout MEFs with an inducible cIAP1 wild-type construct restored SHARPIN recruitment to the TNF-RSC. Considering that cIAP1 re-expression did not reach wild-type expression levels and RIP1 was hence not fully ubiquitinated, SHARPIN and HOIL-1 recruitment to the TNF-RSC were only partially restored as compared to wild-type cells (Figure 15). However, when compared to the inactive mutant of cIAP1, both proteins were clearly recruited. These results indicate that cIAP activity is required and sufficient for LUBAC recruitment to the TNF-RSC. This result is in line with a report by Mahoney et al. who showed that cIAP1 and cIAP2 possess redundant functions in TNF signalling and that the presence of one cIAP protein was sufficient for proper TNFR1 signal transduction (Mahoney et al, 2008). Furthermore, these data show that the ubiquitin-forming activity of cIAPs rather than their mere presence was required to recruit LUBAC to the TNF-RSC.

**Figure 15:** SHARPIN is recruited to cIAP-generated chains. cIAP1/2 double knockout MEFs were reconstituted with wild-type (wt) cIAP1 or the dimerisation mutant F610A lacking enzymatic activity using 4-hydroxy-tamoxifen for 16 hrs. Cells were stimulated with TNF and analysed as previously described.
Conversely, overexpression of SHARPIN together with HOIP stabilised the TNF-RSC as indicated by enhanced cIAP1/2, RIP1, TRAF2 and IKKα recruitment to, and prolonged retention in the TNF-RSC (Figure 16), which was not due to increased expression of the respective proteins (data not shown and Haas et al, 2009). Knockout experiments in which single components of the TNF-RSC were individually depleted showed the significance of each protein for NF-κB and MAPK activation and revealed that the formation of a stable TNF-RSC is a pre-requisite for efficient NF-κB and MAPK activation (reviewed in Silke & Brink, 2010). Therefore, stabilisation of the TNF-RSC by prolonged and enforced recruitment of TNFR1 signalling adaptors by LUBAC allows for efficient TNF-induced target gene transcription and, consequently proliferation and cell survival.

Figure 16: SHARPIN contributes to stabilisation of the TNF-RSC. The TNF-RSC was precipitated from TNF-stimulated HeLa cells stably overexpressing SHARPIN and HOIP or HOIL-1 and HOIP. Protein complexes were subjected to Western blot analysis and were immunoblotted with the indicated antibodies. Overexpression of SHARPIN, HOIL-1 and HOIP was verified in the Input (right panel).
3. Results

3.1.3 SHARPIN stabilises the tripartite LUBAC complex

To analyse the function of SHARPIN in TNF-induced signalling, MEFs from chronic proliferative dermatitis (cpdm) mice were generated. These mice harbour a spontaneous a single base pair deletion in the SHARPIN gene, which leads to the abrogation of SHARPIN expression (see chapter 1.6.2). The macroscopically most obvious alterations observed in cpdm mice include an approximately three to four-fold increase in the spleen weight and the generation of prominent skin lesions starting at four to six weeks following birth (Figure 17). Surprisingly, apart from completely lacking SHARPIN expression, cpdm-derived MEFs also exhibited reduced protein levels of HOIL-1 and HOIP (Figure 18a). HOIL-1 is usually expressed in two variants as assessed by the appearance of a double band on Western Blot analyses, indicating a modification of HOIL-1. However, the upper band was completely absent in cpdm-derived cells, similar to cells with siRNA-mediated silenced HOIP expression (Haas et al, 2009). The mRNA levels of both proteins were even slightly enhanced in cpdm-derived MEFs, suggesting that SHARPIN-mediated down-regulation of HOIL-1 and HOIP was not regulated on the transcriptional level (Figure 18b). Thus, SHARPIN is required for stabilisation of HOIL-1 and HOIP on the protein level, underscoring the existence of a tripartite complex. However, in the absence of SHARPIN a dimeric complex of HOIL-1 and HOIP still existed as assessed by HOIL-1 pull-down experiments (Figure 18c, d). These data indicate the possibility of forming dimeric complexes in the absence of the third component, but also show that for optimal LUBAC expression all three proteins are required. Furthermore, depletion of one component, e.g. SHARPIN, does not promote formation of dimeric complexes but rather leads to degradation of a major portion of HOIL-1 and HOIP proteins.

Figure 17: SHARPIN-deficient cpdm mice. a) SHARPIN deletion results in the development of a severe skin pathology accompanied by retarded growth. Representative photograph of 7-week old wild-type (wt) and cpdm mice is shown. b) Representative image of the spleens of these mice and the respective spleen weight (n=4).
To analyse whether the function to stabilise the tripartite LUBAC was restricted to SHARPIN, fibroblasts from embryos in which exon 1 and exon 2 of the HOIL-1 gene were flanked by loxP sites were generated and analysed. Subsequently to infection with a Cre-expressing virus, HOIL-1 was efficiently excised by recombination in the antibiotic-selected cells (Figure 19a). These cells will be referred to as Hoil-1\(^{-/-}\) MEFs in the following chapters. As shown in Figure 19b, similar to cpdm-derived cells, Hoil-1\(^{-/-}\) fibroblasts also presented significantly lower levels of HOIP and SHARPIN as compared to wild-type MEFs, indicating that all three proteins were required to stabilise the tripartite LUBAC and that deletion of one component resulted in significant, albeit incomplete, loss of the other two proteins. Treatment with the proteasome inhibitor MG132 only marginally increased the levels of the remaining two components in both SHARPIN- and HOIL-1-deficient cells, indicating that other mechanisms than proteasomal degradation account for the down-modulation (data not shown). This observation is in line with the data obtained by the group of K.Iwai (personal communication).
3.1.4 HOIP allows for the recruitment of LUBAC to the TNF-RSC

Given that SHARPIN forms a stimulation-independent complex with HOIL-1 and HOIP, the question arises as to which degree the individual three components contribute to the recruitment of the tripartite complex to the TNF-RSC. To address this question, the TNF-RSC was initially precipitated from cpdm-derived MEFs and analysed for the recruitment of the other two LUBAC components. As shown in Figure 20, despite presenting significantly decreased protein levels of HOIL-1 and HOIP, both proteins were still recruited to the TNF-

![Figure 19: Loss of HOIL-1 destabilises HOIP and SHARPIN protein levels.](image)


![Figure 20: HOIP and HOIL-1 are recruited to the TNF-RSC in the absence of SHARPIN.](image)
3. Results

RSC following TNF treatment, indicating that the tripartite LUBAC complex is not recruited to the TNF-RSC via SHARPIN. Interestingly, although the overall composition of the TNF-RSC, as assessed by levels of TNF-RSC-recruited cIAP1/2 and RIP1, was not severely disturbed at early time points following stimulation, significantly less ABIN-1, which is known to specifically bind to linear ubiquitin chains (Rahighi et al, 2009), was recruited to the TNF-RSC in cpdm-derived MEFs. This result suggests that linear ubiquitination at the TNF-RSC is strongly diminished in the absence of SHARPIN.

Next, HeLa cells were used in which the expression of HOIL-1 was stably silenced by lentiviral delivery of HOIL-1-specific shRNA (Haas et al, 2009). Despite the high knockdown efficiency, down-regulation of HOIL-1 only marginally diminished the recruitment of HOIP and SHARPIN to the TNF-RSC (Figure 21). Interestingly, in contrast to only minor alterations in the recruitment of cIAP1/2 and RIP1, the levels of ABIN-1 at the TNF-RSC were strongly diminished in the absence of HOIL-1, indicating that ABIN-1 requires HOIL-1- (Figure 21), as well as SHARPIN-imparted linear ubiquitination (Figure 20) for its recruitment.

![Figure 21: HOIL-1 is dispensable for the recruitment of HOIP and SHARPIN to the TNF-RSC.](image)

The TNF-RSC was precipitated from HeLa cells in which the expression of HOIL-1 was stably silenced by shRNA. The composition of the TNF-RSC was analysed by Western Blotting using the indicated antibodies.
Lastly, the expression of HOIP was transiently silenced in wild-type MEFs and the composition of the TNF-RSC analysed. As compared to a lack of HOIL-1 or SHARPIN, a decrease in HOIP expression severely diminished the recruitment of HOIL-1 and SHARPIN to the TNF-RSC (Figure 22). However, their recruitment was not completely absent, most likely due to an incomplete knockdown of HOIP and consequently the presence of residual amounts of HOIP at the TNF-RSC following stimulation. Taken together, these data support a major function for HOIP not only for the linear chain forming activity of LUBAC, but furthermore for the recruitment of the tripartite LUBAC to the TNF-RSC.

**Figure 22: HOIP is required for the recruitment of LUBAC to the TNF-RSC.** HOIP expression was silenced in wild-type MEFs for 72 hrs and the TNF-RSC analysed as previously described.
3. Results

3.1.5 SHARPIN is required for proper TNF-induced NF-κB and MAPK activation

Having identified the mechanism of recruitment, the functional consequences of SHARPIN deficiency were next analysed in the context of TNF-induced signalling. As shown in Figure 23a, cpdm-derived fibroblasts stimulated with 50 ng/mL TNF showed impaired phosphorylation and hence degradation of IκBα, the characteristic hallmarks of NF-κB activation. Furthermore, TNF-induced JNK activation was also severely attenuated as assessed by reduced levels of phosphorylated JNK1 and JNK2. This experiment was done in three independently generated MEF cell lines and all of them resulted in the same outcome, underscoring the reproducibility of these results. Additionally, to rule out any disturbances resulting from immortalisation of the cells by large T antigen infection, primary keratinocytes from the tails of ten-day old cpdm mice that have not yet developed any obvious skin lesions were generated. These primary murine keratinocytes also exhibited reduced levels of TNF-induced IκBα phosphorylation and degradation as well as reduced levels of JNK1/2 phosphorylation when compared to wild-type control keratinocytes (Figure 23b), confirming the results obtained in transformed MEF cell lines. Interestingly, levels of HOIL-1 and HOIP were also diminished in cpdm-derived keratinocytes, supporting a cell-type independent function of SHARPIN in stabilisation of LUBAC.

![Figure 23: SHARPIN is required for full NF-κB and JNK1/2 activation. a) Large T antigen transformed wild-type (wt) and cpdm-derived MEFs or (b) primary keratinocytes from 10-day old lesion free mice were stimulated with 50 ng/mL TNF and the lysates subjected to immunoblotting with the indicated antibodies.](image)
3. Results

Besides NF-κB and JNK, p38 activation was also reduced in cpdm-derived fibroblasts as assessed by diminished phosphorylation of p38 (Figure 24a). To confirm this result, a kinase assay was performed in which TNF-stimulated lysates were subjected to immunoprecipitation using an anti-pp38 antibody and subsequently incubated with the p38 target protein ATF2. As shown in Figure 24b, phosphorylation of ATF2 was severely attenuated in cpdm-derived cells, indicating that SHARPIN is required for efficient activation of the p38 MAPK pathway. In contrast, no obvious defect in TNF-induced ERK1/2 activation has been observed (Figure 23a and 24b). On the contrary, levels of phosphorylated ERK1/2 were even slightly enhanced in unstimulated SHARPIN-deficient MEFs and did not significantly alter between wild-type and cpdm-derived cells following TNF stimulation.

Figure 24: TNF-induced p38 activation is diminished in the absence of SHARPIN. a) MEFs of indicated genotypes were stimulated with 100 ng/mL TNF and were lysates analysed by Western Blot. b) Alternatively, cells were stimulated with 50 ng/mL TNF and equal protein levels subjected to pp38 immunoprecipitation followed by a kinase assay. p38 activity was assessed by phosphorylation of its substrate ATF2. Actin served as loading control.

Besides NF-κB and JNK, p38 activation was also reduced in cpdm-derived fibroblasts as assessed by diminished phosphorylation of p38 (Figure 24a). To confirm this result, a kinase assay was performed in which TNF-stimulated lysates were subjected to immunoprecipitation using an anti-pp38 antibody and subsequently incubated with the p38 target protein ATF2. As shown in Figure 24b, phosphorylation of ATF2 was severely attenuated in cpdm-derived cells, indicating that SHARPIN is required for efficient activation of the p38 MAPK pathway. In contrast, no obvious defect in TNF-induced ERK1/2 activation has been observed (Figure 23a and 24b). On the contrary, levels of phosphorylated ERK1/2 were even slightly enhanced in unstimulated SHARPIN-deficient MEFs and did not significantly alter between wild-type and cpdm-derived cells following TNF stimulation.

Figure 25: HOIL-1 knockdown in cpdm-derived MEFs further diminishes, but does not completely abrogate NF-κB activation. HOIL-1 expression was transiently silenced in cpdm-derived MEFs for 72 hrs and cells subsequently stimulated with 50 ng/mL TNF. Non-targeting siRNA was used as control. Lysates were immunoblotted with the respective antibodies.
3. Results

Although a strong defect in NF-κB activation was observed in \textit{cpdm}-derived MEFs, NF-κB signalling was not completely absent. HOIL-1 and HOIP were still recruited to the TNF-RSC in \textit{cpdm}-derived MEFs (Figure 20) and were possibly active at the TNF-RSC. To test the influence of residual HOIL-1 levels on NF-κB activation, its expression in \textit{cpdm}-derived fibroblasts was down-regulated by siRNA and the effects on TNF-induced signalling monitored. As shown in Figure 25, siRNA-mediated silencing of HOIL-1 further decreased phosphorylation and degradation of IκBα, however it did not completely prevent it despite the depletion of HOIL-1 being very efficient. These data indicate that LUBAC is required to enable full activation of NF-κB, but that it is not essential. Hence, LUBAC-independent mechanisms of NF-κB activation are likely to exist.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure26}
\caption{Absence of SHARPIN diminishes and delays TNF-induced p65 nuclear translocation. a) Cells were stimulated with 50 ng/mL TNF for the indicated times, fixed, stained and analysed by confocal microscopy. Green: p65, blue: nuclei (DAPI), turquoise: overlay. b) Quantification of nuclear p65. 30 nuclei from three independent experiments were analysed with the Leica Application Suite for p65 stained nuclear area. Data are presented as mean ± SEM; n=3.}
\end{figure}
3. Results

Phosphorylation of IκBα induces its proteasomal degradation and hence liberation of p50/p65 heterodimers allowing them to translocate to the nucleus and drive transcription of TNF target genes. To analyse whether defects in cpdm-derived cells present at the level of IκBα phosphorylation/degradation translated into diminished NF-κB translocation, cells were stained for p65 location. Indeed, less p65 was detected in the nucleus of TNF-stimulated cpdm-derived fibroblasts as compared to their wild-type controls (Figure 26). Furthermore, translocation of p65 from the cytoplasm of cpdm MEFs to the nuclei was only detected 30 min following stimulation, whereas strong p65 staining was already apparent in the nuclei of wild-type control MEFs after 15 min, indicating that NF-κB activation is diminished and delayed, albeit not completely obliterated in the absence of SHARPIN.

Next, the influence of SHARPIN deficiency on the transcription of TNF target genes was analysed. Therefore, cpdm-derived and their wild-type control MEFs were stimulated for various times with TNF. Subsequently, RNA was extracted, equal amounts of RNA were transcribed into cDNA and the expression levels of the target genes ICAM-1, A20, TNF and IκBα were quantified by real-time PCR (RT-PCR). GAPDH served as internal control and was used for normalisation. Whereas mRNA levels for TNF, ICAM-1 and A20 were basically not induced following TNFR1 stimulation in cpdm-derived MEFs, IκBα was transcribed, albeit to a lesser degree (Figure 27). This result is consistent with a recent report by Tay et al. who showed that the threshold for the expression of some TNF target genes including IκBα is relatively low, whereas others require strong NF-κB activation or concerted actions of NF-κB and MAPKs to be transcribed (Tay et al, 2010). Hence, reduced levels of NF-κB activation observed in cpdm-derived MEFs were sufficient to allow for the expression of IκBα. However,
A20, ICAM-1 and TNF, which also rely on MAPK-activated transcription factors, were not induced due to concomitant defects in stimulating the JNK and p38 MAPK pathways in SHARPIN-deficient cells (Figure 23 and 24).

In line with this result, HeLa cells, in which the expression of SHARPIN, HOIL-1 and HOIP was transiently silenced by RNA interference (RNAi) showed strong defects in TNF-induced MAPK activation, whereas phosphorylation and degradation of IκBα were hardly affected (Figure 28a). This effect is likely to be due to residual levels of the respective protein that has been silenced given the strong effect on TNF-induced IκBα degradation observed in SHARPIN-deficient *cpdm* and *Hoil-1* / / MEFs. Alternatively, HeLa cells might differentially depend on LUBAC-mediated TNF-induced signalling output as compared with fibroblasts, suggesting that the influence of linear ubiquitination is cell-type specific. Hence, these data support a major function of LUBAC in MAPK signalling in cells of different origin and underline the notion that LUBAC-independent mechanisms of NF-κB activation exist.

As previously described, HOIL-1 deficiency also led to decreased TNF-induced NF-κB responses (Tokunaga et al, 2009). Furthermore, JNK and p38 activation were also decreased in *Hoil-1* / / -derived fibroblasts (Figure 28b). However, and in contrast to *cpdm*-derived cells, basal levels of ERK1/2 were not increased in the absence of HOIL-1 and TNF-induced ERK1/2 activation was attenuated as compared to wild-type cells. This is the first indication of different signalling outputs induced by HOIL-1 as compared to SHARPIN deficiency.

![Figure 28: HOIL-1 is required for full NF-κB and MAPK activation](image)

*a) HOIL-1, SHARPIN and HOIP expression were transiently suppressed in HeLa cells by RNAi for 72 hrs. Cells were stimulated with 50 ng/mL TNF and signalling responses analysed with the indicated antibodies. b) Hoil-1* / / MEFs were treated accordingly.*
3. Results

3.1.6 Loss of SHARPIN sensitises to TNF-induced cell death

Although TNF primarily activates proliferation and cell survival, it can also induce cell death under certain conditions. Since the first TNF response, i.e. gene activation, is diminished in the absence of SHARPIN or HOIL-1, it was next analysed whether cell death responses were altered in LUBAC-component-deficient cells. Initially, the TNF-sensitive breast cancer cell line MCF-7 was used, in which the three LUBAC components were transiently silenced by siRNA. Although a function for HOIL-1 and HOIP in the regulation of cellular responses has been demonstrated, the influence of SHARPIN in the context of cell death execution remained undefined (Haas et al, 2009; Tokunaga et al, 2009). As shown in Figure 29, knockdown of HOIL-1, and in particular HOIP and SHARPIN, significantly reduced cell viability, indicating that all three LUBAC components are required to control the TNF-induced signalling output.

Considering that residual amounts of the respective protein to be targeted by siRNA might influence the system, cell death responses in a genetic clean system of knockout fibroblast cell lines were assessed. Absence of SHARPIN significantly reduced cell viability upon treatment with TNF. Whilst wild-type fibroblasts were completely resistant to TNF-mediated cell death, cell viability of cdpm-derived cells was severely diminished even in the presence of low concentrations of TNF (Figure 30a). Although addition of cycloheximide (CHX) increased the extent of TNF-mediated cell death in cdpm-derived MEFs quite substantially (data not shown), CHX and hence the inhibition of protein translation was not required for cell death execution by TNF when SHARPIN was absent.

Figure 29: LUBAC deficiency sensitises to TNF-induced cell death. a) The expression of HOIP, SHARPIN and HOIL-1 was transiently silenced in MCF-7 cells for 72 hrs. b) These cells were then stimulated with increasing concentrations of TNF and cell viability was assessed by Cell Titer Glo analyses. n=2 ± STDEV.
Subsequently, the type of death by which *cpdm*-derived cells were killed by TNF was analysed. It was determined whether it could be blocked by the pan-caspase inhibitor Q-Val-Asp(non-O-methylated)-OPh (QVD) or by the RIP1 kinase inhibitor necrostatin-1. Due to its ability to irreversibly bind to the catalytic centre of caspases, QVD blocks their activity, thereby preventing apoptosis. Necrostatin-1 on the other hand interacts with RIP1 to inhibit its kinase function, thereby preventing necrosome formation and activation, which is a pre-requisite for necroptosis (reviewed in Vandenabeele et al, 2011). To rule out any clonogenic artefacts, five independent transformed as well as primary MEF cell lines were generated from *cpdm* embryos and their cell death responses analysed. TNF-induced cell death in *cpdm*-derived fibroblasts was partially inhibited by QVD or necrostatin-1 alone and best by a combination thereof, indicating that the cell death observed in *cpdm*-derived cells is partially apoptotic and partially necroptotic (Figure 30b,c). Moreover, long-term survival in TNF-treated *cpdm*-derived cells was severely decreased as assessed by the analysis of clonogenic survival (Figure 30d).

Figure 30: SHARPIN-deficient cells are sensitive to TNF-induced cell death, which is partially necroptotic and partially apoptotic in nature. a) Wt and *cpdm*-derived MEFs were stimulated with increasing concentrations of TNF and cell viability was measured by Cell Titer Glow. Large T antigen transformed (b) or primary (c) MEFs were stimulated with 100 ng/mL TNF in the presence of 10 µM QVD (Q), 30 µM necrostatin-1 (N) or a combination thereof and cell death assessed by PI incorporation. n=5 ± SEM. d) 2x10^4 transformed MEFs were seeded per six-well, stimulated with TNF and clonogenic survival assessed 6 days later.
SHARPIN deficiency in \emph{cpdm} mice results in the generation of severe skin lesions characterised by immune cell infiltration and hence inflammation (Gijbels et al 1996; HogenEsch et al, 2001). Increased keratinocyte apoptosis in the skin of these mice has been described (Liang & Sundberg, 2011). To analyse whether the increase in keratinocyte cell death might be TNF-dependent, TNF-mediated cell death responses in primary keratinocytes obtained from ten-day old, lesion-free \emph{cpdm} mice as compared to wild-type control animals were analysed. Indeed, cell viability was decreased in keratinocytes from SHARPIN-deficient \emph{cpdm} mice (Figure 31a). To assess the kind of death keratinocytes underwent when treated with TNF, keratinocytes were pre-incubated with QVD or necrostatin-1 before TNF was added. Similar to the results obtained in MEFs, TNF-induced loss of viability in \emph{cpdm}-derived keratinocytes was partially rescued by QVD and also by necrostatin-1 (Figure 31b), indicating that TNF-induced cell death in keratinocytes was also partially apoptotic and partially necroptotic in nature.

The appearance of skin lesions and inflammatory milieus throughout the body observed in \emph{cpdm} mice were completely prevented by concomitant deletion of the \emph{Tnf} gene (Gerlach et al, 2011). Although heterozygous \emph{Tnf} deficiency significantly delayed the onset of disease progression, \emph{cpdm;Tnf}^{+/−} mice developed dermatitis starting at about 30 weeks of age (data not shown). Interestingly, injection of Enbrel®, a TNFR2-Fc fusion protein known to block the binding of endogenous TNF to TNF-Receptors, did not prevent or even delay the generation of skin lesions (data not shown), even though the treatment was started fifteen days after birth, i.e. at a time when no obvious skin lesions have developed. In these mice, dermatitis occurred with similar kinetics as compared to untreated \emph{cpdm} mice. Thus, the \emph{cpdm} inflammatory phenotype
3. Results

is a TNF-driven disease which results from accumulating defects due to a deregulated TNF signalling output, most likely as a consequence of signals generated by necrotic cells in the skin and possibly other inflamed organs which were killed by TNF.

In line with a report by Tokunaga et al., absence of HOIL-1 sensitised fibroblasts to TNF-induced cell death (Tokunaga et al, 2009) and the level of TNF-induced cell death was comparable to the quantity of cell death obtained in the absence of SHARPIN (Figure 32a). This result is in contrast to the study presented by Ikeda et al. who demonstrated that Hoil-1⁻/⁻ MEFs were not as sensitive towards TNF-induced cell death as cpdm-derived fibroblasts (Ikeda et al, 2011). However, their study was based on fibroblasts obtained from full Hoil-1⁻/⁻ mice whereas the results presented in this thesis were obtained by employing fibroblasts generated from conditional, floxed embryos, in which the Hoil-1 gene was excised ex vivo by Cre-expressing viruses. However, the quality of cell death significantly altered in cpdm- as compared to Hoil-1⁻/⁻ cells given that loss of HOIL-1-induced cell death was not inhibitable by necrostatin-1 alone, but rather relied on the activity of caspases. However, cell death obtained in Hoil-1⁻/⁻-derived fibroblasts could not be completely inhibited by caspase-inhibition, indicating that another form of cell death apart from necroptosis and apoptosis might contribute to TNF-induced sensitisation in the absence of HOIL-1 (Figure 32b). Hence, despite the involvement of both proteins in the regulation of cell death responses, the nature of the ensuing cell death was completely different in the absence of SHARPIN as compared to HOIL-1. This result indicates that SHARPIN and HOIL-1 possess independent, non-redundant functions.

Figure 32: TNF-induced cell death in Hoil-1⁻/⁻ as compared to cpdm-derived fibroblasts is similar in quantity, but severely different in quality. a) Hoil-1⁻/⁻ and cpdm-derived MEFs were stimulated with increasing concentrations of TNF and cell death analysed by PI incorporation. n=4 ± SEM. b) Cells of indicated genotype were pre-treated with 10 µM QVD (Q), 30 µM necrostatin-1 (N) or both in the presence or absence of 100 ng/mL TNF. n=4 ± SEM. An unpaired t-test using the GraphPad software was performed. * indicates p<0.05, ** indicates p<0.005.
3. Results

In line with this finding, TNF-induced generation of apoptotic hallmarks, including cleavage of caspases and their down-stream substrates PARP-1 and Bid, significantly differed between \textit{cpdm}- and \textit{Hoil-1\textsuperscript{-/-}}-derived fibroblasts. As shown in Figure 33, TNF-treatment alone in the absence of CHX rapidly and strongly induced cleavage of caspase-8, caspase-3 and caspase-9 to their active forms p17, p18 and p37/39, respectively and resulted in processing of Bid and PARP-1 in \textit{Hoil-1\textsuperscript{-/-}}-derived cells. In contrast, these apoptotic features were only marginally visible in \textit{cpdm}-derived cells treated with TNF alone. However, addition of CHX significantly enhanced the formation of typical apoptotic hallmarks in \textit{cpdm}-derived cells, but not in \textit{Hoil-1\textsuperscript{-/-}} cells. This result further underscores that cells undergo a differential kind of death in the absence of SHARPIN or HOIL-1, respectively and support the notion that the activity of HOIP is alternatively driven by SHARPIN- and HOIL-1-containing LUBAC complexes.

Taken together, these results show that loss of SHARPIN, and also HOIL-1, result in a cell death-favouring deregulation of TNF-induced signalling and suggest that the inflammatory phenotype observed in \textit{cpdm} mice is due to increased susceptibility to endogenous TNF-induced cell death which is necroptotic in nature. In contrast, though absence of HOIL-1 sensitised cells to TNF-induced death to a similar degree as SHARPIN deficiency, the nature of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure33.png}
\caption{Apoptotic responses significantly alter in \textit{Hoil-1\textsuperscript{-/-}} MEFs as compared to \textit{cpdm}-derived cells. Fibroblasts of the indicated genotypes were incubated with 100 ng/mL TNF in the absence (left panel) of presence (right panel) of 0.5 µg/mL cycloheximide (CHX) for the indicated times and analysed as indicated.}
\end{figure}
3. Results

this cell death was however characterised by rapid caspase cleavage involved in apoptotic, non-immunogenic responses, thereby providing a possible explanation as to why the phenotype of Hoil-1−/− mice does not resemble the one observed in cpdm mice. Hence, these results strongly suggest that HOIL-1 is implicated in the inhibition of apoptosis, whereas the SHARPIN-driven linear ubiquitination activity of HOIP instead prevented necroptotic cell death induction by TNF.

3.1.7 Loss of SHARPIN promotes complex II formation

TNF-induced NF-κB and MAPK activation were strongly diminished in cpdm cells, which rather succumbed to cell death following TNFR1 engagement. In contrast to the TRAIL and CD95L systems, TNF does not induce cell death at the receptor proximal level. Instead, the receptor signalling complex disintegrates and forms an intracellular secondary complex, referred to as complex II (Micheau & Tschopp, 2003). Complex II is a consequence of the membrane-associated complex I and forms with a delayed kinetic with respect to complex I formation and NF-κB and MAPK activation, which are already apparent a few minutes after TNF stimulation. Since these pathways generally induce the transcription of anti-apoptotic genes including c-FLIP and Bcl-2 family members, they are able to inhibit caspase-8 activation.

Figure 34: Loss of SHARPIN induced strong complex II formation. a) TNFR1 expression is not changed in the absence of SHARPIN. Shaded: isotype, black: wt, dotted: cpdm. b) SHARPIN expression was silenced in HeLa cells for 96 hrs. Cells were stimulated with 500 ng/mL TNF in the presence of 0.5 µg/mL CHX for the indicated times and complex II was precipitated with anti-caspase-8 (C20; Santa Cruz) antibodies.
3. Results

and hence cell death induction. However, under certain conditions when proteins involved in pro-survival signalling are mutated, inhibited or absent, complex II formation is increased, thereby subjecting cells to death.

To investigate the mechanism resulting in cell death execution in cpdm-derived cells I initially monitored the expression level of TNFR1 on the surface of SHARPIN-deficient MEFs by flow cytometry. As shown in Figure 34a, surface expression of TNFR1 was not affected by loss of SHARPIN, indicating that levels of TNFR1 are unlikely to be causative for deregulated TNF signalling outputs present in cpdm-derived as compared to wild-type MEFs.

Next, the formation of complex II was analysed in HeLa cells in which SHARPIN had been transiently silenced by specific siRNA oligonucleotides. Cells were stimulated for two and four hours respectively to allow complex II formation to occur and the complex was then precipitated via caspase-8. Caspase-8 does not form part of the membrane-proximal TNF-RSC, therefore allowing specific pull-down of the secondary complex. Depletion of SHARPIN resulted in significantly increased formation of complex II as assessed by increased interaction of caspase-8 with RIP1 and TRADD (Figure 34b). Furthermore, cleavage of RIP1 to a 42 kDa fragment was enhanced when SHARPIN expression was suppressed by siRNA. TNF-induced RIP1 cleavage at residue D324 is a caspase-8-dependent event that correlates with apoptosis execution (Lin et al, 1999). Thus, increased RIP1 cleavage in SHARPIN-depleted cells might

![Figure 35: Absence of any LUBAC component sensitises to TNF-induced cell death which is mediated by increased complex II formation.](image)
contribute to increased sensitivity towards TNF-induced cell death as compared to control cells. Accordingly, pronounced accumulation of the active p18 fragment of caspase-8 in SHARPIN-depleted cells supports the notion that absence of SHARPIN facilitates formation of complex II, resulting in increased caspase-8 activity, thereby promoting enhanced RIP1 cleavage and apoptosis.

Consistently with an increased susceptibility to TNF-induced cell death in HOIL-1- and HOIP-depleted cells (Figure 35c and Haas et al, 2009), stronger complex II formation was also detected in cell lines in which the expression of these two proteins had been silenced (Figure 35a, b). Again, increased interaction of caspase-8 with TRADD, RIP1 and also FADD was observed when one of the LUBAC components was transiently silenced by RNAi. Furthermore, caspase-8 activity was increased in these cells as assessed by accumulation of the cleaved N-terminal fragment of RIP1 and increased levels of the processed p43 c-FLIP fragment.

Several components of the membrane-proximal TNFR1 complex I are known to also form part of complex II, including RIP1, TRADD and possibly TRAF2. Being recruited to the TNF-RSC in a stimulation-dependent manner, it was subsequently analysed whether LUBAC was a novel as yet unidentified component of this secondary complex. As shown in Figure 36, all three components were also identified in complex II following TNF stimulation in the presence of CHX in HeLa cells. Considering that cells were susceptible to TNF-induced cell death in the absence of SHARPIN, HOIL-1 or HOIP, this result suggests that LUBAC linearly ubiquitinates a yet to be identified target, possibly NEMO or RIP1 to inhibit complex II-induced cell death.

**Figure 36**: LUBAC is part of the secondary complex II. HeLa cells were stimulated with 500 ng/mL TNF in the presence of 0.5 μg/mL CHX for the indicated times and complex II was precipitated by anti-caspase-8 pull-down overnight. The immunoblot was analysed with the respective antibodies.
3. Results

Levels of c-FLIP are one of the major determinants that decide between TNF-induced life and death of a cell and c-Flip<sup>−/−</sup> fibroblasts were shown to be TNF-sensitive, underscoring the significance of c-FLIP for cellular responses (Yeh et al., 2000). Surprisingly, c-FLIP<sub>L</sub> levels were significantly down-regulated in three independently generated, unstimulated cpdm-derived fibroblast lines, indicating that the down-regulation was not due to clonal artefacts (Figure 37a). However, similar to HOIL-1 and HOIP, c-FLIP<sub>L</sub> down-regulation was not mediated on the transcriptional level since c-FLIP mRNA levels were even modestly enhanced in unstimulated cpdm-derived fibroblasts (Figure 37b). Although the mechanism resulting in the down-regulation of c-FLIP remains to be determined, decreased c-FLIP levels are likely to be involved in the sensitisation to TNF-induced cell death. In contrast, c-FLIP levels were not reduced but rather marginally increased in Hoil-1<sup>−/−</sup> cells (Figure 37c), demonstrating a further difference between SHARPIN and HOIL-1 deficiency, which might explain differential kinds of TNF-induced cell death.

Taken together, these results show that SHARPIN and HOIL-1 regulate TNF-induced signalling outputs. Loss of one of these LUBAC components shifts the balance from TNF-induced pro-survival signalling in favour of cell death execution. However, the quality of cell death obtained significantly alters in the absence of HOIL-1 as compared to SHARPIN deficiency.

Figure 37: FLIP expression is decreased in cpdm but increased in Hoil-1<sup>−/−</sup> MEFs. a) Three different wild-type (wt) and cpdm-derived MEFs were analysed by immunoblot with the respective antibodies. b) Quantitative analysis of HOIL-1, HOIP and c-FLIP mRNA expression in three different MEF lines. n=3 ± SEM. c) Immunoblot of lysates from the indicated genotypes.
3. Results

3.2 SHARPIN regulates TRAIL- and CD95L-induced signalling

3.2.1 TRAIL- and CD95L-induced cell death responses

3.2.1.1 Loss of SHARPIN sensitises cells to TRAIL-induced death

TNFR1 belongs to the TNFR superfamily and, due to its death domain (DD), shares high homology to TRAIL-R1/2 and CD95. Although caspase-8, caspase-10, c-FLIP and FADD, which are not present in the TNF-RSC, were described as the central components of the TRAIL-R1/2 and CD95 DISCs, recent studies reported on the recruitment of RIP1, TRAF2 and cIAP1/2 to the TRAIL-R1/2 and CD95 DISCs (Geserick et al, 2009; Lin et al, 2000). Thus, the composition of the three receptor signalling complexes shares some degree of homology.

Considering these similarities, it was next analysed whether LUBAC also affected TRAIL- and CD95L-induced signaling. As shown in Figure 38a, absence of SHARPIN sensitised cpdm-derived MEFs to TRAIL-induced cell death. Whereas wild-type fibroblasts known to be completely TRAIL-resistant did not succumb to TRAIL-induced cell death, cpdm-derived

![Graphs showing cell death and viability](image)

**Figure 38: Loss of SHARPIN sensitises to TRAIL-induced apoptosis.** a) Wild-type (wt) and cpdm-derived MEFs were stimulated with increasing TRAIL concentrations and cell death analysed by PI incorporation. n=3 ± SEM. b) Cells were pre-incubated with 10 µM QVD (Q), 30 µM necrostatin-1 (N) or a combination thereof and cell death assessed by PI uptake. c) Primary keratinocytes from lesion-free mice were isolated, stimulated with increasing concentrations of TRAIL and cell viability assessed by Cell Titer Glo. n=2 ± STDEV. d) Clonogenic survival is decreased in SHARPIN-deficient cells. Cells were treated for 6 days.
MEFs died in a dose-dependent manner when incubated with TRAIL. Moreover, primary keratinocytes obtained from lesion-free *cpdm* mice were significantly more sensitive towards TRAIL-induced cell death as compared to wild-type control keratinocytes (Figure 38c).

Intriguingly, in contrast to TNF-induced cell death, TRAIL-mediated cell death was purely apoptotic in nature and necrostatin-1 was unable to inhibit TRAIL-induced cell death (Figure 38b). These data indicate that the signalling events occurring downstream of TNFR1 and TRAIL-R (N.B. mice only express one death-inducing TRAIL-R) are diverse and involve the presence and activity of different downstream proteins. Increased cell death in SHARPIN-deficient fibroblasts translated into decreased clonogenic survival (Figure 38d), suggesting that SHARPIN is an inhibitor of TRAIL-induced apoptosis and that its absence, and hence the depletion of linear ubiquitin chains renders cells susceptible to TRAIL-induced cell death.

### 3.2.1.2 Loss of SHARPIN increases CD95L-induced cell death

The signalling complexes utilised by the TRAIL/TRAIL-R and CD95L/CD95 systems share high similarity and differences in the composition of the respective receptor-associated signalling complexes have so far not been identified. Therefore it was not surprising that *cpdm*-derived fibroblasts also showed enhanced susceptibility towards CD95L-induced cell death (Figure 39a). Although wild-type cells also died when stimulated with high levels of CD95L, the amount of cell death obtained in SHARPIN-deficient MEFs was significantly higher and required lower doses of CD95L, indicating that SHARPIN is required to protect cells from CD95L-induced cell death.

![Figure 39: Loss of SHARPIN sensitisises cells to CD95L-induced cell death. a) Cells were incubated with increasing concentrations of CD95L and cell death measured by PI incorporation. b) Cells were pre-incubated for 1 hr with 10 µM QVD (Q), 30 µM necrostatin-1 (N) or a combination thereof and cell death was assessed by PI uptake. n=3 ± SEM.](image-url)

---

102
To analyse the kind of cell death, i.e. apoptosis versus necroptosis, \textit{cpdm}-derived fibroblasts were pre-incubated with QVD, necrostatin-1 or a combination thereof and the amount of cell death quantified by PI incorporation the next day. Surprisingly, however, CD95L-induced death was partially apoptotic and partially necroptotic and was most efficiently inhibited by a combined use of QVD and necrostatin-1 (Figure 39b). Thus, similar to the TNF system, but different from the TRAIL system, CD95L-mediated cell death relies on the proteolytic activity of caspases as well as on the kinase function of RIP1.

According to the type of cell death TRAIL-treated \textit{cpdm}-MEFs undergo, typical hallmarks of apoptotic cell death were readily detectable, including cleavage of Bid, PARP1 as well as initiator and executor caspases. In contrast, changes in XIAP protein levels were not detected, indicating that sensitivity towards TRAIL was not mediated via alterations on endogenous XIAP protein levels (Figure 40a). Furthermore, low concentrations of CD95L, which did not induce significant amounts of cell death in wild-type fibroblasts, also led to the formation of typical apoptotic hallmarks as shown by the cleavage of caspases, Bid and PARP, hence supporting a role for SHARPIN in the regulation of CD95L-induced cell death.

Similar to the absence of SHARPIN, deletion of HOIL-1 also sensitised fibroblasts to TRAIL- and CD95L-induced death to similar degrees as compared to SHARPIN deficiency (Figure 41a, b). Hence, like SHARPIN, HOIL-1 protects cells from TNF-, but also from TRAIL- and

![Figure 40: Loss of SHARPIN induces typical hallmarks of apoptosis following TRAIL and CD95L stimulation. Fibroblasts of the indicated genotypes were treated with (a) 100 ng/mL TRAIL or (b) 100 ng/mL CD95L for the indicated times and lysates were subjected to immunoblot with the indicated antibodies.](image)
3. Results

CD95L-induced cell death, suggesting a prominent function of linear ubiquitin chains in the regulation of cell death responses. Additionally, clonogenic survival of Hoil-1/- MEFs was similarly decreased as compared to cpdm-derived fibroblasts, indicating that both LUBAC components were equally required for TNF-, TRAIL- and CD95L-induced cell death inhibition and were hence involved in loss of clonogenic survival (Figure 41c).

### Figure 41: HOIL-1 and SHARPIN deficiency sensitise to TRAIL and CD95L-induced cell death independent of endogenous TNF production.

**a, b)** Cells were incubated with increasing concentrations of TRAIL (a) and CD95L (b) and cell death determined by flow cytometry. n=3 ± SEM.

**c)** Clonogenic survival of cells of the indicated genotypes treated with 100 ng/mL TNF, 100 ng/mL TRAIL and 150 ng/mL CD95L for 6 days.

**d)** Cells were incubated with 100 ng/mL TNF, 100 ng/mL TRAIL or 150 ng/mL CD95L in the absence or presence of 30 µg/mL Enbrel (TNFR2-Fc) and cell death analysed the next day.

#### 3.2.1.3 TRAIL- and CD95L-induced death in LUBAC-deficient cells is independent of TNF

TRAIL and CD95L are capable of activating the NF-κB and MAPKs pathways and thereby inducing pro-survival signalling (Kavuri et al, 2011; Varfolomeev et al, 2005). TNF constitutes a prominent NF-κB and MAPK target gene whose expression can be induced by the activation of the TRAIL-R or CD95 systems. To rule out the possibility that TRAIL- and CD95L-induced apoptosis observed in cpdm and Hoil-1/- MEFs was due to an autocrine production of TNF, cells of the different genotypes were stimulated in the presence of excessive doses of the TNFR2-Fc fusion protein Enbrel® (30 µg/mL) to inhibit the potential binding of endogenous
3. Results

TNF produced following TRAIL or CD95L stimulation, respectively. As shown in Figure 41d, addition of the TNF blocker did not alter cell death responses in cpdm and Hoil-1/- MEFs treated with TRAIL or CD95L, indicating that TRAIL- and CD95L-induced cell death occurred independently of TNF. Furthermore, the supernatants of cpdm and Hoil-1/- fibroblasts treated with TRAIL and CD95L (in the presence of QVD and necrostatin-1 to inhibit cell death) did not contain levels of TNF that could be measured by ELISA (data not shown), indicating that autocrine TNF was unlikely to account for the levels of cell death induced by TRAIL and CD95L, respectively. In contrast, as expected, TNF-induced cell death was inhibited in cpdm and Hoil-1/- MEFs in the presence of the TNFR2-Fc fusion protein, indicating that the inhibitor was indeed functional. Thus, the function of LUBAC is not limited to TNF-induced killing, but LUBAC also influences TRAIL- and CD95L death responses.

3.2.1.4 LUBAC is required in other death-inducing signalling pathways

In contrast to ligands of the TNFR superfamily that induce the extrinsic, apoptotic pathway, cell death can also be triggered from within the cell via the intrinsic pathway. Cellular exposure to exogenous stress including DNA-damaging agents results in activation of BH3-only family members which then induce mitochondrial alterations accompanied by cell death responses. Amongst the genotoxic stress inducers, cisplatin is known to crosslink the DNA in order to induce apoptosis, whereas etoposide induces DNA strand breaks by inhibiting the DNA unwinding enzyme topoisomerase II. Although both agents induced cell death in wild-type control fibroblasts when applied at high doses, LUBAC deficient cpdm- or Hoil-1/- MEFS were more sensitive towards these stimuli (Figure 42). Especially low doses of etoposide that only marginally induced cell death in wild-type cells were sufficient to efficiently kill SHARPIN or

![Graphs](image)

**Figure 42: Loss of SHARPIN and HOIL-1 sensitise to DNA damage-induced cell death.** Cells of the indicated genotypes were incubated with increasing concentrations of (a) etoposide or (b) cisplatin for 48 hrs and cell death analysed by flow cytometry. n=3 ± SEM.
3. Results

HOIL-1 deficient fibroblasts. In contrast, differences in cell death responses to cisplatin were not that pronounced between wild-type and LUBAC-deficient cells and were only apparent when cells were treated with low doses of cisplatin. These results are in line with a recent study showing that LUBAC interacted with NEMO following DNA damage and was involved in subsequent cellular responses (Niu et al, 2011). Thus, LUBAC is required for a variety of death responses, ranging from death ligands to genotoxic stress inducing agents.

3.2.2 SHARPIN and HOIL-1 regulate pro-survival signalling by TRAIL and CD95L

Although the major function of TRAIL and CD95L has been ascribed to cell death induction, both death ligands are also capable of activating pro-survival NF-κB and MAPK pathways (Kavuri et al, 2011; Varfolomeev et al, 2005). Considering that TNF-mediated NF-κB and MAPK signalling are deregulated in the absence of SHARPIN and HOIL-1, the influence of LUBAC on TRAIL- and CD95L-induced pro-survival pathway activation was analysed next. Thus, wild-type, cpdm and Hoil-1−/− cells were stimulated with TRAIL and CD95L and the phosphorylation patterns of IκBα, JNK1/2 and ERK1/2 analysed on Western Blot level. As

![Figure 43](image_url): TRAIL and CD95L-induced signalling is impaired in the absence of SHARPIN or HOIL-1. Cpdm-derived (a, b) or Hoil-1−/− (c, d) MEFs were stimulated with 100 ng/mL TRAIL (a, c) or 200 ng/mL CD95L (b, d) for the indicated time points and the activation of NF-κB and MAPKs was analysed as compared to wild-type (wt) MEFs.
shown in Figure 43, phosphorylation and degradation of IκBα was strongly diminished in cpdm-derived fibroblasts following TRAIL as well as CD95L stimulation. In contrast, the absence of SHARPIN on TRAIL-induced JNK1/2 activation was less pronounced and not consistent between individual experiments, suggesting that SHARPIN and hence linear ubiquitination played a minor role in TRAIL-induced JNK activation. CD95L-induced JNK1/2 activation was slightly decreased in the absence of SHARPIN throughout all experiments, suggesting that SHARPIN is required for efficient JNK1/2 activation. In contrast, the ERK1/2 MAPK pathway was slightly enhanced following TRAIL and CD95L stimulation in the absence of SHARPIN. As described earlier for TNF, basal levels of activated ERK1/2 were slightly enhanced in cpdm-derived cells, suggesting that increased basal ERK1/2 signalling might compensate for loss of SHARPIN.

Although TRAIL- and CD95L-induced activation of NF-κB was also diminished in the absence of HOIL-1, the pattern of ERK1/2 activation in Hoil-1/−/− MEFs differed from the one observed in cpdm-derived fibroblasts (Figure 43c and d). Most strikingly, basal levels of ERK1/2 phosphorylation and hence activation were not increased in the absence of HOIL-1. Furthermore, similar to the TNF system, TRAIL- as well as CD95L-stimulation-dependent ERK1/2 phosphorylation were decreased in Hoil-1/−/− MEFs as compared to control cells, indicating that HOIL-1 was required for TRAIL- and CD95L-induced ERK1/2 activation. Consistent with a different kind of cell death to which cpdm- and Hoil-1/−/−-derived cells succumb following TNF treatment, these data demonstrate that SHARPIN and HOIL-1 might also be differentially required for the regulation of non-apoptotic signalling pathways triggered by death ligands.

Taken together, these results imply that HOIL-1 and SHARPIN are both required for the protection of cells from TRAIL- and CD95L-induced cell death. Similar to the TNF system, depletion of either of these LUBAC components also changed death ligand-induced NF-κB and MAPK activation. However, given that TRAIL and CD95L are rather weak inducers of pro-survival signalling, the effects of HOIL-1 and SHARPIN depletion are unlikely to account for the observed sensitisation.

3.2.3 LUBAC is recruited to the TRAIL and CD95 DISC

Previous studies have shown that cIAP1/2 are required to confer resistance to TNF-, TRAIL- and CD95L-induced cell death (Bertrand et al, 2008; McEleny et al, 2004; Wang et al, 1998;
3. Results

Wang et al, 2005). However, the mechanism of cell death inhibition by cIAPs is not completely resolved in the three death receptor systems given that cIAPs are poor direct inhibitors of caspase activity (Eckelman & Salvesen, 2006). Recently, we have shown cIAP1/2 to be required for LUBAC recruitment to the TNF-RSC (Haas et al, 2009 and Figure 14) and, as demonstrated in the previous chapter, LUBAC then shifts the balance of TNF-induced survival versus cell death in favour of the former. To analyse the mechanism by which LUBAC confers resistance to TRAIL- and CD95L-induced cell death, it was analysed whether LUBAC executes its activity at the level of the DISC. Hence, HeLa cells were stimulated for various times with TRAIL, the endogenous, stimulated receptors pulled out and the composition of the TRAIL-R1/R2 DISC analysed by Western blotting. As shown in Figure 44a, TRAIL binding to its receptors induced rapid recruitment of FADD and caspase-8 to the DISC. Caspase-8 then became ubiquitinated over time, a modification that has been linked to enhanced proteolytic activity (Jin et al, 2009). Furthermore, all three LUBAC components were detected in the

Figure 44: LUBAC forms part of the TRAIL-R1/R2 DISC. a) HeLa cells were stimulated with 500 ng/mL moTAP-TRAIL for the indicated times and the DISC subsequently precipitated with anti-FLAG M2 beads. Immunoblots were analysed as indicated. b) Comparison between the TNF-RSC and TRAIL-R1/R2 DISC in HeLa cells stimulated with 500 ng/mL TNF or 500 ng/mL TRAIL, respectively.
3. Results

TRAIL DISC, peaking 30-60 min following stimulation, indicating for the first time that LUBAC did not only form part of the TNF-RSC, but also of the TRAIL DISC (Figure 44a, b). Consistent with earlier reports (Geserick et al, 2009), cIAP1/2 and RIP1 were also recruited to the TRAIL DISC and modified by ubiquitin chains. Whereas RIP1 recruitment to the DISC was rather weak and occurred with delayed kinetics, cIAP1/2 were already bound to the unstimulated receptor and got ubiquitinated over time. This is in agreement with a recent study by Sun et al., who reported on the binding of a complex consisting of cIAP1, DDX3 and glycogen synthase kinase-3 (GSK3) to unstimulated death receptors to confer resistance to the respective death ligand (Sun et al, 2008).

Although LUBAC was apparently recruited to the TRAIL DISC, kinetics and particularly protein quantities were severely different from the TNFR1 system, in which a strong

![Figure 45: LUBAC forms part of the native CD95 DISC. HeLa cells were stimulated with 1 µg/mL Fc-CD95L for the indicated times and the native CD95 DISC precipitated with Protein G beads. Immunoblots were analysed as indicated.](image)
recruitment occurred immediately following receptor engagement and declined after 15-30 min (Figure 44b). In contrast, LUBAC was recruited to the TRAIL DISC rather late peaking at approximately 30-60 min, when the TNF-RSC started to disassemble. Accordingly, severely reduced levels of other adaptor proteins including cIAP1/2 and RIP1 were recruited to the TRAIL DISC when compared to the TNF-RSC, indicating significant differences between these two systems despite employing similar adaptor molecules.

Analogous to the TRAIL DISC, a stimulation- and time-dependent recruitment of LUBAC to the CD95 DISC was also detected. Again, LUBAC binding to the CD95 DISC severely differed from its association with the TNF-RSC, and became detectable only 30-60 min following receptor engagement (Figure 45). Similarly, RIP1 recruitment to the CD95 DISC also peaked approximately 60 min following stimulation when it became readily ubiquitinated. Furthermore, strong ubiquitination of caspase-8 was visible following CD95 trimerisation, which then allowed for strong processing of c-FLIP\textsubscript{L} to its p43 fragment. Taken together, although the composition of the TRAIL DISC, CD95 DISC and the TNF-RSC shares similarity with respect to the source of components recruited to these receptor complexes, the quality of the signalling complexes severely differs, allowing for a preferential induction of cell death or survival pathways, respectively.

3.2.3.1 **LUBAC is recruited to the CD95 and TRAIL DISC in a cIAP1/2-dependent manner**

We recently reported on the recruitment of LUBAC to cIAP1/2-generated ubiquitin chains in the TNF-RSC (Haas et al, 2009). The previous experiments showed that LUBAC was also part of the CD95 and TRAIL DISCs and moreover indicated the presence of cIAP1/2 as well as polyubiquitin chains attached to a variety of DISC constituents. It was therefore next analysed whether cIAP1/2 were also involved in the recruitment of LUBAC to the CD95 and TRAIL-R1/R2 DISCs, respectively. Pre-treatment of HeLa cells with SM-83 (Cossu et al, 2009) efficiently induced auto-ubiquitination and hence degradation of cIAP1/2. In agreement with a recent report by Geserick et al., depletion of cIAP1/2 resulted in increased levels of RIP1 recruited to the TRAIL-R1/R2 DISC (Figure 46a), indicating that cIAP1/2 retain RIP1 in the cytoplasm, thereby preventing its pro-apoptotic/necroptotic activity (Geserick et al, 2009). Although caspase-8, c-FLIP and FADD recruitment to the DISC were not significantly affected by the absence of cIAP1/2, significantly less HOIP, SHARPIN and HOIL-1 were present in IAP antagonist-treated cells, indicating that, similar to the TNF-RSC, LUBAC recruitment to the TRAIL DISC was also cIAP1/2-dependent.
3. Results

Accordingly, analysis of the endogenous CD95 DISC, which was precipitated via the Fc-portion attached to the CD95L sequence, showed reduced levels of SHARPIN, HOIL-1 and HOIP in the absence of cIAPs (Figure 46b). Furthermore, and consistent with previous reports, (Geserick et al, 2009), RIP1 recruitment to the CD95 DISC was strongly increased in cIAP1/2-depleted cells.

Taken together, these data show that the mechanism of recruitment of LUBAC to the three death receptor signalling complexes is very similar. However, as pointed out before, significant differences exist regarding the quality and kinetics of the membrane-proximal complexes which then account for diverse signalling outputs.

3.2.3.2 HOIL-1 absence alters the composition of the TRAIL and CD95 DISC

Having shown that LUBAC was recruited to the TRAIL and CD95 DISCs, the question remained how LUBAC conferred resistance to these death stimuli and whether it acts at the level of DISC formation or down-stream of the DISC. To gain insight into the underlying
mechanism, DISCs were analysed in HeLa cells in which HOIL-1 expression was stably suppressed by RNAi (Haas et al, 2009). Precipitation of the endogenous TRAIL DISC revealed that the absence of HOIL-1 and hence the formation of linear ubiquitin chains strongly altered the formation of the DISC with regards to caspase-8 levels and activity. As shown in Figure 47a, the amount of caspase-8 at the DISC strongly increased in the absence of HOIL-1, and significantly more caspase-8 was modified with ubiquitin chains. Furthermore, caspase-8 ubiquitination was also increased in the CD95 DISC of HeLa cells lacking HOIL-1 expression (Figure 47b). In accordance with Jin et al., increased levels of ubiquitinated caspase-8 correlated with elevated enzymatic activity as assessed by enforced accumulation of the active p18 fragment, and hence increased TRAIL-induced cell death (Jin et al, 2008). Although this result is counter-intuitive given that LUBAC acts as an E3 involved in ubiquitin chain formation, it implies that caspase-8 is not a direct target of LUBAC but rather suggests that LUBAC modifies a yet to be identified protein, which in turn interferes with caspase-8 ubiquitination and hence activation. Additional experiments will be required to solve the mechanism of LUBAC-mediated resistance to TRAIL-induced cell death.

Figure 47: Caspase-8 ubiquitination is increased in the absence of HOIL-1 in the TRAIL-R1/R2 and CD95 DISC. HeLa cells in which the expression of HOIL-1 was stably knocked down and control (ctr) cells were stimulated with (a) 500 ng/mL TRAIL or (b) CD95L for the indicated time and the DISCs precipitated and analysed as indicated.
3. Results

3.3 Summary

Signals emanating from the TNFR1 are quite diverse and can result in pro-survival signalling or cell death, respectively. Deregulated TNF signalling accounts for the generation of a variety of auto-immune diseases, indicating that proper signal transduction is required to allow tissue homeostasis. Thus, it is of great importance to understand the signalling pathways and functions of all relevant proteins involved in TNF-induced signal transduction. Deciphering the complex code of signal generation and propagation induced by this cytokine might guide the future development of therapeutic agents capable of interfering with this process at a particular stage when it functions aberrantly, thereby terminating or enforcing the signal.

In this study, the function of the linear ubiquitin chain assembly complex (LUBAC) with a particular focus on its component SHARPIN was analysed. SHARPIN forms a stimulation-independent complex with HOIL-1 and HOIP that is recruited to the TNF-RSC as well as to the CD95 and TRAIL DISCs following stimulation with the respective ligands. Recruitment to either of these signalling platforms depends on the function of cIAP1/2, which attach ubiquitin chains to target proteins to which the tripartite LUBAC binds via HOIP.

At the TNF-RSC, LUBAC confers stability and allows for full efficient induction of the NF-κB and MAPKs pathways, which subsequently induce a variety of target genes implicated in cell survival and proliferation. Loss of one of the LUBAC components is associated with reduced activation of these pro-survival pathways and instead results in TNF-induced cell death due to facilitated complex II formation. These results place LUBAC at a central position regulating the cellular fate of a cell in response to TNF.

The function of LUBAC is not restricted to the TNF/TNFR1 system but involved in a variety of other pathways, including TRAIL and CD95L-induced signalling. Absence of one LUBAC component results in impaired activation of NF-κB and MAPKs and sensitises the cells to TRAIL- and CD95L-induced cell death, which is independent of TNF. LUBAC is recruited to the TRAIL and CD95 DISCs and its absence from these complexes results in increased ubiquitination and hence activity of caspase-8, indicating that LUBAC is implicated in DISC formation.

Taken together, LUBAC is a new regulator of TNF-, TRAIL- and CD95L-induced signalling and loss of one of its components shifts the balance between life and death of a cell in favour of the latter.
4. Discussion

Post-translational modifications constitute a major event capable of regulating and fine-tuning signalling outputs emanating from distinct signalling platforms. Due to their characteristic of being highly dynamic and largely reversible, post-translational modifications respond to alterations in the cellular microenvironment, thereby controlling the fate of a given cell. For a long time, phosphorylation was the major known form of post-translational modification regulated by a concerted action of kinases and phosphatases. A variety of human cancers is associated with deregulated phosphorylation, most commonly via mutations in the \textit{ras} gene, which accounts for approximately 30% of all human cancers (Downward, 2003). Oncogenic mutation of ras leads to constitutive activation of the ERK1/2 and PI3K (phosphatidylinositol-tris-phosphate kinase) pathways associated with cell proliferation, survival, angiogenesis and metastasis formation (reviewed in Hanahan & Weinberg, 2000).

Apart from phosphorylation, ubiquitination is the post-translational modification system most intensively described in the past decade. It is regulated not only by E3 ubiquitin ligases and deubiquitinating enzymes (DUBs) attaching and removing ubiquitin moieties from target proteins, but also by proteins containing ubiquitin binding domains (UBDs). Deregulation of ubiquitin signalling is associated with the development of a variety of autoimmune disorders, indicating that tight control of ubiquitination, deubiquitination and recruitment of UBD signalling adaptor proteins is required for tissue homeostasis.

In this thesis, I report the identification of a novel E3 ubiquitin ligase complex consisting of the previously described HOIL-1 and HOIP, which form a stable, stimulation-independent complex with SHARPIN (Figure 9). Given its ability to generate poly-ubiquitin chains connected via the N- and C-termini of ubiquitin molecules, respectively, this tripartite complex is referred to as linear ubiquitin chain assembly complex (LUBAC) (Gerlach et al, 2011; Ikeda et al, 2011, Tokunaga et al, 2011). LUBAC is recruited to the TNF-RSC and the TRAIL- and CD95 DISC following stimulation with the respective ligand and is required for full NF-κB and MAPK activation. Absence of a LUBAC component shifts the balance from TNF- (and TRAIL- and CD95L-) induced pro-survival signalling towards cell death induction, thereby promoting an important function for LUBAC in the regulation of death receptor signalling.
4. Discussion

4.1 SHARPIN is the third component of LUBAC and recruited to the TNF-RSC

4.1.1 SHARPIN, HOIL-1 and HOIP stabilise the native TNF-RSC

Signal transduction by members of the TNFR-superfamily relies on the concerted action of a variety of proteins including kinases, ubiquitin ligases, but also adaptor proteins required for the recruitment of these enzymatically active proteins. Using modified tandem affinity purification (moTAP) of the TNF-RSC combined with mass-spectrometry, our group recently reported the identification of HOIL-1 and HOIP (Haas et al, 2009), which together form a complex capable of generating linear ubiquitin chains in vitro (Kirisako et al, 2006). Apart from HOIL-1 and HOIP, the only other possible novel component we identified by mass-spectrometry was SHARPIN, a protein that has not been associated with TNF signalling before. SHARPIN was recruited to the TNF-RSC in a variety of cell lines in a stimulation-dependent manner with kinetics similar to those of HOIL-1 and HOIP (Figure 9). SHARPIN does not possess any enzymatic domain. However, a sequence alignment revealed the presence of UBL and NZF domains at the C-terminus of SHARPIN which are highly homologous to the N-terminus of HOIL-1. Furthermore, pull-down experiments in different cell lines in which HOIL-1, SHARPIN or HOIP were immuno-precipitated revealed that these three proteins form a stimulation-independent complex in the cytosol (Figure 10), suggesting the existence of a tripartite complex. The complex of HOIL-1 and HOIP has initially been described as LUBAC. However, complexes of SHARPIN-HOIP or SHARPIN-HOIL-1-HOIP were also able to form linear ubiquitin chains (Gerlach et al, 2011, Ikeda et al, 2011; Tokunaga et al, 2011), suggesting that the term LUBAC should be expanded to refer to a complex of HOIP, HOIL-1 and/or SHARPIN.

HOIL-1 and HOIP are recruited to cIAP1/2-generated ubiquitin chains (Haas et al, 2009). Considering that SHARPIN forms a stimulation-independent complex with these two proteins, it was not surprising that its recruitment to the TNF-RSC was also dependent on the presence and activity of cIAP1/2 to form ubiquitin chains (Figure 14 and Figure 15). However, it still remains to be determined to which kind of ubiquitin chains LUBAC is recruited. We recently showed that HOIL-1, HOIP and SHARPIN preferentially bind to linear and K63-linked ubiquitin chains as compared with K48-associated ubiquitin moieties (Haas et al, 2009; Gerlach et al, 2011). However, the avidity of the individual LUBAC components for other ubiquitin linkages remains elusive. Dynek et al. showed that cIAP1 in combination with the E2 UbcH5 generates K11-linked ubiquitin chains on RIP1 following TNFR1 stimulation (Dynek et al, 2011). This finding is in line with our data demonstrating that RIP1 is
simultaneously modified by at least four different ubiquitin linkages, i.e. linear, K48, K63 and K11 (Gerlach et al, 2011). Hence, it might well be possible that LUBAC is recruited to cIAP1-generated K11-linked ubiquitin chains attached to RIP1 or possibly other TNF-RSC constituents. Accordingly, the binding affinity of LUBAC to other ubiquitin linkages, including K11 linkages, has to be analysed. Generally, due to the limited availability of such tools, only the involvement of K48-, K63- and linear linked ubiquitin chains has been evaluated until now. However, the binding properties of UBDs to other ubiquitin-linkage types have not been examined. Recently, Dong et al. reported on a new method for the synthesis of distinct ubiquitin chains with high purity and yield which is likely to advance the field of ubiquitin research (Dong et al, 2011). Novel, improved technologies including advances in mass-spectrometry have identified the presence of a variety of other ubiquitin chain linkages in diverse signalling pathways, including K11- in cell cycle progression (Jin et al, 2008), K29- in Notch signalling (Chastagner et al, 2006; Chastagner et al, 2008) and K29/K33-linked ubiquitin chains in the control of AMPK-related kinases (Al-Hakim et al, 2008). These data underline that the rather restricted view of the function of ubiquitination that was propagated during the last decade needs to be thoroughly revised.

Similar to HOIL-1 and HOIP, SHARPIN also bound to ubiquitin chains in vitro (Figure 13). However, it remains to be determined whether this property is of any significance in vivo. Although genetic depletion of HOIL-1 or SHARPIN strongly impaired the protein expression levels of the other two LUBAC components (Figure 19), dimeric complexes of the remaining two LUBAC components were still recruited to the TNF-RSC (Figure 20 and Figure 21). In contrast, depletion of HOIP impaired the recruitment of HOIL-1 and SHARPIN to the TNF-RSC (Figure 22), indicating that the recruitment of LUBAC to cIAP1/2-generated ubiquitin chains was mediated via HOIP. Depletion of the NZF domains from SHARPIN or HOIL-1, which impart ubiquitin binding (Figure 13 and Tokunaga et al, 2009), should not interfere with LUBAC recruitment and activity. However, Tokunaga et al. showed that overexpression of HOIP with a mutant of HOIL-1 lacking the NZF domain was unable to induce NF-κB activation (Tokunaga et al, 2009). Accordingly, a SHARPIN mutant lacking ubiquitin binding ability was unable to activate NF-κB when co-expressed with HOIP (Ikeda et al, 2011). Thus, although HOIL-1 and SHARPIN are dispensable for the recruitment of LUBAC to the TNF-RSC, their respective ubiquitin binding properties are required to activate down-stream signals, possibly by anchoring the tripartite complex at the TNF-RSC. It will be interesting to determine whether a HOIP mutant lacking the NZF1 domain (which mediates ubiquitin binding) will completely abolish the recruitment of LUBAC to the TNF-RSC, supporting the
notion that LUBAC is recruited to the TNF-RSC by an interaction between HOIP and cIAP-generated ubiquitin chains. The mere presence of LUBAC at the TNF-RSC is insufficient for TNFR1 signal transduction, which requires the linear ubiquitin-chain forming activity of HOIP (Haas et al, 2009). However, it is still unknown how HOIP itself gets activated following recruitment. It might well be that components of LUBAC are ubiquitinated once recruited to the TNF-RSC, which subsequently results in HOIP activation. Alternatively, kinases might be implicated in LUBAC activation at the level of TNF-RSC formation by phosphorylation of one or more LUBAC components. Lastly, conformational changes of the tripartite complex occurring upon binding to cIAP-generated chains might account for activation of HOIP’s E3 ligase activity. Mass-spectrometrical as well as two-dimensional (2D)-gel analyses should guide in answering these questions.

Once recruited to the TNF-RSC, LUBAC stabilises the complex and allows for efficient and prolonged retention of RSC-components as indicated upon overexpression of SHARPIN/HOIP or HOIL-1/HOIP complexes (Figure 16). However, at this point it remained obscure as to how LUBAC contributes to complex stabilisation. Precipitating the endogenous TNF-RSC from U937 cells, RIP1 and NEMO were identified as two linearly ubiquitinated targets within the native TNF-RSC, thereby for the first time providing a mechanism by which LUBAC stabilises the TNF-RSC (Gerlach et al, 2011). As yet, only NEMO and RIP1 were found to be decorated with M1-linked ubiquitin chains. However, it is possible that other TNF-RSC components apart from NEMO and RIP1 are also linearly ubiquitinated following TNFR1 stimulation and might therefore also account for LUBAC-mediated TNF-RSC stabilisation and hence efficient signal transduction.

The NEMO spot isolated from 2D-gels contained peptides for only one form of ubiquitin chain linkage, i.e. the linear linkage. According to the size of the protein spot that was analysed (approximately 60-70 kDa), NEMO (48 kDa) was assumed to be modified with a linear di-ubiquitin (14 kDa). Although no other ubiquitin signature peptides were identified in this analysis, it might be possible that NEMO migrating at another molecular weight and/or isoelectrical point contains a different ubiquitin signature. Therefore, a more detailed analysis of the moTAP-isolated TNF-RSC is required. Considering that the mechanisms leading to IKK activation remain poorly understood, the observation that NEMO is linearly ubiquitinated in the TNF-RSC, most likely at lysine residues K285 or K309 (Tokunaga et al, 2009), suggests that these ubiquitin chains serve as platform for the recruitment of additional IKK complexes through binding of the UBAN domain of NEMO (Rahighi et al, 2009).
Increased oligomerisation of the IKK complex might result in trans-autophosphorylation of IKKβ and subsequent NF-κB activation. Additionally, attachment of linear ubiquitin chains to NEMO slightly changes the conformation of the UBAN domain (Rahighi et al, 2009), resulting in re-positioning of IKKα and IKKβ, thereby possibly activating the kinase activities. However, the significance of linear ubiquitination of NEMO has not yet been demonstrated. Once the lysine residue within NEMO carrying the linear di-ubiquitin is identified, mutation of this residue and monitoring of the signalling and cell death responses towards various stimuli should help to determine the biological relevance of this ubiquitination.

In contrast to NEMO, four different ubiquitin signature peptides (K11, K48, K63 and linear) were identified in the same spot with RIP1-derived peptides. This indicates that RIP1 is simultaneously modified by at least four different kinds of ubiquitin chain linkages. Thus, our finding significantly changes the current view on TNFR1 signal transduction as it suggests that each particular chain serves a distinct function resulting in distinct signalling outputs. The concerted, spatio-temporal action of the various ubiquitin chains is required to assure proper signal transmission and hence to balance TNF-induced physiological outcomes.

It has long been a matter of debate why so many diverse DUBs were recruited to the TNF-RSC following stimulation to de-ubiquitinate RIP1. The discovery that at least four different ubiquitin linkage types are present on RIP1 suggests that DUBs of different linkage specificity are required to de-ubiquitinate RIP1 and to thereby terminate the signal. In this context, it is required to extend the analyses of DUB specificity from our limited view on K48, K63 and linear ubiquitin chains and also determine their activities on other linkage types. However, it might not be sufficient to analyse DUB specificities in vitro as it does not necessarily reflect the in vivo situation. A20 for instance preferentially cleaves K48-linked ubiquitin chains in vitro (Lin et al, 2008; Komander et al, 2009) but removes K63-linked ubiquitin chains attached to RIP1 in vivo (Wertz et al, 2004). Hence, the composition of the TNF-RSC has to be examined over time, with a special focus on the appearance and DUB-mediated removal of ubiquitin chains of particular linkage types.

So far, lysine K377 has been described as the only ubiquitin acceptor site in RIP1 (Ea et al, 2006). However, it is so far unknown whether the four individual ubiquitin chain linkages we identified were only attached to this particular lysine residue and form branched chains or whether they were attached to additional lysine residues in RIP1. Once the lysine residue(s) has (have) been identified the function of each chain type should be analysed, i.e. whether it is
4. Discussion

required for NF-κB, JNK1/2, p38 and ERK1/2 activation or cell death inhibition. Mutation of putative lysine residues and subsequent re-expression of these mutants in Rip1\(^{−/−}\) cells should guide the identification of these particular functions. Alternatively, the TNF-RSC could be precipitated from cells expressing ubiquitin mutants in which one particular lysine residue has been exchanged and ubiquitin chains can hence not be prolonged via this particular lysine residue. Recently, Xu et al. applied this approach and showed that ubiquitin mutants lacking the K63-residue exhibited defects in IL-1-induced NF-κB activation but displayed normal TNF-induced responses, indicating that K63-linked ubiquitination is dispensable for TNF signalling but required in the IL-1 pathway (Xu et al, 2009). It will be interesting to determine the cellular responses in different mutants, thereby assessing which particular type of ubiquitin chain is required in which signalling pathway. Kinetic analysis of the TNF-RSC using the moTAP-2D-MRM (multiple reaction monitoring) mass spectrometry approach might help to identify which protein is modified with which ubiquitin chain at which stage following TNFR1 ligation and how this enables the spatio-temporally controlled activation and subsequent inactivation of the components of this complex. The development of ubiquitin chains of different length and linkage, of ubiquitin linkage-specific antibodies and ubiquitin sensors (e.g. TUBEs, tandem repeated ubiquitin entities) which specifically bind to certain ubiquitin linkage types in combination with advances in mass-spectrometry and microscopy-based live cell imaging will be required to understand the complex, dynamic ubiquitin system. Accordingly, the AQUA (absolute quantification) technology will guide in the identification of the stoichiometry of ubiquitin moieties on target proteins and will allow for quantification of ubiquitin chains of different linkages (Kirkpatrick et al, 2006).

4.1.2 LUBAC—a tripartite complex?

The identification of SHARPIN as endogenous, stimulation-independent interaction partner of HOIL-1 and HOIP raises the question of the stoichiometry of this complex. It is currently unknown whether the three proteins form one tripartite complex or whether dimeric complexes with different target specificities exist. Although HOIL-1 possesses a RING domain, overexpression of HOIL-1 and SHARPIN was unable to induce NF-κB activation (Gerlach et al, 2011; Ikeda et al, 2011; Tokunaga et al, 2011), indicating that these two proteins are unlikely to form an active complex involved in signal transduction. Accordingly, although a weak binding of HOIL-1 to SHARPIN was visible when both proteins were overexpressed, their interaction strongly increased in the presence of HOIP (Figure 11a),
suggesting that HOIP binds to both HOIL-1 and SHARPIN, thereby sequestering all three proteins in the same complex. It might be that the binding observed when HOIL-1 and SHARPIN were overexpressed was due to endogenous levels of HOIP. This notion is supported by the finding that an interaction of HOIL-1 with SHARPIN was barely detectable in cells depleted of HOIP (by siRNA and subsequent depletion with anti-HOIP antibodies; Figure 11b). However, a faint interaction between HOIL-1 and SHARPIN was still observed, raising the question whether this was due to remaining HOIP levels, a direct interaction between HOIL-1 and SHARPIN or mediated by a yet unidentified factor. To address this issue, HOIL-1 or SHARPIN should be precipitated from *Hoip*⁻/⁻ cells and the proteins co-precipitated analysed by unbiased proteomic approaches. If no interaction was observed, i.e. SHARPIN and HOIL-1 were unable to directly bind to each other, the existence of an endogenous, tripartite LUBAC complex would be confidently demonstrated.

The protein levels of HOIP and HOIL-1 were severely diminished in the absence of SHARPIN and the levels of HOIP and SHARPIN in the absence of HOIL-1 (Figure 18a and Figure 19b). Down-regulation of HOIL-1 and HOIP was not due to defects in transcription given that their mRNA levels were even slightly enhanced in *cpdm*-derived cells (Figure 18b). Instead, all three proteins seem to be required to stabilise each other and the absence of one component induces rapid degradation of the others. According to the group of K. Iwai and my own observations, down-regulation of HOIP and HOIL-1 in *cpdm*-derived cells was only slightly prevented by treatment with the proteasome inhibitor MG132, indicating that proteasome-independent, as yet unidentified mechanisms account for their degradation.

HOIL-1 generally occurs as a double band on Western blot, indicating that HOIL-1 is modified; based on the size difference between both bands, this second band is most likely a result of mono-ubiquitination of HOIL-1. However, similar to HOIP knockdown (Haas et al, 2009), the upper band of HOIL-1 disappeared in *cpdm*-derived cells, indicating that the presence of both, HOIP and SHARPIN, was required for HOIL-1 modification. Although the significance of this modification on cell signalling remains undetermined, these results further underscore an interdependence between HOIL-1, HOIP and SHARPIN. Despite being present at decreased levels in *cpdm*-derived cells, HOIL-1 and HOIP were still able to interact in the absence of SHARPIN and to be recruited to the TNF-RSC (Figure 20), indicating that dimeric complexes can be formed. However, it remains to be determined whether these complexes are still active. *In vitro* ubiquitination assays so far showed that all three variants were able to form free linear poly-ubiquitin chains or attach linear ubiquitin chains to the target protein.
4. Discussion

NEMO, suggesting that dimeric complexes are sufficient for ubiquitination (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). However, in-vivo evidence that linear ubiquitin chains can still be formed in the absence of HOIL-1 or SHARPIN is still missing. To get insight into this conundrum, the TNF-RSC of Hoil-1\(^{-/-}\) or cpdm MEFs should be analysed by mass-spectrometry and the presence of linear ubiquitin chains investigated. Such analyses would furthermore help to get a better understanding of a possible target specificity of SHARPIN/HOIP and HOIL-1/HOIP complexes, if these complexes indeed existed in vivo. However, considering decreased protein amounts of the remaining two LUBAC components in the absence of the third protein, it is highly likely that most of the endogenous LUBAC complexes are tripartite. Unlike SHARPIN-deficient cpdm mice which develop severe inflammatory and immunological disorders (HogenEsch et al., 1993; HogenEsch et al., 1999), Hoil-1\(^{-/-}\) mice do not develop any prominent inflammatory lesions (Tokunaga et al., 2011; Tokunaga et al., 2009). Hence, although SHARPIN, HOIL-1 and HOIP seem to predominantly form a tripartite complex, SHARPIN might drive the E3 activity of HOIP towards other targets as compared to HOIL-1. Hence, HOIL-1 and SHARPIN do not functionally compensate each other but serve non-redundant roles in enabling particular functions of HOIP, thereby explaining phenotypic differences observed in cpdm and Hoil-1\(^{-/-}\) mice. Taken together, all three proteins are required to stabilise each other and to properly position LUBAC in the TNF-RSC to enable both, HOIL-1- and SHARPIN-guided activities of HOIP by linearly ubiquitinating distinct targets.

4.2 Identification of HOIL-1, HOIP and SHARPIN as novel components of the TRAIL and CD95 DISC

Considering that SHARPIN forms a stimulation-independent complex with HOIL-1 and HOIP, which we recently reported as novel TNF-RSC components it was not surprising to also identify SHARPIN as previously unknown constituent of the TNF-RSC (Figure 9). In contrast, the discovery made in this thesis that HOIL-1, HOIP and SHARPIN also form part of the CD95 and TRAIL DISCs was surprising and represents the first description that linear ubiquitin chains play a role in death receptor signalling. Although significantly lower levels of LUBAC were recruited to these two signalling platforms with delayed kinetics when compared with the TNF-RSC, the identification of LUBAC at the DISC provides new aspects as to how these two pathways transmit their signals. However, a target protein for LUBAC
4. Discussion

has so far not been identified in the CD95 and TRAIL DISCs. It will therefore be required to pull down vast amounts of these complexes and analyse them by mass-spectrometry for the presence of linear ubiquitin chains. First of all, it has to be verified that LUBAC recruitment to the DISC was indeed of functional significance and changed the composition of the DISCs. Pull down experiments revealed that caspase-8 was significantly more ubiquitinated in HOIL-1-depleted HeLa cells as compared with control cells following both TRAIL and CD95L stimulation (Figure 47). At first glance, this result appeared counterintuitive taking into account that LUBAC functions as an E3 ligase and one would therefore expect less ubiquitination in its absence. However, caspase-8 might not be a direct target of LUBAC, but LUBAC might rather ubiquitinate a yet unidentified target protein, which thereby inhibited caspase-8 ubiquitination. Once LUBAC is depleted or functionally inactive, the respective protein is not activated or even not recruited and caspase-8 ubiquitination is therefore not blocked anymore. In line with this thought, Jin et al. demonstrated that cullin3 poly-ubiquitinates caspase-8 following TRAIL stimulation and that ubiquitination of caspase-8 is required to fully activate its proteolytic activity (Jin et al, 2009). Increased levels of caspase-8 ubiquitination correlate with elevated levels of cell death, a scenario observed in LUBAC-depleted cells (Figure 41). However, it will be of major importance to identify the target proteins of LUBAC in the CD95 and TRAIL DISCs, which might be involved in cullin3-mediated caspase-8 inhibition. Alternatively, cullin3 recruitment to the DISC might be inhibited in the presence of LUBAC and reduced levels of cullin3 in the DISC causative for decreased levels of caspase-8 ubiquitination. To test this possibility, DISCs should be precipitated in LUBAC-proficient and -depleted cells and levels of cullin3 investigated. Moreover, it should be studied whether LUBAC activity is required to inhibit caspase-8 ubiquitination or whether its mere presence is sufficient to sterically hinder it. Therefore, DISCs from cells expressing a HOIP mutant lacking enzymatic activity should be analysed and cell death responses monitored.

Taken together, the identification of LUBAC as a novel component of the CD95 and TRAIL DISCs that blocks TRAIL- and CD95L-induced cell death defines linear ubiquitination as major regulator of cell death responses induced by a variety of TNFR superfamily members and highlights that linear ubiquitination has a much broader function than previously assumed.
4.3 LUBAC balances TNF-, TRAIL- and CD95L-induced signalling outputs

4.3.1 LUBAC in TNFR1 signalling

TNF is the key cytokine involved in the initiation of innate immune responses against invading pathogens and binding to its receptor TNFR1 generally associated with cell survival, proliferation and the expression of pro-inflammatory mediators. A variety of molecules have been implicated in the elucidation of a regulated, timely controlled signalling output by the TNFR1 system. In this thesis, SHARPIN, in addition to the previously described HOIL-1 and HOIP (Haas et al, 2009; Tokunaga et al, 2009), is defined as a previously unknown component of the TNF-RSC required for full activation of the NF-κB and MAPK pathways and its absence resulted in severe defects in TNF-induced signalling. However, SHARPIN and HOIL-1 deficiency did not completely abolish the activation of NF-κB, JNK1/2 and p38 but severely impaired their activation (Figure 23, Figure 24 and Figure 28). Consistently, transcription of the bona fide NF-κB target gene IκBα was only slightly decreased in the absence of SHARPIN (Figure 27). This finding is in line with a recent report by Tay et al. who demonstrated that some TNF target genes, including IκBα, require only weak signals to be transcribed, whereas others rely on the concerted action of NF-κB and MAPKs (Tay et al, 2010). Hence, diminished activation of NF-κB in the absence of SHARPIN was sufficient to induce the transcription of IκBα, but the expression of other TNF target genes including A20, ICAM-1 and TNF itself was almost completely abrogated in the absence of SHARPIN due to concomitant defects in TNF-induced JNK1/2 and p38 activation. Considering that dimeric complexes of SHARPIN/HOIP or HOIL-1/HOIP still exist in the absence of HOIL-1 or SHARPIN, respectively, it might be possible that these remaining dimeric complexes were still capable of activating NF-κB and MAPKs. However, although HOIL-1 knockdown in cpdm-derived fibroblasts further decreased activation of these pathways, it did still not completely render them inactive, suggesting the existence of LUBAC-independent mechanisms of NF-κB and MAPK activation. In line with this notion, Hostager et al. recently demonstrated that B cells, in which the gene encoding HOIP was disrupted by somatic cell gene targeting, showed impaired, yet not completely absent CD40-induced activation of NF-κB and JNK1/2 (Hostager et al, 2011). Moreover, TNF-induced signalling in SHARPIN and HOIL-1 deficient cells did not phenocopy each other, indicating that both proteins have non-redundant functions. In particular, cpdm-derived MEFs showed elevated basal levels of phosphorylated ERK1/2, which were even slightly enhanced following TNF stimulation as compared with wild-type control cells (Figure 23a and Figure 24a). In contrast, basal levels of
phospho-ERK1/2 were not increased in Hoil-1\(^{-/-}\) fibroblasts but even showed defects following TNFR1 engagement. The exact mechanism of TNF-induced ERK1/2 activation is only poorly understood, but seems to depend on the MAP3K Tpl2 (Eliopoulos et al., 2003). Although it remains to be determined at which stage Tpl2 is required for TNF signalling or whether any other molecules are implicated in TNF-induced ERK1/2 activation, these results indicate that HOIL-1 and SHARPIN differentially activate diverse signalling pathways, most likely by targeting different proteins for HOIP-mediated ubiquitination. However, the exact mechanisms and differences between SHARPIN- and HOIL-1-activated HOIP complexes need to be addressed in more detail.

Although TNF generally results in cell activation and survival, it is also able to induce cell death if signal transmission is impaired. Consistently, SHARPIN-deficient cpdm-derived or Hoil-1\(^{-/-}\) cells were sensitive to TNF-induced cell death, indicating that loss of linear ubiquitination shifts the balance from pro-survival signalling in favour of cell death execution. In this study, no obvious difference in cell death sensitivity between cpdm and Hoil-1\(^{-/-}\) fibroblasts could be observed. This is in contrast to Ikeda et al. who suggested that absence of SHARPIN resulted in more cell death than absence of HOIL-1 (Ikeda et al., 2011). However, cell death was not quantified in the mentioned study and the presentation of the cell death results with a logarithmic scale was slightly misleading. Although a subtle delay in cell death induction was apparent, both cell types equally succumbed to cell death the following day, indicating that HOIL-1 and SHARPIN conferred a similar degree of resistance to TNF-induced cell death. However, full knockout Hoil-1\(^{-/-}\) MEFs were applied in the study by Ikeda et al. while fibroblasts obtained from embryos in which the Hoil-1 gene was flanked by loxP sites and excised following infection with a Cre-expressing virus were employed in this study. Hence, the differences between these two studies could also be due to the use of differentially generated fibroblasts, in which HOIL-1 was absent chronically as compared to inducibly.

Fibroblasts or primary keratinocytes that die upon TNF stimulation in the absence of SHARPIN exhibited characteristics of apoptotic as well as necroptotic death as assessed by the ability of the RIP1 kinase inhibitor necrostatin-1 and the caspase inhibitor QVD to partially inhibit TNF-induced cell death (Figure 30 and Figure 31). However, cell death was not completely prevented when both inhibitors were applied simultaneously, indicating that either the inhibitors did not completely block caspases and RIP1 or that another form or pathway of cell death might be involved. Given that some cells die in a RIP1-independent, RIP3-dependent manner (Upton et al., 2010), it will be interesting to determine whether
knockdown, knockout or inhibition of the kinase activity of RIP3 in cpdm-derived cells renders them completely resistant to TNF-induced cell death. In contrast to the partial necroptotic nature of cell death in the absence of SHARPIN, the death obtained in *Hoil-1*/* Hoil-1* cells could not be blocked by necrostatin-1, but rather exhibited signs of apoptosis (Figure 32). This is the first example showing a major difference between SHARPIN and HOIL-1 and supports the notion of different functions of HOIP-containing SHARPIN or HOIL-1 complexes, which result in the generation of different phenotypes in mice.

A major obstacle to working on necroptosis is the lack of a marker clearly identifying it as such; as yet, necroptosis is mainly identified based on the absence of apoptotic or autophagic markers in the early stages of cell death (Kroemer et al, 2009). Further complication arises from the occurrence of secondary necrosis at late stages of apoptosis due to inefficient clearance of apoptotic bodies. So far, necrotic cells are mainly identified due to their ability to die in the presence of caspase inhibition or depletion and the dependency of cell death on the kinase activity of RIP1. However, the assessment of cell morphology is so far the only possibility to quantify the fractions of cells that undergo apoptosis versus necroptosis. While a staining for active caspase-3 represents a mean to biochemically quantify apoptosis, no intracellular marker has as yet been described for necroptosis. It is hence required to develop specific markers of cell death that can be readily and easily monitored.

Necrosis has long been thought of as an accidental form of cell death occurring in an unregulated manner. However, early reports already demonstrated that necrosis can be induced by a variety of stimuli including TNF, TRAIL and CD95L (Holler et al, 2000; Laster et al, 1988). However recently, it was discovered that this form of death can, similar to apoptosis, also proceed in a controlled manner and it was hence named necroptosis (Degterev et al, 2005). So far, the kinase activities of RIP1 and RIP3 have been identified as required for necroptosis and RIP1 and RIP3 were shown to form a stimulation-dependent complex (the so-called necrosome). However, down-stream mechanisms resulting in typical necrotic hallmarks remain poorly understood. Generally, the formation of a stronger TNF-induced complex II is considered as a mechanism associated with enhanced TNF-induced cell death. It will hence be of great importance to analyse complex II formation in LUBAC-component-deficient cells. So far, these analyses were only successfully conducted in human HeLa cells in which complex II precipitation is well established. Knockdown of any of the three LUBAC components led to increased complex II formation as assessed by increased interaction of caspase-8 with FADD, TRADD and RIP1 (Figure 35). Furthermore, caspase-8 activity was
increased in these cells compared with non-targeted control cells, and p18 formation and RIP1 cleavage were significantly increased. However, HeLa cells do not express RIP3 and hence do not die necroptotically (Feoktistova et al, 2011; He et al, 2009). Re-expression of RIP3 in these and other RIP3-deficient cells rendered them susceptible to TNF-induced necroptotic death, indicating that RIP3 is the decisive factor required for necroptosis (He et al, 2009). Considering that cpdm-derived cells were prone to both TNF-induced necroptosis and apoptosis whilst Hoil-1/− cells only became sensitive to apoptosis, it will be interesting to analyse the necrosome in SHARPIN-depleted as compared with HOIL-1-depleted RIP3-expressing HeLa cells.

The mechanism leading to increased susceptibility to TNF-, TRAIL- and CD95L-induced cell death in the absence of linear ubiquitin chains remains largely undefined. The identification of LUBAC as a component of TNF-induced complex II (Figure 36), combined with the finding that LUBAC-deficient cells are more prone to TNF-induced cell death (Figure 32), suggests that the presence of linear ubiquitin chains negatively regulates complex II formation and/or activity. However, the LUBAC target responsible for this effect remains to be determined. Oshima et al. described that Abin-1/− cells were more susceptible to TNF-induced cell death due to increased FADD/caspase-8 interaction, which they attributed to enhanced complex II activity (Oshima et al, 2009). However, the precise mechanism as to how ABIN-1 inhibits complex II formation and activity, and thereby interferes with cell death induction, is only poorly described. Considering that the absence of particular linear ubiquitination events in SHARPIN- or HOIL-1-deficient cells inhibited ABIN-1 recruitment to the TNF-RSC (Figure 20 and Figure 21), it might be possible that decreased levels of ABIN-1 in the TNF-RSC allow for efficient formation of complex II and hence the induction of cell death, thereby defining linear ubiquitin chains as negative regulators of complex II generation.

Apart from ABIN-1, c-FLIP is known to inhibit death ligand-induced killing. TNF stimulation generally induces the transcriptional expression of c-FLIP, which is then recruited to complex II, in which it not only inhibits caspase-8-mediated apoptosis (Micheau et al, 2001), but also forms heterodimers with caspase-8 to block RIP1/RIP3-dependent necroptosis (Oberst et al, 2011). This process most likely involves processing of RIP1 (Lin et al, 1999) and RIP3 (Feng et al, 2007). SHARPIN-deficient cells exhibited lower basal levels of c-FLIP (Figure 37). This down-regulation itself might be sufficient for the sensitisation effect observed in cpdm-derived cells. Given that TNF-induced gene transcription was severely inhibited in the absence of SHARPIN (Figure 27), c-FLIP levels are unlikely to increase
4. Discussion

following TNFR1 engagement, further potentiating the failure to block complex II-mediated apoptosis, but also necroptosis. Overexpression of c-FLIP\textsubscript{L} and c-FLIP\textsubscript{S} (whose expression could not be detected by Western blotting) in cpdm-derived fibroblasts will offer insight into whether basal c-FLIP down-regulation is responsible for TNF sensitivity and, if so, whether this is isoform-specific. Accordingly, a constitutively active version of IKK\textbeta allowing for constitutive NF-\kappa B activation should be expressed in cpdm-derived fibroblasts to assess whether defects in NF-\kappa B activation are responsible for TNF-induced death susceptibility. In contrast to the effects seen in cpdm-derived cells, c-FLIP\textsubscript{L} was not down-regulated but even slightly enhanced in Hoil-1\textsuperscript{-/-} cells (Figure 37). Increased c-FLIP\textsubscript{L} levels might account for enforced activity of caspase-8/c-FLIP\textsubscript{L} heterodimers and hence inhibition of necroptosis in Hoil-1\textsuperscript{-/-} cells, which did not die in a necroptotic manner (Figure 32). These results suggest that HOIL-1 drives the activity of HOIP to other targets as compared to SHARPIN and that these targets are rather involved in apoptosis inhibition, whereas SHARPIN-directed HOIP complexes ubiquitinate targets crucial for prevention of necroptosis.

The mechanism of SHARPIN-deficiency-mediated c-FLIP regulation has not yet been investigated. However, only recently it was reported that SHARPIN inhibited the tumour suppressor protein PTEN (phosphatase and tensin homolog) (He et al, 2010). PI3K activation by a variety of stimuli including growth factors leads to the generation of phosphatidylinositol-trisphosphate (PIP\textsubscript{3}), which subsequently recruits PDK1 (phosphoinositol-dependent kinase 1). PDK1 phosphorylates and thereby activates AKT, resulting in enhanced cell survival, proliferation and metabolism. Amongst others, AKT phosphorylates and thereby inactivates the transcription factor FOXO3a (Anderson et al, 1998; Brunet et al, 1999). By an uncompletely understood mechanism, AKT-mediated FOXO3a inhibition leads to an increase in the levels of c-FLIP which is associated with cell death resistance (Skurk et al, 2004). PTEN functions as the phosphatase that counteracts the activity of PI3K, thereby inhibiting AKT activation. Hence, the decreased c-FLIP levels in SHARPIN-deficient cpdm-derived cells might be due to increased PTEN activity resulting in decreased basal AKT activation. Despite showing an interaction between endogenous SHARPIN and PTEN, He et al. did not provide any mechanistic insight as to how SHARPIN inhibited PTEN activity. Considering its ability to form linear ubiquitin chains when complexed with HOIP, PTEN might be a novel LUBAC target whose activity is negatively regulated by linear ubiquitination. However, it remains to be tested whether HOIL-1 and HOIP are also capable of inhibiting PTEN and hence to be determined whether SHARPIN-mediated PTEN inhibition is dependent on linear ubiquitination or, more specifically, on SHARPIN/HOIP-mediated linear ubiquitination.
The E3 ligase Itch has also been implicated in the regulation of cFLIP levels (Chang et al., 2006). Itch becomes activated by JNK1 and was shown to K48-ubiquitinate c-FLIP, thereby inducing its degradation. Therefore, the expression level and activation status of Itch in cpdm-derived cells should be investigated.

Taken together, the identification of TNF-induced linear ubiquitination of RIP1 and NEMO, associated with an inhibition of cell death, refines the model of TNFR1 signalling (Figure 48):

![Figure 48: The influence of SHARPIN-deficiency on TNF signalling.](image)

The E3 ligase Itch has also been implicated in the regulation of cFLIP levels (Chang et al., 2006). Itch becomes activated by JNK1 and was shown to K48-ubiquitinate c-FLIP, thereby inducing its degradation. Therefore, the expression level and activation status of Itch in cpdm-derived cells should be investigated.

Taken together, the identification of TNF-induced linear ubiquitination of RIP1 and NEMO, associated with an inhibition of cell death, refines the model of TNFR1 signalling (Figure 48):
4. Discussion

Following TNFR1 trimerisation, TRADD is recruited via its death domain to TNFR1 and subsequently recruits RIP1 and TRAF2, which in turn recruits cIAP1/2 that then induce ubiquitination of several TNF-RSC components, including RIP1 and cIAP1/2 themselves. Subsequently, the tripartite LUBAC is recruited via HOIP to cIAP-generated chains, activated at the TNF-RSC and, by yet to be determined mechanisms, linearly ubiquitinates RIP1 and possibly other substrates. NEMO, which preferentially binds to linear ubiquitin chains subsequently allows for the recruitment of the IKK complex and, once in the complex, also becomes linearly ubiquitinated. Apart from the UBAN domain of NEMO, cIAP1/2 were shown to efficiently bind to linear ubiquitin chains via their UBA domains (Gyrd-Hansen et al, 2008). Hence, linear ubiquitin chains generated by LUBAC stabilise the TNF-RSC and allow for tight, prolonged retention of TNF-RSC components. Concomitant to IKK recruitment, the TAK1/TAB complex is recruited and becomes activated by as yet incompletely understood mechanisms, which possibly also involve ubiquitination (Wang et al, 2001). Taking into account that K63-linked ubiquitin chains were dispensable (Xu et al, 2009) but that TAK1 is required for TNF-induced signal transduction (Sato et al, 2005), it is unlikely that the recruitment of the TAK1/TAB complex to the TNF-RSC is solely mediated via binding of the NZF of TAB2 or TAB3 to K63-linked ubiquitin chains. Once recruited, TAK1 phosphorylates and hence activates IKKβ, which then leads to activation of NF-κB. Furthermore, TAK1 activates the p38 and JNK1/2 pathways, which, together with NF-κB allows for the transcription of a variety of target genes implicated in cell survival and proliferation. Despite formation of complex II, caspase-8 does not get activated under physiological conditions. However, in the absence of SHARPIN and hence the formation of SHARPIN/HOIP-mediated linear ubiquitin chains, c-FLIP levels are decreased and transcription of pro-survival genes inhibited. This leads to enhanced complex II formation, possibly mediated by less ABIN-1 recruitment, and increased caspase-8 activation (in the absence of c-FLIP), associated with enhanced apoptotic and necroptotic cell death.

4.3.2 LUBAC in TRAIL- and CD95L-induced cell death

While the major function of TNF is associated with survival and proliferation, TRAIL and CD95L are rather implicated in cell death responses. Assessing the effect of LUBAC in these two pathways, it became apparent that loss of SHARPIN or HOIL-1 also sensitised cells to TRAIL- and CD95L-mediated death (Figure 41). As shown in Figure 47, LUBAC recruitment to the TRAIL and CD95 DISC inhibited caspase-8 ubiquitination and hence activation
through a yet to be identified mechanism (Jin et al., 2009). Although SHARPIN and HOIL-1 deficiency were also associated with reduced activation of NF-κB, it remains to be determined whether diminished TRAIL- and CD95L-induced NF-κB activation are causative for the observed sensitisation towards these death ligands. Overexpression of a constitutively active IKKβ mutant will provide the answer to this question. The effect of LUBAC deficiency on TRAIL-induced JNK1/2 activation was less clear and varied between individual experiments, indicating that LUBAC is less likely to be involved in TRAIL-induced JNK1/2 activation. Similar to TNF signalling, TRAIL- and CD95L-induced ERK1/2 activation was slightly enhanced in cpdm-derived, but reduced in Hoil-1−/− fibroblasts, again indicating that HOIL-1 and SHARPIN serve divergent, non-redundant functions.

TRAIL-induced cell death in cpdm-derived fibroblasts was purely of apoptotic nature whereas TNF- and CD95L-induced death was partially apoptotic and partially necroptotic (Figure 38 and Figure 39). In 2000, Holler et al. for the first time described a function of RIP1 in caspase-8-independent TRAIL- and CD95L-induced cell death in T cells (Holler et al., 2000). However, whereas CD95L-induced cell death in the presence of the caspase-inhibitor zvad-fmk was readily detectable in a variety of cell lines and primary T cells, TRAIL only induced caspase-independent cell death when co-administered with the protein synthesis inhibitor CHX, indicating that the synthesis of yet to be identified proteins renders cells susceptible to a purely caspase-dependent form of death. Hence, despite employing the same adaptor and signalling molecules, at least as far as we know to date, the nature of cell death induced by TRAIL significantly differs from the one triggered by CD95L. It will be of great importance to dissect both signalling pathways to understand why TRAIL and CD95L, despite initially appearing similar, differ so profoundly in their cell death responses. Taking the result by Holler et al. into consideration, it is less surprising that TRAIL-induced cell death in SHARPIN-deficient cells was exclusively apoptotic, whereas CD95L-mediated death also involved necroptosis.

CD95L- and TRAIL-induced necroptosis required the presence of FADD, whereas TNF-induced cell death was even increased in Fadd−/− Jurkat T cells (Holler et al., 2000), indicating that different signalling platforms are formed following stimulation by TNF and TRAIL or CD95L. While TNF-induced complex II formation and the requirement of the kinase functions of RIP1/RIP3 for necroptosis induction are well established, little is known about TRAIL- and CD95L-induced necrosome formation. Although RIP1 has been described to be recruited to the TRAIL and CD95 DISCs, particularly in the absence of cIAPs which enable
induction of necroptotic death (Geserick et al, 2009), RIP3 has so far not been detected in the two DISCs. However, He et al. showed that RIP3 was required for TRAIL- and CD95L-induced necroptosis by a yet to be determined mechanism (He et al, 2009). It will therefore be necessary to identify at which level RIP3 exerts its necroptotic function in these pathways, i.e. at the level of DISC formation or further down-stream in secondary cytosolic complexes. Although such a secondary, intracellular complex II has been described following TRAIL stimulation, its function has so far rather been associated with pro-survival NF-κB and MAPK activation (Varfolomeev et al, 2005). Considering that CD95L- and TRAIL-induced necroptosis was FADD-dependent (Holler et al, 2000), it is highly likely that RIP3 would be recruited to a FADD-containing complex, whose formation and activity should be increased in the absence of SHARPIN.

Depletion of cIAP1/2 by IAP antagonists rendered cells susceptible to TRAIL- and CD95L-induced necroptosis, indicating that cIAP1/2 determine the kind of cell death a given cell succumbs to (Geserick et al, 2009). The recruitment of LUBAC to the respective DISCs is mediated by cIAPs and their ubiquitin-chain-forming capacity (Haas et al, 2009). Hence, depletion of cIAP1/2 results in the absence of LUBAC recruitment to the DISCs, which is associated with increased cell death. Contrary to this, the levels of RIP1 in the DISCs increase in the absence of cIAP1/2. Through yet to be identified mechanisms, increased levels of RIP1 modulate the kind of cell death resulting in caspase-8-dependent apoptosis and RIP1-dependent necroptosis.

Taken together, LUBAC is recruited to the TRAIL and CD95 DISC and inhibits apoptosis, most likely by inhibiting efficient caspase-8 ubiquitination. However, the mechanism as to how LUBAC confers protection from CD95L-induced necroptosis remains elusive, but might be due to increased formation and activity of RIP1-RIP3 intracellular signalling platforms in the absence of SHARPIN/HOIP-generated ubiquitin chains. Although CD95L and TRAIL signalling share a variety of similarities, the quality of cell death observed in cpdm-derived cells significantly differs following stimulation with the two death ligands. It is therefore of great importance to functionally dissect the two pathways and identify proteins required for apoptosis versus necroptosis induction.
4. Discussion

4.3.3 Function of LUBAC in other signalling pathways

The identification of NEMO as an endogenous LUBAC target suggests that its function is not limited to TNF-induced signalling but rather involved in a variety of pathways utilising the IKK complex. We and others showed that LUBAC was also required for signalling by CD40L, IL-1 (Gerlach et al, 2011; Tokunaga et al, 2009; Tokunaga et al, 2009), LPS (Ikeda et al, 2011), other Toll-like receptor agonists (Zak et al, 2011) and for anti-viral responses (Inn et al, 2011). These results suggest that, similar to the situation in TNF, TRAIL, CD95 and CD40 activation, LUBAC will be recruited to a signalling platform generated following stimulation with the respective ligands. It will be interesting to analyse these platforms to identify possible other LUBAC targets, thereby getting a better understanding of signal transduction induced by the various stimuli.

The above mentioned pathways are generally involved in immune responses associated with NF-κB and MAPK activation. In contrast to this, but similar to CD95L and TRAIL, genotoxic stress is capable of inducing cell death. As shown in Figure 42, the absence of SHARPIN and HOIL-1 also profoundly sensitised cells to etoposide-induced cell death. Although differences in the quantity of cell death following cisplatin treatment were not that pronounced, small doses of cisplatin also induced more cell death in cpdm-derived and Hoil-1⁻/⁻ cells than in wild-type cells. This observation is in accordance with a recent publication by Niu et al. who showed that LUBAC promotes genotoxic stress-induced NEMO ubiquitination and hence NF-κB activation (Niu et al, 2011). Following DNA damage, LUBAC was reported to interact with NEMO in the cytosol thereby enabling activation of the IKK and TAK1/TAB complex. Absence of HOIL-1 or HOIP strongly diminished this interaction and activation of pro-survival signalling, indicating that LUBAC is not only involved in cell death responses emanating from cytokine and other immune cell receptors, but also required for DNA-damage responses.

Only recently, the two groups of Pascal Meier and Martin Leverkus described a novel intracellular signalling platform which is capable of inducing apoptotic and necroptotic cell death. Given that RIP1 constitutes the critical factor for this signalling platform, it has been dubbed as the “rioptosome” (Feoktistova et al, 2011; Tenev et al, 2011). Following treatment with an IAP antagonist and poly(I:C) or etoposide, RIP1 formed an intracellular complex with RIP3, FADD, and caspase-8/10 which induced necroptosis as well as apoptosis. Formation of the riopptosome and subsequent cell death execution was independent of endogenous production of TNF, TRAIL or CD95L in a variety of cell lines, suggesting that the
rioptosome constitutes a previously unknown signalling platform distinct from death ligand-induced secondary complex II. However, riptosome formation was decreased in the presence of TNF-blocking agents in some cell lines, suggesting that it is cell-type specific and depends on the ability of a given cell type to produce endogenous TNF. Consistently, Biton and Ashkenazi recently showed the formation of an intracellular complex following DNA damage in HeLa cells whose formation was induced by endogenous TNF (Biton & Ashkenazi, 2011). Riptosome activity was negatively regulated by c-FLIP, cIAP1, cIAP2 and XIAP and cells expressing high levels of these molecules were resistant to riptosome formation and cell death. Considering up-regulation of these inhibitors in a variety of tumours, targeting the riptosome might constitute a novel strategy in cancer therapy. Although the role of LUBAC in riptosome formation has so far not been analysed, due to similarities in composition to TNF-induced signalling platforms, it is highly likely that LUBAC also interferes with riptosome activity.

Taken together, its panoply of functions in a variety of signalling pathways defines LUBAC and hence linear ubiquitination as a new regulator of innate immunity and inflammation.

4.4 Deregulated LUBAC signalling

4.4.1 SHARPIN-deficient cpdm mice

The NF-κB pathway regulates cellular responses in physiology and disease. It constitutes a quick detection system capable of recognising potential threats within the microenvironment, which it then rapidly translates into distinct gene expression programs. Expression of pro-inflammatory proteins including cytokines, chemokines, adhesion molecules and enzymes possessing microbicidal activity elicit appropriate immune responses protecting the organism from infection and injury. At the same time, NF-κB signalling leads to cell survival by induction of anti-apoptotic genes and proteins with anti-oxidative function to protect cells from ROS-mediated necrosis (Pham et al, 2004; Sakon et al, 2003). Deregulated NF-κB signalling has been associated with the development of various inflammation-related diseases and cancer. Studies in knockout mice lacking particular components required for NF-κB activation (e.g. IKK components) or termination (e.g. A20 and CYLD) showed that ubiquitous NF-κB inhibition was embryonically lethal (Beg et al, 1995b; Li et al, 1999a; Li et al, 1999b; Makris et al, 2000; Rudolph et al, 2000; Schmidt-Supprian et al, 2000). Conversely, ubiquitous removal of NF-κB inhibitors is associated with the development of
severe inflammation and early postnatal death (Beg et al, 1995a; Klement et al, 1996; Lee et al, 2000). Surprisingly, targeted NF-κB inhibition in non-immune, epithelial or parenchymal cells triggered the development of severe inflammatory conditions, indicating that NF-κB inhibition in certain tissues results in pro-inflammatory outputs by disrupting physiological immune homeostasis. Keratinocyte-specific ablation of IKKβ or NEMO led to the development of severe skin inflammation associated with postnatal death of the mice. The development of skin lesions was shown to depend on TNFR1 signalling given that concomitant deletion of TNFR1 in these epidermis-specific gene-deficient mice completely (IKKβ) or partially (NEMO) rescued the inflammatory phenotype (reviewed in Pasparakis, 2009). Hence, NF-κB inhibition in epidermal keratinocytes disturbs skin immune homeostasis, which is generally maintained by extensive cross-talk between epidermal keratinocytes and stromal and immune cells in the dermis. However, the molecular mechanisms by which NF-κB signalling controls skin homeostasis are poorly defined.

The results obtained in this study show that LUBAC is another complex required for full NF-κB activation. Similar to epidermis-specific IKKβ and NEMO knockout animals (IKKβEKO and NEMOEKO), depletion of SHARPIN in cpdm mice induced the development of overt skin lesions, which first appear three to four weeks after birth (HogenEsch et al, 1993). In contrast, IKKβEKO and NEMOEKO mice already developed severe skin inflammation three to four days following birth resulting in postnatal death (Nenci et al, 2006; Pasparakis et al, 2002), consistent with the absolute requirement of both components for NF-κB activation. Although SHARPIN was required for full NF-κB signalling (Figure 23), cpdm-derived cells did not completely lack IkB phosphorylation, indicating the existence of SHARPIN-independent mechanisms of NF-κB activation. Hence, the severity and occurrence of pathology depends on the requirement of a particular molecule on NF-κB signalling. Macrophages rather than neutrophils and T cells have been demonstrated to be the cells mediating skin inflammation in IKKβEKO and NEMOEKO mice (Pasparakis et al, 2002; Stratis et al, 2006). It remains to be determined whether these cells are also responsible for the pathology observed in cpdm mice.

Concomitant heterozygous deletion of TNF in SHARPIN-mutant mice was sufficient to inhibit the development of inflammation in a variety of organs including skin, liver and oesophagus (Gerlach et al, 2011), indicating that loss of the SHARPIN-driven inflammatory phenotype was TNF-dependent. However, heterozygosity was only capable of delaying the onset of disease progression as skin lesions became apparent several months after birth (J.
4. Discussion

Silke, personal communication). Furthermore, we were unable to inhibit or even delay the inflammatory phenotype by injection of the TNF-blocker Enbrel® (data not shown; N.B: this experiment was conducted together with Eva Rieser), indicating that levels of TNF are decisive for inflammation and disease inevitably progresses once a certain threshold has been exceeded. As yet, it was assumed that TNF-based auto-immune diseases were mediated by elevated levels of TNF-induced pro-inflammatory gene transcription. However, this is unlikely to be the case for the cpdm phenotype: although it constitutes a TNF-driven disease, loss of SHARPIN resulted in decreased TNF-induced gene transcription (Figure 27), suggesting that TNF-induced pro-inflammatory cytokines are less likely to be the cause of inflammation. Accordingly, it remains to be determined whether TNF protein levels were increased in cpdm mice and might therefore account for increased levels of keratinocyte cell death (Liang et al, 2011). Alternatively, the quantity of TNF present in the skin is unaltered and increased sensitivity of keratinocytes is the sole reason for elevated cell death. So far, mRNA levels of TNF were described to be unaltered in cpdm skin samples as compared with skin from wild-type animals (HogenEsch et al, 2001). Furthermore, I was unable to detect significant differences in TNF protein levels between various skin homogenates obtained from wild-type and cpdm mice by ELISA (data not shown), indicating that increased TNF levels are unlikely responsible for elevated cell death quantities. Immuno-histochemical analyses of skin sections may further clarify this issue. Additionally, the source of TNF responsible for inducing cell death has not yet been identified. I did not detect any measurable levels of TNF or IL-1 in keratinocyte cultures (data not shown), suggesting that either the ELISA method was not sensitive enough or that TNF was produced by other cells, for instance epidermis-infiltrating macrophages or mast cells.

TNF-inhibiting drugs are generally applied in the treatment of inflammatory diseases including rheumatoid arthritis and Crohn’s disease (Feldmann et al, 2004) and was thought to inhibit pro-inflammatory TNF-driven gene transcription associated with manifestation of inflammatory conditions. However, TNF not only activates gene transcription, but can, under certain conditions also induce cell death. It is therefore possible that the inflammatory phenotypes observed in IKKβEKO, NEMOEKO or SHARPIN-mutant mice, but also in humans suffering from rheumatoid arthritis or Crohn’s disease are driven by increased rates of cell death. SHARPIN-deficient cpdm-derived fibroblasts and keratinocytes exhibited signs of TNF-mediated apoptosis as well as necroptosis (Figure 30 and Figure 31), a pro-inflammatory form of cell death. Hence, endogenous TNF present in the epidermis of the skin might induce keratinocyte death, which would subsequently release pro-inflammatory molecules, resulting
4. Discussion

in the attraction and activation of immune cells from the dermis and the evocation of inflammation. Deficiency in TNF in \textit{cpdm} mice inhibited keratinocyte death and hence the manifestation of an inflammatory milieu. Consequently, and in contrast to previous assumptions, TNF-blockers used in the treatment of rheumatoid arthritis or Crohn’s disease might not be effective due to inhibition of TNF-mediated gene activation of pro-inflammatory cytokines but rather by preventing TNF-induced pro-inflammatory cell death. If this were the case and inflammation a consequence of cell death, new treatment options for these diseases could be established by the identification of necroptosis inhibitors. The proof-of-principle that necroptosis inhibition can indeed prevent inflammatory conditions was recently provided by numerous reports showing that concomitant RIP3 (or RIP1) knockout in mice deficient for caspase-8 or FADD rescued lethality associated with FADD and caspase-8 depletion (Kaiser et al, 2011; Oberst et al, 2011; Zhang et al, 2011) and prevented colitis induced by intestinal epithelial cell- or keratinocyte-specific FADD deletion (Bonnet et al, 2011; Gunther et al, 2011; Welz et al, 2011). Caspase-8 was shown to form heterodimeric complexes with c-FLIP\textsubscript{L} which inhibit RIP1/RIP3-dependent necroptosis. In the absence of caspase-8, RIP1/RIP3 complexes are no longer inhibited and transmit necroptotic signals leading to inflammatory cell death and hence liver failure. Accordingly, absence of FADD inhibits the formation of a secondary, necroptosis-inhibiting complex consisting of caspase-8 and c-FLIP\textsubscript{L}. Thus, apart from their pro-apoptotic activities, caspase-8 and FADD also exhibit non-apoptotic functions during embryogenesis by inhibiting necroptosis. This mechanism most likely involves cleavage and hence inactivation of RIP1 and RIP3, but might also be regulated by a yet unidentified target of the caspase-8/c-FLIP\textsubscript{L} heterodimers. Considering the increased occurrence of cell death in the skin of \textit{cpdm} mice (Liang & Sundberg, 2011) and the increased susceptibility of \textit{cpdm}-derived cells to TNF-induced necroptosis, it will be interesting to determine whether the inflammatory phenotype observed in \textit{cpdm} mice can be reverted by concomitant RIP3 deficiency. If TNF (or CD95L)-induced necroptosis was the cause for the development of inflammatory skin lesions, \textit{cpdm;Rip3}\textsuperscript{−/−} mice should be protected as keratinocytes would only succumb to apoptotic, non-immunogenic cell death. This finding would further support the notion that patients, in whom necroptotic cell death rather than pro-inflammatory gene induction was the cause for inflammation, could be treated with necroptosis inhibitors. Accordingly, the RIP1 inhibitor necrostatin-1 should be tested for its ability to prevent skin lesion formation. However, treatment of lesion-free \textit{cpdm} mice with Enbrel\textsuperscript{®} starting at fifteen days of age could not prevent the occurrence of skin pathology, indicating that once the threshold has been reached and various cells succumbed to
necroptotic cell death, the vicious circle is initiated and blockage of TNF-induced cell death alone is no longer sufficient to exert a therapeutic effect. Nevertheless, patients might still benefit from a necroptosis-targeting therapy once the inflammation resulting from necroptotic death of keratinocytes has been cleared, for instance by corticosteroid treatment, and the development of new lesions subsequently blocked by inhibition of necroptosis.

Besides Tnf gene deletion, Liang et al. reported that concomitant deletion of the IL-1 Receptor accessory protein (IL-1RAcP) in cpdm mice was also capable of inhibiting the formation of inflammatory skin lesions (Liang et al., 2010). In contrast to the results obtained in this thesis, Liang et al. suggest that SHARPIN plays a negative regulatory role in NF-κB activation as they showed increased constitutive NF-κB activation in the skin of cpdm mice. However, no functional activity assays with isolated cells were performed in that study, which only monitored overall NF-κB activity. Considering that the skin is highly inflamed and populated with activated immune cells, it is not surprising to detect increased overall NF-κB activation. It will be interesting to test whether Il-1Racp-/-;cpdm mice still exhibit elevated levels of keratinocyte death but do not develop skin lesions due to inhibited signal transduction of released pro-inflammatory cytokines including IL-1 and IL-33 utilising the IL-1RAcP. This result would further support the notion of TNF-induced inflammatory cell death being the elicitor of inflammation and IL-1 or related cytokines being the mediators. It should hence also be tested whether blockade of IL-1 by the IL-1R antagonist Anakinra (Kineret®) is capable of inhibiting the development of skin lesions if injected into mice shortly after birth (where TNF-blockade was unable to prevent pathology).

As shown by concomitant deletion of the Tnf gene in cpdm mice, TNF doubtlessly plays a role in skin pathology observed in these mice. Consistent with the necroptotic kind of cell death, it is highly likely that inflammatory cell death is the cause for inflammation. However, it remains to be determined whether, and to which extent, TRAIL and CD95L contribute to the observed phenotype. Considering that CD95L induced apoptotic as well as necroptotic cell death, a role of the CD95 pathway for disease initiation is well possible. It will therefore be interesting to test whether genetic deletion of CD95L or CD95 in cpdm mice is also capable of inhibiting or attenuating the inflammatory phenotype. In contrast, TRAIL-induced cell death in cpdm-derived cells was purely apoptotic in nature. It is therefore less likely that TRAIL contributes to the manifestation of skin pathology. Contrarily, TRAIL application has been shown to alleviate auto-immunity in several models (Cretney et al, 2006; Ikeda et al, 2010; Stratis et al, 2006). Although the mechanism explaining the beneficial role of TRAIL in
these models remains poorly defined, it might be possible that TRAIL shifts the balance from TNF-induced pro-inflammatory necroptosis towards non-immunogenic apoptosis, thereby preventing the manifestation of an inflammatory milieu. Hence, it should be analysed whether TRAIL injection into cpdm mice prevents skin inflammation by altering the kind of cell death keratinocytes succumb to. However, considering that SHARPIN was depleted from all organs, TRAIL application on the other hand might be toxic to cpdm mice if for example hepatocytes deficient in SHARPIN were rendered sensitive to TRAIL-induced apoptosis. Studies on primary hepatocytes isolated from cpdm mice, or indeed the systemic treatment of cpdm mice with TRAIL, will provide the answer to this question.

ABIN-1 has been described as a protein that specifically binds to linear ubiquitin chains (Rahighi et al, 2009) and whose function is associated with the termination of TNF-induced signalling through recruitment of the DUB A20. Reduced levels of ABIN-1 were recruited to the TNF-RSC in the absence of linear ubiquitination (Figure 20 and Figure 21). Hence, although initial TNF-induced NF-κB and MAPK activation were decreased in cpdm and Hoil-1⁻/⁻ cells (Figure 23, Figure 24 and Figure 27), it is possible that, due to reduced ABIN-1 recruitment, and hence decreased A20 activity, the TNF-induced activatory signal cannot be completely shut off in cpdm-derived cells, including in immune cells. As a consequence there would be a constant signalling of low TNF-induced gene activation creating an inflammatory microenvironment that could be causative for auto-inflammation in cpdm mice. It is a challenging task to dissect the separate contributions of cell death and constant, albeit reduced NF-κB and MAPK activation to the formation of the inflammatory phenotype. Generation of skin- and macrophage-specific SHARPIN-deficient mice and crosses with mice deficient in key necroptosis, NF-κB and MAPK pathway components will be very instructive in this regard.

4.4.2 HOIL-1 and HOIP deficiency

Hoil-1⁻/⁻ mice do not develop any obvious skin lesion during maturation (Tokunaga et al, 2011; Tokunaga et al, 2009) and HOIP deficiency is embryonically lethal (unpublished data by the group of K. Iwai). These in-vivo results are in accordance with the biochemical data presented here showing that HOIP is the central component of LUBAC, mediating formation of a tripartite complex, allowing for its recruitment to the TNF-RSC (and possibly other signalling complexes) and endowing the complex with its catalytic, linear ubiquitin chain-forming activity. It will be interesting to test whether TNF-induced NF-κB and MAPK
activation is completely blocked in the absence of HOIP or whether linear ubiquitin-independent mechanisms of activation exist. As mentioned earlier, despite presenting reduced CD40L-induced NF-κB and JNK activation in B cells in which the expression of HOIP was genetically depleted by somatic gene targeting, other pathways were not completely inhibited, arguing for LUBAC-independent mechanisms (Hostager et al, 2011). Considering LUBAC’s pleiotropic effects in various signalling pathways and its ability to confer resistance to death ligands, it is not surprising that the complete absence of linear chain formation in Hoip−/− mice results in embryonic lethality. Furthermore, these phenotypes argue for the existence of remaining linear ubiquitin chains in the absence of SHARPIN or HOIL-1 which were sufficient for embryos to be born but, as in the case of cpdm mice, induced auto-inflammation and immunological abnormalities with age. Moreover, the observation that HOIL-1 and SHARPIN deficiency did not induce identical phenotypes suggests that the HOIL-1-directed HOIP activity has functions that are distinct from the ones of SHARPIN-HOIP complexes. However, possible targets of the respective dimers remain to be identified. Moreover, it has to be tested whether the sensitivity of primary keratinocytes from Hoil-1−/− mice to TNF- and possibly other death ligands is comparable to the quantity and quality observed in cpdm mice. In this respect, Hoil-1−/− animals should be monitored for the presence of active caspase-3 (indicative of apoptosis) and necroptotic cells in the skin. If my hypothesis regarding HOIL-1 as an inhibitor of apoptosis and SHARPIN as an inhibitor of necroptosis is correct, keratinocytes from Hoil-1−/− mice should still die apoptotically but this should not result in inflammation due to the non-immunogenic nature of apoptosis.

Alternatively, phenotypic differences between cpdm and Hoil-1−/− mice could be due to a LUBAC-independent function of SHARPIN. SHARPIN was initially identified as a SHANK-interacting protein whose activity was associated with neuronal signalling (Lim et al, 2001). Only recently, Liang proposed that SHARPIN, independently of its function as LUBAC component, negatively regulates NF-κB signalling by interacting with TRAF2 (Liang, 2011). Despite the unconvincing nature of the data presented in the mentioned study, it is entirely possible that SHARPIN possesses activities unrelated to linear ubiquitination. In line with this notion, Rantala et al. recently reported on an inhibitory function of SHARPIN in β1-integrin activation, which they claimed to be independent of HOIL-1 and HOIP (Rantala et al, 2011). However, it remains a challenging task to differentiate between functions of SHARPIN associated with LUBAC and those that are possibly independent thereof.
4. Discussion

4.4.3 LUBAC inhibition

Since persistent NF-κB activation is associated with a variety of diseases including cancer and auto-immunity, the development of NF-κB inhibitors has been a focus of research for several years. However, NF-κB inhibitors lack specificity and hence interfere with the pleiotropic functions of NF-κB in immunity, inflammation and cellular homeostasis, resulting in non-tolerable side effects. In order to develop cell-type specific inhibitors less prone to the development of adverse effects, a better understanding of the NF-κB pathway is required.

Ubiquitination is a prominent event in NF-κB activation and its deregulation implicated in disease initiation and progression. Thus, similar to kinase inhibitors, inhibition of particular E3 ligases might be therapeutically beneficial if diseases were driven by enhanced ubiquitin-mediated signalling. Enhanced expression of SHARPIN and HOIL-1 were commonly found in a diversity of human tumours (Gustafsson et al, 2010; Jung et al, 2010). Up-regulation of LUBAC components might result in more rapid and progressive tumour growth due to its pro-survival activity, the expression of anti-apoptotic genes and inhibition of cell death. There are several opportunities to interfere with ubiquitin-mediated signalling. Ubiquitin chain formation itself can be blocked by inhibitors binding to the active site of particular ligases, thereby inhibiting their activity. Furthermore, enhancing the expression or activity of DUBs could be a valuable tool to terminate signalling. Alternatively, small-molecules interfering with the binding of UBDs to ubiquitin chains of certain linkage can inhibit the recruitment of downstream signalling adaptors and hence signal transduction. Proof-of-principle has been provided by ubistatins, small-molecule inhibitors capable of binding to K48-linked ubiquitin chains, thereby preventing their recognition and subsequent degradation by the ubiquitin receptors RPN10 and RPN13 of the proteasome (Verma et al, 2004). Although ubistatins were proven inadequate for therapeutic use due to their inability to penetrate cell membranes, this example nevertheless strengthens the notion that ubiquitin-mediated protein-protein interactions can be disrupted by small-molecules. New-generation inhibitors with improved pharmacological qualities might overcome this limitation, however they require high specificity. Tumour cells overexpressing LUBAC components should exhibit enhanced linear ubiquitin chain formation as compared with normal cells. Thus, the application of small molecules preventing binding of UBDs to linear ubiquitin chains, or indeed inhibitors of the enzymatic activity of HOIP, could potentially inhibit tumour growth, without major side effects. Furthermore, the utility of such ligase inhibitors is not restricted to NF-κB, but also targets MAPKs, which are also up-regulated in cancer and auto-inflammatory diseases.
4.5 Outlook

In this study, SHARPIN was identified as a new component of the linear ubiquitin chain assembly complex (LUBAC) interacting with HOIP and HOIL-1. Following TNF, TRAIL, and CD95L stimulation, the tripartite LUBAC complex was recruited to the respective receptor signalling complexes. SHARPIN, HOIL-1 and HOIP were all required for efficient NF-κB and MAPK activation associated with gene transcription of anti-apoptotic as well as pro-inflammatory target genes allowing cells to survive and elicit an immune response at the same time. However, in the absence of SHARPIN and therefore linear ubiquitination pro-survival signalling was disturbed and the balance shifted towards cell death execution. This cell death was partially of necroptotic nature and therefore associated with inflammation. Hence, contrary to the previously assumed notion that TNF generates an inflammatory microenvironment solely due to expression of pro-inflammatory mediators, these data suggest that deregulated TNF-mediated necroptosis might also contribute to pathology. Considering the high incidence of patients suffering from auto-immune or auto-inflammatory conditions and the tremendous cost of current treatment options, the development of necroptosis inhibitors preventing inflammatory cell death or agents shifting inflammatory death towards non-immunogenic, apoptotic death is highly recommended. However, patients have to be categorised according to whether pro-inflammatory cytokines or increased inflammatory cell death are the main drivers of inflammation. Moreover, primary or secondary TNF-non-responders might benefit from such a therapy which is not restricted to TNF-induced necroptotic cell death, but also inhibits necroptosis induced by other death stimuli, including CD95L. Hence, the blockade of death ligand-induced necroptosis opens new opportunities for the treatment of auto-immunity. Given that patients treated with anti-TNF therapies are generally more susceptible to infection, inhibition of necroptosis instead of TNF, a major cytokine involved in innate immunity, illustrates a further therapeutic advantage.

It will furthermore be interesting to screen auto-immune and cancer patients for genetic or epigenetic alterations in the expression or activity of LUBAC and to determine whether disturbed or enhanced linear ubiquitination contributes to disease initiation and progression. If this were the case, development of inhibitors of LUBAC or of the pathological consequences of its functional perturbation may define novel opportunities for the treatment of auto-inflammatory diseases and cancer. However, further studies will be required to understand the physiological roles of linear ubiquitination in more detail and to determine how the knowledge about these processes can be employed to the treatment of patients.
5. Appendix

5.1 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABIN</td>
<td>A20 binding inhibitor of NF-kappaB</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation induced cell death</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosin-monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
</tr>
<tr>
<td>APF-1</td>
<td>ATP-dependent proteolysis factor-1</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease-activating factor-1</td>
</tr>
<tr>
<td>APC/C</td>
<td>Anaphase-Promoting Complex</td>
</tr>
<tr>
<td>APF-1</td>
<td>ATP-dependent proteolysis factor</td>
</tr>
<tr>
<td>AQUA</td>
<td>Absolute quantification</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis signal-regulating kinase-1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosin-triphosphate</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2 antagonist of cell death</td>
</tr>
<tr>
<td>BAFF</td>
<td>B cell-activating factor</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl2-antagonist/killer</td>
</tr>
<tr>
<td>BARD</td>
<td>BRCA1-associated RING domain protein</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl2-associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>B-cell lymphoma extra large</td>
</tr>
<tr>
<td>BCMA</td>
<td>B cell maturation antigen</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl-2 homology 3</td>
</tr>
<tr>
<td>Bid</td>
<td>BH3-interacting domain death agonist</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2-interacting mediator of cell death</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculoviral IAP repeat</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast cancer</td>
</tr>
</tbody>
</table>
5. Appendix

C     Cysteine
CAD   Caspase Activated DNase
Caspase Cysteinyl-aspartate specific proteases
CD    Cluster of differentiation
CD40L CD40 ligand
Cezanne Cellular Zn finger anti-NF-κB
c-FLIP Cellular FLICE-inhibitory protein
CHX   Cycloheximide
cIAP   Cellular inhibitor of apoptosis protein
CIM    cIAP1/2 interacting motif
ConA  Concanavalin A
cpdm  Chronic proliferative dermatitis
CRD   Cysteine rich domain
CREB  cAMP response element-binding
CYLD  Familial Cylindromatosis Protein
CXCL  Chemokine (C-X-C motif) ligand
Da    Dalton
DAMP  Danger-associated molecular pattern
Dc    Decoy-Receptor
DC    Dendritic cell
DD    Death domain
DED   Death-effector domains
DIABLO Direct IAP binding protein with low PI
DISC  Death inducing signalling complex
DMEM  Dulbecco’s modified Eagle’s medium
dsRNA Double stranded ribonuclease acid
DUB   Deubiquitinases
EBNA  EBV-specific nuclear antigen
EBV   Epstein-Barr virus
ELISA Enzyme-linked immunosorbent assay
ER    Endoplasmic reticulum
5. Appendix

ERK Extracellular signal related kinase
FADD Fas-associated death domain
FACS Fluorescence activated cell sorting
FCS Fetal calf serum
G Glycine
GAPDH Glyceraldehyde 3-phosphate dehydrogenase
GC Germinal centre
GCK Germinal centre kinase
GCKR Germinal centre kinase related
GITR Glucocorticoid-induced TNF-R family receptor
gld Generalised lymphoproliferative disease
GLUD1 Glutamate dehydrogenase 1
GLUL Glutamata ammonia ligase
GM-CSF Granulocyte macrophage colony-stimulating factor
GTP Guanosine-triphosphate
HBS Hepes buffered saline
HECT Homologous with E6-associated protein C-terminus
HEK Human embryonic kidney
HOIL-1 Haeme-oxidized IRP2 ubiquitin ligase 1
HOIP HOIL-1-interacting protein
HPRT Hypoxanthine-guanine phosphoribosyltransferase gene
HRP Horseradish-peroxidase
HUVEC Human umbilical vein endothelial cell
HVEM Herpes virus entry mediator
IBR In Between RING
ICAM Intercellular adhesion molecule
IFN Interferon
Ig Immunoglobulin
IkB Inhibitor of kappaB
IKK Inhibitor of kappaB kinase
IL Interleukin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthetase</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1R-associated kinase</td>
</tr>
<tr>
<td>JAMM</td>
<td>JAB1/MPN/MOV34 metalloprotease</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>KD</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>lpr</td>
<td>Lymphoproliferative disease</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Lt</td>
<td>Lymphotoxin</td>
</tr>
<tr>
<td>LUBAC</td>
<td>Linear ubiquitin chain assembly complex</td>
</tr>
<tr>
<td>M</td>
<td>Methionine</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>Myeloid leukaemia cell differentiation 1</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MEK</td>
<td>MAP/ERK kinase kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MKP</td>
<td>MAPK phosphatase</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane permeabilisation</td>
</tr>
<tr>
<td>moTAP</td>
<td>Modified tandem affinity purification</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappaB</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB inducing kinase</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NLK</td>
<td>Nemo-like kinase</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation sequence</td>
</tr>
<tr>
<td>NZF</td>
<td>Npl4-type zinc finger</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>OPD</td>
<td>O-Phenylene-diaminedihydrochloride</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OTU</td>
<td>Ovarian tumour proteases</td>
</tr>
<tr>
<td>P</td>
<td>Proline</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamid gel electrophoresis</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PARP-1</td>
<td>Poly(ADP)-Ribose-Polymerase-1</td>
</tr>
<tr>
<td>PLAD</td>
<td>pre-ligand assembly domain</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDB</td>
<td>Pull-down buffer</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLAD</td>
<td>Pre-ligand-binding assembly domain</td>
</tr>
<tr>
<td>Pru</td>
<td>Plextrin receptor for ubiquitin</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PS</td>
<td>Protease cleavage site</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl-serine</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 up-regulated modulator of apoptosis</td>
</tr>
<tr>
<td>PYGL</td>
<td>Glycogen phosphorylase</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of NF-κB</td>
</tr>
<tr>
<td>RBCK1</td>
<td>RBCC protein interacting with PKC 1</td>
</tr>
<tr>
<td>RBR</td>
<td>RING-IBR-RING</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>RHIM</td>
<td>RIP homotypic interaction motif</td>
</tr>
<tr>
<td>RING</td>
<td>Really Interesting New Gene</td>
</tr>
<tr>
<td>RIP1</td>
<td>Receptor interacting protein 1</td>
</tr>
</tbody>
</table>
RNAi  RNA interference
RNF  RING finger protein
ROS  Reactive oxygen species
RPMI  Roswell Park Memorial Institute medium
RSC  Receptor signalling complex
RTK  Receptor tyrosine kinase
S  Serine
SAPK  Stress-activated protein kinases
SCF  Stem-cell factor
SCFβ-TrCP  Skp1, Cul1, Roc1 and the F-box protein β-TrCP
SEM  Standard error of the mean
SHARPIN  SHANK-associated RH-domain-interacting protein
SLE  Systemic lupus erythematosus
SM  Smac mimetic
Smac  Second mitochondria-derived activator of caspases
SOS  Son of sevenless
T  Threonine
TAB  TAK1 binding protein
TACE  TNFα converting enzyme
TACI  Transmembrane activator and CAML interactor
TAD  Transactivation domain
TAK1  TGF-β-activated kinase 1
TAP  Tandem-affinity purification
TAX1BP1  Tax 1 binding protein 1
TCR  T cell receptor
T_H  T helper cells
THD  TNF homology domain
TIM  TRAF-interacting motif
Tpil2  Tumour progression locus 2
TLR  Toll-like receptor
TMD  Transmembrane domain
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF-R1-associated death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF-receptor associated factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TrCp</td>
<td>Transducin repeat-containing protein</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TWEAK</td>
<td>TNF-like weak inducer of apoptosis</td>
</tr>
<tr>
<td>UBA</td>
<td>Ubiquitin-associated</td>
</tr>
<tr>
<td>UBAN</td>
<td>Ubiquitin-binding domains found in ABINs and NEMO</td>
</tr>
<tr>
<td>UBD</td>
<td>Ubiquitin-binding domain</td>
</tr>
<tr>
<td>UBL</td>
<td>Ubiquitin-like</td>
</tr>
<tr>
<td>UCH</td>
<td>Ubiquitin C-terminal hydrolase</td>
</tr>
<tr>
<td>UEV</td>
<td>Ubiquitin-conjugating enzyme variant</td>
</tr>
<tr>
<td>UIM</td>
<td>Ubiquitin-interacting motif</td>
</tr>
<tr>
<td>USP</td>
<td>Ubiquitin-specific proteases</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>XEDAR</td>
<td>X-linked EDA-A2 receptor</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-chromosome linked inhibitor of apoptosis</td>
</tr>
<tr>
<td>ZF</td>
<td>Zinc finger</td>
</tr>
</tbody>
</table>
5.2 List of Figures

Figure 1: TNFR1 signal transduction.................................................................6
Figure 2: Members of the NF-κB and IκB family. .............................................11
Figure 3: MAPK signalling.............................................................................16
Figure 4: The extrinsic and intrinsic apoptotic pathways.................................23
Figure 5: Schematic representation of caspase-3 activation..............................24
Figure 6: TNF-induced necrosome formation..................................................33
Figure 7: The ubiquitin system........................................................................37
Figure 8: Schematic representation of the targeted allele used to generate conditional Hoil-1−/− mice..............................................................74
Figure 9: SHARPIN forms part of the native TNF-RSC....................................75
Figure 10: SHARPIN forms a stimulation-independent tripartite complex with HOIL-1 and HOIP..................................................76
Figure 11: HOIP facilitates the interaction between HOIL-1 and SHARPIN.........77
Figure 12: SHARPIN and HOIP interact via their UBL and NZF domains, respectively..........................................................78
Figure 13: SHARPIN binds to ubiquitin via its NZF domain..............................78
Figure 14: SHARPIN is recruited to the TNF-RSC via cIAP1/2.........................79
Figure 15: SHARPIN is recruited to cIAP1/2 generated chains.........................80
Figure 16: SHARPIN contributes to stabilisation of the TNF-RSC....................81
Figure 17: SHARPIN-deficient cpdm mice......................................................82
Figure 18: Loss of SHARPIN destabilises HOIL-1 and HOIP on the protein level, but still allows for their interaction..................................................83
Figure 19: Loss of HOIL-1 destabilises HOIP and SHARPIN protein levels........84
Figure 20: HOIP and HOIL-1 are recruited to the TNF-RSC in the absence of SHARPIN.................................................................84
Figure 21: HOIL-1 is dispensable for the recruitment of HOIP and SHARPIN to the TNF-RSC.................................................................85
Figure 22: HOIP is required for the recruitment of LUBAC to the TNF-RSC........86
Figure 23: SHARPIN is required for full NF-κB and JNK1/2 activation.............87
Figure 24: Diminished p38 activation in the absence of SHARPIN....................88
Figure 25: HOIL-1 knockdown in cpdm-derived MEFs further potentiates defects in NF-κB activation........................................................................88
Figure 26: Absence of SHARPIN diminishes and delays TNF-induced p65 nuclear translocation. ................................................................. ............................................................. 89
Figure 27: TNF-induced gene transcription is impaired in cpdm-derived MEFs. .......... 90
Figure 28: HOIL-1 is required for full NF-κB and MAPK activation............................... 91
Figure 29: LUBAC deficiency sensitises to TNF-induced cell death. ............................... 92
Figure 30: SHARPIN-deficient cells are sensitive to TNF-induced cell death, which is partially necroptotic and partially apoptotic................................................................. 93
Figure 31: Primary cpdm-derived keratinocytes are sensitive towards TNF-induced cell death. ............................................................................................................................... 94
Figure 32: TNF-induced cell death in Hoil-1−/− as compared to cpdm-derived fibroblasts is similar in quantity, but severely different in quality................................................. 95
Figure 33: Apoptotic responses significantly alter in Hoil-1−/− MEFs as compared to cpdm-derived cells. .................................................................................................................. 96
Figure 34: Loss of SHARPIN induced strong complex II formation. .......................... 97
Figure 35: Absence of any LUBAC component sensitises to TNF-induced cell death mediated by increased complex II formation. ......................................................... 98
Figure 36: LUBAC is part of the secondary complex II.................................................... 99
Figure 37: FLIP expression is decreased in cpdm but increased in Hoil-1−/− MEFs. .... 100
Figure 38: Loss of SHARPIN sensitises to TRAIL-induced apoptosis......................... 101
Figure 39: Loss of SHARPIN sensitises cells to CD95L-induced cell death................ 102
Figure 40: Loss of SHARPIN induces typical hallmarks of apoptosis following TRAIL and CD95L stimulation.............................................................................................. 103
Figure 41: HOIL-1 and SHARPIN deficiency sensitise to TRAIL and CD95L-induced cell death independent of endogenous TNF production................................. 104
Figure 42: Loss of SHARPIN and HOIL-1 sensitise to DNA damage-induced cell death. ................................................................................................................................. 105
Figure 43: TRAIL and CD95L-induced signalling is impaired in the absence of SHARPIN or HOIL-1. .................................................................................................................. 106
Figure 44: LUBAC forms part of the TRAIL-R1/R2 DISC.............................................. 108
Figure 45: LUBAC forms part of the native CD95 DISC............................................... 109
Figure 46: LUBAC recruitment to the TRAIL-R1/R2 and CD95 DISC depends on cIAP1/2. ................................................................................................................................. 111
Figure 47: Caspase-8 ubiquitination is increased in the absence of HOIL-1 in the TRAIL-R1/R2 and CD95 DISC. ................................................................. 112
Figure 48: The influence of SHARPIN-deficiency on TNF signalling.................128

5.3 List of Tables

Table 2.1: Unconjugated antibodies........................................................................58
Table 2.2: Conjugated antibodies ...........................................................................59
Table 2.3: Oligonucleotide sequences and corresponding Probe numbers according to the Roche Universal Probe Library...............................................71

5.4 List of Publications


* contributed equally
6. References


6. References


Cordova SM, Papenfuss K, Walczak H (2009b) From biochemical principles of apoptosis induction by TRAIL to application in tumour therapy. *Results Probl Cell Differ* **49**: 115-143


6. References


Lee SY, Reichlin A, Santana A, Sokol KA, Nussenzweig MC, Choi Y (1997) TRAF2 is essential for JNK but not NF-kappaB activation and regulates lymphocyte proliferation and survival. *Immunity* 7(5): 703-713


Niu J, Shi Y, Iwai K, Wu ZH (2011) LUBAC regulates NF-kappaB activation upon genotoxic stress by promoting linear ubiquitination of NEMO. *EMBO J*


6. References


6. References


Wroblewski VJ, Witcher DR, Becker GW, Davis KA, Dou S, Micanovic R, Newton CM, Noblit TW, Richardson JM, Song HY, Hale JE (2003) Decoy receptor 3 (DeR3) is proteolytically processed to a metabolic fragment having differential activities against Fas ligand and LIGHT. *Biochem Pharmacol* **65**(4): 657-667

6. References


176


