Characterisation of SHARPIN as a third component of the linear ubiquitin chain assembly complex (LUBAC)

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Anna Schmukle
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1 Summary

Tumour necrosis factor (TNF) is an important cytokine with great physiological relevance and effects ranging from pro-inflammatory to immuno-regulatory functions. On a cellular level, it induces signalling processes by crosslinking its receptors and by initiating the formation of an intracellular, multi-protein receptor-signalling complex (RSC). Investigation of the TNF-RSC by modified tandem affinity purification (moTAP) and mass spectrometry revealed the presence of three novel components in this complex: heme-oxidised IRP2 ubiquitin ligase-1 (HOIL-1), HOIL-1-interacting protein (HOIP) and SHANK-associated RH-domain-interacting protein (SHARPIN). Previous studies showed that HOIL-1 and HOIP form an E3-complex that mediates the generation of linearly linked ubiquitin chains and is hence referred to as linear ubiquitin chain assembly complex (LUBAC). Identification of peptides specific for SHARPIN in the mass spectrometric analysis of the native TNF-RSC together with its sequence similarity to HOIL-1 raised the questions whether SHARPIN contributes functionally to TNF-signalling and/or the E3-activity of LUBAC.

In this thesis, it could be shown that all three proteins are specifically recruited to the TNF-RSC in a cIAP1/2-dependent manner. As SHARPIN, HOIL-1 and HOIP can bind ubiquitin chains this suggests that the three proteins are recruited via cIAP1/2-generated ubiquitin chains. In addition, HOIP is required for presence of SHARPIN and HOIL-1 in the TNF-RSC. This, together with the finding that these three factors form a stimulation-independent protein complex in the cytosol, indicates that LUBAC is recruited to the TNF-RSC as a tripartite complex via its central component HOIP.

In-vitro ubiquitination assays showed that SHARPIN is not only a physical but also a functional component of LUBAC. HOIP can generate ubiquitin chains when combined with either SHARPIN, HOIL-1 or both and was shown to exclusively generate linear linkages via a HECT-like mechanism. NEMO was identified as a common target of all possible LUBAC-combinations *in vitro* and, in line with this, the activity of LUBAC is required for full activation of NF-κB following TNF stimulation.

The results obtained in this thesis identify SHARPIN as a third component of LUBAC, an E3-complex that is specifically recruited to the TNF-RSC and regulates TNF signalling by modifying specific target proteins with linearly linked ubiquitin chains.

I Declaration

I, Anna Schmukle, 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, 30.04.2012

Anna Schmukle

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2 Introduction

2.1 The ubiquitin system

Ubiquitin is a small, relatively heat-stable polypeptide of 76 aminoacids that was first isolated from bovine thymus (Goldstein, 1974; Schlesinger et al., 1975) and was found to be identical to an essential component of an energy-dependent protein degradation system that had been referred to as ATP-dependent proteolysis factor 1 (APF-1) (Ciechanover et al., 1978; Wilkinson et al., 1980). Ubiquitin is covalently linked via its C-terminus to the amino-groups of lysine residues or the N-terminus of other proteins by the concerted action of three classes of proteins, a ubiquitin activating enzyme (E1), a ubiquitin conjugating protein (E2) and a ubiquitin ligase (E3) (Figure 1 and (Ciechanover et al., 1982; Hershko et al., 1983).

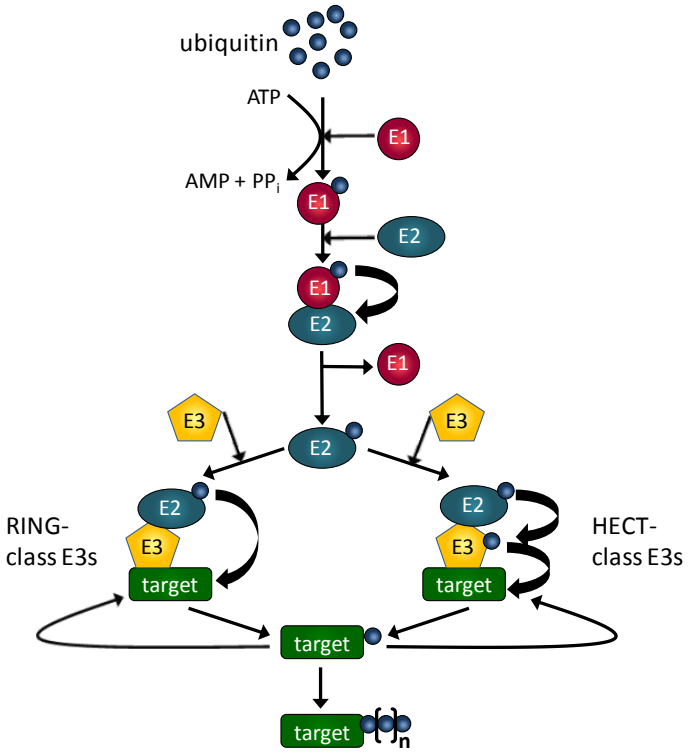


Figure 1: Schematic representation of the ubiquitination process. In a first ATP-dependent step ubiquitin is activated by the E1. In a second reaction it is transferred onto a catalytic cysteine within the E2 and with the help of an E3 it is conjugated to an aminogroup, usually the ε-aminogroup of a lysine residue, within the target protein. Depending on the class of E3 participating in the process the last step can involve the formation of an E3-ubiquitin thioester intermediate or can occur independently thereof. PP_i: inorganic phosphate, RING: really interesting new gene, HECT: Homologous to the E6-AP Carboxyl Terminus.

Ubiquitin itself contains 7 internal lysine-residues, which in addition to the α-aminogroup of methionine 1 (M1) can be used as conjugation points. Therefore the formation of

differentially linked chains is possible and these chains differ both structurally and functionally (Komander, 2009; Peng et al., 2003; Virdee et al., 2010). In the context of protein degradation the target is often modified with ubiquitin chains linked via lysine (K) 48 and this leads to its recognition by the proteasome and its degradation (Chau et al., 1989; Ciechanover et al., 1980; Hershko et al., 1980). Tagging of target proteins can be reversed by the action of ubiquitin specific proteases known as deubiquitinases (DUBs) (Clague et al., 2012; Komander et al., 2009a; Reyes-Turcu et al., 2009). Ubiquitin is encoded on 4 highly conserved genes. Two of these encode linear polyubiquitin in which ubiquitin molecules are linked to each other from ‘‘head-to-tail,’’ and the other two genes encode ubiquitin fused to ribosomal subunits. Hence, one important function exerted by specific members of the DUB family is to co- or posttranslationally cleave the resulting ubiquitin fusion proteins into single ubiquitin moieties (Baker and Board, 1987; Finley et al., 1987; Ozkaynak et al., 1984; Reyes-Turcu et al., 2009; Wiborg et al., 1985).

Ubiquitin is the prototype of a family of proteins that, in spite of variable sequences, display a remarkably similar structure, referred to as the β-GRASP fold. This structure contains a domain with two β-sheets followed by an α-helix and another two β-sheets (Schulman and Harper, 2009; van der Veen and Ploegh, 2012; Vijay-Kumar et al., 1987). Due to the similarities to ubiquitin, the structure and the group of proteins containing it are also referred to as the ubiquitin-like (UBL)-fold and -family, respectively. Members of the UBL-family include, amongst others, NEDD8, ATG8, SUMO1-3, FAT10 and ISG15 that, like ubiquitin, can be covalently conjugated to target proteins via an isopeptide bond. The mechanisms of tagging proteins with the different UBLs are similar but rely on specific enzymes and only the process of ubiquitination will be discussed here (van der Veen and Ploegh, 2012).

2.1.1 The ubiquitination process

The ubiquitination process involves three steps. In the initial step ubiquitin is activated in an ATP-dependent manner by the E1. The second step involves the formation of an energy-rich thioester bond between ubiquitin and the E2 and in the final step, which is facilitated by an E3, an isopeptide bond between the C-terminus of ubiquitin and an aminogroup in the target protein is established and ubiquitin thus becomes covalently linked to the substrate.

2.1.1.1 Ubiquitin-activating enzymes (E1s)

In humans there are two ubiquitin-specific E1s, UBA1 and UBA6 (Chiu et al., 2007; Jin et al., 2007; Pelzer et al., 2007). The two proteins are distantly related and share about 40 % sequence identity. Whereas UBA1 is involved in charging a variety of E2s with ubiquitin, UBA6 is more restricted in its activity and charges the UBA6-specific E2 USE1. In addition the two enzymes seem to differ in their catalytic efficiencies *in vivo*. Almost all UBA1 molecules as well as its substrates are in their activated or charged forms at steady state whereas only 50 % of the UBA6 and USE1 molecules are activated or charged under the same conditions (Jin et al., 2007).

Structurally, E1s are characterised by three domains, the adenylation-domain, the catalytic cysteine containing domain and the C-terminal ubiquitin fold domain (UFD) (Schulman and Harper, 2009). The adenylation-domain is responsible for the initial binding of the substrates ubiquitin and ATP•Mg^{2+} and for the acyl-adenylation of ubiquitin. It is a pseudo-symmetric domain that resembles the prokaryotic proteins molybdopterin biosynthetic enzyme B (MoeB) and thiamine biosynthesis protein F (ThiF) (Lake et al., 2001; Lee and Schindelin, 2008). These bacterial proteins are involved in the C-terminal acyl-adenylation of molybdopterin converting factor subunit 1 (MoaD) and thiamine biosynthesis protein S (ThiS), thus enabling them to form a C-terminal thiocarboxylate which facilitates the insertion of sulphur into the organic cofactors thiamine and molybdopterin respectively (Hochstrasser, 2000; Rajagopalan, 1997; Taylor et al., 1998). Human E1s resemble MoeB and ThiF not only structurally but also in the mechanism catalysing the adenylation of their substrates (Pickart and Eddins, 2004). Generally the reaction mediated by the E1 can be divided into two steps, the initial formation of a ubiquitin-adenylate intermediate and the following generation of an E1-Ub thioester. The activation of ubiquitin is initiated by the E1 binding to its substrates. Structural studies on MoeB and ThiF suggest that one of their two symmetrical catalytic centres binds a surface on the substrate that corresponds to the hydrophobic L8-I44-H68-V70 patch in ubiquitin whereas the other contributes to the stabilisation of this interaction. The monomer that is not involved in nucleotide binding contains a conserved arginine that is involved in binding ATP and an aspartate residue coordinating Mg^{2+} (Lee and Schindelin, 2008; Pickart and Eddins, 2004; Schulman and Harper, 2009) (Figure 2, upper panel).

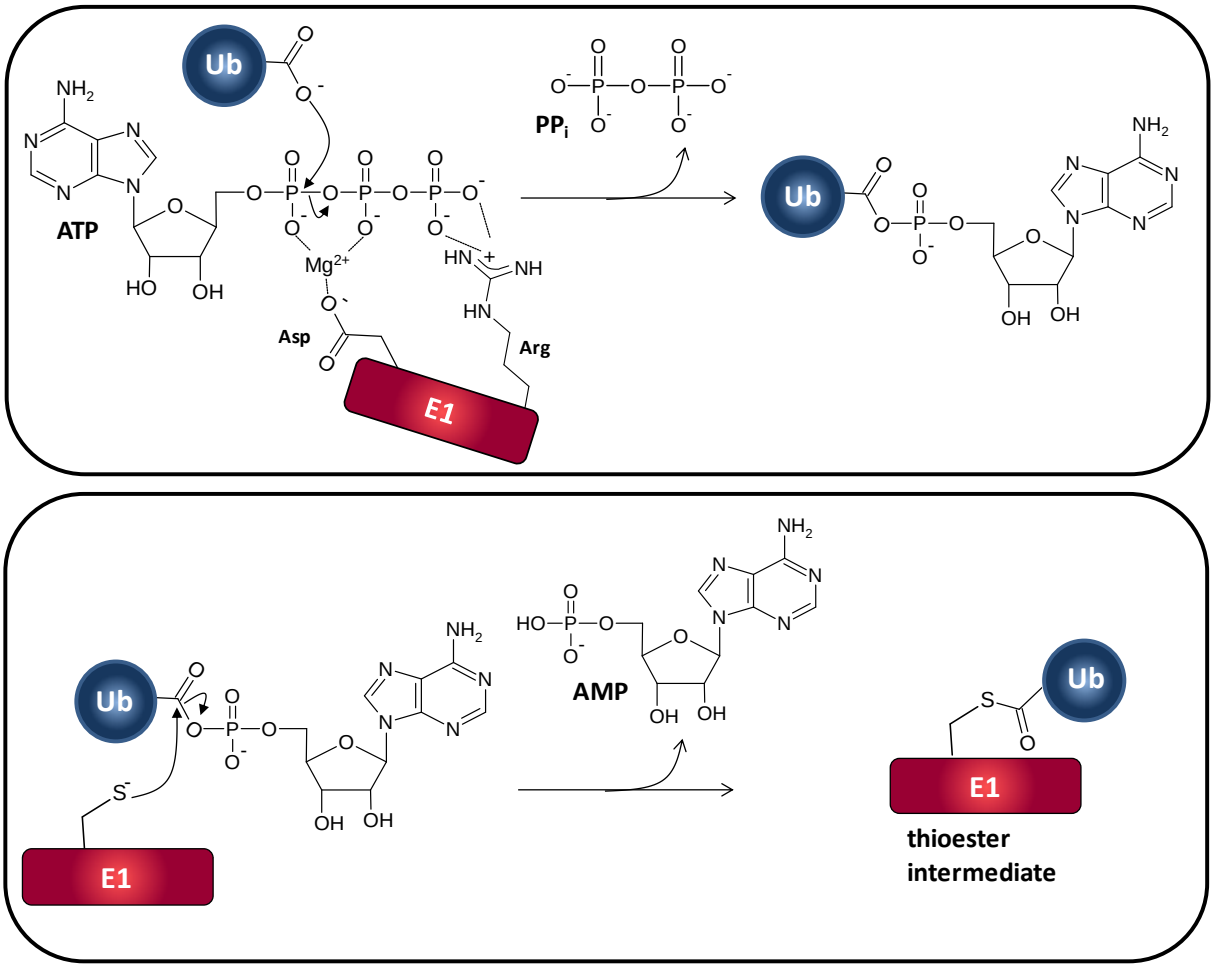


Figure 2: Activation of ubiquitin by the E1. In the first step the E1 binds ATP•Mg^{2+} and catalyses the C-terminal acyl-adenylation of ubiquitin (upper panel). In the second part of the reaction (lower panel), the catalytic cysteine of the E1 attacks the ubiquitin-adenylate and a thioester linkage is formed between the C-terminus of ubiquitin and the catalytic cysteine. The individual steps are reversible in principle and the reaction is driven by the release of inorganic phosphate (PP_i) and AMP.

The key catalytic residues including the Mg^{2+}-coordinating aspartate and basic residues that provide electrostatic stabilization to the departing pyrophosphate product are conserved in E1 enzymes from *E. coli* to humans. The conserved arginine that contributes to ATP-binding is located in a distant region of the linear sequence of UBA1 (Pickart and Eddins, 2004). By making positive contacts with residue 72 of ubiquitin UBA1 can distinguish its substrate from the UBL-protein NEDD8 which at this position contains an alanine instead of the arginine present in ubiquitin (Lee and Schindelin, 2008; Walden et al., 2003a; Walden et al., 2003b). This ensures that only the correct substrate can enter the ubiquitination cascade. Once all substrates are bound the E1 facilitates the attack of the C-terminal carboxylate oxygen of ubiquitin at the α-phosphate of ATP. This results in the C-terminal adenylation of ubiquitin which stays non-covalently associated with the E1 (E1~Ub(A)) and the release of

inorganic phosphate (PP_i) (Haas and Rose, 1982; Haas et al., 1982; Haas et al., 1983). The ubiquitin-adenylate is attacked by the catalytic cysteine of UBA1 which, under the elimination of AMP, results in an E1-Ub(T) complex that is covalently linked by a thioester bond between the C-terminus of ubiquitin and the catalytic centre of the E1 (Figure 2, lower panel) (Ciechanover et al., 1982; Ciechanover et al., 1981; Haas et al., 1982). Although a general base poised to deprotonate the catalytic cysteine has not been identified, a network of polar and charged side chains surrounding the thioester bound was found to be critically involved in the catalysis (Huang et al., 2007; Schulman and Harper, 2009). Both steps of this process, i.e. the adenylation of ubiquitin and the formation of the thioester bond, are in principle reversible but the release of PP_i and AMP drives the progression through the reaction (Haas and Rose, 1982; Haas et al., 1982).

Once the thioester intermediate has been established UBA1 catalyses the adenylation of a second ubiquitin which remains non-covalently associated with the adenylation domain. UBA1 thereby becomes asymmetrically associated with two ubiquitin molecules (E-Ub(T)~Ub(A)) (Figure 3; (Haas and Rose, 1982; Schulman and Harper, 2009).

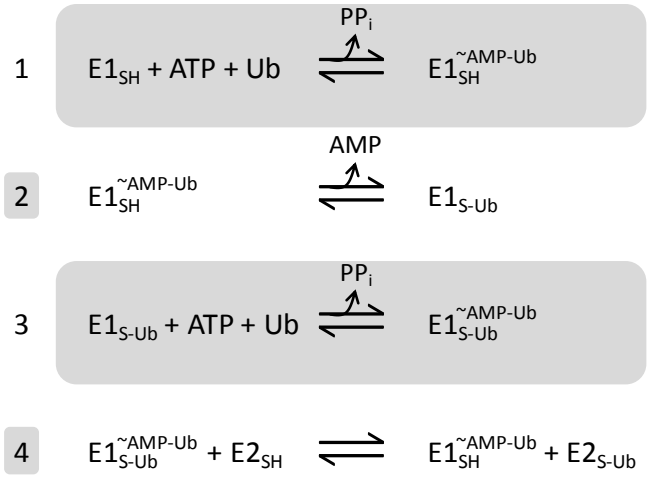


Figure 3: Schematic representation of the different steps of the reaction catalysed by the E1. Step 1 shows the activation of ubiquitin by the formation of ubiquitin-adenylate, step 2 represents the thioester formation, step 3 depicts the additional association with a second adenylated ubiquitin and step 4 shows the transfer of ubiquitin to the E2. Modified from (Haas and Rose, 1982).

In order for the ubiquitination reaction to proceed UBA1 needs to associate with an E2 and transfer the activated ubiquitin to its catalytic centre. Although UBA1 can work with a variety of different E2s it needs to be ensured that E2s are not charged with the wrong kind of UBL. UBA1 therefore needs to select only ubiquitin-specific E2s. This is achieved mainly by interactions between the UFD of the E1 and the N-terminal sequence of the E2's catalytic domain (Huang et al., 2007; Lee and Schindelin, 2008; Wenzel et al., 2011b). Subtle differences between the UFDs of UBA1 and UBA6 allow them to interact with different E2s

(Jin et al., 2007). The interaction between E2s and the E1 in its uncharged state is usually weak as shown by the observation that UBA1 can be separated from the E2 by gelfiltration (Hershko et al., 1983). On the other hand doubly charged UBA1 (E1-Ub(T)~Ub(A)) binds uncharged E2s with nanomolar affinities (Haas et al., 1988). This increased affinity could be due to the availability of additional binding sites. Indeed, the ubiquitin thioester itself may act as an interaction point. Furthermore, a rotation in the E1's UFD could unmask an additional E2 binding surface in the adenylation domain of the E1 and make a negatively charged groove in the UFD accessible. This feature can be recognised by two conserved lysine residues that are present in the α1-helix of all ubiquitin-specific E2s but absent from E2s involved in the conjugation of other UBLs (Huang et al., 2007; Lee and Schindelin, 2008; Ye and Rape, 2009). The conformational changes induced in the E1 by its charging with ubiquitin therefore contribute to both affinity and specificity for the E2. In addition, the rotation of the UFD and possibly the catalytic domain could also bring the ubiquitin thioester into close proximity to the catalytic centre of the E2 and may thus be a prerequisite for the ubiquitin transfer to occur (Huang et al., 2007; Schulman and Harper, 2009). In this thioester transfer reaction the C-terminus of the covalently bound ubiquitin is transferred from the E1 to the catalytic cysteine of the E2. By elimination of the E1-ubiquitin-bond one of the binding sites for the E2 is lost and the thioester-linked E2-ubiquitin complex is released from the E1. This enables the charged E2 to interact with an E3 which, due to a structural overlap in the E1- and E3-binding sites on the E2, is not possible in the presence of an E1 (Eletr et al., 2005). In addition, the dissociation of the E1-E2 complex enables the E1 to participate in additional rounds of ubiquitin-activation and transfer.

2.1.1.2 Ubiquitin-conjugating enzymes (E2s)

Ubiquitin conjugating enzymes are characterised by a conserved catalytic domain of approximately 150 aminoacids that assumes a compact structure in the shape of an elongated ellipsoid and that is referred to as Ubc domain. This domain contains 4 α-helices, a short 3_{10} helix, i.e. a right handed helix in which the stabilising hydrogen-bonds are formed between the CO-group of one aminoacid and the NH-group of the aminoacid three residues later, and a 4-stranded antiparallel β-sheet (Lin et al., 2002; Wenzel et al., 2011b; Ye and Rape, 2009). Apart from the catalytic cysteine, the enzymatic reaction also involves other essential residues as for example an HPN-motif approximately 10 aminoacids N-terminal of the catalytic cysteine in which histidine serves a structural role and asparagine is involved in mediating the formation of an isopeptide bond between ubiquitin and the substrate. These conserved aminoacids are located in a groove on the bottom of the domain which is formed by the loop connecting helix 2 and helix 3 and the loop proximal to the catalytic cysteine (Wenzel et al., 2011b; Wu et al., 2003; Ye and Rape, 2009). In addition to the signature Ubc domain, E2s can also contain additional domains that vary in size and structure and that can contribute to E3- or substrate binding or regulate the intrinsic activity of the E2. According to the presence or absence of these additional features E2s can be allocated to 4 subclasses: class I contains only the Ubc-domain, class II has a C-terminal extension, class III E2s are characterised by additional domains in their N-terminus and class IV comprises of E2s that have additional domains in both their N- and their C-terminus (van Wijk and Timmers, 2010). However, this classification does not permit predictions concerning the functionality of the E2. Alternatively, E2s can be classed into those initiating chain formation, those that interact with ubiquitin moieties already attached to a substrate and that exclusively promote chain elongation and those that can fulfil both functions. In the group of chain-initiating E2s it can further be distinguished between those E2s that target lysine residues in an unspecific manner and therefore serve as general activators of the ubiquitination process and those that recognise a sequence or motif in the proximity of the aminogroup to be targeted and that therefore act in a more substrate-specific manner (Ye and Rape, 2009). Finally, E2s could be divided into groups based on their ability to catalyse the formation of isopeptide bonds. Most E2s can transfer the activated ubiquitin to either cysteine residues on an E3 thereby forming another thioester intermediate or to lysine residues of target proteins or ubiquitin through formation of an isopeptide-bond. On the other hand it was

recently found that UBE2L3 lacks lysine reactivity and the formation of an E3-ubiquitin thioester is therefore obligatory in reactions catalysed by this E2. It is possible that other E2s have similar requirements but, due to the concept being quite recent, this has not been fully established (Wenzel et al., 2011a). Because the E2-thioester bond is relatively stable and in order to ensure substrate specificity, E2s need to cooperate with E3s in transferring ubiquitin (Song et al., 2009). Although N- or C-terminal domains can contribute to some extent, the E3-binding is mainly mediated by the Ubc-domain of an E2. Specifically, polar and charged residues in helix 1, hydrophobic residues in loop 7 and, depending on the E3, a conserved hydrophobic residue in loop 4 mediate this interaction. Variations in these interacting parts generate specificity of the E2-E3 combinations (Ye and Rape, 2009) meaning that not every E2 can interact with every E3 and vice versa. However, as expected from the respective numbers (there are about 38 E2s and 600-1000 E3s encoded in the human genome) E2-E3 pairs are usually not exclusive. Most E2s can bind different E3s while many E3s can also accommodate different E2s. As described previously, the E2-E3 interaction generally needs to be dissolved for the E2 to be recharged by the E1. E2-E3 complexes are therefore of a transient nature, with the binding partners displaying only low affinities in the micromolar range for each other (Wenzel et al., 2011b; Yin et al., 2009b). Nevertheless, these associations are sufficient for the catalysis to occur. The structural and catalytic requirements for a transfer of ubiquitin between thiols or from a thiol to an amine should include an oxyanion hole that can stabilise the charged intermediate formed during the attack of the thiol- or aminogroup. In addition a general base might be required to deprotonate the attacking group which may be especially relevant for the ε-aminogroup of lysine which has a high pK_a. However, most of these features have not been identified in E2s (Pickart and Eddins, 2004). It is therefore possible that E1 or E3 contribute certain catalytic elements in the respective reactions. Although binding of an E3 substantially increases the rate of ubiquitin discharge from the E2's catalytic centre, the fact that the binding of the E3 occurs at a surface of the E2 that is distant from its active site makes this direct cooperation seem less likely (Das et al., 2009b; Deshaies and Joazeiro, 2009; Ozkan et al., 2005; Zheng et al., 2000). On the other hand binding of the E3 or a substrate might induce conformational changes in the E2 that might cause potential cryptic groups in the active centre to adopt a catalytic conformation. A strictly conserved asparagine (Asn) residue for example could then form part of the oxyanion hole (Ozkan et al., 2005; Wu et al., 2003). A schematic

representation of ubiquitin being transferred from the E2 to a target or the catalytic cysteine of an E3 is shown in Figure 4.

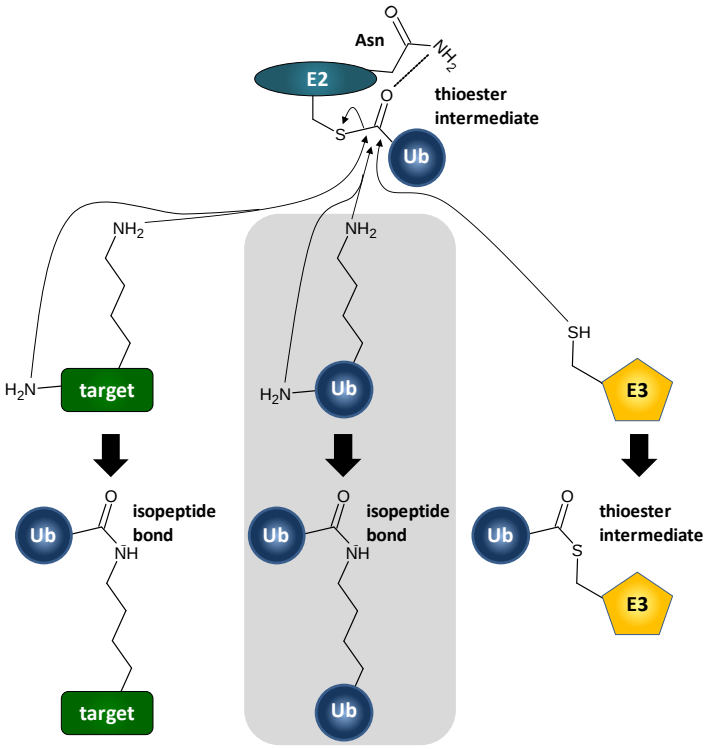


Figure 4: Schematic representation of the ubiquitin-transfer from an E2 to a target or an E3. An E2 that was charged by the corresponding E1 carries activated ubiquitin in a thioester bond. This can be attacked by the catalytic cysteine of an E3 (right part) or by an aminogroup of a target protein or a ubiquitin-molecule already associated with a substrate. Usually the aminogroup involved in the formation stems from a lysine residue (shown here) but the α-aminogroup can also be involved (resulting bond not shown). The catalytic residues present in the E2 have not been entirely clarified but a conserved Asn residue is thought to be involved in stabilising the intermediate.

In this reaction E2s are not only carriers that transfer ubiquitin from the E1 to the E3 or the substrate but they also have an important regulatory role. Due to the internal lysine residues present in ubiquitin the formation of inter-ubiquitin linkages and thus ubiquitin-chains is possible. The function of these chains depends both on length and linkage type (Hochstrasser, 2006) and E2s are involved in determining both these features. The length of a ubiquitin chain depends on the availability of chain-elongating E2s and on the processivity of the ubiquitination reaction. The term processivity is defined as the number of ubiquitin molecules transferred to a substrate during a single round of its association with the E3 (Hochstrasser, 2006). E2s can influence this factor because it depends on efficient chain initiation, which is usually the rate limiting step in a ubiquitination reaction (Deshaies and Joazeiro, 2009). This first step can be supported by substrate binding sites within the E2. The reaction can be further optimised by atypical E2-E3 interactions that enable recharging of the E2 without the need to fully dissociate from the E3. Other factors that can enhance the processivity and thereby favour the generation of longer chains include the oligomerisation

of E2s or even the pre-assembly of ubiquitin chains on the active site of the E2 which can then be transferred *en bloc* to the substrate (Brzovic et al., 2006; Li et al., 2007; Li et al., 2009b; Ravid and Hochstrasser, 2007). In addition, non-covalent interactions between an E2 and ubiquitin may force the acceptor ubiquitin to adopt a certain orientation making only a particular aminogroup accessible to the active centre thereby determining linkage specificity. This influence on chain topology exerted by the E2 is especially relevant in reactions that proceed without the formation of a covalent E3-ubiquitin intermediate (Eddins et al., 2006; Nagy and Dikic, 2010; VanDemark et al., 2001).

2.1.1.3 Ubiquitin ligases (E3s)

Although E2s can have substrate-binding features, modification of targets with ubiquitin usually depends on a third class of proteins, the ubiquitin ligases (E3). All E3s share E2-binding properties and the ability to recognise specific substrates. However, in the case of E3-complexes, these two functions may be performed by different subunits of the protein assembly (Pickart and Eddins, 2004). Due to the necessity to ensure specific recognition of a plethora of different substrates, a large number of E3s exists which can be subdivided into two major classes, the RING/U-box- and the HECT-ligases. The former group comprises approximately 95 % of all predicted E3s (Li et al., 2008). Although both subgroups facilitate the transfer of ubiquitin from the charged E2 to the target protein, members of the two families differ both structurally and in the mechanism they employ. While RING-E3s act as a scaffold bridging the E2 and the substrate, HECT-ligases contain a catalytic cysteine and form a thioester intermediate with ubiquitin before transferring it onto the target (Figure 1). Thus the active centre mediating the formation of the final (iso)peptide-bond differs depending on the E3 involved with aminolysis being catalysed by HECT-ligases themselves whereas the active centre of the E2 is responsible in RING-dependent reactions (Deshaies and Joazeiro, 2009; Rotin and Kumar, 2009).

2.1.1.3.1 HECT-ligases

The founding member of this family of proteins is the human papilloma virus (HPV) E6 associated protein (E6AP) (Huibregtse et al., 1995). Proteins are classified as members of this family based on the presence of a bilobal HECT-domain, which is formed by ~350 aminoacids and which is homologous to E6AP's C-terminus. The N-terminal lobe of this domain (N-lobe) mediates E2 binding whereas the C-terminal lobe (C-lobe) contains the catalytic cysteine (Huibregtse et al., 1995; Pickart, 2001). Apart from this signature domain, which is generally located in the C-terminal part of the E3, most members of the HECT-family contain additional structures in their N-terminus that can for example be involved in substrate binding (Rotin and Kumar, 2009). According to the specific N-terminal domains HECT-ligases can be subcategorised into three groups. Nedd4 ligases are characterised by a C2 domain, which binds to phospholipids and targets the protein to membranes, and by two to four WW-domains that typically bind PY-motifs in substrates thereby mediating protein-protein interactions (Kanelis et al., 2001; Staub et al., 1996). HECT-E3s containing regulator of chromosome condensation 1 (RBCC1)-like domains (RLDs) are referred to as HERC-ligases. The RLD assumes a 7-bladed β-propeller fold and apart from mediating interaction with chromatin might also serve as a guanine exchange factor (GEF) for the small GTPase Ran (Garcia-Gonzalo and Rosa, 2005; Renault et al., 2001; Renault et al., 1998). E3s of this class can further be subdivided into large HERCs which contain multiple RLDs and small HERCs which contain only a single copy of this domain. Finally, HECT-ligases containing other N-terminal features such as ankyrin repeats, zinc fingers, UBA-, PHD- or RING-domains are assigned to the group of "other HECTs" (Rotin and Kumar, 2009).

Like E2s, HECT-E3s accept ubiquitin on a catalytic cysteine by forming a thioester intermediate which is then attacked by the aminogroup of a target residue resulting. This results in the formation of an isopeptide-bond and thus in the ubiquitination of the target (Pickart and Eddins, 2004; Scheffner et al., 1995). As in the case of E2s (see section 2.1.1.2), the active site of HECT-ligases lacks well-positioned candidates for the anticipated general base and the oxyanion hole (Pickart and Eddins, 2004). It is therefore likely that conformational changes induced by substrate- or ubiquitin-binding lead to a rearrangement in the catalytic centre. The need for a change in conformation is further emphasised by the observation that, if the native conformation was maintained, the catalytic centre of the E2 would be separated from the E3's catalytic cysteine by a distance too great to enable

ubiquitin transfer (Verdecia et al., 2003). Due to their active role in transferring ubiquitin specificity for a certain chain topology can be an intrinsic, E2-independent property of HECT-E3s (Kim and Huibregtse, 2009).

2.1.1.3.2 RING- and U-box-E3 ligases

Members of the RING-family are characterised by a RING domain which, unlike the HECT-domain can be located anywhere within the protein. This characteristic domain is a cysteine- and histidine-rich domain that was first identified in really interesting new gene 1 (RING1) (Freemont et al., 1991). It consists of a central α-helix and several small β-strands that are separated by variable loops (Pickart and Eddins, 2004). On one surface a shallow hydrophobic groove is formed by the central helix and the loops. The overall primary sequence of this domain is not well conserved. However, characteristic cysteine and histidine residues, which are buried in the domain's core where they coordinate two zinc ions, and their spacing are largely invariant. Although swapping of cysteine and histidine residues or replacement by other zinc-coordinating residues is possible in certain cases, the canonical RING-sequence can be summarised as C-X_2-C-X_{9-39}-C-X_{1-3}-H-X_{2-3}-C-X_2-C-X_{4-48}-C-X_2-C (Deshaies and Joazeiro, 2009). The zinc-coordinating residues are interleaved, yielding a rigid globular structure (see Figure 5A) (Barlow et al., 1994; Borden et al., 1995). A similar overall structure is assumed by the UFD2-homology (U-box) domain. UFD2 is a yeast protein that is involved in the elongation of ubiquitin chains and is hence categorised as an E4 (Koegl et al., 1999). Other proteins containing this domain can however also act as E3s in a manner very similar to that of RING-containing E3s (Jiang et al., 2001). The U-box domain does not coordinate zinc ions but like the RING-domain it has a hydrophobic core and two interaction centres. Charged or polar residues, that correspond to the zinc-coordinating residues of the RING in their function and position, form salt bridges and hydrogen-bonds whereby the domain adopts an overall RING-like fold (Ohi et al., 2003). Functionally, the RING-domain was first thought to be involved in DNA-binding but many RING-containing proteins were soon found to act as ubiquitin ligases (Freemont et al., 1991; Joazeiro et al., 1999; Lorick et al., 1999; Xie and Varshavsky, 1999). Indeed most RING-proteins seem to posses the ability to act as an E3. However, there are a few exceptions. In these cases heterodimerisation with an active RING-E3, mediated by the RING-domain itself or other domains present in the respective proteins can greatly increase the activity of the latter

protein (Hashizume et al., 2001; Linares et al., 2003; Wang et al., 2004). Furthermore, dimerisation of the E3 can increase the processivity of ubiquitination by favouring the recruitment of multiple E2s or by inducing conformational changes required for activity (Feltham et al., 2011; Feltham et al., 2010; Tang et al., 2007). Unlike HECT-ligases, RING-E3s do not form a covalent intermediate with ubiquitin (Joazeiro and Weissman, 2000). Instead they mediate ubiquitination of a substrate by inducing sufficient proximity between the E2 which binds to the hydrophobic groove on the surface of the RING-domain (Zheng et al., 2000) and the target protein. It is thought that the rigidity of the RING-domain is well suited for fixing the E2 and the substrate in a favourable position. In addition, binding of the E3 induces conformational changes in the E2 (see section 2.1.1.2) and thus RING-E3s indirectly favour ubiquitination (Pickart and Eddins, 2004; Zheng et al., 2000). The lysine targeted by this reaction is likely to be chosen based on its ability to access the thioester-bond in the catalytic centre of the E2 rather than by the surrounding primary sequence. However a certain influence could be exerted by the proximal residues as nearby basic residues could lower the pK_a of the aminogroup and thus enhance its reactivity (Deshaies and Joazeiro, 2009). An influence of the E3 on the selection of the target residue seems likely and could be mediated by binding the substrate in a way that makes only a certain residue accessible for ubiquitination. However, conserved residues on the E3 that would be suitable to guide substrates in this way have not been identified (Pickart and Eddins, 2004). On the other hand, domains outside the RING-domain might also contribute to binding and orienting the target. Indeed about three quarters of all RING-ligases are predicted to have additional domains such as SH2-, SH3-, PDZ- or UBL-domains (Li et al., 2008). Based on these domains and on sequence homology subclasses of RING-proteins can be established, the largest classes being the TRIM/RBCC family which is characterised by a B-box and a coiled-coil domain (Meroni and Diez-Roux, 2005; Sardiello et al., 2008) and the RBR/TRIAD group of E3s which comprises those E3s that contain two RING-domains separated by an in-between-RING (IBR) domain (Eisenhaber et al., 2007; Marin and Ferrus, 2002).

The founding member of the latter subfamily was Parkin, a protein that, when mutated and inactivated, predisposed to several forms of Parkinson's disease (Foroud et al., 2003; Kitada et al., 1998; Oliveira et al., 2003a; Oliveira et al., 2003b; Pankratz et al., 2003). Structurally it contains an N-terminal UBL-domain followed by two typical RING-domains (C_3HC_4) and an intermediate IBR which is characterised by a C_6HC pattern (Kitada et al., 1998; Morett and

Bork, 1999; van der Reijden et al., 1999). The sequence of RING1-IBR-RING2 comprises around 200 aminoacids, and is referred to as RBR-domain (Eisenhaber et al., 2007; Marin and Ferrus, 2002). This structural element, like isolated RING-domains, can be located anywhere in a protein and is the defining feature of the RBR-family (Wenzel and Klevit, 2012). In humans this group comprises only approximately 15 members so far. However, the RBR-proteins constitute an ancient and highly diversified family, members of which can be found in fungi, plants and animals (Marin, 2009; Marin and Ferrus, 2002; Marin et al., 2004). Many of the RBR-proteins were shown to act as E3s. The three parts of the RBR-domain are all cysteine- and histidine-containing zinc-coordinating features but they nevertheless show certain differences. Comparison of the general formulas for RING1 (C-X_2-C-$X_{10–24}$-C-$X_{1–6}$-H-X_2-C-X_2-C-$X_{14–25}$-C-$X_{2–9}$-C) and RING2 (C-X_2-C-$X_{9–11}$-C-X-H-X_2-C-$X_{1–4}$-C-X_4-C-X_2-C) shows that the RING1 domain is longer, usually around 60 aminoacids, and shows greater tolerance towards changes in sequence or size of its loops than the RING2 which comprises only approximately 40 residues. The IBR-RING is intermediate in size (~50 aminoacids) and like the RING1 more variable in the loops connecting the zinc coordinating residues (Eisenhaber et al., 2007; Marin and Ferrus, 2002). On the other hand the identity of these cysteine and histidine residues is more strictly conserved in the N-terminal domains. Indeed, structural studies revealed that the RING2-domain of ARIH1 (also known as human homolog of *Drosophila* ariadne-1 (HHARI)), assumes an atypical conformation coordinating only a single zinc ion (Figure 5B; (Capili et al., 2004).

A recent study found that the RING2 domain of ARIH1 is required for E3 activity and involved in formation of a ubiquitin thioester (Wenzel et al., 2011a). Accordingly, a HECT-like mechanism was suggested for all RBR-E3s (Figure 5C and (Wenzel and Klevit, 2012)). Whereas the functions of RING1- and RING2-domains consist of E2-binding and carrying out the enzymatic reaction, respectively, the relevance of the IBR-domain is less well understood. It is possible that it has a structural role in bringing RING1 and RING2 into close proximity thus allowing the catalytic cysteine to attack the thioester bond of the RING1-bound E2-ubiquitin complex (Wenzel and Klevit, 2012). However additional studies will have to clarify whether this is the only function of this domain.

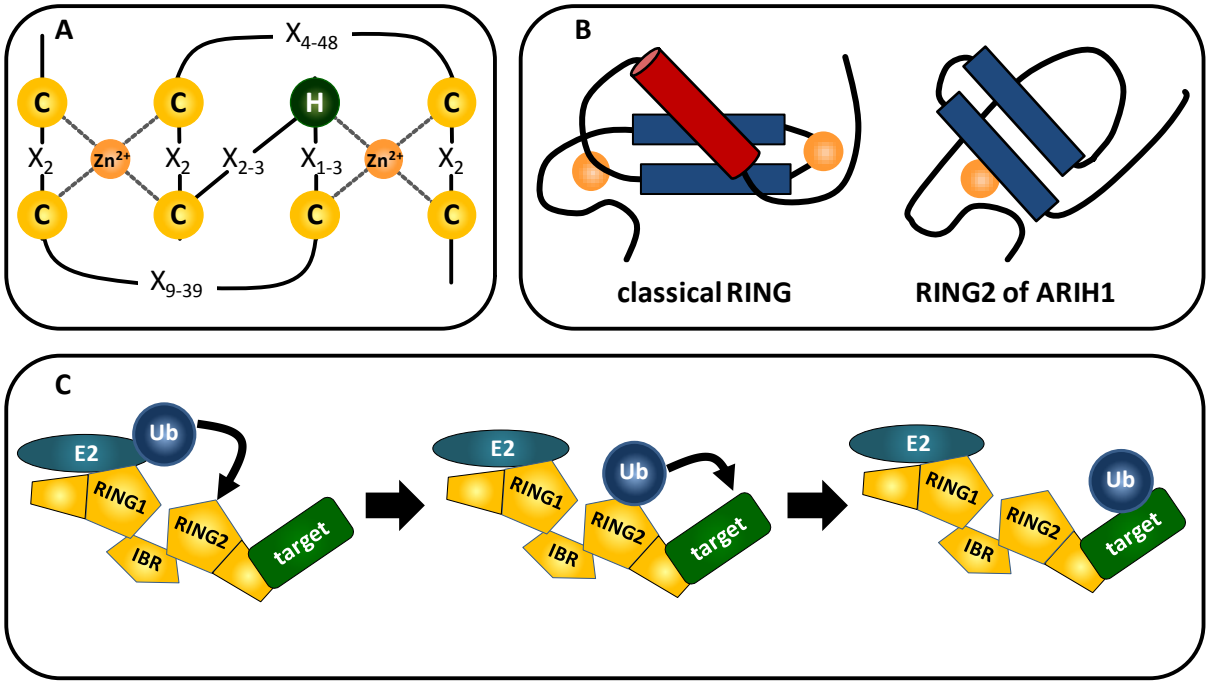


Figure 5: Schematic representation of classical and atypical RING-domains and of the HECT-like mechanism adopted by RBR-E3s. A: Representation of a classical RING-domain. A sequence of 7 cysteine (C) and one histidine (H) residues forms an interleaved structure coordinating two zinc residues. X indicates interspacing aminoacids. The backbone is shown as a black line; non-covalent interactions are depicted as dashed lines. **B:** Schematic structure of a classical RING coordinating two zinc ions as compared to the RING2 domain of ARIH1 which does not show a canonical RING fold and only binds one zinc ion. Modified from (Capili et al., 2004). **C:** RBR E3s act via a HECT-like mechanism. Ubiquitin is transferred from the E2-ubiqutin complex that is bound by the RING1-domain to a catalytic cysteine present in the RING2 of the E3 before being conjugated to the target protein.

Overall, RBRs can be categorised as RING-HECT-hybrids, and due to their direct involvement in ubiquitin transfer they are likely to determine the topology of the generated ubiquitin chain independently of the E2 involved and are thus critically involved in determining the fate of their target proteins.

2.1.2 Different forms of ubiquitination

Like phosphorylation, ubiquitination leads to a covalent posttranslational modification of a target protein. The attachment of a ubiquitin moiety can occur at one (monoubiquitination) or several sites (multi-monoubiquitination). In contrast to phosphorylation, ubiquitination has a further layer of complexity as chains of ubiquitin can be generated. This results from the possibility to attach the C-terminus of a ubiquitin molecule to the N-terminus or one of the 7 internal lysine residue of the preceding moiety and allows the formation of eight different types of polyubiquitin (Behrends and Harper, 2011). Chains in which identical lysine residues mediate linkage of sequential ubiquitin molecules, e.g. the C-terminus of the

distal ubiquitin forms an isopeptide-bond with lysine 48 of the proximal ubiquitin throughout the chain, are referred to as homotypic chains (Figure 6).

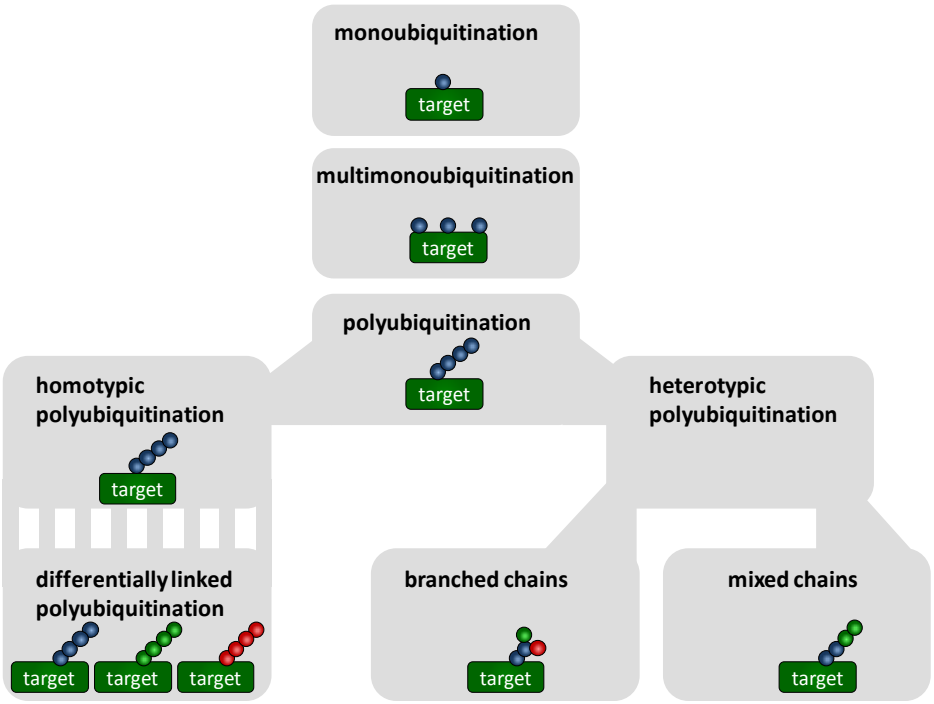


Figure 6: Types of ubiquitination. A target protein can be modified on one or several sites by single ubiquitin molecules or by ubiquitin chains. Polyubiquitin can be linked via one of the seven lysine residues of ubiquitin or its N-terminus resulting in differentially linked chains. Different linkages can also be combined in one chain.

K48-linked chains, that were the first to be functionally characterised (Chau et al., 1989) are referred to as canonical whereas all other linkage types are usually referred to as non-canonical or atypical chains. This nomenclature can vary slightly as K63-linked ubiquitin, the second linkage to be associated with a functional outcome (Deng et al., 2000; Spence et al., 1995), is sometimes counted among the canonical forms of ubiquitination. In addition to the homotypic chains the formation of heterotypic ubiquitin conjugates is possible. In the latter not only one but different lysine residues are used to conjugate ubiquitin molecules thus creating a chain that contains alternating linkage types. The group of heterotypic polyubiquitin chains also comprises branched or forked linkages in which a single ubiquitin is extended at two or more lysine residues (Ben-Saadon et al., 2006; Kim et al., 2007; Peng et al., 2003).

Modification of a target by covalent conjugation of ubiquitin molecules can change its fate by exerting an allosteric effect on its activity as for example in the activation or inactivation of an enzyme by ubiquitination-induced conformational changes. Alternatively, ubiquitination can enable interactions with proteins that specifically bind ubiquitin via ubiquitin-binding domains (UBDs) (Dikic et al., 2009; Komander, 2009; Scaglione et al., 2007; Todi et al., 2009). There are at least 20 different types of UBDs, including for example

ubiquitin binding zinc fingers (ZnF) (UBZ), nuclear protein localisation 4 ZnFs (NZFs), ubiquitin conjugating (Ubc) domains, typically found in E2s, ubiquitin interacting motifs (UIMs) and ubiquitin-associated (UBA) domains. The individual domains employ different structural elements and can recognise different surfaces on the ubiquitin molecule. Their interaction with conformations generated by a specific linkage between two ubiquitin molecules allows them to specifically bind one but not another type of ubiquitin chains (Dikic et al., 2009; Hurley et al., 2006). Despite the structural similarities in a UBD-family, specific recognition of a particular linkage type can usually not be attributed to UBDs of only a single family and in addition different members of the same family can show different linkage specificities (Dikic et al., 2009; Raasi et al., 2005). Although UBDs usually show very low affinities for isolated ubiquitin molecules they can efficiently mediate the interaction between ubiquitin and the UBD-containing protein, also referred to as a ubiquitin receptor (Hurley et al., 2006). Efficient binding in spite of low affinity is usually due to increased avidity which can be accomplished by the presence of multiple UBDs in a protein, by oligomerisation of ubiquitin receptors or by accumulation of ubiquitin-receptors and -chains in cellular compartments or in multi-protein complexes. Binding of ubiquitin receptors to a ubiquitinated protein can alter the activity, localisation or stability of the modified target protein. Due to the ability of ubiquitin-interacting proteins to differentiate between the different forms of ubiquitination (Dikic et al., 2009; Raasi et al., 2005) the fate of a target depends on the type of modification it is subject to. This applies not only to the difference between mono- and polyubiquitination but also to differentially linked ubiquitin chains. An additional aspect that contributes to the specific outcomes of ubiquitination with the individual forms of ubiquitination relates to different linkages being more or less refractory towards the disassembly by particular DUBs. These proteases usually also contain UBDs allowing them to interact with ubiquitin chains of specific linkage types thus enabling them to efficiently and specifically cleave the (iso)peptide bonds linking the individual ubiquitin molecules (Bremm et al., 2010; Faesen et al., 2011; Komander et al., 2009a; Virdee et al., 2010).

2.1.2.1 Monoubiquitination

When compared to other posttranslational modifications such as phosphorylations, ubiquitination involves the attachment of a relatively large molecule to the target. Although the bigger surface area of ubiquitin provides a variety of potential interaction sites (Searle et al., 2012; Winget and Mayor, 2010) most UBDs bind to a hydrophobic area surrounding Ile44 (Ile44 patch) of ubiquitin, rendering the recognition of monoubiquitin by different UBDs mutually exclusive. This exclusivity can prevent a single ubiquitin molecule from triggering different and possibly opposing signals at the same time. However, it is possible that wherever cooperation of two ubiquitin-binding proteins is needed, their UBDs may recognise different features in the ubiquitin molecule allowing the receptors to be recruited at the same time (Dikic et al., 2009). Therefore, attachment of a single ubiquitin molecule to one or several sites in a target protein can not only be the initiating step of a polyubiquitination (Windheim et al., 2008) but may in itself already alter the fate of the substrate. In the DNA-damage response for example, monoubiquitination of histones or of the DNA sliding clamp proliferating-cell nuclear antigen (PCNA) is of functional relevance. In yeast the ubiquitination status of PCNA is decisive for the method by which replication passes damaged DNA. Monoubiquitination of PCNA triggers translesion synthesis, which is more error prone than the template switching pathway initiated by K63-ubiquitinated PCNA (Hoege et al., 2002; Ulrich and Jentsch, 2000). Multiple-monoubiquitination is also implicated in internalisation and subsequent lysosomal degradation of cell surface receptors (Haglund et al., 2003). Here some of the subunits of the multimeric endosomal-sorting complex required for transport (ESCRT) machinery contain UBDs which are responsible for ubiquitinated cargoes to be recruited and to be transported to multivesicular bodies (MVBs) where they are degraded in a lysosomal manner (Hirano et al., 2006; Raiborg and Stenmark, 2009; Williams and Urbe, 2007). A role for multi-monoubiquitination in the context of protein degradation is indicated by the finding that processing of the nuclear factor kappa B (NF-κB) precursor p105 to the NF-κB subunit p50 can be initiated by the conjugation of single ubiquitin moieties rather than of a polyubiquitin chain (Kravtsova-Ivantsiv et al., 2009). In addition, following genotoxic stress monoubiquitination of phosphorylated and SUMOylated NF-κB essential modulator (NEMO, also known as IKKγ), the regulatory subunit of the inhibitor of kappa B kinase (IKK) complex, in the nucleus leads to its export to the

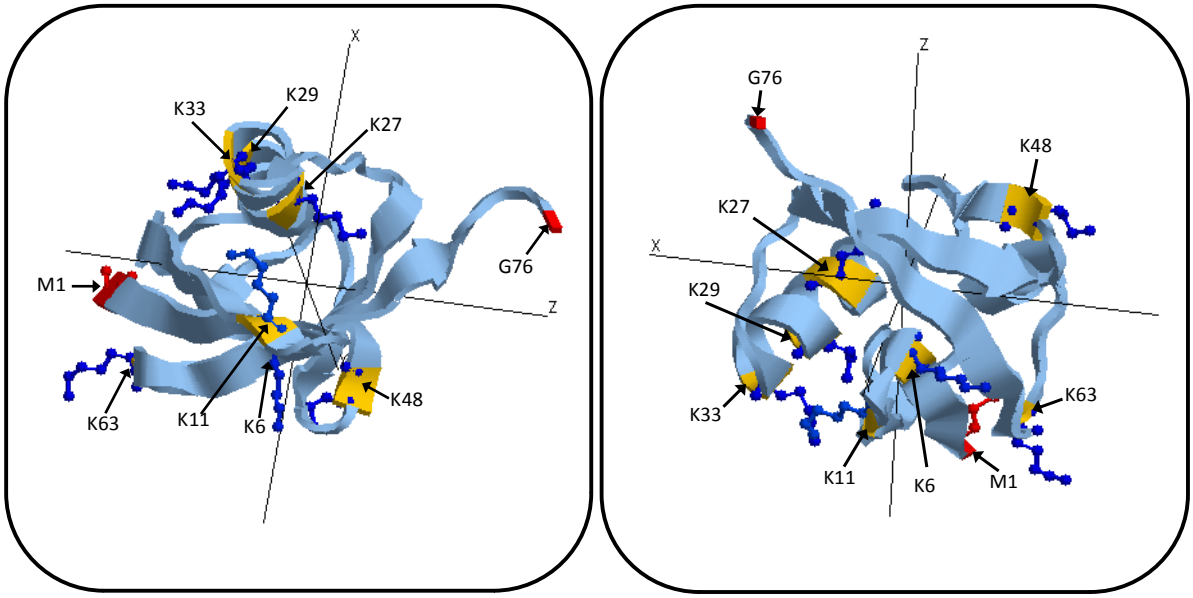
cytoplasm where it can mediate the induction of the NF-κB pathway (Hadian and Krappmann, 2011; Huang et al., 2003).

2.1.2.2 Polyubiquitination

As mentioned previously the generation of ubiquitin chains can occur by the formation of an (iso)peptide-bond between the carboxyl-group of the incoming ubiquitin moiety and an aminogroup of the preceding molecule. The latter group can be provided by the N-terminal methionine (M1) or by one of the internal lysine residues (K6, K11, K27, K29, K33, K48 or K63). Studies in both yeast and cell lines found that all possible linkage types exist *in vivo*. Different studies found K48 to be the most abundant linkage type with 29 % and 52 % of all ubiquitin chains being linked in this manner in yeast and HEK293 cells respectively (Dammer et al., 2011; Xu et al., 2009b). The percentages of the other chain types were estimated as 11 % (K6), 28 % (K11), 9 % (K27), 3 % (K29), 4 % (K33) and 16 % (K63) in yeast (Xu et al., 2009b) and 2 % (K11), 8 % (K29), 38 % (K63) of all ubiquitin chains were found to be linked in an atypical manner in HEK293 cells where the amounts of K6-, K27-, K33- and M1-linked chains accounted for less than 0.5 % (Dammer et al., 2011). Due to technical limitations of the analysis and because certain linkages are formed in a stimulation- or cell cycle dependent manner, the observed percentages may not represent the physiological relevance of the individual chain types. Branched chains including for example K29 and K33 linkages were also identified and it was found that in contrast to all other homomeric chains these forked chains cannot be degraded by the proteasome (Kim et al., 2007). Indeed, inhibition of the proteasome increases the amount of all linkages in HeLa cells (Meierhofer et al., 2008) and of all chain types apart from K63-linked chains in yeast. In addition, all linkages could signal for degradation by purified proteasomes *in vitro* (Xu et al., 2009b). Although this could indicate that all linkages apart from K63 target for proteasomal degradation and are thus functionally redundant, it is likely that recognition of different chains by proteins that regulate the transport to the proteasome and by proteasomal subunits themselves differs both qualitatively and quantitatively. In addition, differential DUB-sensitivity may allow transient non-proteolytic functions to be mediated by some, i.e. the more stable, but not by other, i.e. the more DUB-sensitive, linkage types. Along these lines specific binding of proteasome-independent UBDs may protect selected chain types from transport to the proteasome as well as from DUB-mediated disassembly. Indeed

specific receptors, proteases and functions have been described for the different linkage types (Dikic et al., 2009; Komander et al., 2009a). Specific recognition is enabled by the fact that differentially linked ubiquitin chains differ not only in the linkage between individual ubiquitin molecules but also in their overall conformation (Virdee et al., 2010) thus creating specific binding surfaces that are absent from monoubiquitin or other chain types. These different topologies result from the fact that all potential linkage points in the ubiquitin molecule are located on different surfaces and point into different directions. The exceptions are M1 and K63 which are in close proximity (Figure 7 and (Vijay-Kumar et al., 1987). Accordingly, K63- and linear chains assume a similar extended conformation in which the individual ubiquitin moieties do not make contact. However, the two chain types can be differentiated by the chemical characteristics of the linkage. M1 chains are linked by a peptide- instead of an isopeptide bond and are thus conformationally more restrained (Komander et al., 2009b). On the other hand K6-, K11- and K48-linked chains have a different structure. K6-linked chains assume an asymmetric compact conformation in which the Ile44 patch of the proximal ubiquitin which contains the lysine residue involved in the linkage binds to another hydrophobic surface containing L71, I36 and I8, the so called Ile36 patch, on the distal ubiquitin. The resulting structure is further fixed by additional interactions and the Ile44 patch of the distal ubiquitin remains accessible thus allowing interaction with UBDs (Virdee et al., 2010). Two different conformations exist for K11 chains. One of the structures involves an asymmetric interaction between the areas surrounding Glu24 on the distal and K29 and K33 in the proximal ubiquitin moiety. In the other the ubiquitin molecules interact via their respective Ile36 patches. Both conformations exist in an equilibrium and contribute to a higher order assembly in crystals (Bremm et al., 2010; Matsumoto et al., 2010). Finally, K48 chains assume a conformation in which the two Ile44 patches of subsequent ubiquitin moieties interact with the linking residues being closely packed against the ubiquitin units. In tetramers further contacts between molecules exist leading to an overall pseudo-tetragonal structure. Nevertheless, interactions with K48-linked chains are possible as an equilibrium with more open conformations exists and because UBA-domains can insert between the Ile44-patches of interacting molecules (Cook et al., 1992; Trempe et al., 2005; Varadan et al., 2004; Varadan et al., 2005). The structures of the other linkage types have not been determined to date

but due to the location of the linking residues as well as to distinct cellular functions (see below) it can be assumed that they all adopt specific conformations.



linkage type	M1	K6	K11	K27	K29	K33	K48	K63
structure	linear, extended	asymmetric, compact	compact	?	?	?	kinked, compact	linear, extended
function	signalling	DNA repair	Cell cycle, proteasomal degradation, signalling	signalling, degradation	lysosomal degradation	kinase modification	proteasomal degradation	signalling, DNA repair

Figure 7: Structural features of ubiquitin and of different ubiquitin chains. Upper panels: Two different angles of the ubiquitin structure (PDB: 1UBQ; (Vijay-Kumar et al., 1987) reveal that the different potential linkage points for the formation of ubiquitin chains, i.e. the N-terminal methionine 1 (M1, depicted in red) and the seven internal lysine residues (K6-K63, shown in yellow with dark blue side chains), are located on different surfaces of the molecule and face into different directions. **Lower panel:** Table summarising structural and functional differences between the different linkage types. Modified from (Komander, 2009) under incorporation of information obtained from (Virdee et al., 2010) and (Bremm et al., 2010).

2.1.2.2.1 K6-linked chains

K6-linked chains have been implicated in different cellular functions. In the context of DNA damage K6-chains are made by an E3-complex consisting of breast cancer breast cancer 1, early onset (BRCA1) and BRCA1-associated RING domain protein 1 (BARD1) and might also be involved in recruitment of this complex to DNA lesions via its subunit receptor associated protein (RAP80) (Morris and Solomon, 2004; Nishikawa et al., 2004; Sobhian et al., 2007; Wu-Baer et al., 2003). Independently, K6-linkages could also be attached to NEMO thereby favouring efficient IKK-phosphorylation and activation in the context of tumour necrosis factor (TNF)-signalling (Tang et al., 2003b) or be conjugated to misfolded mutants of DJ-1

and alpha-synuclein (α-syn) by TNF-receptor associated factor (TRAF) 6 thereby causing accumulation of these Parkinson's disease associated proteins in cytoplasmic aggregates (Zucchelli et al., 2010).

2.1.2.2.2 K11-linkages

As mentioned previously K11-linkages were found to account for 28 % of all ubiquitin chains in yeast but only for 2 % in human cells. The low amount of K11-linkages identified in HEK293 cells may be due to the abundance of this linkage type being strongly cell cycle dependent, reaching maximum levels during mitosis (Jin et al., 2008; Matsumoto et al., 2010). This is in accordance with a functional role of this chain type as a signal for proteasomal degradation of cell cycle related proteins such as cyclin B1, cyclin A and Securin. Modification of these substrates is mediated by the anaphase-promoting complex/cyclosome (APC/C) in cooperation with the K11-specific E2 UBE2S and with UBE2C (Jin et al., 2008; Kirkpatrick et al., 2006; Williamson et al., 2009). K11-linked ubiquitination of cyclin B1 was shown to be sufficient for its proteasomal degradation *in vitro* (Jin et al., 2008) and although specific UBDs that may be involved in shuttling K11-ubiquitinated proteins to the proteasome have not been identified, a physiological role for this modification is suggested by the finding that depletion of both UBE2C and UBE2S causes mitotic arrest (Williamson et al., 2009). In addition, K11-linked chains have been suggested to be functionally important in the endoplasmatic reticulum associated degradation (ERAD) pathway (Xu et al., 2009b). Furthermore, a potentially non-proteolytic function was suggested in the context of TNF-signalling. It was described that cellular inhibitor of apoptosis (cIAP) 1 in conjunction with the promiscuous E2 UBE2D can attach K11-linked chains to RIP1 (Dynek et al., 2010) and K11-linkages were found to be associated with RIP1 in the native TNF-receptor signalling complex (RSC) (Gerlach et al., 2011). It has not been clarified whether this leads to RIP1 degradation and thus termination of signal transduction emanating from the receptor complex or whether K11-chains have a proteasome-independent function in this context. The latter notion is supported by the finding that the UBD of NEMO can bind to K11-dimers with similar affinity as to K63-linked dimers (Dynek et al., 2010) indicating that K11-chains could be a potential recruitment platform for the IKK-complex. In addition, Cezanne, a DUB that was described to negatively regulate TNF-induced NF-κB activation was found to preferentially cleave K11-linked chains (Bremm et al., 2010;

Enesa et al., 2008) further implying that K11-chains have a signal-promoting rather than a degradative function in the context of TNF-induced signal transduction.

2.1.2.2.3 K27-linked chains

Due to the close proximity of lysines K27, K29 and K33 their analysis by mass spectrometry is challenging and this has somewhat dampened the investigation of these linkages types (Komander, 2009). Accordingly, little is known concerning the structure and function of K27-linked chains. Like K6-linked chains they were reported to be attached to misfolded DJ-1 and α-synuclein favouring their aggregation (Zucchelli et al., 2010). In addition, it was reported that upon viral infection, the E3 ligase tripartite motif containing 23 (TRIM23) conjugates K27-linked to NEMO thereby promoting NF-κB activation and the production of antiviral IFNβ (Arimoto et al., 2010). In another context, attachment of the same linkage type to the same substrate was reported to have an entirely different outcome. In this case, the bacterium *Shigella* was found to hijack the ubiquitin conjugation system of the host cell leading to K27-ubiquitination of NEMO followed by its degradation. This led to a dampening of the host's proinflammatory response (Ashida et al., 2010). Further investigation is needed to clarify the structure and function of K27-linked chains and especially to established whether this chain type preferentially targets proteins for proteasomal degradation or whether it fulfils other, signalling related tasks.

2.1.2.2.4 Ubiquitin chains conjugated via K29

Together with K6- and K27-chains K29-linked ubiquitin was reported to be conjugated to DJ-1 and α-synuclein by TRAF6 in the context of Parkinson's disease (Zucchelli et al., 2010). Furthermore, another member of the TRAF family, TRAF7 was found to generate this linkage type on NEMO and the NF-κB subunit p65 leading to their lysosomal degradation and to a down-regulation of NF-κB signalling (Zotti et al., 2011). K29-linked chains were also reported to target Deltex (DTX) for lysosomal degradation when attached to this protein by the action of the E3 ligase E3 ubiquitin-protein ligase Itchy homolog (ITCH) (Chastagner et al., 2006). Furthermore, K29-linked chains were also implicated in the ubiquitin fusion degradation pathway where the E3 Ufd5 is responsible for their generation (Johnson et al., 1995). In addition, it was shown that the DUB TRABID preferentially cleaves K29-linked chains (Virdee et al., 2010) providing an indirect hint that ubiquitin of this linkage type might play a role in the Wnt-pathway where TRABID was found to play a role (Tran et al., 2008). Finally, several

members of the AMP-activated protein kinase (AMPK) family are inactivated by K29-linked ubiquitination as this modification seems to prevent the phosphorylation required for kinase activity (Al-Hakim et al., 2008).

2.1.2.2.5 K33-linkages

Although they are not well characterised, K33-linked chains were found to collaborate with K29-linked chains in the regulation of AMP kinases (Al-Hakim et al., 2008). Furthermore, a role in TCR-signalling for this linkage type was suggested. Here, TCRζ was reported to be targeted by Casitas B-lineage lymphoma proto-oncogene b (Cbl-b) and ITCH with the resulting ubiquitination inhibiting phosphorylation of TCRζ and preventing its association with the downstream tyrosine kinase (zeta-chain associated protein of 70kDa) ZAP-70 thereby restricting TCR signalling (Huang et al., 2010).

2.1.2.2.6 K48-linked ubiquitin chains

Ubiquitin chains conjugated via K48 represent the best characterised linkage type. It was recognised early on in ubiquitin research that ubiquitin's function as a tag targeting proteins for proteasomal degradation is usually not mediated by the conjugation of single ubiquitin molecules but by attachment of K48-linked chains consisting of at least 4 ubiquitin molecules (Chau et al., 1989; Thrower et al., 2000). This can be mediated by a variety of E2-E3 combinations amongst them those involving the K48-specific E2s UBE2R1 or UBE2K (Haldeman et al., 1997; Petroski and Deshaies, 2005). Once modified the ubiquitinated protein is recognised by a set of specific ubiquitin-binding proteins, Rad23, Dsk2, and Ddi1, which bind ubiquitin via UBA-domains whereas their N-terminal UBL-domain binds the proteasome (Chen and Madura, 2002; Elsasser et al., 2002; Funakoshi et al., 2002; Rao and Sastry, 2002). These ubiquitin receptors may therefore capture substrates remotely and then mediate their shuttling to the proteasome (Finley, 2009). In the proteasome itself S5a (hRpn10) and Rpn13, two additional ubiquitin receptors that bind ubiquitin via ubiquitin interaction motifs (UIMs) or a pleckstrin-like receptor for ubiquitin (Pru) domain might take over the binding of the ubiquitin chains (Deveraux et al., 1994; Husnjak et al., 2008; Kang et al., 2007; Schreiner et al., 2008; Wang et al., 2005). Alternatively, ubiquitin chains of sufficient length may bind shuttling and proteasome intrinsic receptors at the same time or stay associated with the shuttling receptors which are in turn bound by the proteasomal receptors via their UBL-domains (Finley, 2009). Once a ubiquitinated substrate has been

recognised by the regulatory subunit (19S) of the proteasome, ubiquitin chains are removed by proteasome intrinsic proteases, the substrate is unfolded and threaded through a narrow channel into the core particle (20S) where it is hydrolysed resulting in a variety of peptides (Ardley et al., 2001; Goldberg et al., 2002; Groll et al., 2000; Kisselev et al., 1999; Lee et al., 2002; Lee et al., 2001; Navon and Goldberg, 2001). Thus, due to its role in protein degradation, K48-linked ubiquitin impacts diverse processes such as removal of aged or misfolded proteins thereby regulating protein homeostasis, the cell cycle which depends on specific degradation of different proteins in the different phases of the progression through the cycle and different signalling pathways which are regulated both positively and negatively by the proteasomal degradation of antagonists or agonists, respectively.

2.1.2.2.7 Ubiquitin chains conjugated via lysine 63

K63-linked chains were the first type of polyubiquitin to be shown to have non-proteolytic functions when they were found to play role in the DNA damage response (Spence et al., 1995). An E2 complex consisting of UBE2N and UBE2V2 was shown to specifically generate K63-linked chains even in the absence of an E3. This specificity is generated by a non-covalent interaction between UBE2V2, which structurally resembles an E2 but lacks a catalytic cysteine, and the acceptor ubiquitin. The interactions between ubiquitin and the E2 complex position ubiquitin in a way that results in selective presentation of K63 to the active site (Deng et al., 2000; Eddins et al., 2006; VanDemark et al., 2001).

In addition to its implication in different DNA-damage related pathways (Hoege et al., 2002; Spence et al., 1995), K63-linked chains may assist or even replace monoubiquitination in the context of endocytosis as they may be recognised more efficiently by the relevant ubiquitin receptors (see section 2.1.2.1 and (Duncan et al., 2006; Geetha et al., 2005). A role for K63-linked chains is further supported by the implication of the K63-specifc DUB AMSH in this process (McCullough et al., 2004; Sato et al., 2008). An important field regulated by K63-linked ubiquitin is that of intracellular signal transduction. This linkage type was suggested to play a role in the Wnt- and interferon-response factor signalling pathways (Komander, 2009) and has a firmly established role in signalling initiated by different cytokines (Harhaj and Dixit, 2012; Jiang and Chen, 2011). It was shown that K63-linkages serve as recruitment platforms in the context of different receptor associated or -induced complexes (Conze et al., 2008; Ea et al., 2006; Kanayama et al., 2004; Oeckinghaus et al., 2007; Wu et al., 2006).

Genetic depletion of UBE2N as well as replacement of wild-type ubiquitin with a mutant incapable of generating K63-linked chains showed that this linkage type is indispensable for IL-1- but not TNF-induced signal transduction, although it may still collaborate with other chain types in the context of the latter stimulus (Xu et al., 2009a; Yamamoto et al., 2006). Mechanistically it was shown that K63-linked chains are often generated by TRAFs or IAPs and that they are responsible for the recruitment of the preformed IKK- and TGFβ-activated kinase (TAK)1-binding protein (TAB)–TAK1-kinase complexes (Ea et al., 2006; Kanayama et al., 2004; Lee et al., 2004; Silke and Brink, 2010; Wu et al., 2006). Especially the TAB–TAK1 complex depends on K63-linked ubiquitin for its recruitment, as its regulatory subunit, TAB2, has been shown to preferentially bind this linkage type (Kulathu et al., 2009). Thus, in the context of intracellular signal transduction, K63-linked chains are indirectly and possibly also directly (Fan et al., 2010) involved in the activation of kinases initiating the NF-κB and mitogen activated protein kinase (MAPK) pathways.

2.1.2.2.8 M1-linked or linear chains

As mentioned in section 1.1 ubiquitin is translated as a polyubiquitin precursor, i.e. in form of a polypeptide in which ubiquitin molecules are linked via their C- and N-termini, respectively. Co- and posttranslational cleavage of these chains by specific DUBs generates the cellular pool of monoubiquitin (Baker and Board, 1987; Finley et al., 1987; Komander et al., 2009a; Ozkaynak et al., 1984; Reyes-Turcu et al., 2009; Wiborg et al., 1985). However, it was recently shown that a specialised E3 ligase complex referred to as linear ubiquitin chain assembly complex (LUBAC) is able to re-establish M1-linked chains from this pool of monoubiquitin (Kirisako et al., 2006) thus creating an eighth and uniquely lysine independent form of ubiquitin linkage. As M1-linked chains structurally resemble K63-linked chains (see section 2.1.2.2) functional similarities could have been anticipated. However, the first function of linear chains to be uncovered was their implication in proteasomal degradation of a model substrate (Kirisako et al., 2006). This observation was supported by studies that found that fusion of a non-cleavable linear tetra-ubiquitin chain to a target protein can lead to the degradation of this protein (Prakash et al., 2009; Zhao and Ulrich, 2010) and that linear chains can bind the same proteasomal receptors as K48-linked chains (Thrower et al., 2000). In a signalling context the activity of LUBAC was linked to the degradation of TRIM25 and hence to suppression of type I interferon induction following

stimulation of RIG-I (Inn et al., 2011). On the other hand it was shown that LUBAC can ubiquitinate NEMO (Tokunaga et al., 2009) and that the UBAN (ubiquitin-binding in NEMO and ABIN (A20-binding inhibitor of NF-κB)-domain of this protein binds M1-linked ubiquitin dimers with much higher affinity than K63-linked dimers (Lo et al., 2009; Rahighi et al., 2009). This selectivity can be explained by contacts between the UBAN domain and surfaces of the dimer specific to this linkage type (Rahighi et al., 2009). Presence on and recognition by NEMO, the regulatory subunit of the IKK-complex implies that linear chains have a role in the NF-κB pathway. Indeed, down-regulation or absence of LUBAC-components was found to affect signal transduction induced by TNF, IL-1, CD40, lipopolysaccharide (LPS), lymphotoxin-β receptor (LTβR), or by genotoxic stress (Gerlach et al., 2011; Ikeda et al., 2011; Niu et al., 2011; Tokunaga et al., 2011). Both modification of NEMO with linear chains and the ability of this protein to bind M1-linked chains seem to be important in this context. Mutants preventing NEMO's modification (K285,309R) were unable to restore IL-1- or LUBAC-induced NF-κB induction in NEMO-deficient cells (Tokunaga et al., 2009) whereas mutations that abolished NEMO's ability to bind linear chains without preventing its association with K63-linked chains reduce NF-κB activation in response to TNF (Hadian et al., 2011; Rahighi et al., 2009). Furthermore, mutations affecting ubiquitin binding by NEMO are associated with X-linked ectodermal dysplasia and immunodeficiency (Rahighi et al., 2009). Finally, linear chains may also serve as a recruitment platform for negative regulators of signalling processes such as the UBAN-containing proteins ABIN1 and Optineurin which were reported to interfere with TNF-induced NF-κB activation by competing with NEMO for binding to ubiquitinated RIP1 (Zhu et al., 2007) and by assisting the DUBs A20 and CYLD (cylindromatosis) in down-regulating TNF-signalling (Harhaj and Dixit, 2011).

2.1.3 Physiological and pathophysiological roles of the ubiquitin system

Due to its important regulatory role in a variety of intracellular processes and its involvement in many signalling pathways, ubiquitination has an impact on many biological processes. This is exemplified by its involvement in defending the organism from invading pathogens, where recognition and clearance of the pathogen by the innate immune system as well as activation of the adaptive immune response are subject to regulation by the ubiquitin system. Recognition of pathogens is often mediated by the binding of pathogen associated molecular patterns (PAMPs) by pathogen-recognition receptors of the Toll-like-

receptor (TLR), the retinoic acid inducible gene I (RIG-I)-like receptor (RLR) or the Nucleotide Oligomerisation Domain (NOD)-like receptor (NLR) families. Activation of these receptors triggers intracellular pathways leading to NF-κB- or interferon regulatory factor (IRF)-dependent expression of effector molecules such as proinflammatory cytokines or type I interferons (IFNs) that counteract the infection directly and indirectly by eliciting an adaptive immune response (Jiang and Chen, 2011; Kawai and Akira, 2011). The pathways leading to the activation of these transcription factors involve the assembly of multi-protein complexes which initiate signalling and which require non-proteolytic ubiquitin chains for their assembly and stability (Schmukle and Walczak, 2012). Furthermore, downstream signalling may depend on the removal of inhibitory proteins as for example the inhibitor of kappa B (IκB) which in the unstimulated state binds NF-κB dimers and prevents their translocation to the nucleus. Following its phosphorylation by the IKK-complex (Regnier et al., 1997) IκB is recognised by the substrate binding subunit, β-transducin repeat-containing protein (βTRCP), of the Skp, Cullin, Fbox (SCF) E3-complex and is subsequently ubiquitinated and degraded by the proteasome (Alkalay et al., 1995; Chen et al., 1995; Palombella et al., 1994; Spencer et al., 1999; Tan et al., 1999; Winston et al., 1999; Yaron et al., 1997; Yaron et al., 1998). In context of the adaptive immune system, intracellular signalling initiated by the T- or B-cell receptors, by CD40- or by BAFF which also involves the activation of the NF-κB and MAPK pathways is subject to the same type of regulation (Jiang and Chen, 2011).

Furthermore, selective autophagy, which represents a cell-autonomous effector mechanism of the innate immune system and fights intracellular bacteria by isolating them in double-membrane surrounded autophagosomes thus depriving them of nutrients and targeting them to microbicidal autolysosomes, depends on ubiquitination (Randow, 2011). *S.typhimurium, streptococcus pyogenes* as well as other bacteria acquire a dense ubiquitin coat when entering the cytosol. The ubiquitin molecules may be conjugated to bacterial proteins directly or to host-proteins associating with the bacterium by a so far unidentified E3. This ubiquitination is sensed by specific autophagy receptors, that, in a manner comparable to the proteasome shuttling receptors (see section 2.1.2.2.6), target their cargo to the autophagosomes. This process is based on the ability of the autophagy receptors, as for example p62, NDP52 and Optineurin (Thurston et al., 2009; Wild et al., 2011; Zheng et

al., 2009), to simultaneously bind ubiquitin and LC3/Atg8, a ubiquitin-like protein covalently coupled to the membranes of phagophores, the double-membrane precursors of autophagosomes (Fengsrud et al., 2000), thus mediating the interaction between cargo and engulfing membrane (Randow, 2011).

Finally, the surface expression and peptide-loading of MHC molecules is regulated by ubiquitination. Ubiquitination of MHC class II molecules which present antigenic peptides to T-helper cells leads to their endocytosis and lysosomal degradation (Shin et al., 2006; van Niel et al., 2008). In addition, incorrectly folded MHC class I molecules are degraded via the ER-associated degradation (ERAD) pathway in a ubiquitin-dependent manner (Burr et al., 2011) and even more importantly the antigenic peptides presented to cytotoxic T-cells by MHC class I molecules are generated by proteasomal processing of intracellular proteins (Michalek et al., 1993; Rock and Goldberg, 1999).

On the other hand, pathogens often employ the ubiquitin system to manipulate or evade an immune response. Mechanisms involved can include the degradation of host proteins critically involved in raising an appropriate immune response as for example components of the NF-κB or interferon pathways or MHC molecules. Alternatively, pathogens can aim to prevent or counteract specific ubiquitination events for example by the expression of DUBs (Jiang and Chen, 2011; Randow, 2011; Randow and Lehner, 2009). To mediate these effects, pathogens can either express specific proteins suitable for the respective task or adaptor proteins allowing them to hijack specific components of the host's ubiquitination system. Specific example for the mentioned schemes, include the ubiquitin dependent degradation of the NF-κB subunit p65 by the gammaherpesvirus MUHV.4 protein ORF3 (Rodrigues et al., 2009), the ubiquitination of NEMO that is mediated by the IpaH9.8 protein expressed by *Shigella felxneri* (Ashida et al., 2010) and the degradation of IRF3 and IRF7 which are targeted by the rotavirus protein NS1 and the KSHV protein replication and transcription activator (RTA) (Barro and Patton, 2005; Bauhofer et al., 2007; Yu and Lai, 2005). Furthermore, in a case of E3-hijacking, the highly conserved V-proteins of several paramyxoviruses, including mumps virus, human parainfluenza virus type 2 (HPIV2) and simian virus 5 (SV5), target signal transducer and activator of transcription (STAT)s, important mediators of IFN-signal transduction, for ubiquitination and degradation by recruiting them to DNA Damage Binding Protein 1 (DDB1), the substrate binding subunit of the Cul4A E3 ligase-complex (Li et al., 2006b; Parisien et al., 2002).

To evade the action of cytotoxic T-cells, human CMV (cytomegalovirus) encodes proteins, US2 and US11, which, probably indirectly, initiate the ubiquitination and degradation of MHC class I molecules via the ERAD pathway (Lilley and Ploegh, 2004; Wiertz et al., 1996; Ye et al., 2004). Similarly, K3, a protein expressed by Kaposi's sarcoma virus (KSHV) and the canonical member of the viral RING-CH ligase family, associates with and ubiquitinates MHC class I in a post-ER compartment, leading to its internalization, ESCRT-dependent sorting and lysosomal degradation (Cadwell and Coscoy, 2005; Lehner et al., 2005; Wang et al., 2007). Finally, strategies to prevent ubiquitination events generally or specifically are employed by bacteria and viruses. The proteins CHBP from *Burkholderia pseudomallei* and its homologue Cif from *Escherichia coli* deamidate specific glutamine residue within ubiquitin and thus prevent the formation of ubiquitin chains (Cui et al., 2010) in general. Individual ubiquitination events can be more specifically counteracted by expression of DUBs or by adaptor proteins that recruit the host's proteases to a designated target. An example of a virally encoded DUB is $UL36^{USP}$ which by a so far unknown mechanism affects pathogenicity but not replication of viruses (Bottcher et al., 2008; Jarosinski et al., 2007). Overall this shows that the immune system, as an example of many complex processes is highly regulated by ubiquitination and that this can also be exploited by pathogens.

Due to its implication in intracellular signalling and in the regulation of physiological processes, deregulations affecting the ubiquitin system have been associated with a set of different diseases. Although the association of a faulty proteasomal degradation process with the pathogenesis of different disorders is the best studied aspect of this connection, alterations at any stage of the ubiquitination system can have detrimental results.

Replacement of the C-terminal glycine in ubiquitin itself by a 20-residue extension can result from a dinucleotide deletion on the mRNA-level. Expression of this ubiquitin mutant (Ub+1) was associated with Alzheimer's disease (van Leeuwen et al., 1998). Mechanistically, for a protein to be degraded by the proteasome, it needs to interact with the proteasomal machinery via a ubiquitin moiety and to have a tail that is long enough to be able to reach the inner proteolytic chamber. Ub+1 can be incorporated into ubiquitin chains but its C-terminal extension is too short for the molecule to be degraded. Therefore Ub+1 binds the proteasome but cannot be processes, thus exerting an inhibitory effect on the degradation machinery which may cause or at least aggravate the accumulation of insoluble protein deposits observed in Alzheimer's disease (Shabek et al., 2009). Alterations in a substrate

that changes its ubiquitination pattern can also be the cause for disease as exemplified by a mutation in the epithelial Na^+ channel ENaC that prevents its interaction with the E3 ligase Nedd4-2. This leads to an accumulation of the ion channel and to a deregulation of Na^+-reabsorption. This is associated with a condition referred to as Liddle's syndrome which is characterized by early onset of severe hypertension (Rotin and Schild, 2008; Staub et al., 1997; Zhou et al., 2007). On the other hand, not only disturbances in the covalent attachment of ubiquitin chains to specific substrates but also the non-covalent binding to nonproteolytic ubiquitin chains can have deleterious effects. Several studies have identified mutations in the UBAN motif of NEMO, that inhibit its ability to bind ubiquitin chains and alter its specificity of binding linear ubiquitin chains, in patients with anhidrotic ectodermal dysplasia and immunodeficiency (Doffinger et al., 2001; Filipe-Santos et al., 2006; Rahighi et al., 2009). Although missense mutations in UBA1 are associated with spinal muscular atrophy, a motor neuron disorder in which the ubiquitin-dependent degradation of proteins in this cell type is affected (Ramser et al., 2008), and mutations in the E2 UBE2A are linked with an X-linked mental retardation syndrome (Budny et al., 2010; de Leeuw et al., 2010; Nascimento et al., 2006), the most common part of the ubiquitination process to be affected are the ubiquitin ligases. This can be attributed to the large number of proteins included in this group and to their role of providing the ubiquitination process with substrate selectivity. Mutations in different E3s can affect different targets and processes and accordingly alterations in particular E3s have been associated with a multitude of diseases. Loss of function in the HECT-E3 E6AP is implicated in the development of Angelman syndrome, a neurodevelopmental disorder characterised by mental retardation, seizures and movement disorders (Kishino et al., 1997; Rougeulle et al., 1997; Vu and Hoffman, 1997). On the other hand, a gain of function of this E3 in complex with the viral protein E6 leads to the destabilisation of tumour suppressors, most importantly p53, thus promoting proliferation, transformation and eventually the development of HPV-induced cervical cancer (Scheffner et al., 1993; Scheffner and Staub, 2007). BRCA1 mutations lead to a high incidence of breast and ovarian cancers (Futreal et al., 1994) and alterations in many other E3s have also been implicated in causing cancer (Kirkin and Dikic, 2011). This can be explained by a multilayered mechanism which includes the ubiquitin-dependent regulation of oncogenic proteins and the role of ubiquitin in maintaining a balance between pro-survival and cell death pathways (Lipkowitz and Weissman, 2011; Vucic et al., 2011). In

addition, a loss of function in E3s or an overall down-regulation of proteasomal protein degradation has been associated with neurodegenerative disorders such as Parkinson's disease, where mutation of the E3 ligase Parkin causes the accumulation of misfolded, insoluble aggregates (Foroud et al., 2003; Kitada et al., 1998; Lucking et al., 2000; Oliveira et al., 2003a; Oliveira et al., 2003b; Pankratz et al., 2003).

2.2 The TNF/TNFR-system

2.2.1 The TNF/TNFR-superfamilies

As indicated by its name TNF was originally identified as a product of macrophages that causes lysis of tumour cells (Carswell et al., 1975). Its cDNA was cloned in 1984 (Marmenout et al., 1985; Pennica et al., 1984) and many related proteins were identified in the following years. TNF thus became the founding member of a cytokine family referred to as the TNF-superfamily (TNF-SF). With the exception of lymphotoxin alpha (LTα) and vascular endothelial growth inhibitor (VEGI), which are secreted, members of this family generally are transmembrane proteins with a single transmembrane domain and a C-terminal extracellular domain. They are hence classified as type II transmembrane proteins (Aggarwal, 2003). Soluble forms of the different members of the TNF-SF can be generated by proteolytic cleavage of the transmembrane proteins by a specific set of proteases (Black et al., 1997; Lum et al., 1999; Nocentini et al., 1997; Powell et al., 1999). The extracellular part of the transmembrane forms which is retained in the soluble variants and which is also referred to as TNF-homology domain is responsible for the ability of the different ligands to bind to their respective receptors. It also mediates the assembly of non-covalent ligand-trimers. The individual chains interact via hydrophobic surfaces and the aromatic residues involved account for most of the 20-30 % sequence similarity observed between the different members of the TNF-SF (Fesik, 2000; Locksley et al., 2001). Although mono- or dimeric forms of the different ligands may exist, their biological activity is mediated by the trimeric forms (Black et al., 1997; Cabal-Hierro and Lazo, 2012). This is due to the requirement for a 3:3-stoichiometry in ligand-receptor complexes to activate the receptors. Both forms of the ligands can engage the respective receptors but depending on the specific cytokine and receptor the soluble variant can have agonistic or antagonistic effects (Aggarwal, 2003). The receptors mediating the effects of TNF-SF cytokines belong to the TNF receptor superfamily (TNFR-SF). They are type I transmembrane proteins that typically

contain one to six cysteine rich domains (CRDs) (Wajant et al., 2003). These pseudorepeats are usually approximately 40 aminoacids long and contain six highly conserved cysteine residues that form three intrachain disulfide bonds (Locksley et al., 2001; Smith et al., 1994). The resulting scaffold of disulfide bridges causes the receptor to adopt an elongated conformation. This shape allows the receptor to fit in the lateral grooves between protomers of the ligand trimer which in turn permits the crosslinking of three receptor monomers by a trimeric ligand into an active 3:3-complex (Banner et al., 1993). Although ligand-independent assembly of receptors via a pre-ligand assembly domain (PLAD) in their distal CRD was observed (Chan et al., 2000; Siegel et al., 2000) the resulting complexes assume a conformation that differs from that of the ligand-induced multimers and pre-assembled receptors therefore still require ligand binding, which might induce conformational changes or allow formation of higher order multimers, for their activation (Locksley et al., 2001; Naismith et al., 1996; Naismith and Sprang, 1995). As receptors of the TNFR-SF generally lack enzymatic activity in their intracellular domains they rely on the activation-induced recruitment of adaptor proteins to initiate signalling. Based on the differences in their intracellular domains three subgroups of the TNFR-SF, the death domain (DD)- and the TRAF-interaction motif (TIM)-containing groups as well as the receptors lacking a functional intracellular domain, can be defined which differ in the intracellular adaptors recruited (Hehlgans and Pfeffer, 2005; Locksley et al., 2001). The DD-comprising receptors, like TNFR1, CD95, TRAILR1 and TRAILR2, recruit TNF receptor associated protein with a DD (TRADD) or Fas associated protein with a DD (FADD) and can therefore induce both gene-induction and programmed cell death in the form of apoptosis or potentially necroptosis (Laster et al., 1988; Vanlangenakker et al., 2012), whereas the second group of receptors as for example TNFR2, CD40, fibroblast growth factor inducible 14 (Fn14) or B cell activation factor receptor (BAFFR), recruits TRAFs and initiates signalling which is largely restricted to prosurvival outcomes. Finally, the third group comprises receptors like TRAILR3, TRAILR4 and Osteoprotegerin (OPG) that lack functional intracellular domains. Individually and together members of the TNF/TNFR-superfamilies have prominent roles in a variety of biological processes including organogenesis and regulation of the immune system (Aggarwal, 2003; Hehlgans and Pfeffer, 2005).

2.2.2 TNF

The human TNF gene is located on chromosome 6p21.3 and contains at least four NF-κB binding motifs and cJun N-terminal kinase (JNK)-responsive elements in its promoter region (Collart et al., 1990; Tsai et al., 2000). These elements are required for full TNF expression as shown by the observation that its transcription is down-regulated both in JNK1/2-deficient mouse embryonic fibroblasts (MEFs), macrophages and T-cells (Das et al., 2009a; Ventura et al., 2003) and in RelB-deficient macrophages (Weih et al., 1997). Furthermore, TNF expression is regulated on the mRNA level by an AU-rich element (ARE) in the 3'untranlsated region (UTR) which recruits tristetrapolin, a zinc finger protein that accelerates the turnover of the mRNA. This destabilising effect can be counteracted by binding of different proteins that mediate mRNA stability and translation (Carballo et al., 1998; Kontoyiannis et al., 1999). Especially the p38 MAPK pathway has been implicated in this stabilisation and thus in promoting TNF expression (Dumitru et al., 2000; Hitti et al., 2006). Translation of the mRNA leads to a generation of a 26 kDa protein consisting of 233 aminoacids which is directed to the plasmamembrane by a signal peptide comprising its first 76 residues (Kriegler et al., 1988; Pennica et al., 1984). The membrane integrated form can then be cleaved by a metalloprotease referred to as TNF alpha converting enzyme (TACE). This cleavage leads to release from the membrane and thus to the formation of soluble TNF (Black et al., 1997; Kriegler et al., 1988). Production of TNF occurs mainly in macrophages but also in a variety of other cell types including lymphoid cells, mast cells, endothelial cells, fibroblasts and in neuronal tissues (Carswell et al., 1975; Wajant et al., 2003; Williamson et al., 1983). Due to the stimulation-dependent regulatory mechanisms that control the expression of TNF and its role in regulating the innate immune system, TNF is produced in response to a variety of proinflammatory stimuli as for example triggering of TLRs. The release of large amounts of TNF in response to LPS or other bacterial products is in line with a role of this protein in defending the host against invading pathogens. Using genetic deletion or neutralisation of TNF with antibodies (e.g. Infliximab) or soluble receptor molecules (e.g. Enbrel), it could be shown that infections with different pathogens such as *Listeria monocytogenes, Mycobacteria tuberculosis, M. avium, Salmonella typhimurium* or *Toxoplasma gondi*i are exacerbated in the absence of TNF (Bean et al., 1999; Ehlers, 2003; Pfeffer et al., 1993). Mechanistically, TNF promotes an efficient immune response by inducing the production of proinflammatory cytokines and by increasing the expression of

adhesion molecules that are essential for the interaction between endothelial cells and lymphocytes and that hence regulate trafficking of circulating immune cells into tissues (Roach et al., 2002). Finally, TNF increases the phagocytic ability of macrophages (Bekker et al., 2001). In spite of its important role in promoting immune responses, TNF can also be harmful to the host. Deregulated, prolonged exposure to TNF can result in chronic inflammation, a wasting syndrome referred to as cachexia or, when the TNF-levels are high, lead to shock (Beutler et al., 1985a; Beutler et al., 1985b; Kettelhut et al., 1987; Tracey et al., 1986). Even in the context of an infection TNF is not always beneficial as shown by the finding that TNF- or TNFR1-deficient mice show a delayed pathological response when challenged with certain pathogens such as *Citrobacter rodentium* or *Mycobacterium bovis* (Goncalves et al., 2001; Zganiacz et al., 2004). While these opposing effects of TNF in different infection models can be attributed to the different pathogens investigated it was also show that spatio-temporal differences in TNF production can have decisive effects on the outcome. In a mouse model in which caecal ligation and puncture lead to abdominal infection and immunoparalysis, it was shown that the effect of TNF-injection during the immunoparalysis, which prevents efficient production of endogenous TNF, can be beneficial or detrimental depending on location and timing (Echtenacher et al., 2003). Taken together this shows that the overall effect of TNF depends on the tissue, on the precise cellular context and on the timing and duration of the stimulus (Wajant et al., 2003). In addition to its role in promoting and regulating immune responses, TNF also affects organogenesis and architecture of lymphoid tissues. Although the development of TNF-deficient mice is largely normal, they lack primary B cell follicles, organised follicular dendritic cell (DC) networks and germinal centres (Pasparakis et al., 1996; Pasparakis et al., 1997) indicating that TNF is essential in establishing these features.

2.2.3 TNFR1 versus TNFR2

The multiple biological and pathological effects exerted by TNF are mediated by its binding to TNFR1 (also known as p55 TNFR or TNFRSF1a) and TNFR2 (also p75 TNFR or TNFRSF1b) which were both cloned in 1990 (Gray et al., 1990; Heller et al., 1990; Loetscher et al., 1990a; Loetscher et al., 1990b; Schall et al., 1990). These receptors are single membrane proteins belonging to the TNFR-SF. They share approximately 28 % homology which can be attributed almost exclusively to their extracellular domains (Dembic et al., 1990; MacEwan,

2002). Here both receptors contain four CRDs of which CRD1 comprises a PLAD whereas CRD2 and CRD3 are responsible for ligand binding (Banner et al., 1993; Chan et al., 2000; Eck and Sprang, 1989; Mukai et al., 2010). However, structural differences between the two receptors result in an altered mode of ligand recognition (Mukai et al., 2010) which could be a possible explanation as to why soluble TNF can efficiently activate TNFR1 but not TNFR2 (Grell et al., 1998; Grell et al., 1995; Krippner-Heidenreich et al., 2002) although both receptors can bind soluble and membrane-bound TNF. The different half-lives of the resulting receptor-ligand complexes could contribute to the specific activation of TNFR2 by the membrane-bound form of the ligand and might also be responsible for the kinetic differences observed regarding the activation of the two receptors (MacEwan, 2002). Furthermore, the two receptors also differ in their expression patterns with TNFR1 being constitutively and widely expressed whereas the expression of TNFR2 is highly regulated and restricted to certain cell types which mostly belong to the immune system (Dembic et al., 1990; Erikstein et al., 1991; Hohmann et al., 1990; Naume et al., 1991; Tannenbaum et al., 1993). Most importantly the intracellular domains of TNFR1 and TNFR2 differ significantly. While TNFR1 contains a globular bundle of α-helices in its C-terminus that form a DD, a sequence of a few aminoacids in TNFR2 can be bound by adaptor proteins through charged residues and thus serves as a TIM (Locksley et al., 2001; Rothe et al., 1994b; Tartaglia et al., 1993a). Therefore the two receptors belong to different subgroups of the TNFR-SF and signal transduction and biological outcomes initiated can differ significantly. Nevertheless there are overlapping functions and crosstalk between the receptors and it thus depends on the context if TNFR1 and TNFR2 have similar, distinct or even opposing outcomes (Cabal-Hierro and Lazo, 2012; Faustman and Davis, 2010; MacEwan, 2002). Overall activation of the two receptors has been implicated in both physiological and pathophysiological situations. TNFR1-deficient mice show enhanced sensitivity to certain pathogens as for example *Mycobacteria tuberculosis, Lysteria monocytogenes* or *Leishmania major* (Flynn et al., 1995; Rothe et al., 1994a; Vieira et al., 1996). It was also found that TNFR1$^{-/-}$ mice die from necrotising encephalitis when orally infected with a strain of *toxoplamsa gondii* that displays low virulence and does not kill wild-type or TNFR2-deficient mice (Deckert-Schluter et al., 1998). This indicates that TNF's role in defending the host against these pathogens is mediated by TNFR1. In addition, this receptor seems to be involved in liver regeneration following partial hepatectomy (Yamada et al., 1997). On the

other hand TNFR2 also has specific protective roles as for example by regulating antigen induced differentiation of T cells (Grell et al., 1999; Kim et al., 2006; Kim and Teh, 2001), by contributing to the elimination of autoreactive T cells in diabetic immunity thereby facilitating pancreatic regeneration (Kodama et al., 2005; Ryu et al., 2001) and by playing a role in angiogenesis (Goto et al., 2006). Furthermore, TNFR2 seems to have a role in cardioprotection as demonstrated by the findings that TNFR2$^{-/-}$ mice are more prone to heart failure and show reduced survival after infarction (Monden et al., 2007) and that TNFR2 signalling protects heart cells from ischemia in isolated heart preparations from female mice (Wang et al., 2008). Another beneficial role of TNFR2 may be relevant in demyelating disorders as for example multiple sclerosis. Systemic blocking of TNF in patients suffering from this kind of disorder increased lesions in the central nervous system and disease activity (Kassiotis and Kollias, 2001) indicating that TNF has a protective role in this context. This beneficial effect could be mediated by TNFR2 as it was shown that regeneration of oligodendrocyte precursors, that form myelin, can be induced in a TNFR2-dependent manner (Arnett et al., 2001). In addition, it was found that TNFR2 is involved in the repair of the striatum following viral encephalitis in a mouse model (Rodriguez et al., 2009) and that it promotes neuroprotection in the context of retinal ischemia in mice (Fontaine et al., 2002). In the former model TNFR1 serves a similar but locally distinct function by mediating the repair of the hippocampus (Rodriguez et al., 2009). On the other hand it seems to counteract TNFR2 in retinal ischemia as it was associated with neurodegeneration in this context (Fontaine et al., 2002). However, activation of TNFR2 does not have an exclusively beneficial role and polymorphism and deregulation in the expression of this receptor have been implicated in a variety of pathological conditions. This included familiar rheumatoid arthritis, Cohn's disease, ulcerative colitis and systemic lupus erythematosus (SLE) (Barton et al., 2001; Dieude et al., 2002; Komata et al., 1999; Pierik et al., 2004; Sashio et al., 2002). TNFR2 was also found to play a role in experimental hepatitis, in graft-versus-host-disease and in the neurovascular damage arising in a model of cerebral malaria (Ishikawa et al., 2002; Kusters et al., 1997; Stoelcker et al., 2002). Furthermore, an up-regulation of TNFR2 correlates with a bad prognosis in renal cell carcinoma (Al-Lamki et al., 2010).

All the distinct but overlapping functions of TNFR1 and TNFR2 are initiated by intracellular signal transduction cascades that emanate from the receptors upon TNF-stimulation. As in

their functional outcomes the two TNF receptors share the activation of certain common signalling events such as the NF-κB and MAPK-pathways, but also differ in certain aspects as for example the direct induction of programmed cell death.

2.2.3.1 TNFR1 signalling

Binding of a TNF-trimer to TNFR1 induced trimerisation of the receptor. It was shown that the receptor multimers formed upon activation exclusively contain TNFR1 and that no heterotrimerisation with TNFR2 could be observed (Idriss and Naismith, 2000; Locksley et al., 2001; Moosmayer et al., 1994). In addition to promoting trimerisation ligand binding potentially also triggers conformational changes in preformed receptor multimers. The activated receptors then initiate intracellular signalling cascades which eventually result in a biological outcome. Depending on the cellular context, TNFR1 activation can lead to induction of proinflammatory cytokines, cell proliferation, differentiation or cell death (Chen and Goeddel, 2002; Wajant et al., 2003). Because TNFR1, like other members of the TNFR-SF, does not exhibit enzymatic activity it relies on the recruitment of cytoplasmic proteins which form a receptor-associated complex from which all signalling pathways emanate and which is thus referred to as receptor signalling complex (RSC).

2.2.3.1.1 The TNFR1-SC

Upon stimulation TRADD is recruited to the intracellular domain of TNFR1 via homotypic interactions of their DDs (Hsu et al., 1995). The serine threonine kinase receptor interacting protein 1 (RIP1) can associate with TNFR1 in an equivalent manner (Ermolaeva et al., 2008; Haas et al., 2009; Pobezinskaya et al., 2008) but could also indirectly bind to the receptor via TRADD (Hsu et al., 1996a). The latter protein serves as an adaptor for TRAF2 and this interaction is mediated via the C-terminal TRAF-domain present in TRAF2 which is also responsible for homotrimerisation (Hsu et al., 1996b; Shu et al., 1996). Indeed, TRAF2 forms mushroom-shaped trimers in which each off the protomers is capable of interacting with one TRADD molecule (Park et al., 2000). An alternative mode of TRAF2 recruitment seems to exist as suggested by the observation that TRAF2 is absent from the TNF-RSC in TRADD-deficient MEFs (Ermolaeva et al., 2008; Haas et al., 2009; Pobezinskaya et al., 2008) but not in TRADD-deficient macrophages (Pobezinskaya et al., 2008). It was suggested that in this case RIP1 may be the relevant adaptor. However, in presence of TRADD, RIP1 is dispensable for TRAF2 recruitment in MEFs (Haas et al., 2009). The TRAF2 related protein TRAF5 is often

referred to as a component of the TNF-RSC although its recruitment has not been shown to date (Wajant and Scheurich, 2011). TRAF2 associates with the cIAP1 and cIAP2 with high efficacy and thus serves as an adaptor for their association with the TNF-RSC (Mace et al., 2010; Shu et al., 1996; Zheng et al., 2010). Structural studies revealed that a TRAF2 trimer interacts with one cIAP2 molecule in an asymmetric manner and it was also shown that a cIAP-interaction motif (CIM) comprising aminoacids 283-293 of TRAF2 and the BIR1-domain of cIAP mediate this interaction and are thus required for the recruitment of cIAP to the TNFR (Samuel et al., 2006; Varfolomeev et al., 2008; Vince et al., 2009). Once this core complex is assembled several components are modified by ubiquitin chains of a non-proteolytic nature. TRAF2, cIAP1 and cIAP2 have all been suggested to be the relevant E3 involved in generating these ubiquitin chains (Bertrand et al., 2008; Ea et al., 2006; Varfolomeev et al., 2008; Wertz et al., 2004). However, the ability of TRAF2 to contribute to these modifications is controversial (Alvarez et al., 2010; Yin et al., 2009a). Functionally, these ubiquitination events are a prerequisite for the recruitment of downstream components such as the TAB/TAK- and IKK-complexes (Ea et al., 2006; Kanayama et al., 2004; Li et al., 2006a; Wu et al., 2006). Especially, K63-linked chains on RIP1 have been implicated in this task but several lines of evidence suggest that ubiquitinated RIP1 may not be responsible for recruiting downstream effectors on its own. It was found that the activity of UBE2N as well as K63-linkages are dispensable for TNF-induced IKK-activation (Xu et al., 2009a), components of the TNF-RSC other than RIP1 were shown to be ubiquitinated (Bertrand et al., 2008; Fan et al., 2010; Li et al., 2009a; Shi and Kehrl, 2003) and additional ubiquitin linkages, including K11-linked chains (Dynek et al., 2010; Gerlach et al., 2011) were found in the complex. It is however established that ubiquitin is responsible for or at least considerably contributes to the recruitment of kinase complexes and thus to the initiation of signal transduction (Chen, 2012; Gautheron and Courtois, 2010; Kanarek and Ben-Neriah, 2012; Wajant and Scheurich, 2011). The assembly of the TNF-RSC and ubiquitination events mediated by cIAP and possibly TRAF2 are depicted schematically in Figure 8.

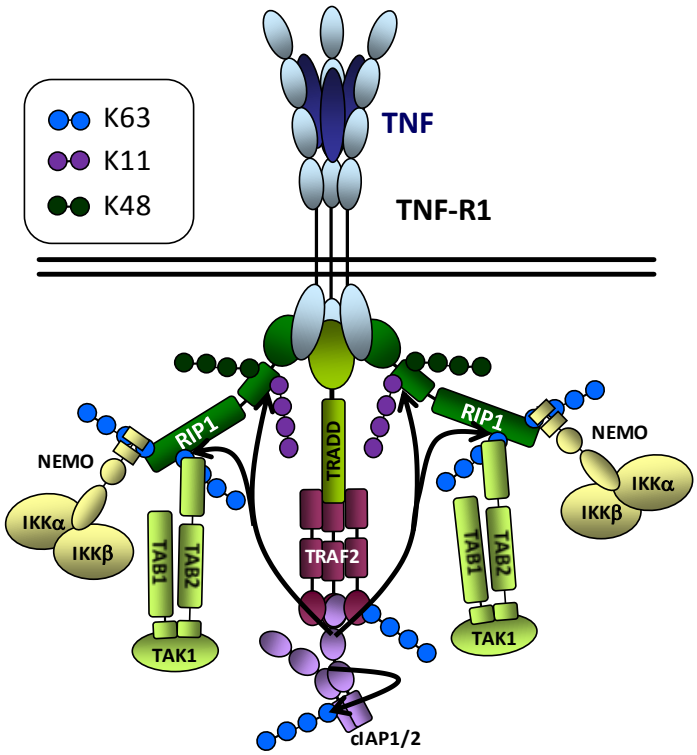


Figure 8: Schematic representation of the TNFR1 associated signalling complex. Upon crosslinking by TNF, TNFR1 recruits TRADD and RIP1 via homotypic interaction of the respective DDs. TRADD then serves as an adaptor for recruitment of TRAF2 which in turn recruits cIAP1 and cIAP2. These E3s then mediate ubiquitination of different complex components with several types of ubiquitin chains including K63- and K11-linkages. These non-proteolytic poly-ubiquitins then act as a recruitment platform for the preassembled TAB/TAK- and IKK-complexes which upon their activation initiate downstream signalling.

2.2.3.1.2 TNFR1-induced signal transduction

TNFR1 activation induces a variety of downstream signalling pathways. The best studied are the activation of NF-κB and MAPKs, especially p38 and JNK (Karin and Gallagher, 2009) and the induction of programmed cell death. However, other events such as the activation of AKT (protein kinase B, PKB) or protein kinase C (PKC) have been reported as well (Osawa et al., 2001; Ozes et al., 1999; Schutze et al., 1990; Wiegmann et al., 1992).

The term NF-κB refers not to a single protein but to a family of homo- and heterodimeric transcription factors formed by the combination of members of the Rel family which comprises RelA (p65), RelB, cRel, p52 and p50 (Hoffmann and Baltimore, 2006; Oeckinghaus and Ghosh, 2009). All five Rel proteins are characterised by a Rel homology domain (RHD) which confers the ability to dimerise and to bind DNA (Chen et al., 1998; Ghosh et al., 1995; Muller and Harrison, 1995; Muller et al., 1995). In addition, RelA, RelB and cRel contain a transactivation domain (TAD) which is absent from p52 and p50 (Hayden and Ghosh, 2008).

Therefore, some of the potential NF-κB dimers lack transcriptional activity and thus serve an inhibitory function unless they gain the ability to act as a transcription factor by association with additional components (Ballard et al., 1992; Bours et al., 1993; Franzoso et al., 1992; Kunsch et al., 1992; Mercurio et al., 1992; Ryseck et al., 1992; Schmid et al., 1991; Schmitz and Baeuerle, 1991). In a resting state, preformed NF-κB dimers exist but they are prevented from translocating to the nucleus and exerting their transcriptional activity by members of the IκB family (Baeuerle and Baltimore, 1988; Baeuerle et al., 1988; Kanarek et al., 2010). Proteins of this family are characterised by five to seven ankyrin repeats and include the typical members IκBα, IκBβ, IκBε, the atypical proteins IκBζ and BCL3 as well as p100 and p105 which are the precursors of the NF-κB subunits p52 and p50 respectively (Ghosh et al., 1990; Meyer et al., 1991; Naumann et al., 1993a; Naumann et al., 1993b; Oeckinghaus and Ghosh, 2009; Schmid et al., 1991). Hence, the essential step in NF-κB activation is freeing the dimeric transcription factor from its inhibitor. This process involves phosphorylation, ubiquitination and proteasomal degradation of IκB. Once the kinase complex consisting of IKKα, IKKβ and the regulatory subunit NEMO has been recruited to the TNF-RSC via the interaction of NEMO with ubiquitin chains and potentially with RIP1 (Ea et al., 2006; Wu et al., 2006; Zhang et al., 2000) the kinase subunits are activated. Mechanistically this may involve conformational changes induced in NEMO when it binds to ubiquitin that translate onto the kinase subunits, trans-autophosphorylation of IKKs favoured by clustering of kinases in the TNF-RSC or phosphorylation of IKK by an upstream kinase (Oeckinghaus and Ghosh, 2009; Rahighi et al., 2009; Tang et al., 2003a). Although RIP1 itself is a kinase, its activity was shown to be dispensable for NF-κB activation (Lee et al., 2004) and therefore the kinases responsible for IKK phosphorylation and activation have been suggested to be MAPK/ERK kinase kinase 1 (MEKK1), MEKK2, MEKK3 or TAK1 (Lee et al., 1998; Schmidt et al., 2003; Shim et al., 2005; Yang et al., 2001). While absence or down-regulation of each of these kinases individually or in combination affects TNF-induced NF-κB activation, there is currently no clear understanding of their interplay or potential redundancy. Once IKKβ, the main IKK in the canonical pathway of NF-κB activation (Chu et al., 1999; Hacker and Karin, 2006; Hu et al., 1999; Li et al., 1999) has become phosphorylated in its activation loop and is thus active, it phosphorylated IκBα at serines 32 and 36 (Brown et al., 1995; DiDonato et al., 1996; Ghosh and Baltimore, 1990; Traenckner et

al., 1995). Other canonical IκBs are targeted in a similar manner and in all cases phosphorylation generates a degron motif that is recognised by the $SCF^{\beta TRCP}$-E3 complex which then mediates the conjugation of K48-linked ubiquitin to lysines 21 and 22 of IκBα (Hatakeyama et al., 1999; Kanarek et al., 2010; Scherer et al., 1995; Shirane et al., 1999; Wu and Ghosh, 1999; Yaron et al., 1997; Yaron et al., 1998). Ubiquitinated IκBs are recognised and degraded by the 26S proteasome thereby uncovering the nuclear localisation sequence of previously bound NF-κB dimers and allowing them to translocate to the nucleus where they can bind specific κB-sites and initiate the transcription of target genes (Kanarek et al., 2010). Among these targets are several inhibitors of NF-κB activation which in a negative feedback loop ensure the timely termination of the signal as failure to down-regulate NF-κB activity can result in chronic inflammation and tumour formation (Ben-Neriah and Karin, 2011). These negative regulators include IκBα which can bind NF-κB dimers and lead to their export from the nucleus via its nuclear export sequence (NES) (Huxford et al., 1998; Jacobs and Harrison, 1998; Le Bail et al., 1993; Malek et al., 1998), as well as A20 and CYLD, two DUBs that remove non-proteolytic ubiquitin chains from the apical complex. This results in interactions with downstream components being lost and eventually in termination of the signal (Brummelkamp et al., 2003; Heyninck and Beyaert, 2005; Jono et al., 2004; Kovalenko et al., 2003; Krikos et al., 1992; Trompouki et al., 2003; Wertz et al., 2004).

In parallel to activating NF-κB, TNF also induces different MAPK pathways. MAPKs are serine threonine kinases that can be activated by a variety of cellular stresses and stimuli in a hierarchical cascade of phosphorylation events (Cargnello and Roux, 2011; Schaeffer and Weber, 1999; Wajant et al., 2003). Specific scaffolding proteins mediate the interaction between different components of the pathway thus allowing the downstream MAPK to be phosphorylated within a conserved Threonine-X-Tyrosine motif by a specific MAPK kinase (MAPKK, MAP2K, MAPK/ERK kinase (MEK), MKK) which in turn was activated by its MAPKK kinase (MAPKKK, MAP3K, MEKK). Once activated MAPKs can regulate downstream kinases and initiate activation of transcription factors thus translating extracellular stimuli into a wide range of cellular responses. In mammalian cells several MAPK modules, including the extracellular signal regulated kinases 1/2 (ERK1/2)-, the JNK- and the p38-cascades, have been identified (Cargnello and Roux, 2011). In the context of TNFR1 signalling the most potently activated MAPKs are JNK and p38 (Karin and Gallagher, 2009). Following TNF

stimulation different JNK isoforms, especially the widely expressed JNK1 and JNK2 which are also known as stress-activated protein kinase (SAPK)γ and SAPKα, respectively, are phosphorylated and activated (Derijard et al., 1994; Kyriakis et al., 1994; Westwick et al., 1994). This depends on the MAP2Ks MKK4 and especially MKK7 as shown by the finding that although MKK4 is required for maximal JNK activation it cannot carry out this activation on its own in MKK7-deficient MEFs (Derijard et al., 1995; Tournier et al., 2001; Tournier et al., 1997). The upstream MAP3K for this pathway has not been unambiguously identified but a role for TAK1, MEKK1 or MEKK3 has been suggested (Blank et al., 1996; Minden et al., 1994; Ninomiya-Tsuji et al., 1999; Xia et al., 2000). Furthermore, an additional class of kinases, the MAP4Ks may contribute to JNK activation by phosphorylating the MAP3Ks. For example it was shown over-expression of group I germinal centre kinases (GCKs), which can be differentiated from group II GCKs by the their regulatory domains, can lead to MAP3K phosphorylation and that GCK and GCK-related (GCKR) can activate MEKK1 (Kyriakis, 1999; Shi and Kehrl, 1997; Yao et al., 1999; Yuasa et al., 1998). The system is further complicated by a second phase of prolonged JNK activation that depends on a different set of proteins, including apoptosis signal-regulating kinase 1 (ASK1) (Tobiume et al., 2001). Whereas the first transient wave of JNK signalling leads to the activation of AP-1, a family of heterodimeric transcription factors composed of Jun, Fos, JDP and ATF proteins, prolonged activation of this MAPK has been associated with production of reactive oxygen species (ROS) and the induction of cell death (Roulston et al., 1998; Tobiume et al., 2001; Ventura et al., 2004; Ventura et al., 2003).

Activation of the MAPK p38 follows the same scheme of events but involves different kinases. Although there are four p38 isoforms (α,β,γ,δ), p38α is the most widely and highly expressed variant and therefore the best characterised member of this subgroup of MAPKs (Cuadrado and Nebreda, 2010; Han et al., 1994; Jiang et al., 1996; Jiang et al., 1997; Lee et al., 1994; Mertens et al., 1996; Rouse et al., 1994). As in the case of the JNK-module, MEKK1-3, TAK1, ASK1 and other potential MAP3Ks have been implicated in the initiation of the p38 pathway (Blank et al., 1996; Ninomiya-Tsuji et al., 1999); (Cargnello and Roux, 2011; Minden et al., 1994; Xia et al., 2000). The MAP2Ks activating p38 by phosphorylating it in its activation loop are MKK3, MKK6 and to a certain extent MKK4 (Derijard et al., 1995; Enslen et al., 1998; Han et al., 1996; Johnson and Lapadat, 2002; Stein et al., 1996). Once activated p38 phosphorylates a multitude of cytoplasmic and nuclear proteins, amongst them, as in

the case of JNK, components of AP-1 transcription factors which then mediate the manifold outcomes of this pathway as for example the production of proinflammatory cytokines (Cuadrado and Nebreda, 2010). Importantly, the NF-κB and MAPK pathways following TNF stimulation are not independent events but regulate the biological outcome of the stimulus by a complex crosstalk and interplay (De Smaele et al., 2001; Papa et al., 2004; Schwabe and Brenner, 2006; Tang et al., 2001). This overall effect is also determined by a different arm of TNF-signalling which, in contrast to the gene-inducing pathways that mainly result in a pro-survival outcome, induces cell death in the form of apoptosis or necroptosis (Figure 9).

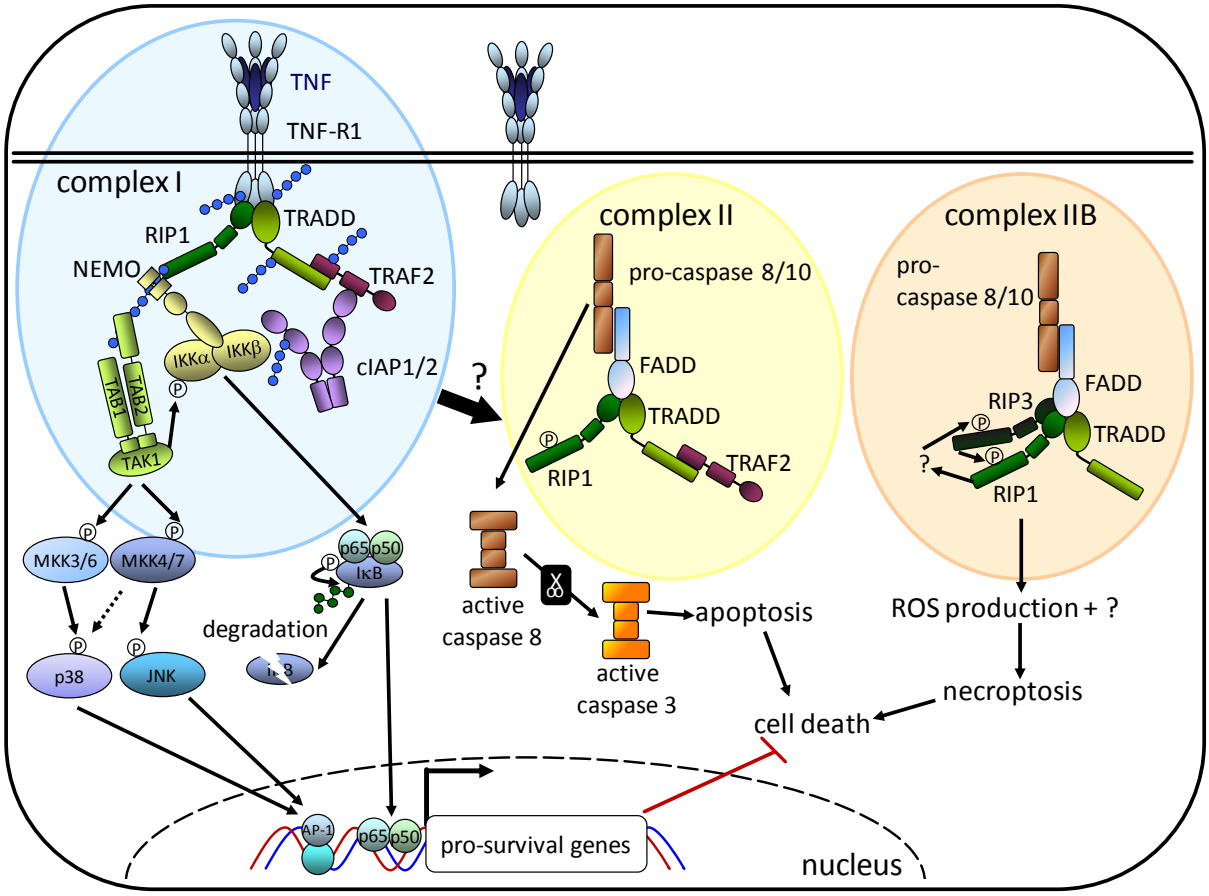


Figure 9: Schematic representation of TNF-signalling. Upon cross-linking by TNF, TNFR1 recruits TRADD, RIP1, TRAF2, cIAP1/2 and the TAB/TAK- and IKK-complexes. This receptor associated complex (TNF-RSC or complex I) initiates pathways inducing the activation of NF-κB and MAPKs which eventually leads to the expression of pro-survival genes. Extended TNF stimulation can result in the formation of secondary, death-inducing complexes (complex II and complex IIB) and kill the cell in two different ways which are referred to as apoptosis or necroptosis, respectively. See text for details.

Both forms of programmed cell death are initiated not at the TNF-RSC itself but at secondary cytoplasmic complexes (Cho et al., 2009; He et al., 2009; Micheau and Tschopp, 2003; Zhang et al., 2009). Upon dissociation from TNFR1, TRADD can either recruit or be replaced by FADD thus allowing procaspases 8 and 10 to associate with the complex, which

is referred to as complex II, where they are activated (Micheau and Tschopp, 2003). The active initiator caspase dimers can then directly cleave and activate effector caspases as for example caspase 3 or by cleaving Bid can engage a mitochondrial amplification loop. The latter induces the permeabilisation of the outer mitochondrial membrane and via the release of cytochrome C from the mitochondria leads to formation of the apoptosome, an activation platform for the initiator caspase 9 which can again activate effector caspases (Kantari and Walczak, 2011). These effectors can then cleave a great number of substrates thereby executing a form of cell death, called apoptosis, which is characterised by no intracellular content being released and which is thus considered non-immunogenic. Alternatively, a different secondary death-inducing complex, referred to as complex IIB or necrosome, can be formed especially in situations when the activity of caspases is blocked. In this case the main mediators of death induction are the kinases RIP1 and RIP3. The cell death pathway emanating from complex IIB is less well characterised than apoptosis induction by complex II but is thought to involve phosphorylation of downstream components and the production of ROS. Overall this pathway induces a type of cell death that is referred to as necroptosis and that results in bursting of the cell and therefore creates a pro-inflammatory milieu (Cho et al., 2009; Festjens et al., 2006; He et al., 2009; Vanlangenakker et al., 2012; Zhang et al., 2009). The transition from the TNF-RSC (complex I) to either of the death-inducing complexes has not been characterised well to date. The formation of the secondary complexes occurs upon prolonged stimulation with TNF and the deubiquitination of RIP1 seems to be an essential step in this process (O'Donnell et al., 2007). In addition, the formation of the necrosome and the induction of necroptosis requires the kinase activities of RIP1 and RIP3 (Cho et al., 2009; He et al., 2009; Holler et al., 2000; Vandenabeele et al., 2010). Other factors controlling the formation of death inducing complexes and the execution of cell death programmes include the crosstalk between the different TNF-induced signalling pathways. Pro-survival proteins that are up-regulated via the gene expression inducing pathways activated upon TNF stimulation can prevent aberrant death of a cell. This group includes the cellular FLICE like inhibitory protein (cFLIP) which on the one hand is recruited to complex II where it prevents the activation of caspase 8 homodimers thereby blocking the apoptosis pathway and which on the other hand, in a heterodimer with caspase 8, mediates RIP1 cleavage thus preventing necroptosis (Hu et al., 1997; Kreuz et al., 2001; Oberst et al., 2011). Overall TNF induces a complex

network of signalling pathways (Figure 9) that allows this cytokine to initiate its various biological effects. For the correct balance between the different arms to be maintained a tight regulation is required and many of the regulatory mechanisms are dependent on ubiquitination or deubiquitination events mediated by E3s such as cIAP1/2 or TRAF2 or DUBs like A20 or CYLD (Harhaj and Dixit, 2012; Wajant and Scheurich, 2011).

2.2.3.2 TNFR2 signalling

Although some of the components contributing to both TNFR1- and TNFR2-mediated signal transduction were first found to be associated with TNFR2, the complex associated with this receptor and the signalling pathways emanating from this protein assembly are much less understood than in the case of TNFR1. It was shown that TRAF2 interacts directly with the intracellular domain of TNFR2 and can thus serve as an adaptor for TRAF1, TRAF3, cIAP1 and cIAP2 (Rothe et al., 1995b; Rothe et al., 1994b). It can be assumed that the TAB/TAK- and IKK-complexes could be recruited in a similar ubiquitin-dependent manner as described for TNFR1-signalling although little data exists to support this hypothesis. Downstream of the signal initiating complex there are many similarities between TNFR1- and TNFR2-induced signalling. Like TNFR1, TNFR2 was reported to trigger the activation of NF-κB and JNK although with different kinetics and possibly less efficiently (Haridas et al., 1998; Jupp et al., 2001; Laegreid et al., 1994; Rothe et al., 1995b; Vandenabeele et al., 1995). In contrast to TNFR1, TNFR2 can also activate the non-canonical pathway of NF-κB activation (Rauert et al., 2010). Here, TRAF2 and cIAP1 are degraded in a cIAP1- and K48-ubiquitination dependent manner. Because they are components of a destruction complex targeting the NF-κB inducing kinase (NIK), this kinase is stabilised and can mediate phosphorylation of IKKα and p100 thereby initiating p100 processing to p52 and thus the generation of an active transcription factor (Li et al., 2002; Wu et al., 2005). In addition, TNFR2 can modulate TNFR1-induced cell death (Fotin-Mleczek et al., 2002; Grell et al., 1999; Vercammen et al., 1995; Weiss et al., 1997). Multiple mechanisms could contribute to this crosstalk. One of them is referred to as “ligand-passing” and suggests that by binding TNF, TNFR2 increases the local concentration of this cytokine in the vicinity of TNFR1 and eventually transfers the ligand to this receptor thereby favouring its prolonged stimulation and hence the induction of cell death (Tartaglia et al., 1993b). Another model includes the induction of TNF expression by activation of gene-expression inducing pathways upon TNFR2 stimulation. The

TNFR2 mediated degradation of TRAF2 and cIAP may in addition cause a shift in the balance between pro-survival and death-inducing signalling induced by TNFR1 and thus sensitise cells to TNFR1 induced cytotoxicity (Grell et al., 1999; Vercammen et al., 1995). In spite of its more restricted expression pattern TNFR2 can therefore significantly alter the outcome of TNF stimulation and changes in the TNFR1:TNFR2 ratio caused by an increase or decrease in the highly regulated expression of TNFR2 may therefore be a mechanism in controlling the overall signalling output (Wajant et al., 2003).

2.3 Three novel components of the TNF-RSC

In spite of the regulatory role exerted by TNFR2 most aspects of TNF-signalling are mediated by TNFR1 and all the different pathways contributing to the signal transduction network initiated by this receptor emanate from the receptor associated complex. A detailed knowledge of all the components of this multi-protein assembly is therefore crucial to gain an understanding of how signalling is initiated and regulated upon TNF stimulation. In an attempt to fully elucidate the composition of this complex, the native TNF-RSC was purified using a modified tandem affinity purification (moTAP) procedure. A form of TNF that contained a triple FLAG-tag followed by a PreScission cleavage site and a biotinylated Avi-tag was used to stimulate U937 cells and to isolate the TNFR-associated complex in a two-step precipitation process (Haas et al., 2009). Mass-spectrometric analysis of the purified complex identified not only most of the previously known TNF-RSC constituents but also revealed the presence of three novel components, SHANK-associated RH domain protein SHARPIN), heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-1) and HOIL-1 interacting protein (HOIP) (Gerlach et al., 2011; Haas et al., 2009).

2.3.1 SHARPIN

SHARPIN is a protein of 387 aminoacids that was first identified as an interactor of SHANK1 in a yeast-two-hybrid screen of a rat brain cDNA library (Lim et al., 2001). It was found that SHARPIN interacts with the ankyrin repeats of SHANK1 via its C-terminal part that contains an NZF- and an UBL-domain (Figure 10) while its N-terminal part mediates homodimerisation. It was therefore suggested that SHARPIN plays a role in regulating the complexity of the SHANK-based protein network in the postsynaptic density of excitatory synapses in the brain (Lim et al., 2001). However, expression of SHAPRIN is not restricted to

the brain and the first study investigating this protein in mice found it to be up-regulated in the gastric fundus of W/W^V mice that have a mutation in the tyrosine kinase KIT and a role for SHARPIN in the function of the interstitial cells of Cajal (ICC), which are gastrointestinal (GI) pacemaker cells that generate and propagate electrical slow waves, was suggested (Daigo et al., 2003). Later a spontaneous mutation in the *Sharpin* gene was found to be causative for the *chronic proliferative dermatitis mutation* (*cpdm*) phenotype (Wang et al., 2012). Cpdm mice are characterised by severe inflammation of several organs, especially the skin, by eosinophil accumulation, defects in lymphoid organ development and an altered Th1-Th2 balance with an increase of type 2 cytokines and impaired Th1 cytokine production (HogenEsch et al., 1993; HogenEsch et al., 1999; HogenEsch et al., 2001). In line with this SHARPIN was reported to be important for the production of proinflammatory cytokines and for the induction of Th1 differentiation by dendritic cells (Wang et al., 2012). On the intracellular level SHARPIN was reported to be a regulator of several different signalling pathways. It was found to associate with the phosphatase eyes-absent (EYA-1) which can also act as a transcriptional cofactor and to enhance its activity. This interaction was reported to be important for craniofacial development in zebrafish (Landgraf et al., 2010). In addition SHARPIN was reported to regulate the mitochondrial apoptosis pathway (Liang and Sundberg, 2011) and to be required for TLR2 induced signalling as shown by alterations in the phosphorylation of ERK and the translocation of p65 to the nucleus in the absence of SHARPIN (Zak et al., 2011). On the other hand, SHARPIN was also described to inhibit NF-κB activation (Liang et al., 2011) and to act as a negative regulator of integrin signalling by associating directly with the intracellular region of α-integrins thus impairing their binding to talin and kindlin thereby preventing β1-integrins from switching into their active conformation (Rantala et al., 2011). Furthermore, it was shown to negatively affect the phosphatase activity of PTEN in human tumour cell lines and to promote tumorigenesis (He et al., 2010).

2.3.2 HOIL-1

HOIL-1 was identified as an E3 ligase for oxidised iron regulatory protein 2 (IRP2) of 498 aminoacids (Yamanaka et al., 2003) and as a hepatitis B virus X-associated protein (XAP3) (Cong et al., 1997). Following a yeast two-hybrid screen of a rat cDNA library it was described as a RBCC protein interacting with PKC (RBCK1) (Tokunaga et al., 1998a) and finally it was found as a UbcM4-interacting protein (UIP28) in a murine system (Martinez-Noel et al., 1999). In addition, another start codon was identified in the mRNA of RBCK1 10 codons upstream of the original initiation site and the resulting protein was dubbed HOIL-1L (Kirisako et al., 2006). However, there is very limited data on differential expression patterns or functions of the two forms and the terms RBCK1 and HOIL-1 are widely used to refer to the longer form even by protein databases. In this thesis the 510 aminoacid form of the protein will be referred to as HOIL-1. Structurally, this protein includes an N-terminal UBL-domain, an NZF, two RING domains and an IBR domain (Figure 10), making it part of the RBR-family (Kirisako et al., 2006; Marin et al., 2004). The structures of the UBL- and NZF domain were solved and it was shown that the UBL assumes a ubiquitin fold followed by an α-helical segment (Uekusa et al., 2011) while the NZF contains a zinc-coordinating core and an additional α-helical NZF tail. This combination allows it to bind to M1-linked ubiquitin specifically by interacting with the Ile44 patch on the distal ubiquitin and a Phe4 centred hydrophobic patch on the proximal ubiquitin (Sato et al., 2011). Functionally, different activities have been reported for HOIL-1. It was described to shuttle between nucleus and cytoplasm and to possess transcriptional activity (Tatematsu et al., 1998; Tatematsu et al., 2005; Tokunaga et al., 1998b). Reported targets include estrogen receptor (ER) alpha and cyclin B1 which mediate an effect of HOIL-1 on cell cycle progression and on proliferation in ERα positive breast cancer cells (Gustafsson et al., 2010). This transcriptional activity was reported to be inhibited by the splice variant RBCK2, which lacks the RING domains of HOIL-1 and by tethering its active counterpart in the cytoplasm prevents it from acting as a transcription factor (Tokunaga et al., 1998b; Yoshimoto et al., 2005). Furthermore, transcriptional activity was described to be enhanced by PKA and to be repressed by MEK1 or MEKK1 (Tatematsu et al., 1998). Finally, phosphorylation by PKCβ was reported to cause auto-ubiquitination of HOIL-1 leading to its proteasomal degradation (Tatematsu et al., 2008). This ability to attach supposedly K48-linked chains to target proteins is the other activity by which HOIL-1 affects signalling. Targets include the transcription factors Bach-1

(Zenke-Kawasaki et al., 2007) and interferon regulatory factor (IRF3) (Zhang et al., 2008) as well as TAB2 and TAB3 (Tian et al., 2007). Degradation of these proteins was reported to cause an inhibitory effect of HOIL-1 on the antiviral interferon production and on NF-κB signalling, respectively (Tian et al., 2007; Zhang et al., 2008). Like the transcriptional activity the ability of HOIL-1 to act as an E3 is regulated by interaction with RBCK2 and by phosphorylation by PKC which was reported to lead to cleavage and thus inactivation of HOIL-1 (Nakamura et al., 2006; Tatematsu et al., 2008).

2.3.3 HOIP

HOIP which is also known as RNF31, zinc IBR finger UBA domain (ZIBRA) or putative ARIADNE like ubiquitin ligase (PAUL) was identified as a potential E3 ligase which is highly expressed on the mRNA level in different kinds of cancers (Thompson et al., 2004) and as an interactor of the muscle-specific receptor tyrosine kinase (MuSK) (Bromann et al., 2004). Like HOIL-1 it contains two RING-domains and an IBR-domain which makes it a member of the RBR-family of E3 ligases (Marin et al., 2004). In addition, HOIP contains a ZnF-, two NZF- and a UBA-domain (Figure 10). It was reported that HOIP associates with the atypical orphan receptor DAX-1 and in complex with this protein acts as a corepressor at the promoters of the StAR and CYP19 genes by interacting with the nuclear orphan receptor steroidogenic factor 1 (SF-1). Thus HOIP was suggested to regulate metabolism and steroid hormone synthesis and was also implicated in the transcriptional regulation of the Wnt pathway (Ehrlund et al., 2009; Ehrlund et al., 2012). Furthermore, HOIP was found to be recruited to the CD40-RSC and experiments using cells deficient in HOIP or expressing a variant lacking E3 activity showed that HOIP is required for NF-κB- and JNK-activation by this receptor (Gerlach et al., 2011; Hostager et al., 2010; Hostager et al., 2011).

2.3.4 LUBAC

In 2006 it was found that HOIL-1 and HOIP interact and form an E3-complex of approximately 600 kDa. The interaction was mapped to their UBL- and UBA-domains respectively (Kirisako et al., 2006) and structural analysis revealed that the two domains bind to each other in a non-canonical manner using different surfaces than the ones usually involved in UBL-UBA-interactions (Yagi et al., 2012). The distinguishing property of this E3-complex is that it can generate M1-linked ubiquitin chains as demonstrated by its ability to use lysine-less (K0) but not methylated ubiquitin as a substrate. Due to this role in

generating linear ubiquitin chains, the complex was dubbed linear ubiquitin chains assembly complex (LUBAC) (Kirisako et al., 2006). It was found that the E3-activity of the complex resides in the RING-domains of HOIP but not HOIL-1 (Kirisako et al., 2006). As described in section 2.1.2.2.8 linear chains were first described to have a role in proteasomal degradation and LUBAC was thereby implicated in the regulation of the RIG-I pathway and of conventional PKC (Inn et al., 2011; Kirisako et al., 2006; Nakamura et al., 2006; Prakash et al., 2009; Thrower et al., 2000; Zhao and Ulrich, 2010). Later it was found that LUBAC is a positive regulator of NF-κB activation in the context of different stimuli such as TNF, IL-1 and genotoxic stress (Haas et al., 2009; Niu et al., 2011; Tokunaga et al., 2009) and that it also regulates other signalling pathways such as the activation of JNK downstream of TNF-stimulation (Haas et al., 2009).

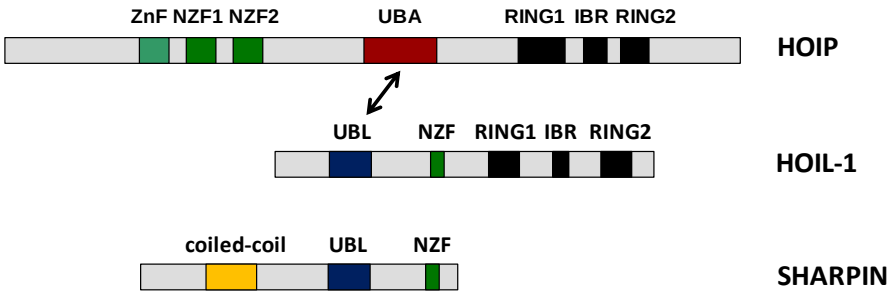


Figure 10: Schematic representation of the domain structures of SHARPIN, HOIL-1 and HOIP. There is significant sequence homology (45 % identity) between the carboxyl terminus of SHARPIN and the amino terminus of HOIL-1 (Lim et al., 2001), each of which contains a UBL- and an NZF-domain. Both HOIL-1 and HOIP contain an RBR-motif making them members of the RBR-family of E3s (Marin et al., 2004). It was found that these two proteins interact via their UBL- and UBA-domains respectively thereby forming the E3 complex referred to as LUBAC (Kirisako et al., 2006). ZnF: zinc finger; NZF: Npl4 zinc finger; UBL: ubiquitin-like domain; UBA: ubiquitin-associated domain; IBR: in-between RING domain. RING: really interesting new gene.

3 Aims

The identification of peptides for SHARPIN together with HOIL-1 and HOIP in the mass-spectrometric analysis of the native TNF-RSC raised the question whether it is a functional component of this complex.

This study therefore aimed to investigate whether SHARPIN, along with the other two novel components found by mass-spectrometry, i.e. HOIL-1 and HOIP, is recruited to TNF-receptors in a stimulation-dependent manner and to unravel which other components of the complex this recruitment depends on. In this context the question how SHARPIN influences TNF-induced signalling was to be answered.

Furthermore, the presence of SHARPIN alongside the LUBAC-components HOIL-1 and HOIP together with the similarity of its sequence to that of HOIL-1 indicated the possibility that SHARPIN might affect the activity of LUBAC. To test this hypothesis it was to be investigated whether SHARPIN interacts with this E3-complex. In case such an interaction was identified, a potential role for SHARPIN as a modulator of LUBAC's activity was to be analysed.

In brief the aims of this study were to

1. investigate the mechanism by which SHARPIN, HOIL-1 and HOIP are recruited to the TNF-RSC
2. test the influence of SHARPIN on TNF-induced signalling
3. analyse a potential role of SHARPIN as an active part or an inhibitor of LUBAC
4. further characterise the activity of LUBAC

4 Materials and Methods

4.1 Materials

4.1.1 Chemicals and reagents

All chemicals and biologically reactive reagents were purchased from Roth, Sigma/Aldrich (Fluka), Merck (Calbiochem), Invitrogen (Gibco; Molecular Probes), AppliChem, Pierce, Honeywell (Riedel-de Häen) or Amersham Biosciences in pA quality unless indicated otherwise.

4.1.2 Specific inhibitors

4.1.2.1 Inhibitors used in the purification of recombinant proteins

All protease inhibitors used in the purification of recombinant proteins were purchased from Sigma. Specifically the following inhibitors were used: AEBSF, Aprotinin, E-64, Leupeptin and Pepstatin.

4.1.2.2 Inhibitors used in the lysis of eukaryotic cells

Proteases were inhibited by using the Complete Protease Inhibitor Cocktail obtained from Roche; Phosphatase Inhibitor Cocktail from Sigma was employed to impair the activity of phosphatases.

4.1.3 Buffers and solutions

4.1.3.1 General buffers

- 1x PBS:
  137 mM NaCl
  8.1 mM Na_2HPO_4
  2.7 mM KCl
  1.5 mM KH_2PO_4
  pH 7.4

4.1.3.2 Buffers used in the cloning and purification of recombinant proteins

- TAE buffer (10x):
  400 mM Tris/HCl
  200 mM Acetic Acid
  10 mM EDTA

- Bacteria lysis buffer (GST-purification):
  1x PBS
  70 µM AEBSF
  5 µM E-64
  1.2 µg/mL Aprotinin
  1 µM Pepstatin
  1 mM DTT

- GST-wash buffer:
  1x PBS
  1 mM DTT

- GST-elution buffer:
  1x PBS
  50 mM reduced glutathione

- PreScission cleavage buffer:
  50 mM Tris-HCl
  150 mM NaCl
  1 mM EDTA
  1 mM DTT
  pH 7.0

- Dialysis buffer (ligands)
  50 mM Trizma Base
  100 mM NaCl
  0.02 % Tween 20 (v/v)
  2 mM DTT
  0.5 M L-Arginine

- Dialysis buffer (other proteins):
  1x PBS
  1 mM DTT

- Bacteria lysis buffer (His-tagged proteins):
  50 mM Tris/HCl
  200 mM NaCl
  100 mM KCl
  10 % Glycerol (v/v)
  0,5 % Triton X100
  2 mM β-Mercaptoethanol
  70 µM AEBSF
  5 µM E-64
  1.2 µg/mL Aprotinin
  1 µM Pepstatin A

- His-wash buffer
  50 mM Tris/HCl
  300 mM NaCl
  25 mM imidazole
  pH 7.5

- His-elution buffer
  50 mM Tris/HCl
  300 mM NaCl
  500 mM imidazole
  pH 7.5

4.1.3.3. Buffers used for freezing, transfecting and lysing eukaryotic cells

- Freezing medium:	90 % FCS (v/v) 10 % DMSO (v/v)
- 2x HBS:	50 mM HEPES 280 mM NaCl 1.5 mM Na_2HPO_4 x 2 H_2O pH 7.0
- IP-lysis buffer:	30 mM Trizma Base 120 mM NaCl 2 mM EDTA 2 mM KCl 10 % Glycerol (v/v) 1 % Triton X-100 (v/v) pH 7.4 + Complete protease inhibitor cocktail

4.1.3.4. Buffers used for gelfiltration

- Lysis buffer gelfiltration:	50 mM Tris/HCl (pH 7.5) 1 mM $MgCl_2$ 1 mM DTT + Complete protease inhibitor cocktail
- Running buffer gelfiltration:	50 mM Tris/HCl (pH 7.5) 1 mM $MgCl_2$ 1 mM DTT 150 mM NaCl + Complete protease inhibitor cocktail

4.1.3.5. Buffers for SDS-PAGE and western blotting

- Running buffer (MOPS)	1:20 dilution of 20x MOPS (Invitrogen)
- Transfer buffer:	192 mM Glycine 25 mM Trizma Base 0.01 % EDTA (w/v) 20 % Methanol (v/v)
- Blocking buffer:	1x PBS 5 % milk powder (w/v) 0.05 % Tween-20 (v/v)
- Wash buffer:	1x PBS 0.05 % Tween-20 (v/v)
- Stripping buffer:	50 mM Glycine pH 2.3

4.1.3.6. Buffers for Luciferase assays

- Passive lysis buffer:	1:5 dilution of 5x passive lysis buffer (Promega)
- Luciferase assay buffer A:	20 mM HEPES 33.3 mM DTT 8 mM MgCl2 130 µM EDTA 530 µM ATP 470 µM Luciferin 270 µM Coenzyme A pH 7.8
- Luciferase assay buffer B:	15 mM $Na_4P_2O_7$ 7.5 mM NaOAc 400 mM $NaSO_4$ 10 mM CDTA 25 µM APMBT 1 % Methanol (v/v) 2 µM Benzyl-Coelenterazin pH 5.0
- MTT solution:	2.5 mg/mL MTT in PBS

4.1.3.7 Buffers for ubiquitin-related in-vitro assays

- Pull-down buffer:	150 mM NaCl 50 mM Trizma Base (pH 7.5) 5 mM DTT 0.1 % NP-40 (v/v)
- Ubiquitylation buffer:	20 mM Trizma Base (pH 7.5) 5 mM $MgCl_2$ 2 mM DTT
- DUB dilution buffer:	150 mM NaCl 25 mM Tris (pH 7.5) 10 mM DTT
- 10x DUB reaction buffer:	500 mM NaCl 500 mM Tris (pH 7.5) 50 mM DTT

4.1.4 Antibodies

4.1.4.1 Primary antibodies

Table 1: Primary antibodies. WB: western blotting; IP: immunoprecipitation

antibody	isotype	Source	application(s)
anti-Actin (A5441)	mIgG1	Sigma	WB
anti-cIAP2	rat	Gift from John Silke	WB
anti-cIAP Pan (MAB3400)	mIgG2a	R&D Systems	WB
anti-FLAG (M2)	mIgG1	Sigma	WB, IP
anti-GAPDH	mIgG1	Abcam	WB
anti-GST	goat	GE Healthcare	WB
anti-HOIL-1	mIgG2a	Produced in the lab	WB, IP
anti-HOIP	rabbit	Sigma	WB, IP
anti-HOIP	rabbit	Eurogentech	WB
anti-IκBα (C-15)	mIgG1	Santa Cruz	WB
anti-myc (9E10)	mIgG1	Abcam	WB, IP
anti-NEMO (FL419)	rabbit	Santa Cruz	WB
anti-NEMO (B3)	mIgG1	Santa Cruz	WB
anti-pIκB (5A5)	mIgG1	Cell Signaling	WB
anti-pJNK (98F2)	rabbit	Cell Signaling	WB
anti-pp38(3D7)	rabbit	Cell Signaling	WB
anti-RIP1	mIgG1	Pharmingen	IP
anti-RIP1 (clone 38)	mIgG2a	BD Biosciences	WB
anti-SHARPIN	mIgG1	Produced in the lab	WB, IP
anti-SHARPIN	rabbit	Gift from Ivan Dikic	WB
anti-TNF-R1 (H-5)	mIgG2b	Santa Cruz	WB, IP
anti-TNF-R1 (ab19139)	rabbit	Abcam	WB
anti-TNFR2	rabbit	Cell Signaling	IP
anti-TNFR2	rat	Pharmingen	WB
anti-TRADD (clone 37)	mIgG1	BD Biosciences	WB
anti-TRADD (H-278)	rabbit	Santa Cruz	WB
anti-TRAF2 (C-20)	rabbit	Santa Cruz	WB
anti-TRAF2 (AP1040)	rabbit	Calbiochem	WB
anti-TRAF6	IgG1	Cell Signaling	WB
anti-TRAF6	rabbit	Epitomics	WB
anti-UBE2D3	IgG1	Abnova	WB
anti-Ubiquitin (FK1)	mIgM	Biomol	WB
anti-Ubiquitin (FK2)	mIgG1	Biomol	WB
anti-Ubiquitin (#07-375)	rabbit	Millipore	WB
anti-V5 (V5-10)	rabbit	MBL	WB, IP
anti-V5	mIgG2a	Santa Cruz	WB

4.1.4.2 Secondary antibodies

- Goat-anti-mIgG1-HRP (SouthernBiotech)
- Goat-anti-mIgG2a-HRP (SouthernBiotech)
- Goat-anti-mIgG2b-HRP (SouthernBiotech)
- Goat-anti-mIgM-HRP (SouthernBiotech)
- Goat-anti-rat-HRP (SouthernBiotech)
- Rabbit-anti-goat-IgG-HRP (Santa Cruz)
- Goat-anti-rabbit-IgG-HRP (SouthernBiotech)

4.1.5 Beads for precipitations

- anti-Myc agarose (Sigma)
- anti- NEMO beads (Santa Cruz)
- anti-V5 Agarose (clone V5-10) (Sigma)
- FLAG M2 affinity Gel (Sigma)
- glutathione beads (GE Healthcare)
- Protein G Sepharose (GE Healthcare)
- ubiquitin-coupled agarose (Boston Biochem)

4.1.6 Recombinant proteins

- K48-linked tetra-ubiquitin (Enzo Life Sciences)
- K63-linked tetra-ubiquitin (Enzo Life Sciences)
- linear tetra-ubiquitin (Enzo Life Sciences)
- PreScission Protease (GE Healthcare)
- Thrombin (GE Healthcare)
- TRAF2 (Signal Chem)
- USP2 catalytic domain (Enzo Life Sciences)
- UBE1 (Enzo Life Sciences)
- E2s (Enzo Life Sciences)

4.1.7 Commercially available kits and solutions

- ABsolute QPCR ROX Mix (ABgene)
- BCA Protein Assay (Pierce)
- ECL Western Blotting Detection (GE Healthcare)

- ECL Western Blotting Detection Plus (GE Healthcare)
- Chemoluminescent Substrate SuperSignal West Dura (Pierce)
- Chemoluminescent Substrate SuperSignal West FEMTO (Pierce)
- E.Z.N.A. Plasmid Mini Kit (Omega Bio-Tek)
- E.Z.N.A. Plasmid Maxi Kit (Omega Bio-Tek)
- QIAquick Gel Extraction kit (Qiagen)
- QIAquich PCR purification kit (Qiagen)
- RevertAidTMH Minus first strand cDNA synthesis kit (Fermentas)
- TNT Quick coupled Transcription/Translation System (Promega)
- TrypanBlue (Serva)
- pcDNA3.1V5/His TOPO Directional Expression kit (Invitrogen)
- SuperScript III Reverse Transcriptase (Invitrogen)
- tissue lysates (IMGENEX)

4.1.8 Bacteria strains

DG1 chemically competent cells (Eurogentech) were used for plasmid amplification and expression of recombinant proteins was performed in BL21(DE3) cells (Invitrogen).

4.1.9 Cell culture media and antibiotics

- Ampicillin (Roth)
- β-mercaptoethanol (Invitrogen)
- Dulbecco's Modified Eagle Medium (Invitrogen)
- Hygromycin B (Boehringer)
- Insulin (Sigma)
- Penicillin/Streptomycin (Invitrogen)
- RPMI 1640 (Invitrogen)
- Sodium Pyruvate (Invitrogen)
- Trypsin/EDTA solution (Invitrogen)

4.1.10 Cell lines

Table 2: Cell lines

cell line	Description	reference
HEK 293T	Human embryonic kidney cells transformed with sheared adenovirus 5 DNA.	(Graham et al., 1977)
HEK293-NF-κB	Cell line purchased from Panomics that was obtained by cotransfection of a luciferase reporter construct containing multiple copies of the NF-κB response element and a hygromycin selection marker into HEK293 cells, followed by hygromycin selection.	Panomics
HeLa	Cell line isolated from an adenocarcinoma of the cervix in 1952.	(Scherer et al., 1953)
HT1080	Sarcoma cell line, derived from a 35-year-old male with fibrosarcoma.	(Rasheed et al., 1974)
HaCat	Cell line resulting from spontaneous in-vitro transformation of keratinocytes from histologically normal skin.	(Boukamp et al., 1988)
Saos-2	Osteosarcoma cell line with epithelial morphology that was isolated from an 11-year-old female patient.	(Fogh et al., 1977a; Fogh et al., 1977b)
IGROV-1	Cell line originating from an ovarian carcinoma of a 47-year-old woman.	(Benard et al., 1985)
DKO-4	Colorectal adenocarcinoma cell line derived from DLD-1 cells by targeted disruption of its mutant Ki-Ras allele.	(Shirasawa et al., 1993)

DU145	Cell line that was originally derived from a human prostate adenocarcinoma that metastasized to the brain.	(Stone et al., 1978)
A549	This line was initiated in 1972 by culturing of lung carcinomatous tissue from a 58-year-old male.	(Giard et al., 1973)
MCF-7	Cell line obtained by pleural effusion from a female patient with metastatic breast cancer.	(Brooks et al., 1973)
JA-3	The Jurkat cell line was first established from the peripheral blood of a 14-year old boy suffering from T cell leukemia.	(Schneider and Schwenk, 1977; Schneider et al., 1977)
Raji	Human lymphoblast-like cell line which was established from a Burkitt's lymphoma of the left maxilla of an 11-year-old male.	(Pulvertaft, 1964)
U937	Cell line derived from malignant cells obtained from the pleural effusion of a patient with histiocytic lymphoma. Terminal monocytic differentiation can be induced.	(Sundstrom and Nilsson, 1976)
THP-1	Cell line with distinct monocytic markers that was derived from the peripheral blood of a patient with acute monocytic leukemia.	(Tsuchiya et al., 1980)
MEF	Wt and knockout MEFs were generated from E15 embryos in accordance with standard procedures and were infected with SV40 large T antigen-expressing lentivirus. Cells with inducible expression of cIAP1 or TRAF2 were described previously.	(Feltham et al., 2010; Mace et al., 2008; Vince et al., 2009)

4.1.11 Additional materials

- Cuvettes (Greiner Bio One)
- Cryogenic vials (Nunc)
- Dialysis Membrane 12,000-14,000 MWCO (Roth)
- GSTrap FF (GE Healthcare)
- HisTrap FF (GE Healthcare)
- Hybond ECL Nitrocellulose Membrane (Amersham Bioscience)
- NuPAGE 4-12 % Bis-Tris Gels (Invitrogen)
- 4x LDS-Sample buffer (Invitrogen)
- PCR tubes (StarLab)
- Plastic pipettes (5 mL, 10 mL and 25 mL) (StarLab)
- Pipette tips (0.1-10, 1-200, 101-1000 µL) (StarLab)
- 50 mL Reagent Reservoir (Corning Inc.)
- Safe-Lock Reaction Tubes (1.5mL, 2 mL) (Eppendorf)
- SeeBlueTM Plus2 Pre-Stained Standard (Invitrogen)
- SmartLadder DNA Standard (Eurogentech)
- Sterile filter (0.22 µm and 0.45 µm pore size) (Millipore)
- Superdex 200 10/300 GL column (GE Healthcare)
- Tissue culture equipment (TPP)
- Whatman paper (Schleicher&Schuell)
- X-Ray film HyperfilmTM ECL (Amersham Bioscience)

4.1.12 Instruments

- Biofuge Stratos (Heraeus)
- Blotting equipment X cell IITM (Novex)
- Electrophoresis chamber (Biorad)
- GelSystem Flexi 4040 Biostep
- Hyper Processor X-Ray film Developer (Amersham Bioscience)
- Incubator Stericult 200 (Forma Scientific)
- Microscope Axiovant 25 (Zeiss)
- Mithras Luminometer LB 940 (Berthold Technologies)
- Multifuge 3S-R (Heraeus)

- Multiskan Ascent (Thermo Labsystems)
- Multitron Incubator Shaker (Appropriate Technical Resources)
- NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies)
- Photometer Ultrospec 3100 pro (Amersham Bioscience)
- Sonifier (Branson Ultrasonics Corporation)
- Mastercycler Pro (Eppendorf)
- Äkta Purifier (GE Healthcare)
- peristaltic pump P1 (Pharmacia Fine Chemicals)

4.2 Methods

4.2.1 Methods in Molecular Biology

4.2.1.1 RNA purification

Total RNA 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).

4.2.1.2 cDNA preparation

A reverse transcription was performed on 5 µg total RNA for 1 h at 42°C using the RevertAidTM H Minus first strand cDNA synthesis kit (Fermentas) according to the manufacturer's instructions.

4.2.1.3 Quantitative Real-time PCR

Quantitative Real-time PCR was performed with the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) using the ABsoluteTM 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. For the RT-PCR reaction, cDNA was diluted 1:10. Amplification was carried out in stages of incubation at 95°C for 15 min following 40 cycles of 95°C for 15 s and 60°C for 60 s. The mRNA levels were measured as double determinations and normalised with reference to the amount of housekeeping gene transcripts (GAPDH or HPRT1). The following gene-specific primers were used: HOIP: cttctgtgtgcgctgcaa and ttctggaagtcctcacagctc;

HOIL-1: gcctgaggtctccccaac and ggtgacggtgtgcatctg; SHARPIN: cggaagcctccacactca and tccctgccagctcttctc; GAPDH: agccacatcgctcagaca and gcccaatacgaccaaatcc; HPRT1: tgaccttgatttattttgcatacc and cgagcaagacgttcagtcct.

4.2.1.4 Polymerase Chain Reaction (PCR)

All PCRs were set up in a volume of 50 µL, which contained 1-50 ng plasmid-DNA or cDNA as template, 0.4 µM of each oligonucleotide, 200 µM of each dNTP, 2.5 U Polymerase in 1x Polymerase Buffer. The polymerase used was PfuUltra (Stratagene). If possible, the synthetic oligonucleotides were designed with 40-60 % GC content, no internal structure or complementarity at the 3'-ends. Annealing temperatures were chosen ~5-10°C lower than the melting temperature Tm and elongation times were calculated based on an elongation rate of 1 kb/min.

The following oligonucleotides were used:

Table 3: Oligonucleotides used in PCR reactions. Restriction sites are underlined, stop codons are in bold print and reverse primers are presented in upper case.

insert	vector	Primers	restriction sites used
SHARPIN	pcDNA3.1 D/V5-His-TOPO	- CACCatggcgccgccagcg - GGTGGAAGCTGCAGCAAGGG	TOPO cloning
SHARPIN	pGEX6-p2	- atggcgccgccagcgggc - atatGCGGCCGC**tca**GGTGGAAGCTGCAGCAAGGGGGTCC	SmaI + NotI
SHARPIN-V5	pGEX6-p2	- atggcgccgccagcgggc - atatGCGGCCGC**tca**CGTAGAATCGAGACC	SmaI + NotI
HOIL-1	pcDNA3.1 D/V5-His-TOPO	- CACCatggacgagaagaccaagaaagc - GTG GCA GTT CTG ACA GCT TGG	TOPO cloning
HOIL-1	pGEX6-p2	- atggacgagaagaccaagaaagc - atatGCGGCCGC**tca**GTG GCA GTT CTG ACA GCT TGG	SmaI + NotI
HOIL-1-V5	pGEX6-p2	- atatGAATTCatatggacgagaagaccaagaaagc - atatATTTAAAT**tca**CGTAGAATCGAGACCGAGGAGAGG	EcoRI + SwaI (PCR) / SmaI (vector)
HOIP	pGEX6-p2	- atgccgggggaggaag - atatGCGGCCGC**tca**CTTCCGCCTGCGGG	SmaI + NotI
HOIP-V5	pGEX6-p2	- atatGAATTCatatgcccgggggaggaagagg - atatATTTAAAT**tca**CGTAGAATCGAGACCGAGGAGAGG	EcoRI + SwaI (PCR) / SmaI (vector)
HOIP ZnF only	pGEX6-p2	- agtgctcatttgccctggcactgtgc - atatGCGGCCGC**tca**ACAGCCTCGGGGCCGATCACAGG	SmaI + NotI
HOIP NZF1 only	pGEX6-p2	- gcacggggtcggtgggcc - atatGCGGCCGC**tca**GGCCAGCCGAGGTCGCTCACATATGG	SmaI + NotI

HOIP NZF2 only	pGEX6-p2	- agagtcaagtctggtactgtattcactgtacc - atatGCGGCCGC**tca**GGGGCTACTAGTCCGGTTGC	SmaI + NotI
cIAP1	pGEX6-p2	- atgcacaaaactgcctccc - atatGCGGCCGC**tca**AGAGAGAAATGTACGAACAGTACCC	SmaI + NotI
cIAP2	pGEX6-p2	- atgaacatagtagaaaacagcatattcttatcaaatttg - atatGCGGCCGC**tca**TGAAAGAAATGTACGAACTGTACCCTT	SmaI + NotI
RNF5	pGEX6-p2	- atggcagcagcggaggaggagg - atatGCGGCCGC**tca**aatactgagcagccaaaaaaagaagaagatgg	SmaI + NotI
PARKIN	pGEX6-p2	- atgatagtgtttgtcaggttcaactcc - atatGCGGCCGC**tca**CACGTCGAACCAGTGGTC	SmaI + NotI
PARKIN RBR only	pGEX6-p2	- atgcggaacatcacttgcattacg - atatGCGGCCGC**tca**CCACTCGCAGCCAC	SmaI + NotI
ARIH1 RBR only	pGEX6-p2	- atggcacaggatatgccttgtc - atatGCGGCCGC**tca**CCATGGGCCAAGACAC	SmaI + NotI
ARIH2	pGEX6-p2	- atgtcagtggacatgaatagccag - atatGCGGCCGC**tca**GGTGTCATGGAAATCTTTCAGCAG	SmaI + NotI
ARIH2 RBR only	pGEX6-p2	- atgcctcaccactgtgcag - atatGCGGCCGC**tca**CCAATCTCCTAGACACATCC	SmaI + NotI
RNF19A	pGEX6-p2	- atgcaagaacaagaaataggttttatctctaaatataatg - atatGCGGCCGC**tca**AATTTCAGTCTGAATTGCAACTTTTAATTCC	SmaI + NotI
RNF144B	pGEX6-p2	- atgggctcagctggtagg - atatGCGGCCGC**tca**GGTTGTGGATGGGTCGTG	SmaI + NotI
RNF216	pGEX6-p2	- atggaagagggaaacaacaatgaag - atatGCGGCCGC**tca**GAAGCGATGCCGCGG	SmaI + NotI
RNF14	pGEX6-p2	- atgtcgtcagaagatcgagaagc - atatGCGGCCGC**tca**GTCTTCTACCTCATCTTCCCAAATATCG	SmaI + NotI
PTEN	pcDNA3.1 D/V5-His-TOPO	- CACCatgacagccatcatcaaagagatcgttagc - GAC TTT TGT AAT TTG TGT ATG CTG ATC TTC ATC AAA AGG	TOPO cloning
ABIN-1	pcDNA3.1 D/V5-His-TOPO	- CACCatggaagggagaggaccgtaccg - CTG AGG CCC CTC ACG GTC ATT TTT TGG	TOPO cloning

4.2.1.5 Site-directed Mutagenesis

To introduce deletions or point mutations by PCR, primers containing the point mutation were designed. These oligonucleotides contained the desired mutations with ~10-15 bases of correct sequence on both sides. Following the PCR, the reaction was treated with DpnI endonuclease for 1 hour at room temperature to cut unmethylated DNA. The linearised, mutated plasmid was then transformed into competent bacteria (Section 4.2.1.10).

The oligonucleotides used to introduce mutations were:

Table 4: Oligonucleotides used to introduce mutations. Restriction sites are underlined; introduced mutations are shown in upper case and bold print.

protein	mutation	primers
SHARPIN	deletion of UBL-domain	- caggatggggaccctgctttcctc - ggggccaggtgggaagcaggcctc
SHARPIN	deletion of NZF-domain	- acttgggacccccttgctgcagcttc - tggactgggcaggctggaggcagc
SHARPIN	C353,356S	- ccagctggtcct**C**tccttcct**C**caccttcatcaatgcc - ggcattgatgaaggtg**G**aggaagga**G**aggaccagctgg
SHARPIN	C367,370S	- ccagaccgccctggct**C**tgagat**C**tgtagcacccagagg - cctctgggtgctaca**G**atctca**G**agccagggcggtctgg
HOIP	deletion of the UBA-domain	- cgcctagagcccttccgccag - gccatgacgatccagccaggc
HOIP	C699,702S	- cgcttgcttgcccaggagt**C**tgccgtgt**C**tggctgggccctgccccac - gtggggcagggcccagcca**G**acacggca**G**actcctgggcaagcaagcg
HOIP	C871,874S	- ggaaaacggcattgact**C**ccccaaat**C**caagttctcgtacgcc - ggcgtacgagaacttgGatttggggGagtcaatgccgttttcc
HOIP	C885S	- cgagagcctagagccag - atatgcatgc**T**gcctcctc

4.2.1.6 DNA digestion and restriction analysis

Sequence-specific cleavage of DNA molecules was performed using specific restriction endonucleases of the FastDigest® system obtained from Fermentas. Restriction reactions were incubated at 37°C for 1 hour.

4.2.1.7 Agarose gel electrophoresis of nucleic acids

The analysis or separation of DNA fragments was performed by agarose gel electrophoresis. Gels were prepared by dissolving 1-2 % agarose (w/v) in TAE buffer. DNA fragments were resolved by electrophoresis which was performed in a gel chamber filled with TAE buffer at 10 Volts per cm of electrodal distance. After electrophoretic separation the gel was incubated for 30 min in TAE buffer + ethidium bromide (400 ng/mL) and DNA fragments were visualised by UV light (λ = 254 nm).

4.2.1.8 Gel extraction of DNA fragments

The appropriate bands were cut out from the agarose gel and the isolation of DNA was achieved using the QIAquick Gel Extraction kit (Qiagen) according to manufacturer's

instructions. DNA was eluted in 30-50 µL ddH_2O and directly employed in following applications.

4.2.1.9 Ligation of DNA fragments

Digested vector and insert were mixed in ratios ranging from 1:3 to 1:7. The ligation reaction was carried out in 11 µL total volume containing 1 µL T4 DNA ligase (NEB), 1 µL PEG4000 and 1.1 µL 10x Ligase buffer (NEB). The reaction volume was brought up to 11 µL with ddH_2O. Ligation reactions were incubated at room temperature for at least 1 hour and between 2 and 5 µL were directly used to transform competent bacteria.

4.2.1.10 Transformation of competent E. coli bacteria

Competent bacteria were thawed on ice and an appropriate amount of plasmid DNA or of a ligation reaction were added to the bacteria followed by incubation on ice for 30 min. After a heat shock that was achieved by incubating the bacteria at 42°C for 1 min the bacteria were cooled on ice for 5 min. 300 µL SOC medium were added and the bacteria suspension was incubated at 37°C for 1 hour. Afterwards, bacteria were plated on LB agar plates containing the antibiotic required for selection and incubated at 37°C overnight.

4.2.1.11 Isolation of Plasmid-DNA

For plasmid isolation on an analytical scale 5 mL LB medium (+ antibiotic) were inoculated with a single bacterial colony and incubated at 37°C overnight. Next, the plasmid DNA was isolated using the QIAprep Spin Mini Kit (Qiagen) according to the manufacturer's instructions. For the production of larger amounts of plasmid DNA, 400-500 mL LB medium, supplemented with an appropriate antibiotic, were inoculated and the culture was grown at 37°C and under constant shaking (170rpm) overnight. The preparation of plasmid DNA was carried out with the E.Z.N.A. Plasmid purification Maxi kit (Omega bio-tek) as per manufacturer's protocol.

4.2.2 Methods of Cell Biology

4.2.2.1 Cell culturing conditions

All adherent cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) in a humidified atmosphere containing 10 % CO_2 at 37 °C. For MCF-7 cells 1 % pyruvate and 0.1 % human insulin were added to the cultivation medium, while the medium for DKO-4 and HaCat cells was supplemented with

2 mM Glutamine. A 1x Trypsin/EDTA solution was used to regularly detach and dilute the adherent growing cells. Suspension cells were maintained in RPMI with 10 % FBS at 5 % CO_2 and 37°C. Cell density was determined with a Neubauer chamber slide. Cells were centrifuged with a refrigerated centrifuge (Heraeus Multifuge 3 S-R) for 4 min at 300 x g and 4°C.

4.2.2.2 Freezing and thawing of eukaryotic cells

For freezing, cells were harvested, pelleted and resuspended in freezing medium at a concentration of approximately $2x10^6$ cells/mL. The cryogenic vials were then slowly cooled down to -80°C and transferred to liquid nitrogen for long-term storage.

Cells were thawed rapidly to 37°C and fresh culture medium was added immediately. Cells were centrifuged and resuspended in fresh medium and transferred to cell culture flasks.

4.2.2.3 Transfection of adherent cells with plasmid DNA or siRNA

HEK293 NF-κB cells were transfected using FuGene 6 (Roche Applied Science) according to the manufacturer's protocol. HEK293T cells were transfected by the calcium phosphate method. For this purpose, 450 µL H2O were added to a solution of plasmid DNA and 50 µL $CaCl_2$ (2 M). 500 µL HBS (2x) were added dropwise to the DNA/ $CaCl_2$ mix. After 30 min incubation at room temperature, the transfection mixture was added slowly to the cell medium which had previously been supplemented with Chloroquine at a concentration of 25 µM. Transient knockdowns were performed using siRNAs obtained from Dharmacon (Thermo Fisher) which were transfected using Dharmafect I (Thermo Fisher) according to the manufacturer's protocol. Briefly, per six-well, 1.5 µL Dharmafect were mixed with 200 µL DMEM without FBS for 10 min at room temperature. Subsequently, 2.2 µL siRNA with a concentration of 20 µM were added and incubated for 30 min at RT. The mix was added dropwise to the cells and 1 mL of culture medium was added. For the knockdown of SHARPIN and HOIL-1 the respective siRNA smartpools were employed whereas for HOIP a single siRNA (#4) was used. The shRNA for HOIL-1 had the sequence 5'-CCACAACACTCATCTGTCAAA-3´.

4.2.2.4 Inducible Protein Expression

cIAP1/2-deficient MEFs reconstituted with an inducible lentiviral system for the expression of wild-type or mutant cIAP1 as well as TRAF2/5-deficient MEFs containing an inducible

system for TRAF2 mutants were kindly provided by John Silke (Mace et al., 2008; Vince et al., 2009). Expression of the respective proteins and their mutants was induced by the addition of 4-hydroxy-tamoxifen at a final concentration of 20 nM for 20 hours.

4.2.2.5 Treatment with Smac mimetics

SM-164 (Smac059) was synthesised and kindly provided by Pierfausto Seneci and Leonardo Manzoni (Cossu et al., 2009). Before TNF stimulation, cells were pre-treated with SM-164 at a final concentration of 100 nM in cell culture medium for 2 hours at 37°C.

4.2.3 Biochemical methods

4.2.3.1 Determination of protein content

To determine the protein concentration of cell lysates, the bicinchoninic acid (BCA)-containing protein assay was applied (Pierce). For this purpose, 2.5 µL of the lysate was incubated in 0.5 mL BCA solution at 60°C for 20 min, followed by measuring light absorption at 540 nm.

4.2.3.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of proteins was performed using 4-12 % Bis-Tris-NuPAGE gels from Invitrogen. Samples were mixed with LDS Sample buffer containing a final concentration of 200 mM DTT and heated for 10 min at 75°C. The SeeBlueTM Plus2 Pre-Stained marker (Novex) was used as a molecular weight standard. The electrophoretic separation was carried out at a constant voltage of 125 V for 10 min and subsequently 185 V for 55 min using MOPS as a running buffer. For samples resulting from in-vitro ubiquitylation assays the second separation step was reduced to 47 min at 175 V.

4.2.3.3 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 constant voltage of 30 V and a maximum current of maximum 200 mA per gel for 2 hours and 15 minutes. Afterwards, membranes were incubated for 1 hour with blocking buffer at room temperature to occupy non-specific protein binding sites.

Subsequently, membranes were subjected to immunoprobing with primary and secondary

horseradish-peroxidase (HRP)-conjugated antibodies. Proteins were visualised using the ECL® detection system (Amersham Biosciences).

4.2.3.4 Stripping of immunoblot membranes

If nitrocellulose membranes had to be incubated with alternative antibodies, bound immunoglobulins were removed by incubating the nitrocellulose membranes with stripping buffer at room temperature for 12 min. The membranes were then rinsed with PBST, followed by incubation in blocking solution and probing as described in 4.2.3.3.

4.2.3.5 Quantification of band intensities

Intensities of the bands obtained by western blotting were quantified using the ImageJ software. Briefly, using the rectangle selection tool a rectangle (higher than wide) was drawn around the band of interest. This box was then copied until all bands to be analysed were selected. In a profile plot, the peak representing the actual band was separated from the background using the straight line selection tool. The area under these peaks as given by the programme was taken as the intensity of the respective band. These intensities were used to normalise the intensities of the HOIL-1 bands in lysates and IPs to the intensities of the bands corresponding to ACTIN in the lysates and SHARPIN in the IP respectively. Based on these normalised values the down-regulation of HOIL-1 in the lysates was compared to the percentage that remained associated with SHARPIN.

4.2.3.6 Generation and in-vitro translation of SHARPIN and HOIP mutants

For *in vitro* binding assays, V5-His-tagged versions of SHARPIN, HOIP and different deletion mutants (HOIP ΔC terminus (residues 1–654), HOIP ΔUBA (deletion of residues 564–615), HOIP ΔN-terminus (residues 494–end), SHARPIN ΔUBL (deletion of residues 219–289), SHARPIN ΔNZF (deletion of residues 348–377), SHARPIN NZFmut1 (point mutations of cysteines 353 and 356 to serine) and SHARPIN NZFmut2 (point mutations of cysteines 367 and 370 to serine) were generated by PCR and cloned into pcDNA3.1 (Invitrogen). The specific point-mutants were obtained by site-directed mutagenesis. All mutations and deletions were verified by sequencing and the proteins were generated *in vitro* using the TNT Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instructions. Briefly, 1.2 µg of each expression vector was added to an aliquot of the TNT Quick MasterMix and incubated in a reaction volume of 50 µL at 30°C for

80 min. Equal protein production was controlled by western blotting (input: 1 µL of reaction mix).

4.2.3.7 Expression and Purification of GST-tagged recombinant proteins

Recombinant proteins and their mutants (SHARPIN-UBL-only (residues 218–314), HOIP-ZnF-only (residues 298–329), HOIP-NZF1-only (residues 350–379), HOIP-NZF2-only (residues 408-438), Parkin-RBR-only (residues 234-453), ARIH1-RBR-only (residues 181-379), ARIH2-RBR-only (residues 136-330)) were expressed from a pGEX-6P2-vector (GE Healthcare Life Sciences). The DNA constructs were transformed into *E. coli* BL21(DE3) (Invitrogen) and 500 mL LB/Amp medium were inoculated with 10 mL overnight culture of a single BL21 colony and grown until an OD600 of approximately 0.6. Protein expression was induced with 1 mM IPTG (Isopropyl-β-D-thiogalactoside). For proteins containing many zinc-coordinating domains the growth medium was supplemented with 200 mM $ZnSO_4$. The bacteria suspension was incubated at 18°C and 170 rpm for 16 hours, centrifuged at 4600 rpm and 4°C for 15 min, and the supernatant was discarded. The bacterial pellet was resuspended in 10 mL Bacteria lysis buffer and the lysate was incubated on ice for 15 min after addition of lysozyme (50 µg/mL). The bacteria were sonicated six times for 20 s, incubated on ice for 10 min and centrifuged at 15000 rpm and 4°C for 30 min. The supernatant was then filtered using 0.45 µm syringe filters and applied to a 1 mL GSTrap column (GE Healthcare) which had been equilibrated with 10 mL PBS + 1 mM DTT. After application of the sample which was performed at a flow rate of 0.5 mL/min, the column was washed with 10 mL GST-wash buffer. Elution of the GST fusion proteins was carried out at 4°C using 10 mL GST-elution buffer and the eluate was collected in 1 mL fractions. Alternatively, 160 u PreScission (GE Healthcare Life Sciences) were loaded onto the column in 1 mL cleavage buffer and incubated for at least 4 hours at 4°C. Protein elution was determined by Coomassie staining. Protein containing fractions were dialysed in 5 L of dialysis buffer overnight. If the protein had been eluted using the elution buffer the GST-tag could be removed by incubation with 1 u PreScission protease per 100 µg protein.

4.2.3.8 Expression and Purification of human HF-TNF

His-Flag-tagged TNF was expressed from a pQE32 expression vector (Qiagen). Protein expression was performed as described above and the bacterial pellet was resuspended in bacterial lysis buffer for His-tagged proteins. Lysis and sample preparation were performed

as described for GST-tagged proteins and His/FLAG-TNF (HF-TNF) was purified using a HisTrap FF Agarose column (GE Healthcare Life Sciences) according to manufacturer's instructions. The expression and purification of HF-TNF was controlled by SDS-PAGE and Coomassie staining. Dialysed fractions, containing HF-TNF in dialysis buffer containing L-Arginine were stored at -80°C.

4.2.3.9 TNF-RSC precipitation

For analytical RSC analysis, 2-5 x 10^7 U937 or HeLa cells per sample were either treated with 1 μg FLAG-tagged TNF per mL stimulation medium or left untreated. Cells were then lysed in IP-lysis buffer for 30 min at 4°C on a head-to-head shaker. Afterwards, the lysates were centrifuged at 15.000 x g for 30 min, protein content was assessed using the BCA-assay (section 4.2.3.1) and protein concentrations for the different samples were adjusted. 0.5 μg FLAG-TNF was added to the non-stimulated control. FLAG-TNF was precipitated using M2 beads (Sigma) for 16 hours. Alternatively, 2 μg anti-TNFR1 or anti-TNFR2 antibody were precoupled to ProteinG beads for at least 2 hours at room temperature, unbound antibody was washed off and the antibody-coupled beads were used to precipitate the TNF-RSC. In all cases the beads were washed 5 times with 1 mL IP-lysis buffer and eluted with SDS-Sample buffer. The TNF-RSC were analysed by immunoblotting using the indicated antibodies.

4.2.3.10 Treatment of the TNF-RSC with recombinant USP2

The TNF-RSC was precipitated from 2x10^8 U937 cells as described under 4.2.3.9. Following the precipitation, the beads were washed 5x in DUB reaction buffer and split into 4 samples. As controls, one sample was left untreated and one was incubated in 1x DUB reaction buffer for 2 hours at 37°C. The remaining two samples were subject to DUB treatment as described previously (Komander et al., 2009b). For this purpose, the catalytic domain of USP2 (Enzo Life Sciences) was diluted to a concentration of 0.2 μg/μL in DUB dilution buffer and pre-incubated for 10 min at room temperature. The TNF-RSC coupled beads were treated with 2 or 4 μg of the activated DUB in a total volume of 30 μL for 2 hours at 37°C. The reaction was stopped by addition of reducing sample buffer and analysed by western blotting.

4.2.3.11 Size exclusion chromatography

HeLa cells were lysed in lysis buffer for gelfiltration by repeated passing through a syringe needle. After adding an equal volume of lysis buffer containing 300 mM NaCl, lysates were

centrifuged at 100,000 g for 60 min to obtain S100 lysates. S100 lysates were separated via a Superdex 200 10/300 GL column (GE Healthcare Life Sciences) in gelfiltration running buffer using an ÄKTA chromatography system (GE Healthcare Life Sciences).

4.2.3.12 Protein interaction studies

For in-vivo interaction studies, 7 x 10^6 HEK293T cells were transfected with 5 µg of the respective DNAs by standard calcium phosphate transfection. Cells were harvested 24 hours post transfection and lysed in 1 mL IP-lysis buffer for 30 min at 4°C. Subsequently, lysates were centrifuged at 15,000 x g for 30 min. Beads coupled to antibodies specific for a tag or one of the transfected proteins itself were used for immunoprecipitations which were performed at 4°C for 16 hours. The beads were washed 5 times with IP-lysis buffer and the proteins were eluted in 35 µL 2x LDS sample buffer containing DTT as a reducing agent. Precipitated proteins were analysed by immunoblotting.

4.2.3.13 Ubiquitin pull-down assays

Lysates from unstimulated HeLa or U937 cells were incubated with control beads or ubiquitin-coupled agarose (Boston Biochem) at 4°C overnight. The beads were washed and dried and the proteins were eluted using 2x LDS Sample buffer. Binding was assessed by western blotting. The mapping of the ubiquitin binding domain in SHARPIN was performed in a similar manner. Here the ubiquitin-coupled beads were used to precipitate in-vitro translated SHARPIN or mutants thereof. Investigation of linkage specificity was performed by incubating 10 µg of purified C-terminally V5-tagged SHARPIN, HOIL-1 or HOIP with anti-V5-beads in 600 µL Pull-down buffer (PDB) at 4°C for 6 hours. Beads were washed three times with PDB, split into aliquots and incubated at 4°C overnight with 2 µg recombinant K48-, K63- or linear-linked ubiquitin chains in 450 µL PDB. The beads were washed five times with PDB. The bound proteins were eluted using 2x LDS sample buffer and subsequently subjected to SDS-PAGE and immunoblotting using ubiquitin specific antibodies.

4.2.3.14 NF-κB Luciferase assay

HEK293-NF-κB cells were seeded into 6-well plates and cultured for 10 to 12 hours in DMEM + 10 % FCS before transfection. Cells were cotransfected with 1 µg/well of the pCMV-RLuc plasmid (Stratagene) and the plasmids encoding the respective LUBAC components using FuGENE 6 (Roche). Alternatively transient knockdown was performed as described in

section 4.2.2.3. 16 to 24 hours after transfection with plasmid DNA or 72 hours after siRNA transfection, cells were detached and seeded in 96 well plates. The next day cells were stimulated with TNF for 4 hours or left untreated. Afterwards, cells were lysed with 1x Passive lysis buffer (Promega) and the luminescence was measured using a microplate reader (Mithras LB940; Berthold Technologies). Firefly luciferase data were either normalised to values obtained in a MTT-viability assay (knock-down experiments) or to Renilla luciferase activity (over-expression experiments).

4.2.3.15 MTT-viability assay

The MTT [3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyl-tetrazoliumbromide] method is based on the reduction of the yellow soluble tetrazolium salt by mitochondrial dehydrogenases of the respiratory chain leading to the formation of blue formazan crystals (Gerlier and Thomasset, 1986). Cells were seeded on a 96 well plate in parallel with those required for the luciferase assay. After the addition of 25 µL MTT solution, the cells were incubated at 37°C for 2 hours. Afterwards, the medium was discarded and the MTT reaction was stopped by adding 100 µL of an acetic acid solution (5 % acetic acid in isopropanol). The reduction of the substrate was assessed by measuring the absorption at a wavelength of 570 nm.

4.2.3.16 In-vitro ubiquitylation assay

For in-vitro ubiquitylation assays, 0.8 mg recombinant E3 were incubated with 5 µg ubiquitin (wild-type, His-tagged or K0), 200 ng E1 (UBE1), 300 ng E2, 1x ERS (Boston Biochem) in ubiquitylation buffer. If TRAF2 was used as an E3, sphingosine-1-phosphate was added to a final concentration of 100 nM. After 2 hours incubation at 37°C the reaction was stopped by adding reducing sample buffer. The samples were analysed by western blotting.

For in-vitro ubiquitylation of potential target proteins 293T cells were transfected with plasmids encoding a tagged version of the respective protein using calcium phosphate and proteins were immunoprecipitated using beads coupled to antibodies specific for either the tag or the protein itself. The beads were washed, dried and added to the reaction mix. The assay was performed as described above.

5 Results

5.1 SHARPIN, HOIL-1 and HOIP are recruited to TNFR1 upon stimulation

A first step in the investigation of the three novel components identified by mass spectrometry was to verify their presence in the TNF-RSC and to test whether they are recruited in a stimulation-dependent manner or if they are already associated with unstimulated receptors. For this purpose U937 cells were stimulated with His-FLAG (HF)-TNF for different times and the receptor complex was precipitated by an anti-FLAG immunoprecipitation. To control for unspecific, stimulation-independent binding or a direct interaction between LUBAC and TNFR1, TNF was also added post lysis to the lysate of untreated cells. Analysis by western blotting revealed that recruitment of SHARPIN, HOIL-1 and HOIP is a stimulation-dependent event (Figure 11).

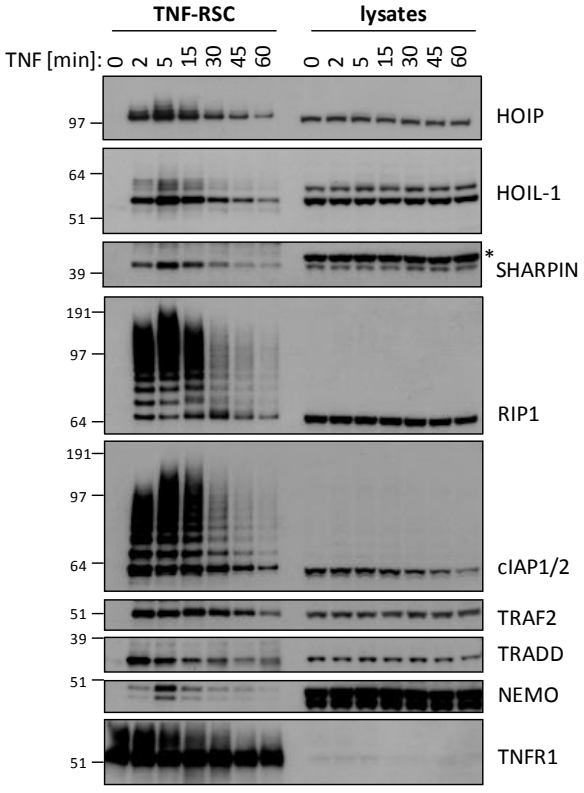


Figure 11: SHARPIN, HOIL-1 and HOIP are recruited to the TNF-RSC in a stimulation-dependent manner. $5x10^7$ U937 cells per sample were stimulated with 1μg/mL HF-TNF for the indicated times. The TNF-RSC was precipitated using anti-FLAG beads and analysed by western blotting for the presence of both the three novel proteins and of bona-fide components of this complex using specific antibodies. The asterisk indicates an unspecific band recognised by the anti-SHARPIN-antibody.

It also showed that the three proteins are already present after two minutes of stimulation and are recruited with similar kinetics. Their levels reach a maximum at five minutes and start decreasing after about 15 minutes. This coincides with the recruitment of other components like RIP1, cIAP1/2 and NEMO, which also reach maximal levels, and in the case of RIP1 and cIAP1/2 maximal modification after five minutes and which start disappearing from the complex after 30 minutes of stimulation. The kinetics of recruitment for TRADD seem to be slightly different. The latter protein seems to reach its maximum levels already at two minutes of stimulation whereas. Overall this result shows that, together with other components of the TNF-RSC, SHARPIN, HOIL-1 and HOIP are recruited to the TNF receptor in a stimulation-dependent manner.

As the mass spectrometric analysis was performed on the TNF-RSC isolated from U937 cells, a cell line which expresses both TNFR1 and TNFR2 (Haridas et al., 1998; Shu et al., 1996), the question arose which of the two receptors is essential for the recruitment of SHARPIN, HOIL-1 and HOIP. In line with the finding that soluble TNF fully activates TNFR1 but not TNFR2 (Grell et al., 1998; Grell et al., 1995; Krippner-Heidenreich et al., 2002), only peptides for TNFR1 were identified in the mass-spectrometric analysis of the TNF-RSC which had been precipitated using soluble TNF (Gerlach et al., 2011; Haas et al., 2009). This provides a good indication that SHARPIN, HOIL-1 and HOIP are recruited to TNFR1 rather than TNFR2. However, for a more direct analysis of this aspect, the TNF-RSC precipitated from U937 cells was compared to that of HeLa cells which, in contrast to U937 cells, only express TNFR1 (Haridas et al., 1998; Shu et al., 1996); PhD thesis Dr. C. H. Emmerich). In spite of much lower expression levels of HOIL-1 in HeLa cells, SHARPIN, HOIL-1 and HOIP were detectable in the TNF-RSC isolated from these cells (Figure 12), indicating that TNFR2 is not essential for their presence in TNF-precipitations.

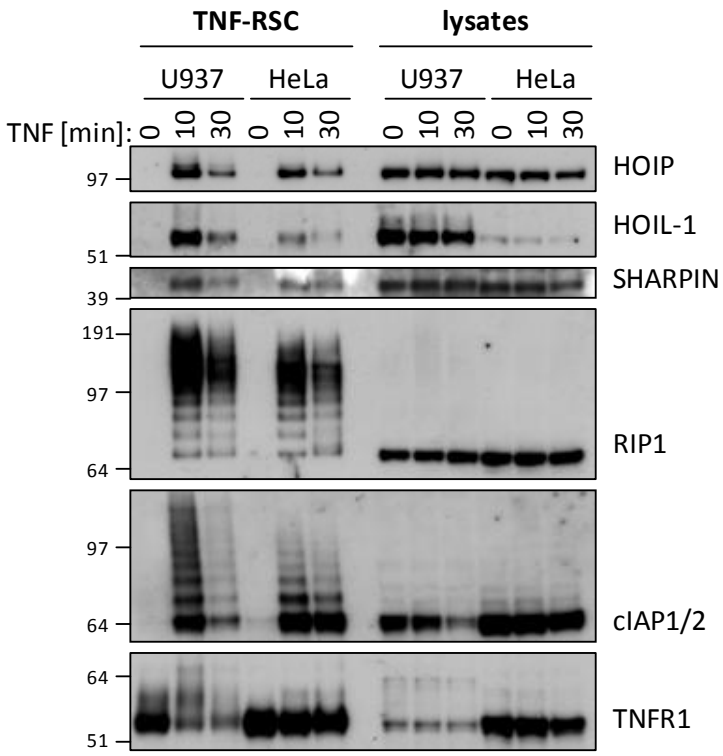


Figure 12: SHARPIN, HOIL-1 and HOIP are recruited to the TNF-RSC of both U937 and HeLa cells. U937 or HeLa cells were stimulated with 1μg/mL TNF for ten or thirty minutes. After lysis, protein levels were adjusted to the same levels and the TNF-associated complex was pulled out in an anti-FLAG immunoprecipitation. The resulting TNF-RSC was analysed by western blotting using the indicated antibodies.

To further investigate this point, lysates of U937 cells stimulated with TNF for five, fifteen or thirty minutes were divided into two parts and each fraction was subject to precipitation of either TNFR1 or TNFR2 using specific antibodies. Comparable levels of TRAF2, a protein that was described to associate with both TNFR1 and TNFR2 (Shu et al., 1996), were detectable in both receptor complexes after fifteen minutes (Figure 13). In the precipitation of TNFR1, TRAF2 was already present after five minutes and could hardly be detected anymore after thirty minutes. On the other hand, the levels of TRAF2 detectable in the TNFR2-complex after five minutes were very low but the association remained stable at thirty minutes. A similar picture could be observed for cIAPs, additional components common to the two receptor complexes (Shu et al., 1996), although the levels recruited to TNFR2 were lower than the ones detectable on TNFR1. This indicates that the kinetics of complex formation differ between the two receptors, with TNFR2 showing a slight delay in recruitment of intracellular proteins. Another difference between the two complexes is constituted by the fact that SHARPIN, HOIL-1 and HOIP, like RIP1, a specific component of the TNFR1- but not the TNFR2-associated complex (Hsu et al., 1996a), could only be detected in the

precipitation of TNFR1. Overall, these findings establish that SHARPIN, HOIL-1 and HOIP are recruited to TNFR1. However, a potential recruitment to TNFR2, especially under conditions in which this receptor is efficiently stimulated by membrane-bound TNF, cannot be fully excluded on the basis of these results.

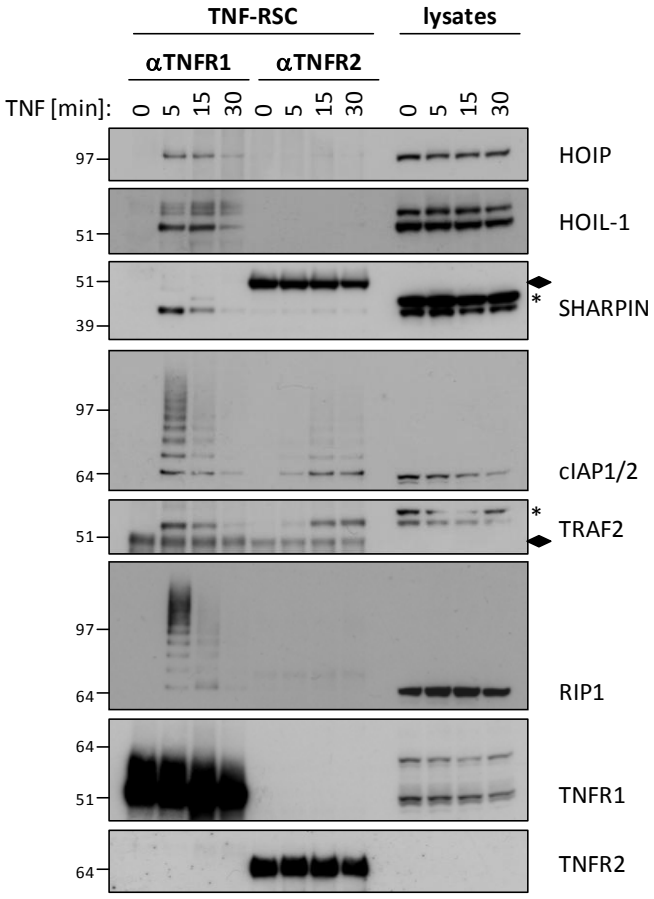


Figure 13: SHARPIN, HOIL-1 and HOIP are not present in the TNFR2-SC. U937 cells were stimulated with TNF for the indicated times. Lysates were separated into two parts and subject to precipitation using antibodies specific for TNFR1 or TNFR2, respectively. The proteins associated with the two receptors were analysed by western blotting. Black rhombi indicate the position of the heavy chain and unspecific bands recognised by the anti-SHARPIN and anti-TRAF2-antibodies respectively are marked by asterisks.

5.2 Down-regulation of SHARPIN, HOIL-1 or HOIP inhibits TNF-signalling

Having identified SHARPIN, HOIL-1 and HOIP as components of the TNF-RSC, the next step was to analyse the functional relevance of this recruitment for downstream signalling events. To this end, expression levels of the three proteins were down-regulated by RNA interference in HEK293-NF-κB cells, a cell line that stably expresses luciferase under a NF-κB-responsive promotor. Following the knockdown-procedure, a part of the cells was taken to determine the efficiency of the down-regulation. The rest of the cells were stimulated with different concentrations of TNF. As shown in Figure 14, down-regulation of

any of the three proteins decreased luciferase expression following TNF-stimulation with knockdown of HOIP being the most efficient in inhibiting TNF-induced NF-κB activation. At 100 ng/mL TNF HOIP knockdown decreased luciferase-activity to 44 % of the control whereas RNAi for SHARPIN and HOIL-1 led to a reduction to 66 % and 49 %, respectively.

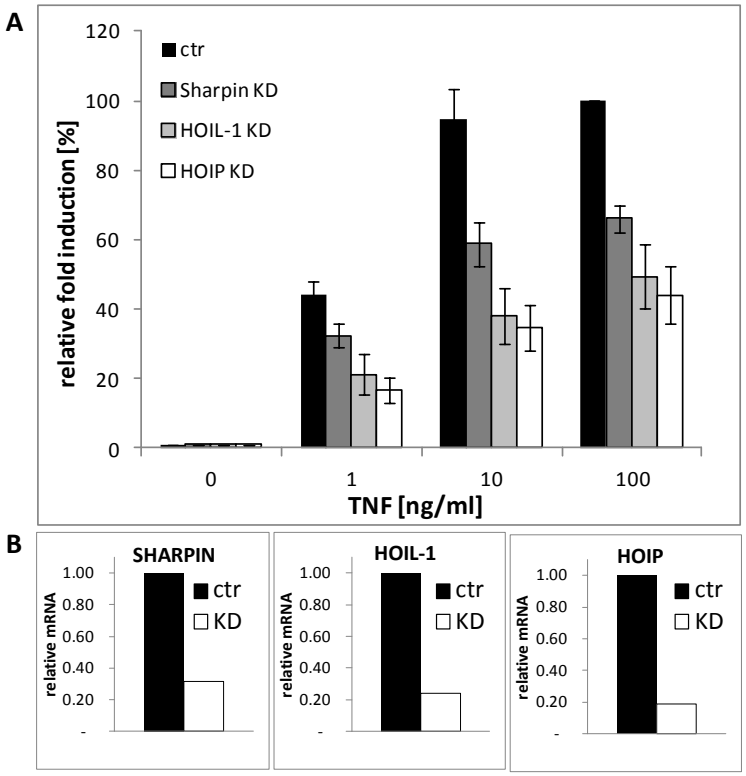


Figure 14: Down-regulation of SHARPIN, HOIL-1 or HOIP reduces TNF-induced NF-κB activation. HEK293-NF-κB cells were transfected with siRNAs targeting SHARPIN, HOIL-1 or HOIP, respectively. **A:** TNF-induced NF-κB activation was assessed in a luciferase-reporter assay, following stimulation with the indicated concentrations of TNF for four hours. Values were normalised to the values obtained in a MTT-viability assay and values reached for stimulation with 100 ng/mL TNF were set to 100 %. Results are shown as mean +/- SEM, n=4. **B:** Knockdown efficiency was determined by qPCR. Results for one representative experiment are shown.

In line with this, phosphorylation of p38, JNK and IκB were almost completely abolished when HOIP expression was down-regulated in HeLa cells (Figure 15A), indicating that not only the activation of NF-κB but also that of other TNF-induced signalling pathways is affected by down-regulation of this component. Knockdown of SHARPIN or HOIL-1 was not as efficient in blocking the different pathways. However, the phosphorylations indicating the activation of the different signalling cascades were also slightly decreased. This suggests that of the three, HOIP is the most important for TNF-induced signalling. However, a pronounced decrease in the protein levels of SHARPIN and especially HOIL-1 can be observed when HOIP is down-regulated by siRNA (Figure 15A). This is unlikely to be an off-

target effect of the siRNA, as the qPCR analysis did not show an effect of the siRNA on the mRNA levels of SHARPIN or HOIL-1 (Figure 15B). Furthermore a similar down-regulation on protein- but not on mRNA level was described to occur in mouse embryonic fibroblasts (MEFs) deficient for SHARPIN or HOIL-1 (Gerlach et al., 2011; Tokunaga et al., 2011). Overall this demonstrates that a decrease in levels of SHARPIN, HOIL-1 or HOIP but especially their co-down-regulation efficiently inhibits TNF-induced signalling cascades. This suggests that recruitment of the three proteins to TNFR1 is an event essential for TNF-induced signalling to occur at its full strength.

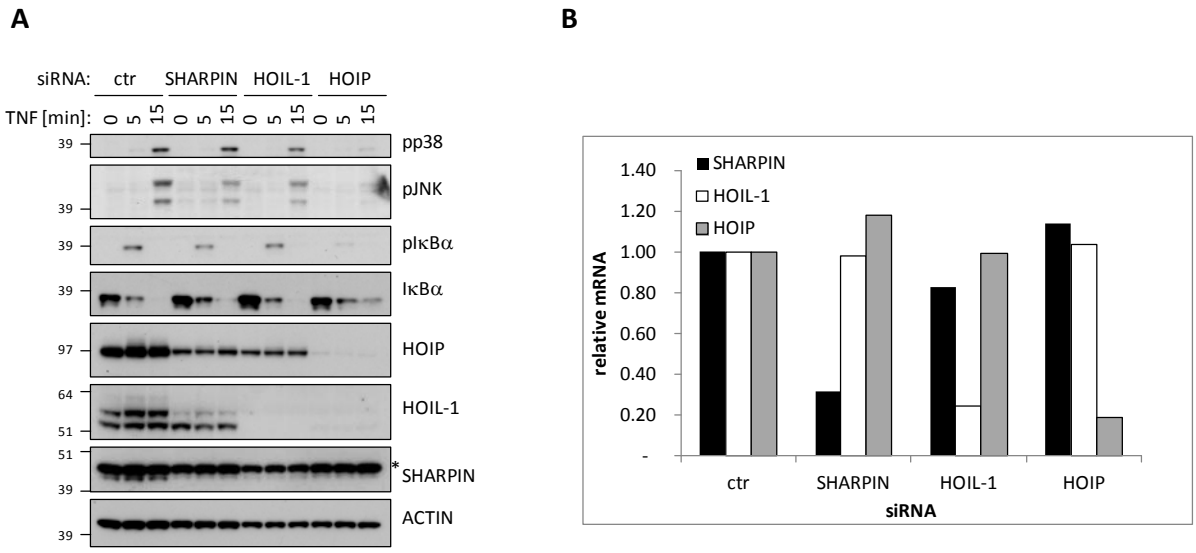


Figure 15: Other TNF-induced signalling cascades are also affected by down-regulation of SHARPIN, HOIL-1 and HOIP. A: Protein levels of SHARPIN, HOIL-1 and HOIP were down-regulated in HeLa cells using RNA interference. Cells were then stimulated with 25 ng/mL TNF for five or fifteen minutes and events indicating the activation of signal transduction were analysed using western blotting. **B:** Down-regulation of SHARPIN and HOIL-1 by HOIP knockdown is not an off-target effect. As determined by qPCR the knockdown of either SHARPIN, HOIL-1 or HOIP does not lead to co-down-regulation of the other two on mRNA-level.

5.3 Recruitment of SHARPIN, HOIL-1 and HOIP to the TNF-RSC is interdependent and requires the presence of cIAP1/2

To follow up this finding, the recruitment of SHARPIN, HOIL-1 and HOIP to the TNF-RSC was to be investigated further with the aim of identifying the mechanism of recruitment. By analysing the TNF-RSC precipitated from MEFs deficient for individual components of this complex, it was established that HOIL-1 requires the presence of cIAPs for its recruitment (Haas et al., 2009). To test whether the same was true for SHARPIN and HOIP, HeLa cells were treated with the Smac-mimetic compound SM-164, a substance that leads to the auto-ubiquitination and degradation of cIAP1 and cIAP2 (Lu et al., 2008). The TNF-RSC was

isolated from these cells and compared to that of cells that had been treated with DMSO as a control. This comparison revealed that in absence of cIAP1/2 neither HOIL-1 nor SHARPIN or HOIP are recruited (Figure 16).

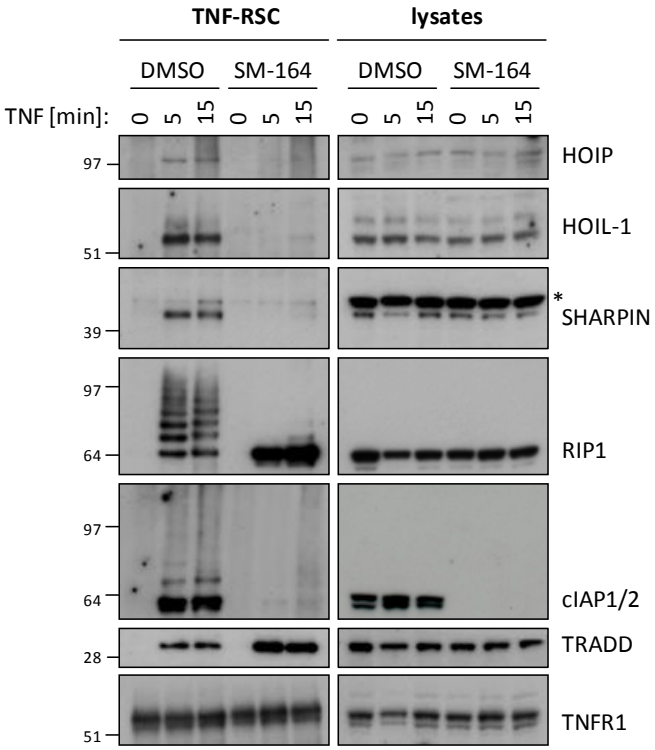


Figure 16: SHARPIN, HOIL-1 and HOIP are recruited to the TNF-RSC in a cIAP-dependent manner. HeLa cells were pre-treated for two hours with the Smac-mimetic compound SM-164 (100 nM) which leads to degradation of cIAP1 and cIAP2. The TNF-RSC was precipitated following TNF-stimulation using an anti-TNFR1-antibody and compared to that of control treated cells (DMSO).

As cIAPs are E3s able of generating ubiquitin-chains on RIP1, themselves and potentially other components of the TNF-RSC (Bertrand et al., 2008; Lopez and Meier, 2010; Varfolomeev et al., 2008; Wertz et al., 2004), the question arose whether this activity is required for recruitment of the three proteins. To test this, MEFs deficient for cIAP1 and cIAP2 that contain a tamoxifen-inducible construct encoding either wild-type cIAP1 or mutants of this protein that cannot dimerise (F610, V576E) or bind their corresponding E2 (V567A/D57) (Feltham et al., 2011; Feltham et al., 2010; Mace et al., 2008) were treated with 20 nM 4-hydroxy-tamoxifen (4-HT). As can be seen in Figure 17A, this efficiently induced the expression of cIAP1 or its mutants. In Figure 17B the result of a TNF-RSC precipitation from reconstituted cells is shown. Even though re-expression of wild-type cIAP1 was not efficient enough to fully rescue RIP1 ubiquitination, it was sufficient for HOIL-1 recruitment to occur. In contrast, none of the mutants was able to restore RIP1

modification or HOIL-1 recruitment, indicating that not only presence of cIAPs but also their activity is required for either of these events to occur. Due to technical limitations in the detection of SHARPIN and HOIP in the murine system, recruitment of these proteins was not investigated in this assay.

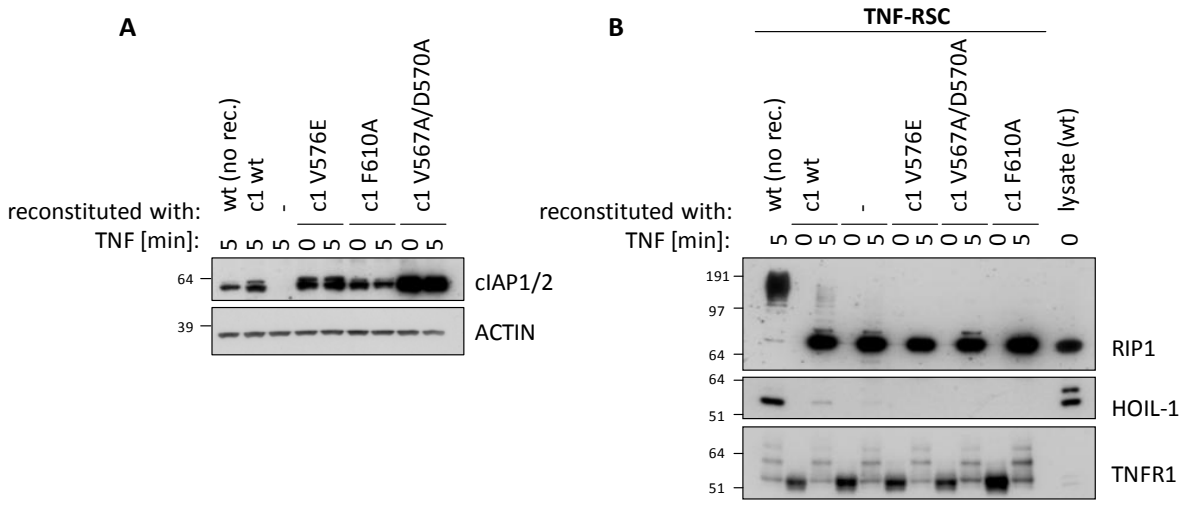


Figure 17: Recruitment of HOIL-1 to the TNF-RSC depends on the activity of cIAP. A: Expression of cIAP1 was induced in cIAP1/2$^{-/-}$ MEFs containing constructs for the re-constitution with either wild-type (wt) cIAP1 or mutants of this protein that are unable to dimerise (V576E, F610A) or to bind the corresponding E2 (V567A/D570A) (Feltham et al., 2011; Feltham et al., 2010; Mace et al., 2008) by incubation with 20nM 4-hydroxy-tamoxifen. **B:** The TNF-RSC was precipitated from cIAP1/2$^{-/-}$ MEFs reconstituted with different cIAP1 mutants and the recruitment of HOIL-1 was analysed by western blotting.

Making use of a similar inducible system, in which expression of TRAF2 or its mutants can be switched on using tamoxifen, it was shown that cIAP requires presence of TRAF2 and specifically of a cIAP-interaction motif (CIM) within TRAF2 for its recruitment to the TNF-RSC whereas the RING-domain of TRAF2 is dispensable (Figure 18). These results also indicate that the RING-domain of TRAF2 is insufficient to mediate the modification of RIP1 and that TRAF2 instead serves a role in recruiting cIAP1, which in turn is responsible for ubiquitinating RIP1.

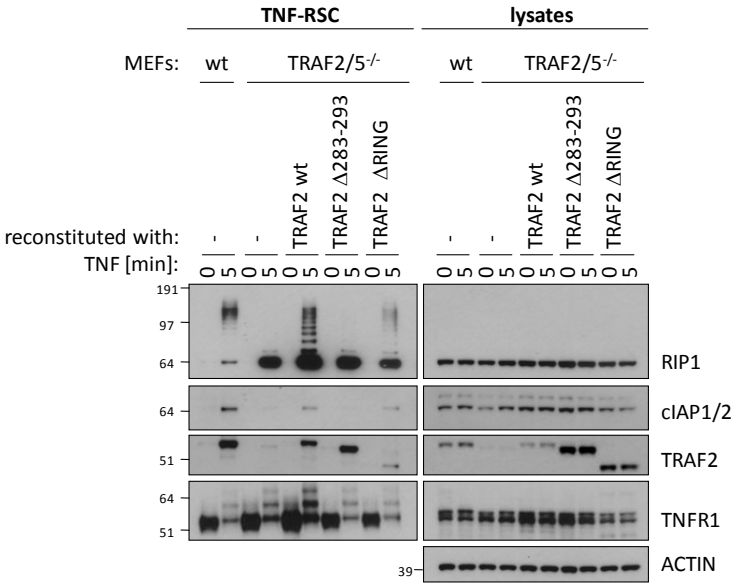


Figure 18: cIAP requires a cIAP-interaction motif (CIM) within TRAF2 for its recruitment to the TNF-RSC. TRAF2/5$^{-/-}$ MEFs reconstituted with constructs for the tamoxifen-inducible expression of TRAF2 mutants (Vince et al., 2009), were used to precipitate the TNF-RSC in presence or absence of 4-HT. Modification of RIP1 and recruitment of cIAP in presence of the different mutants was assessed by western blotting.

Together this indicates that recruitment of HOIL-1 and potentially of SHARPIN and HOIP depends on the activity of cIAPs, which in turn are recruited to the TNFR-complex via their interaction with TRAF2. As cIAPs generate ubiquitin chains and this activity is required for recruitment of HOIL-1, it was next investigated whether SHARPIN, HOIL-1 and HOIP have ubiquitin-binding properties. As a first approach to this question, ubiquitin-coupled agarose beads were compared to control beads in their ability to precipitate SHARPIN, HOIL-1 and HOIP from unstimulated lysates of HeLa (Figure 19A, left panel) or U937 (Figure 19A, right panel) cells. It could be shown that all three proteins associate specifically with ubiquitin-coupled but not with the control beads. To further investigate this property of SHARPIN, HOIL-1 and HOIP recombinant versions of the three proteins with an N-terminal GST- and a C-terminal V5-tag were produced in *E.coli*. After it had been used for purification, the GST-tag was removed and the proteins were precipitated via their C-terminal V5-tag. The predominant non-proteolytic ubiquitin linkage reported to be generated by cIAPs are K63-linked chains. Therefore, K63-linked tetra-ubiquitin was added to immobilised SHARPIN, HOIL-1 and HOIP and the co-precipitation of K63-linked ubiquitin chains was analysed by western blotting (Figure 19B). Association of ubiquitin with all three proteins but not with anti-V5-beads alone was detectable, indicating that SHARPIN, HOIL-1 and HOIP all have the ability to bind K63-linked ubiquitin. However, it can be observed that although the levels of HOIP are much lower than those of SHARPIN and HOIL-1, the amount of ubiquitin co-

precipitated with the former protein is the highest. This suggests that HOIP has a greater overall affinity for ubiquitin or that SHARPIN and HOIL-1 preferentially bind other ubiquitin chain types.

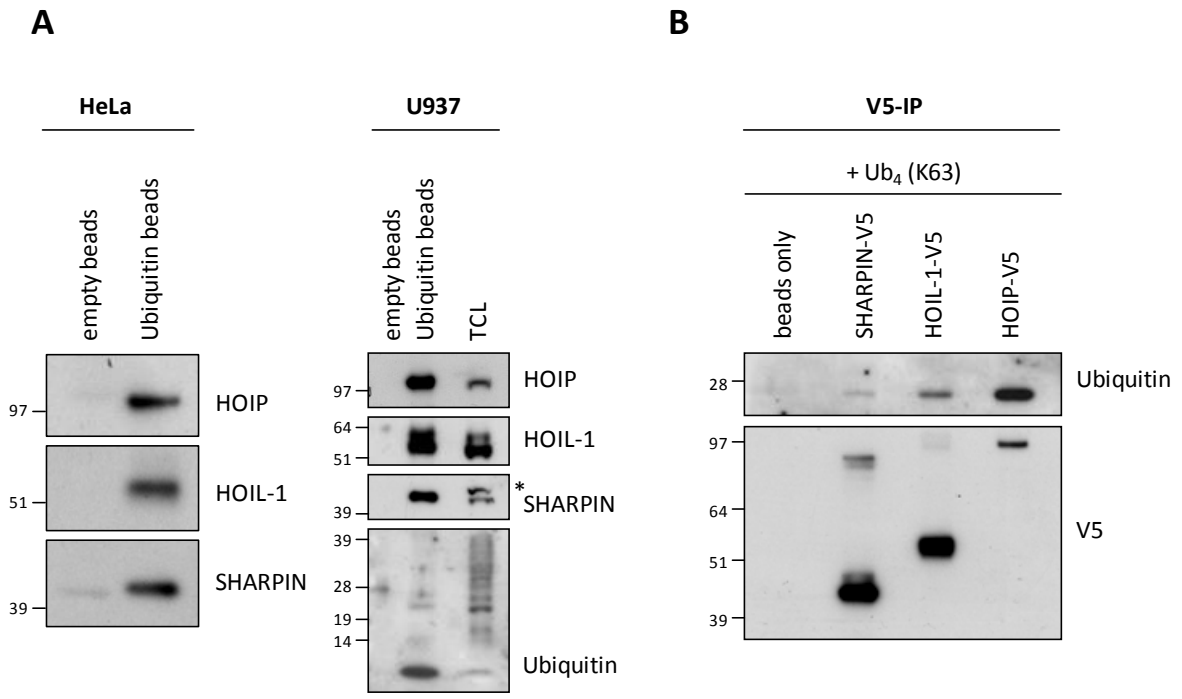


Figure 19: SHARPIN, HOIL-1 and HOIP can bind to ubiquitin. A: Control beads and ubiquitin-coupled agarose were compared in their capacity to precipitate SHARPIN, HOIL-1 and HOIP from lysates of unstimulated HeLa (left panel) or U937 cells (right panel). Association of the three proteins with the two types of beads was analysed by western blotting. **B:** C-terminally V5-tagged forms of SHARPIN, HOIL-1 and HOIP were coupled to anti-V5 beads, K63-linked tetra-ubiquitin (Ub_4 K63) was added and co-precipitation was determined on western blot level.

Indeed, a recent report demonstrated that the NZF-domain of HOIL-1 shows a strong specificity for linear chains (Sato et al., 2011). In order to investigate whether a similar preference exists for SHARPIN, the recombinant protein was precipitated using a specific antibody. K48-, K63- or linearly linked tetra-ubiquitin chains were added and co-precipitation was assessed by western blotting (Figure 20). This analysis revealed that K63-linked and linear chains were co-precipitated with SHARPIN, whereas binding of K48-linkages could not be detected (Figure 20A). It is important to note that different linkage types are not equally well detectable with antibodies recognising total ubiquitin (Figure 20B).

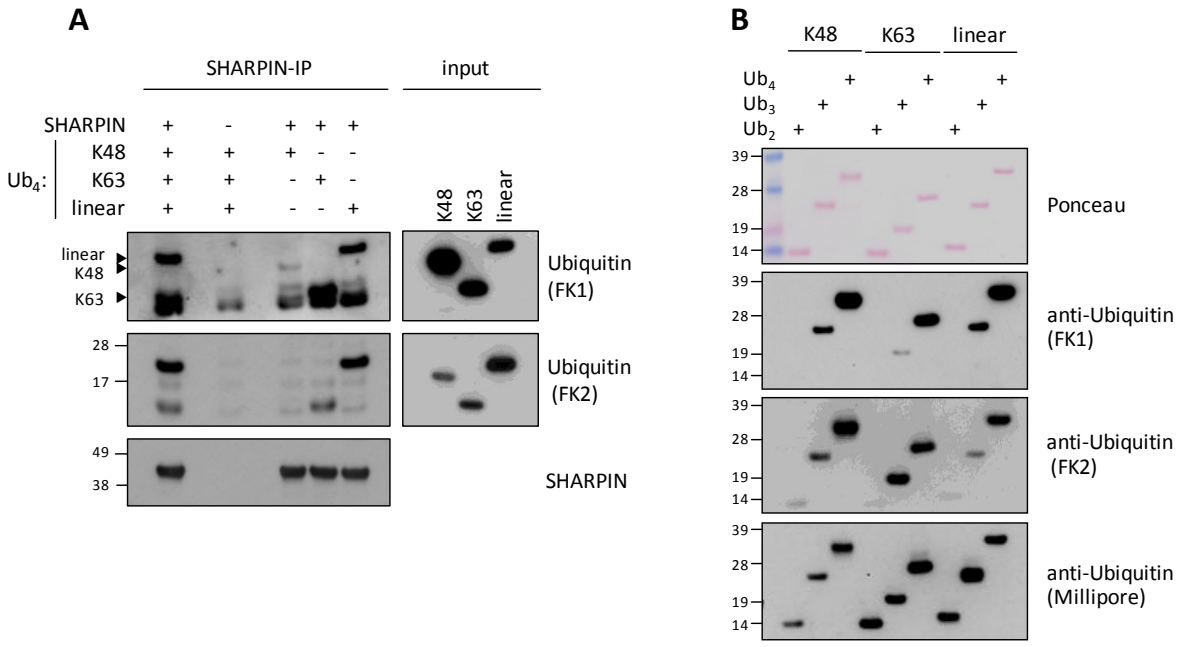


Figure 20: SHARPIN shows a preference for K63- and M1-linked tetra-ubiquitins. A: Untagged recombinant SHARPIN purified from *E.coli* was precipitated using a specific antibody and tetra-ubiquitin of different linkage types was added as indicated. To assess binding of the different linkages, precipitations were separated by SDS-PAGE and the corresponding western blots were probed with two anti-ubiquitin-antibodies (clones FK1 and FK2, Biomol) and an anti-SHARPIN antibody. **B:** Ubiquitin-chains of different lengths and linkage types were resolved by SDS-PAGE, transferred to membranes by western blotting and the corresponding membranes were probed with different anti-ubiquitin antibodies. Equivalent loading was verified by staining with PonceauS.

Although no band representing K48-linked tetra-ubiquitin was visible in the lanes corresponding to co-precipitation of this linkage type in the western blots shown in Figure 20A, a strong band was present in the input samples, suggesting that the lack of detectable co-precipitation of K48-linkages was not due to a problem with antibody specificity. Instead it indicates a preference of SHARPIN K63- and linearly linked ubiquitins which is in line with a recent publication (Sato et al., 2011). The ubiquitin-binding motifs of HOIL-1 and HOIP were allocated to their NZF- and NZF1- domains, respectively (Ikeda et al., 2011). To map the parts of SHARPIN involved in ubiquitin binding, ubiquitin-coupled agarose beads were used to precipitate in vitro-translated versions of SHARPIN, in which deletion- or point-mutations had been introduced (Figure 21A). This assay showed that both the wild-type protein and a mutant in which the UBL-domain had been deleted (ΔUBL) efficiently bound to the ubiquitin-beads (Figure 21B). In contrast, much lower levels of mutants in which the NZF-domain had been deleted (ΔNZF) or in which specific residues within that domain had been mutated (NZF1mut: C353,356S; NZF2mut: C367,370S) were found to be associated with the beads. This indicates that SHARPIN binds ubiquitin mainly via its NZF domain.

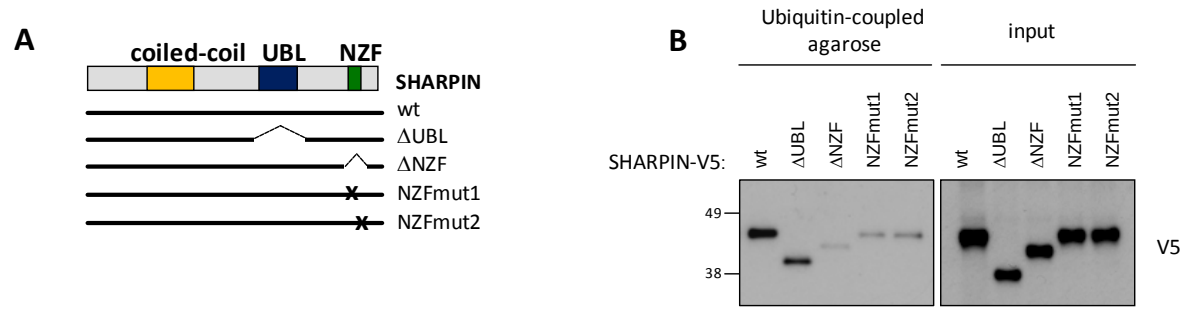


Figure 21: SHARPIN binds to ubiquitin via its NZF-domain. A: Schematic representation of SHARPIN and mutant employed in C. **B:** Ubiquitin-coupled agarose beads were used to assess the binding of V5-tagged in-vitro translated versions of SHARPIN to ubiquitin. Cysteines 353 and 356 were mutated to serine in NZFmut1 and NZFmut2 is a C367,370S mutant of SHARPIN. Co-precipitation was analysed by western blotting.

The observations that SHARPIN, HOIL-1 and HOIP are recruited to the TNF-RSC with similar kinetics (Figure 10) and that all three proteins require cIAPs and potentially ubiquitin chains generated by this E3 for their presence in the TNFR-associated complex (Figures 16-21), led me to investigate whether their recruitment is interdependent.

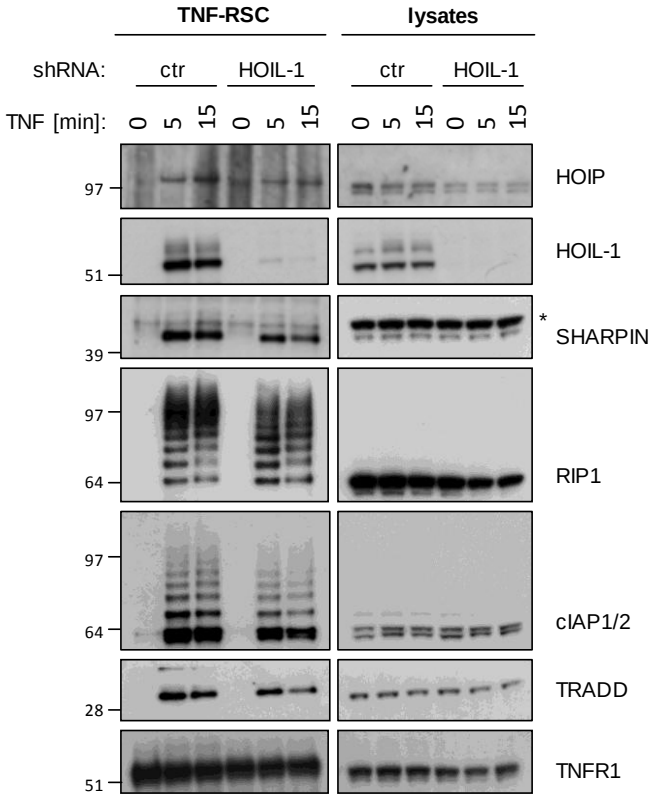


Figure 22: SHARPIN and HOIP are still recruited to the TNF-RSC in absence of HOIL-1. The TNF-RSC was isolated using an anti-TNFR1-antibody from HeLa cells in which expression of HOIL-1 was stably down-regulated by shRNA. Levels of SHARPIN and HOIP in the complex were compared to those associated with the receptor in control cells by western blotting. The asterisk indicates an unspecific band that is recognised by the anti-SHARPIN antibody.

To assess the role of HOIL-1 in association of SHARPIN and HOIP with the TNF-induced signalling complex, expression of HOIL-1 was stably down-regulated in HeLa cells using

shRNA. The TNF-RSC isolated from these cells was then compared to that of control cells (Figure 22). Comparative western blotting shows that, even though HOIL-1 expression was efficiently down-regulated, the levels of SHARPIN and HOIP remain within the same range as in control cells indicating that HOIL-1 is dispensable for their recruitment.

A similar approach was taken to investigate a potential role for SHARPIN as an adaptor for HOIL-1 and HOIP. Again, the levels of the other two proteins within the TNF-RSC were not markedly reduced in comparison to the control (Figure 23). In addition, a similar analysis in SHARPIN-deficient MEFs, isolated from cpdm mice, supports a SHARPIN-independent mechanism of recruitment for HOIL-1 and HOIP (Gerlach et al., 2011).

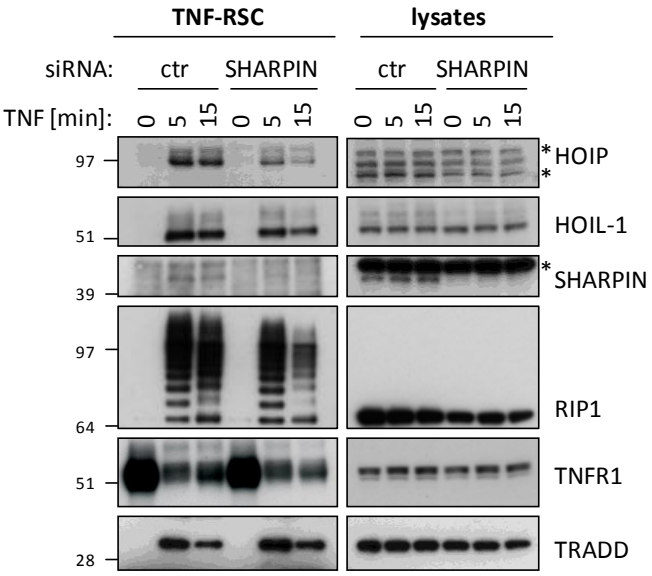


Figure 23: Knockdown of SHARPIN does not prevent recruitment of HOIL-1 and HOIP to the TNF-RSC. SHARPIN-expression was transiently down-regulated in HeLa cells by siRNA. Following stimulation with HF-TNF, the TNF-RSC was pulled out using anti-FLAG-beads and levels of complex-components present in the precipitation were analysed by western blotting. Asterisks mark cross-reactive bands detected by the anti-SHARPIN and anti-HOIP antibodies, respectively.

Lastly, HOIP was transiently down-regulated using RNA interference in HeLa cells and recruitment of all three proteins to the TNF-RSC was analysed by western blotting. As shown in Figure 24, absence of HOIP prevented the association of SHARPIN and HOIL-1 with the complex, demonstrating that HOIP acts an adaptor for the other two.

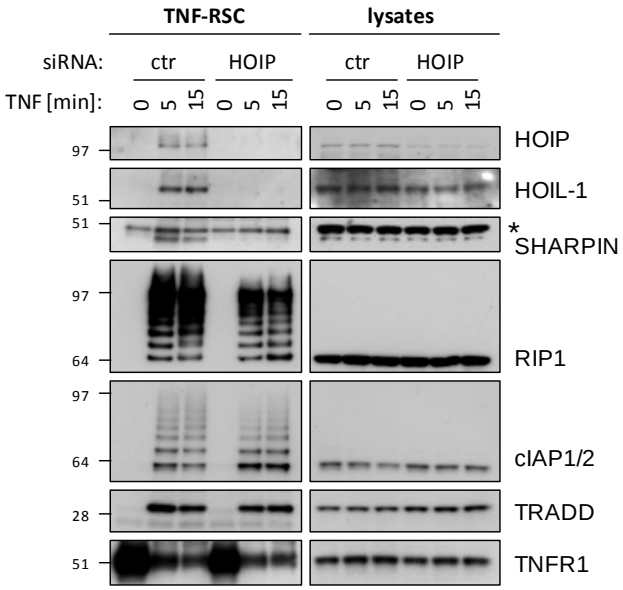


Figure 24: HOIP is required for the recruitment of SHARPIN and HOIL-1 to the TNF-RSC. HOIP expression was down-regulated in HeLa cells using RNA interference and the TNF-RSC was precipitated and analysed as in Figure 13. Asterisk marks an unspecific band detected by the anti-SHARPIN antibody.

5.4 SHARPIN, HOIL-1 and HOIP form a stimulation-independent complex

This central role of HOIP raised the question whether SHARPIN, HOIL-1 and HOIP form a complex in the absence of stimulation, which is then recruited to the TNF-RSC as a pre-formed unit. To address this, lysates of unstimulated HeLa cells were separated by gelfiltration chromatography and protein containing fractions (Figure 25, upper panel) were analysed by western blotting for the presence of SHARPIN, HOIL-1 and HOIP. An unseparated lysate control was included on all blots to ensure equal exposure times. While especially HOIP and SHARPIN partially eluted in the fractions corresponding to the molecular weights of their monomeric forms (fractions 22-25 and 33+34, respectively), all three proteins were also present in high molecular weight fractions (fractions 13-17), suggesting that even in the absence of a stimulus they are present in a pre-formed protein complex (Figure 25, lower panel). On the other hand the unspecific band recognised by the anti-SHARPIN antibody remained in the low molecular weight fractions. This is in line with a previous report showing that HOIL-1 and HOIP are present in a complex of about 600 kDa in unstimulated HeLa and SH-SY5Y cells and that exogenous co-expression of HOIL-1 and HOIP shifts both proteins to these high-molecular weight fractions (Kirisako et al., 2006).

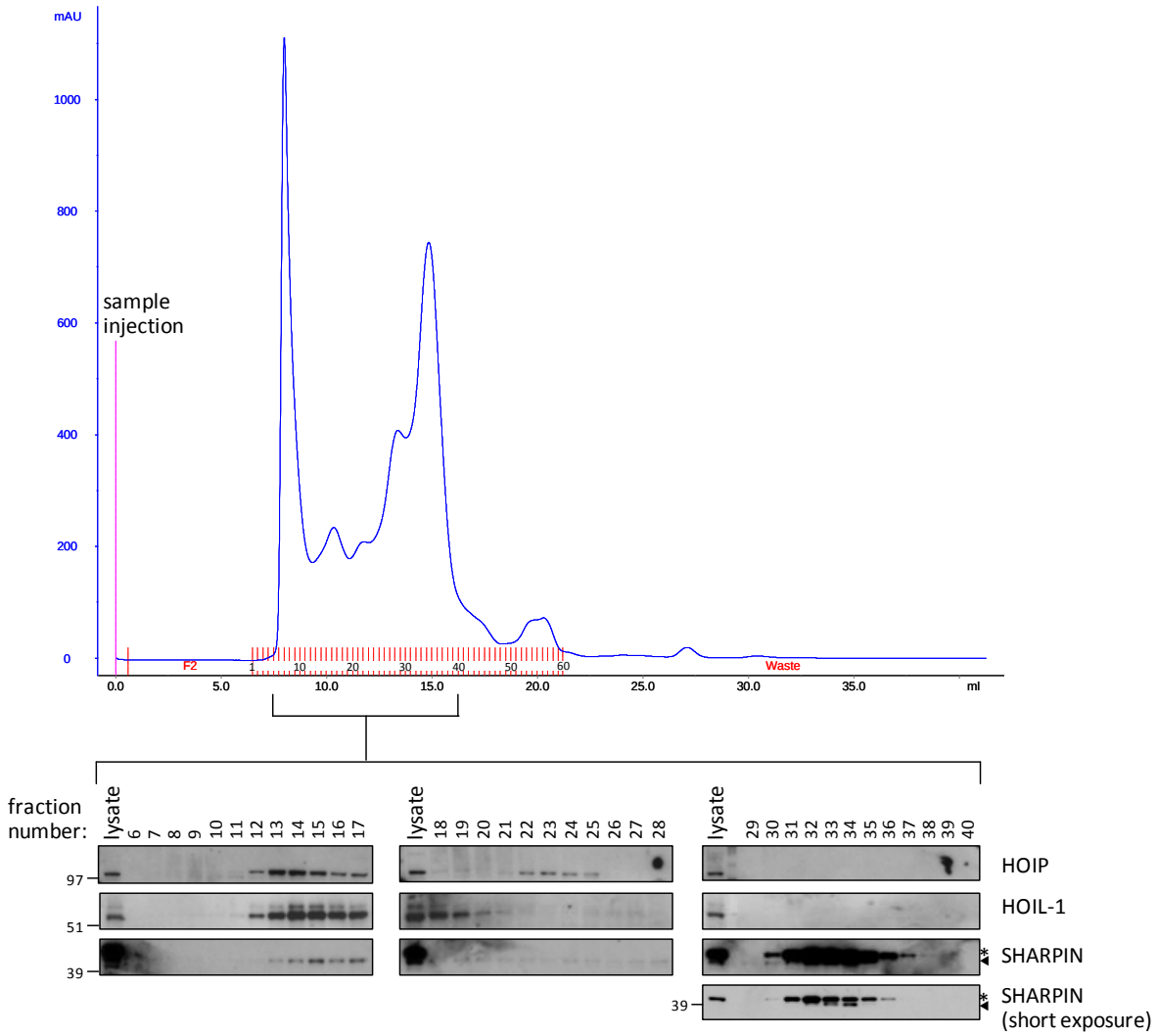


Figure 25: SHARPIN, HOIL-1 and HOIP co-elute in high molecular-weight-fractions. S100 lysates of unstimulated HeLa cells were separated on a Superdex 200 10/300 GL column (GE Healthcare Life Sciences). The upper panel represents the elution profile with elution volume and fraction numbers being shown on the horizontal axis, whereas the vertical axis presents protein content in arbitrary units. The lower panel shows analysis of protein containing fractions by western blotting.

To further test the possibility of a stimulation-independent interaction of SHARPIN with HOIL-1 and HOIP, an N-terminally myc-tagged version of SHARPIN, was over-expressed in HEK293T cells either alone or in combination with C-terminally V5-tagged forms of HOIL-1 and HOIP. Immunoprecipitation with anti-V5 coupled beads followed by western blotting revealed that SHARPIN was co-precipitated with HOIL-1 and even more strongly with HOIP (Figure 26A). In order to verify this interaction in a more physiological setting, U937 cells were left untreated or stimulated with TNF for five minutes. The lysates of these cells were split into five parts of which three were used to precipitate SHARPIN, HOIL-1 and HOIP using specific antibodies, respectively, whereas the other two served as controls for unspecific binding to the beads or the antibodies. As shown in Figure 26B, precipitation of any of the

three proteins also pulled out the other two and the levels of the co-precipitated protein did not change upon TNF-stimulation. This demonstrates that in an unstimulated state endogenous SHARPIN, HOIL-1 and HOIP are associated with each other. However, this data was insufficient to judge whether the interaction between the three is direct. To clarify whether another factor is required to mediate the association of the three, recombinant versions of SHARPIN, HOIL-1 and HOIP were precipitated using specific antibodies and co-precipitation of the other two was analysed by western blotting (Figure 26C).

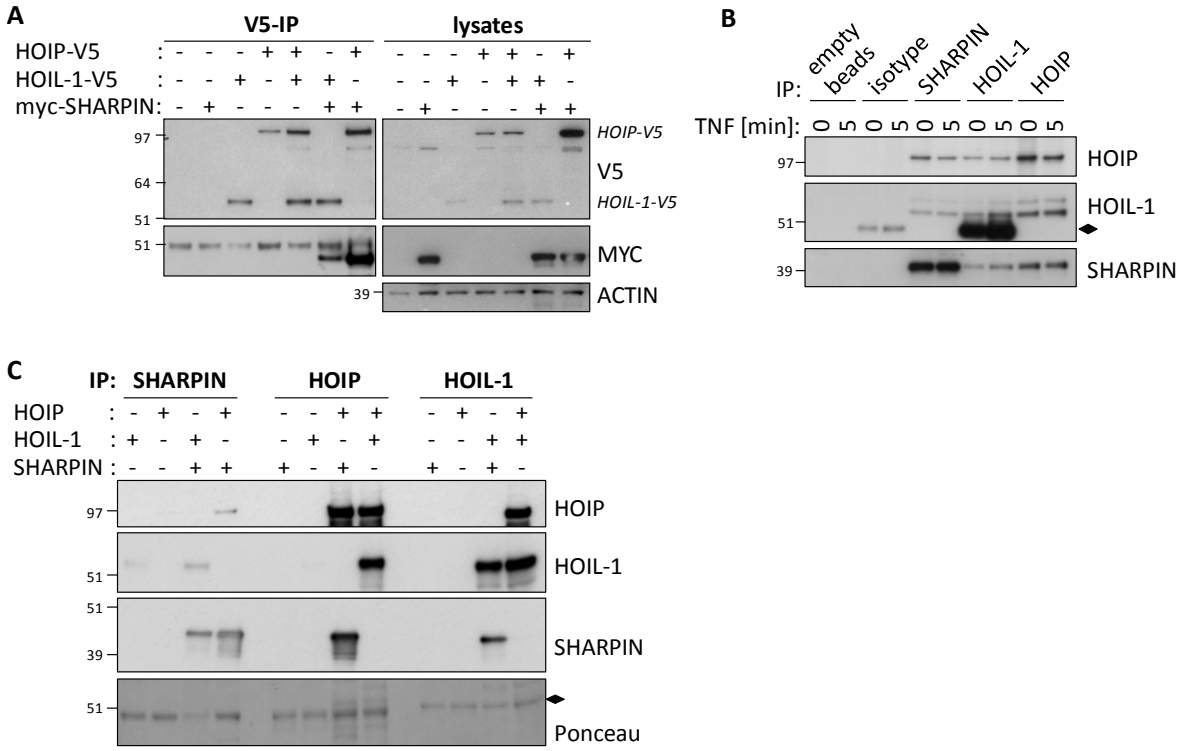


Figure 26: SHARPIN, HOIL-1 and HOIP interact in a direct and stimulation-independent manner. A: V5-tagged forms of HOIL-1 and HOIP were over-expressed in HEK293T cells either alone or in combination with SHARPIN and precipitated using anti V5-beads. Co-precipitation of SHARPIN was assessed on western blot level. **B:** U937 cells were stimulated with TNF for five minutes or left untreated. Lysates were split into five parts and precipitations using empty beads, an isotype control (IgG2a) or anti-SHARPIN, anti-HOIL-1 or anti-HOIP antibodies. Presence of the three proteins in the different pulldowns was determined by western blotting. **C:** Recombinant forms of SHAPRIN, HOIL-1 or HOIP were added to either the precipitations of the respectively other two or to beads containing the specific antibodies used for pull-down but not their specific antigen. The heavy chain as detected by PonceauS staining is used as a loading control. Black rhombi mark bands representing the heavy chain.

Both HOIL-1 and HOIP were associated with SHARPIN (lanes 3 and 4) but were not detectable on beads coupled to the anti-SHARPIN antibody (lanes 1+2), indicating that a specific and direct interaction occurs between the proteins. Similarly, interaction of SHARPIN and HOIL-1 with recombinant HOIP (lanes 7+8) and of SHARPIN and HOIP with

HOIL-1 (lanes 11+12) could be detected. These results demonstrate that SHARPIN, HOIL-1 and HOIP can associate with each other in a direct manner and that this interaction occurs in cells in a stimulation-independent manner. However, the data are not sufficient to distinguish between the existence of one complex containing all three proteins and that of different complexes containing only two of the proteins, respectively. Since SHARPIN and HOIL-1 show high sequence similarity (Lim et al., 2001) it seemed possible that HOIP associated with either of the two in a tissue- or cell line specific manner. Expression of HOIL-1 in a variety of tissues (Tokunaga et al., 1998b) and its association with HOIP in several cell lines (Kirisako et al., 2006; Tokunaga et al., 2009) were reported.

To test, whether SHARPIN shows a similar expression pattern, lysates of different human tissues were analysed by western blotting. Although quantitative statements are not possible due to different expression levels of GAPDH and ACTIN in the tissues analysed, the western blot depicted in Figure 27 shows that SHARPIN is widely expressed across tissues.

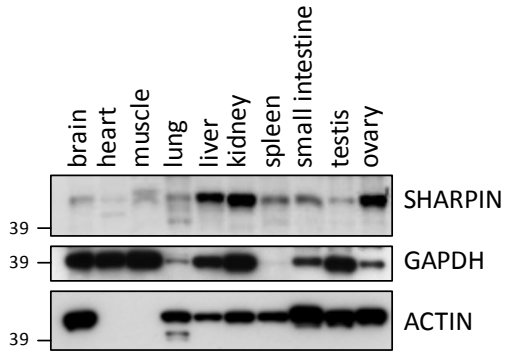


Figure 27: SHARPIN is widely expressed in normal human tissues. Lysates of normal human tissues (IMGENEX) were analysed by western blotting for the presence of SHARPIN. Membranes were probed with anti-GAPDH and anti-ACTIN antibodies as a loading control.

To test whether SHARPIN, HOIL-1 or HOIP exclusively binds only one of the other two in certain tissues, fourteen cell lines of different tissue origins (table 1) were used to precipitate each of the three proteins using specific antibodies respectively.

Table 5: Origin of cell lines used in Figure 28. Information according to ATCC, Benard et al and Boukamp et al. (Benard et al., 1985; Boukamp et al., 1988)**.**

cell line	disease	organ / cell type
HEK 293T	-	human embyonic kindey
HeLa	adenocarcinoma	cervix
HT1080	fibrosarcoma	connective tissue
HaCat	-	skin/keratinocyte
Saos-2	osteosarcoma	bone
IGROV-1	carcinoma	ovary
DKO-4	colorectal adenocarcinoma	colon
Du145	carcinoma	prostate
A549	carcinoma	lung
MCF-7	adenocarcinoma	mammary gland, breast
JA-3	acute T cell leukemia	T lymphocyte
Raji	Burkitt's lymphoma	B lymphocyte
U937	histiocytic lymphoma	monocyte / macrophage
THP-1	acute monocytic leukemia	monocyte

Although expression levels seem to differ slightly between cell lines, with very low levels of HOIL-1 in HEK293T and HeLa cells being the most notable difference, precipitation of any of the three proteins led to the co-precipitation of the other two in all cell lines tested (Figure 28).

While a tissue-specific formation of different dipartite complexes cannot be fully excluded on the basis of this result, it is a clear indication that stimulation-independent association of SHARPIN, HOIL-1 and HOIP is not a cell type specific event. Based on these results, a further investigation of the interactions between the three proteins was undertaken.

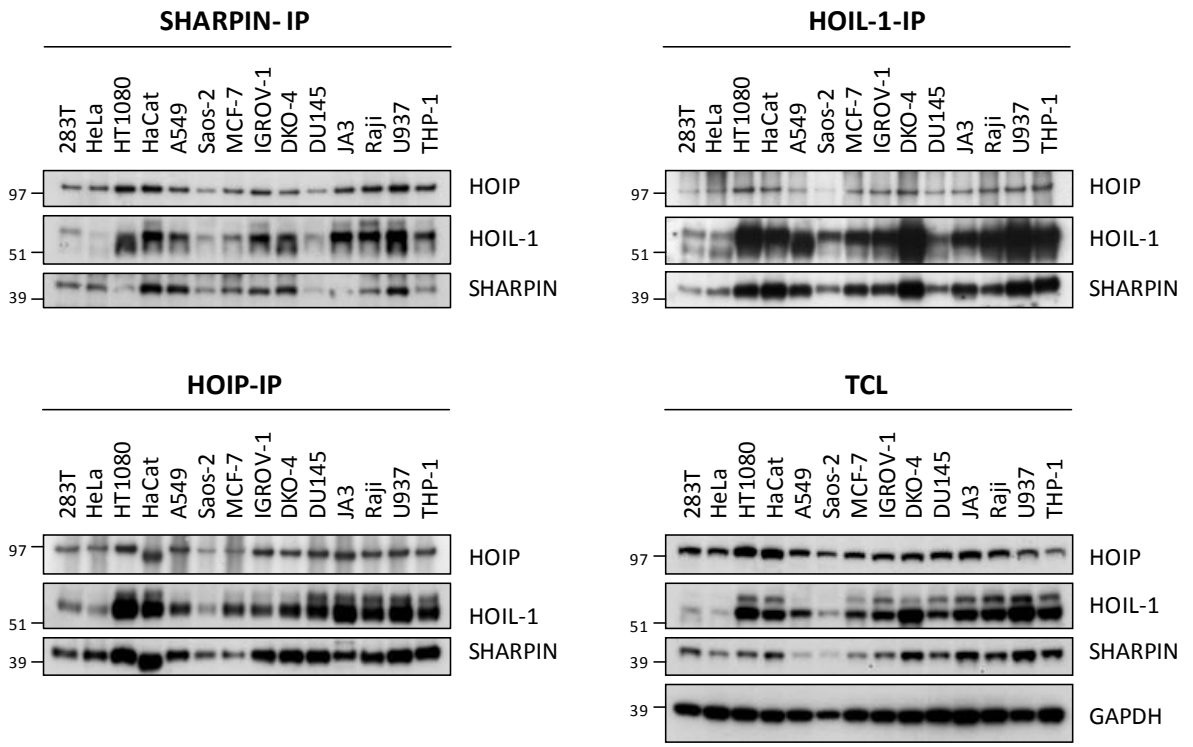
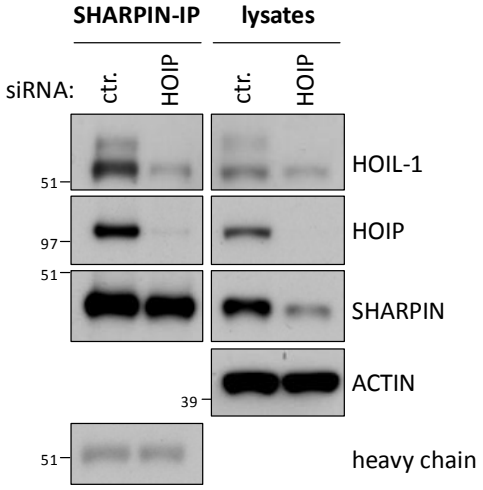


Figure 28: SHARPIN, HOIL-1 and HOIP co-precipitate in a variety of cell lines. Lysates of the indicated fourteen cell lines of different tissue origins were employed to assess protein levels of SHARPIN, HOIL-1 and HOIP by western blotting (lower right panel) or to precipitate each of the three proteins using specific antibodies. Association of the remaining two proteins with beads coupled to the third was again analysed on western blot level.

It can be noted in Figure 26A that more SHARPIN was co-precipitated with HOIP than with HOIL-1. To follow up on this point, HOIP expression was down-regulated using siRNA and the amount of HOIL-1 bound to SHARPIN, which was pulled out using a specific antibody, was estimated by western blotting (Figure 29A). In absence of detectable HOIP, the amount of HOIL-1 associated with SHARPIN was clearly decreased. However, as observed previously (Figure 15), down-regulation of HOIP severely affected the levels of SHARPIN and HOIL-1. To account for this co-down-regulation and to be able to judge whether there is a role for HOIP in mediating the interaction between SHARPIN and HOIL-1, the bands representing HOIL-1 were quantified using the ImageJ software (Figure 29B and C). The intensities were then normalised to those representing ACTIN in the lysates or SHARPIN in the immunoprecipitations. When the band intensities of the control were set to 100 % in both lysates and precipitations the amount of HOIL-1 in HOIP knockdown cells was reduced to 29 % and 18 %, respectively. This shows that although the absence of HOIP already leads to a down-regulation of HOIL-1 (by 71 %) in total cell extracts the interaction between SHARPIN and HOIL-1 is even further influenced by the absence of HOIP (82 % reduction)

compared to control cells. Although it cannot be distinguished if a direct binding between SHARPIN and HOIL-1 is taking place in the knockdown situation or if the remaining HOIP is sufficient to mediate this association, this result indicates that HOIP increases the interaction between the other two.

A



B

	IP		lysates	
siRNA	ctr.	HOIP	ctr.	HOIP
HOIL-1	38661	5713	13570	3888
ACTIN			29759	28987
SHARPIN	43390	36084		
relative ACTIN			1	0.97
relative SHARPIN	1	0.83		
HOIL-1/ACTIN			13570	3991
HOIL-1/SHARPIN	38661	6869		
relative HOIL-1 (lysates)			1	0.29
relative HOIL-1 (IP)	1	0.18		

C

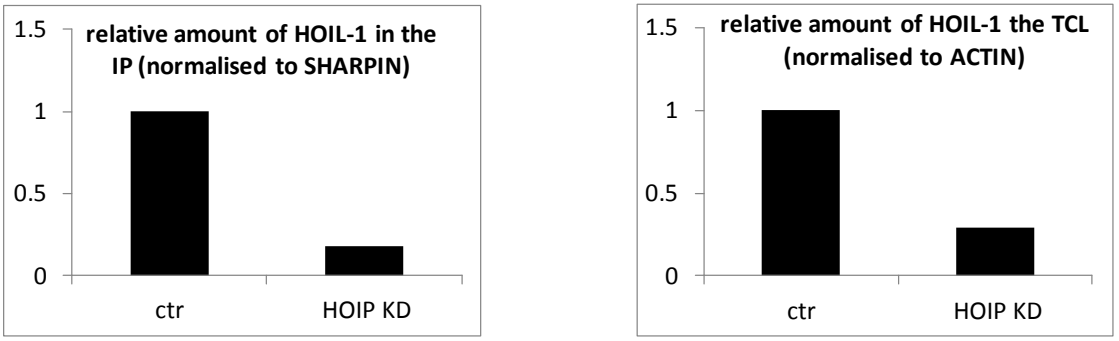


Figure 29: HOIP enhances the interaction between SHARPIN and HOIL-1 in HeLa cells. Expression levels of HOIP were down-regulated in HeLa cells and SHARPIN was precipitated using a specific antibody**. A:** Western blot showing the co-precipitation of HOIL-1 in presence or absence of HOIP. **B and C:** Quantification of bands from A and calculation of relative HOIL-1 levels in immunoprecipitations and lysates.

This was confirmed by co-precipitation of recombinant HOIL-1 with V5-tagged SHARPIN in absence or presence of increasing amounts of HOIP. Again, a weak direct interaction between HOIL-1 and SHARPIN could be detected and an increase in this association was observed in the presence of HOIP indicating that this protein can mediate the interaction between the other two (Figure 30).

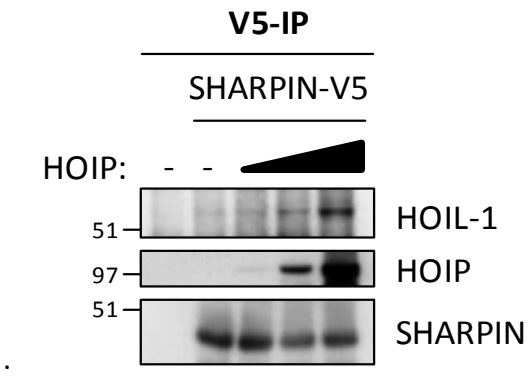


Figure 30: HOIP increases the interaction of HOIL-1 and SHARPIN *in vitro*. 10µg of recombinant V5-tagged SHARPIN per sample were used to couple the protein to beads. 3µg of HOIL-1 were added to either empty beads (first lane) or to the SHARPIN-coupled beads in the absence of HOIP (second lane) or in presence of different amounts (0.05µg, 0.5µg, 5µg) of HOIP. Co-precipitation was assessed on western blot level.

This provides additional evidence for the existence of a tripartite complex. Furthermore, it raised the question as to how HOIP binds to SHARPIN and HOIL-1 at the same time. The interaction between HOIL-1 and HOIP was reported to be mediated via their UBL- and UBA-domains respectively (Kirisako et al., 2006). In order to map which parts of HOIP are involved in binding SHARPIN, in-vitro-translated versions of HOIP lacking the UBA-domain, the N-terminus or the C-terminus (Figure 31A), were tested for their ability to bind recombinant SHARPIN.

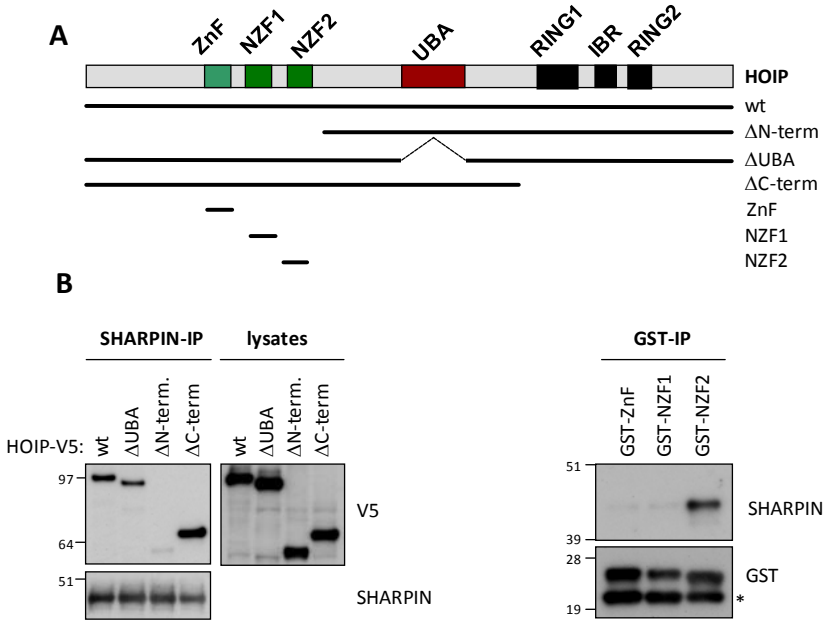


Figure 31: The NZF2-domain in the N-terminal part of HOIP is sufficient to mediate the interaction with SHARPIN. A: Schematic representation of HOIP mutants employed in B. **B:** In-vitro translated mutations of HOIP lacking the UBA-domain, the N-terminus or the C-terminus were tested for their ability to bind recombinant SHARPIN in a co-precipitation assay using a specific anti-SHARPIN antibody (left panel). Right panel: recombinant forms of the ZnF, NZF1 and NZF2 domain of HOIP were pulled down via their N-terminal GST-tag, recombinant SHARPIN was added and binding was assessed by western blotting.

As shown in Figure 31B deletion of its N-terminus abolished co-precipitation of HOIP, indicating that this part is essential for the association with SHARPIN. To further allocate the SHARPIN-binding motif to one of the three zinc-fingers present in the N-terminal part of HOIP, GST-tagged versions of these domains were purified from *E.coli*, coupled to glutathione-beads and tested for co-precipitation of SHARPIN (Figure 31C). This experiment revealed that the NZF2-domain was sufficient to pull down recombinant SHARPIN, whereas no binding of this protein to the ZnF- or NZF1-domain could be detected.

To identify the corresponding domain in SHARPIN that is recognised by HOIP's NZF2, recombinant HOIP was coupled to beads using a specific antibody and co-precipitation of in-vitro-translated SHARPIN mutants (Figure 32A) was analysed by western blotting. As shown in Figure 32B, binding efficiency was greatly reduced when the UBL-domain of SHARPIN was deleted.

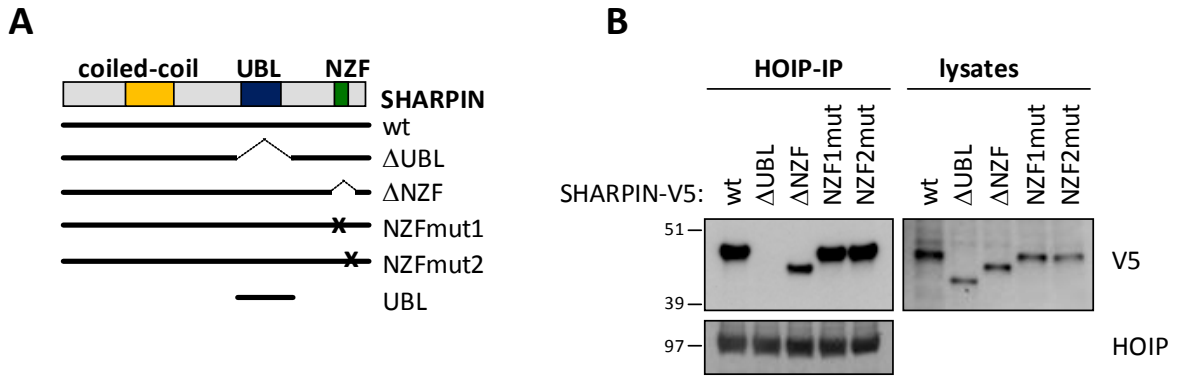


Figure 32: Deletion of SHARPIN's UBL-domain prevents its interaction with HOIP. A: Schematic representation of SHARPIN and of mutants employed in this assay. **B:** Recombinant HOIP was pulled down using a specific antibody and tested for its capacity to co-precipitate mutants of SHARPIN in which the UBL-domain or the NZF had been deleted or in which zinc-coordinating cysteines had been mutated to serine (NZFmut1= C353,356S; NZFmut2= C367,370S).

Indeed a GST-tagged recombinant form of this domain was sufficient to bind V5-tagged HOIP in precipitations performed using glutathione- (Figure 33, left panel) or anti-V5-beads (Figure 33, right panel).

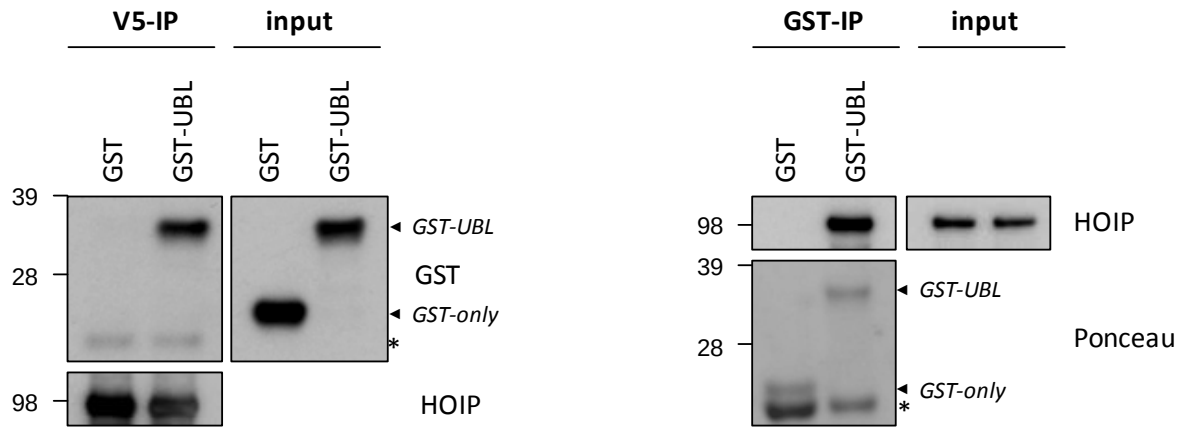


Figure 33: The UBL-domain of SHARPIN is sufficient to mediate the interaction with HOIP. Anti-V5-beads were used to precipitate recombinant HOIP and co-precipitation of GST-only or a GST-tagged form of SHARPIN's UBL-domain was analysed by western blotting (left panel). The reciprocal experiment was performed using glutathione-beads to precipitate GST-only or the N-terminally GST-tagged UBL-domain of SHARPIN. Binding of HOIP was determined using a specific antibody on western blot level. Precipitation of GST and the UBL-domain was controlled by PonceauS-staining.

Taken together, it could be shown that the interaction between HOIP and SHARPIN is mediated via their NZF2- and UBL-domains, respectively.

5.5 LUBAC exclusively generates linear ubiquitin chains *in vitro*

A high-molecular weight complex consisting of HOIL-1 and HOIP was previously described to act as an E3 generating linear ubiquitin chains (Kirisako et al., 2006). Furthermore, this activity was correlated to the ability of inducing NF-κB activation upon over-expression (Haas et al., 2009; Tokunaga et al., 2009). To investigate the effect SHARPIN has on the processes mediated by LUBAC, the three proteins were ectopically expressed in HEK293-NF-κB cells and the resulting luciferase expression was assessed in an activity assay. As shown in Figure 34, none of the proteins induced NF-κB activation when expressed on its own and neither did the combination of SHARPIN and HOIL-1. However, combination of HOIP with either SHARPIN, HOIL-1 or both induced luciferase expression, providing evidence that also in the context of LUBAC's activity SHARPIN fulfils a role similar to that of HOIL-1.

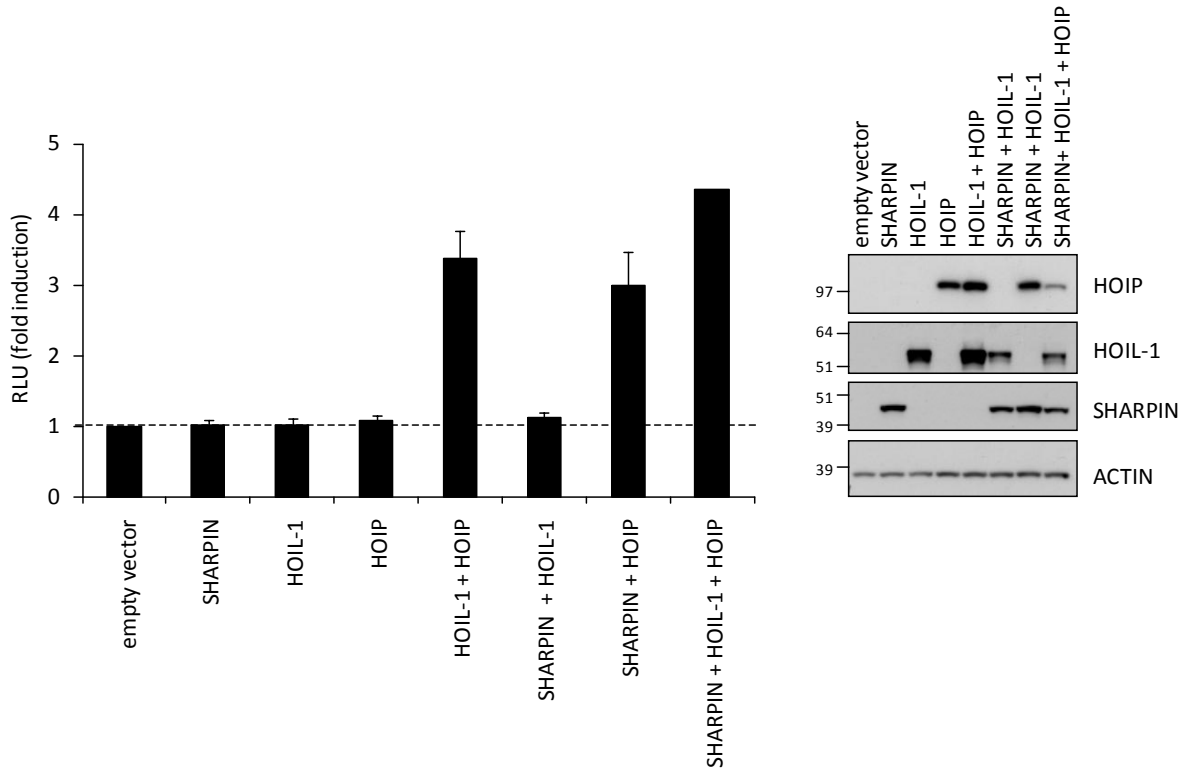


Figure 34: Over-expression of HOIP in combination with SHARPIN and/or HOIL-1 induces NF-κB activation in a luciferase-reporter assay. HEK293-NF-κB cells were transfected with SHARPIN, HOIL-1 and HOIP either alone or in combination. Luciferase-expression was measure in a luciferase-assay and normalised to renilla-activity. Fold inductions in the relative luminescence units (RLU) were calculated and results are shown as mean +/- SEM; n=5 (left panel). The right panel shows a representative western blot to control for successful over-expression of the three proteins.

To be able to directly investigate its role in the generation of linear ubiquitin chains by LUBAC, an in-vitro ubiquitination assay using recombinant proteins and mono-ubiquitin as a model substrate was set up. By stopping the reaction after the times indicated in Figure 35A, the incubation time necessary for efficient chain formation was established. The assay showed that the combination of HOIL-1 and HOIP, in line with it being previously described to act as an E3 (Kirisako et al., 2006), was active in generating ubiquitin chains as seen by the appearance of poly-ubiquitin conjugates after twenty and more strongly after forty minutes (Figure 35A, lanes 7+8). This conjugation was specifically mediated by HOIL-1 and HOIP because even after sixteen hours the combination of E1 and E2 was unable to catalyse the reaction in the absence of an E3 (lane 1+2). Based on the signal strength for the ubiquitin-chains generated and on practicability an incubation time of two hours was deemed optimal and therefore applied in all following experiments of this type. Having set up the system to analyse it, SHARPIN's ability to promote the formation of ubiquitin conjugates was assessed (Figure 35B). Again, combinations of HOIP with SHARPIN and/or

HOIL-1 but not the combination of SHARPIN and HOIL-1 or HOIP alone were active. Thus, the capacity to promote formation of poly-ubiquitin conjugates correlated well with the ability or inability of these combinations to induce NF-κB activation as established in Figure 34.

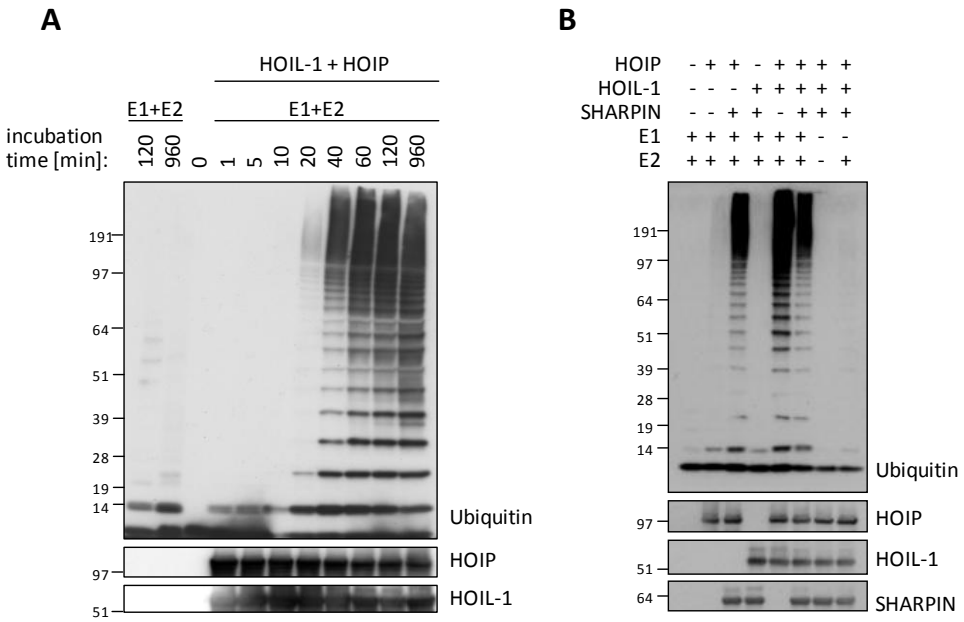


Figure 35: HOIP generates ubiquitin chains when complexed with SHARPIN and/or HOIL-1. A: An in-vitro assay was performed as described under 4.2.3.16. Incubation time for in-vitro ubiquitination assays was optimised by stopping the reaction catalysed by the E3-complex consisting of HOIL-1 and HOIP after the indicated times. Formation of ubiquitin polymers was determined on western blot level. **B:** Different combinations of untagged HOIP and GST-tagged SHARPIN were tested for their ability to mediate the conjugation of ubiquitin moieties in the presence or absence of E1 and E2 in an in-vitro assay as described under A.

The E3-complex consisting of HOIL-1 and HOIP, LUBAC, was found to be able to generate ubiquitin chains from K0-ubiquitin, in which all lysine residues were mutated to arginine, and it was concluded that these chains are linked via the N-terminal methionine (Kirisako et al., 2006). It can be observed in Figure 35B, that the bands for the ubiquitin conjugates generated by SHARPIN-containing E3-combinations run at the same apparent molecular weight as the ones generate by HOIL-1 and HOIP. This indicates that these chains are also M1-linked because different linkage types, especially of lower order ubiquitin oligomers, can be distinguished due to their different electrophoretic mobilities (Komander et al., 2009b). For the purpose of assessing more directly if SHARPIN supports the formation of linear chains or if it shifts linkage specificity towards a different type, the active E3-combinations identified in Figure 35B were compared to TRAF6 in their ability to use N-terminally His-

tagged ubiquitin, in which the N-terminal aminogroup of M1 is rendered inaccessible by the conjugation of six histidine-residues while all lysine residues remain unchanged, as a substrate. Although all E3s were active in promoting the formation of ubiquitin-chains using wild-type ubiquitin, conjugation of His-ubiquitin could only be observed for TRAF6 (Figure 36A). This demonstrates that HOIP in combination with SHARPIN and/or HOIL-1 is not only able to generate linear ubiquitin chains but is restricted to catalysing the formation of only this linkage type. In line with this strict linkage specificity, addition of increasing amounts of His-tagged ubiquitin reduces the formation of poly-ubiquitin chains by HOIL-1 and HOIP (Figure 36B), indicating that N-terminally tagged ubiquitin acts as a competitive inhibitor of LUBAC.

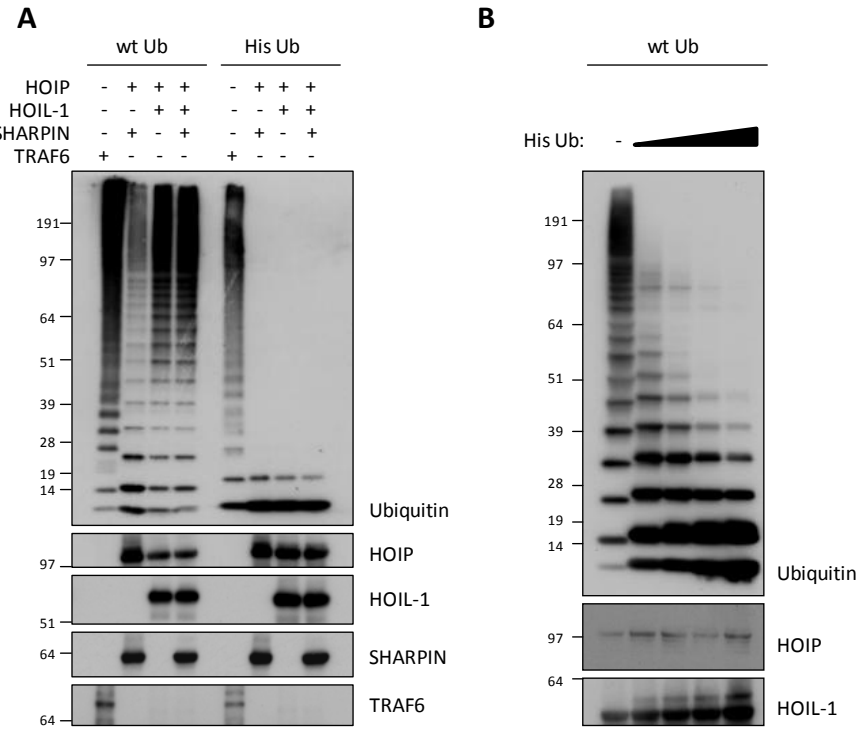


Figure 36: All LUBAC combinations exclusively generate linear linkages. A: HOIP in combination with SHARPIN and/or HOIL-1 was compared to TRAF6 in its ability to generate ubiquitin chains from wild-type (wt Ub) or His-tagged (His Ub) ubiquitin in an in-vitro ubiquitination assay. **B:** Increasing amounts of His-tagged ubiquitin (0.5, 1, 2, 10μg) were added to reactions of an in vitro-assay of HOIL-1 and HOIP generating polyubiquitin conjugates.

Overall, this data shows that LUBAC is unable to generate any linkage type other than linear chains and that SHAPRIN is both a structural and a functional component of this E3 complex. The term LUBAC will hence be used from here onwards to refer to the tripartite, linear ubiquitin chain generating complex consisting of SHARPIN, HOIL-1 and HOIP.

In addition, in Figure 36B an additional band can be noticed in the anti HOIL-1 blot thus creating a pattern that resembles the appearance of HOIL-1 in cell lysates (Figures 10, 13, 28). This gave rise to questions concerning the identity, location and function of this modification.

5.6 Modifications of LUBAC components

A common principle in the regulation of enzymes is the addition of post-translational modifications. In several previous analyses it can be noticed that LUBAC-components are indeed modified (e.g. Figures 10, 13, 15 and 22). The fact that LUBAC is an E3 together with the sizes of the modified proteins in relation to their unmodified forms suggests that the type of modification could potentially be ubiquitination. To test this hypothesis, the TNF-RSC was precipitated from U937 cells and the precipitate was split into four parts of which two were treated with different amounts of the catalytic domain of USP2 (Figure 37). This deubiquitinase was described to remove a wide range of ubiquitin linkages while not affecting modifications with the ubiquitin-like protein NEDD8 (Kim et al., 2011). Treatment with USP2 was efficient as can be seen by the almost complete de-modification of RIP1 and cIAP2. Furthermore, modifications of SHARPIN, HOIL-1 and HOIP were also removed by DUB-treatment, indicating that LUBAC-components are indeed ubiquitinated within the TNF-RSC.

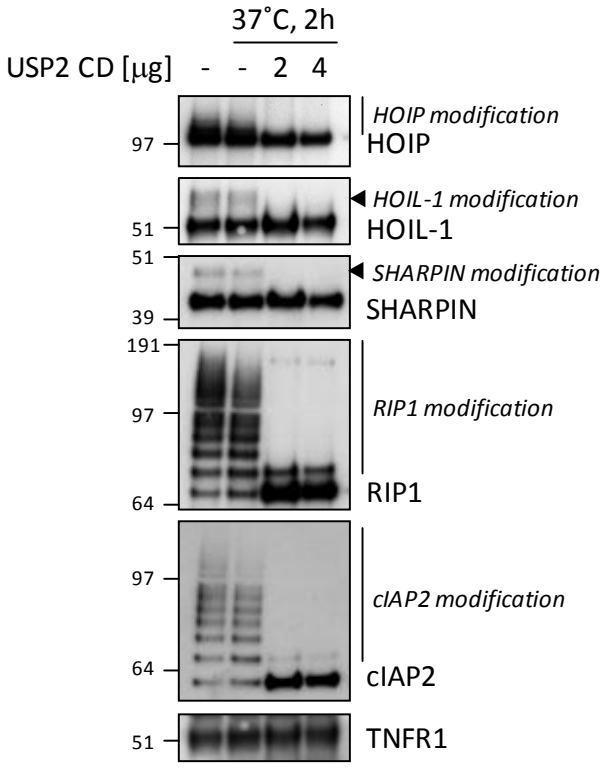


Figure 37: Treatment with the catalytic domain of USP2 removes modifications form LUBAC components present in the TNF-RSC. $2x10^8$ U937 cells were stimulated with TNF for five minutes. The TNF-RSC was isolated using M2-beads, the precipitate was split into four parts of which one was left untreated, one was incubated in DUB-buffer for two hours at 37˚C and the remaining two were treated with 2 or 4 µg of the catalytic domain (CD) of USP2 (Enzo Life Sciences), respectively. Reactions were stopped by addition of reducing sample buffer and protein modifications were analysed on western blot level.

The strength of the band representing modified HOIL-1 in relation to its unmodified form, as observed in several experiments, indicates that a higher percentage of this protein is modified as compared to SHARPIN and HOIP. Based on this distribution I chose to investigate the modification of HOIL-1 in more detail. To study the influence of TNF-stimulation and to investigate a potential role for SHARPIN and HOIP in mediating this modification, HeLa cells in which expression of HOIP (Figure 38A) or SHARPIN (Figure 38B) had been down-regulated by siRNA, were treated with TNF for five or fifteen minutes and the corresponding lysates were analysed on western blot level. No significant change can be observed in the upper band of HOIL-1 upon stimulation with TNF, indicating that HOIL-1 modification is a stimulation-independent event. However, down-regulation of SHARPIN or HOIP led to a clear decrease in this modification (Figure 38A and B). To be able to directly compare the effects the down-regulation of the two proteins has on HOIL-1 modification, HeLa (Figure 38C, left panel) and A549 cells (Figure 38C, right panel) were transfected with siRNAs targeting SHARPIN or HOIP. Again, it could be observed that a decrease of HOIP protein levels was accompanied by a strong reduction in the HOIL-1 modification. In HeLa cells knockdown of SHARPIN also led to a decrease in the intensity of the band representing a modification on HOIL-1, albeit to a lesser degree than down-regulation of HOIP. In contrast, in A549 cells this modification seems to be largely unaffected by a decrease in SHARPIN levels. This suggests that SHARPIN may have a, potentially cell line specific, role in mediating the modification of HOIL-1 but that it is not able to modify HOIL-1 on its own. Presence of HOIP on the other hand is essential for HOIL-1 modification to occur.

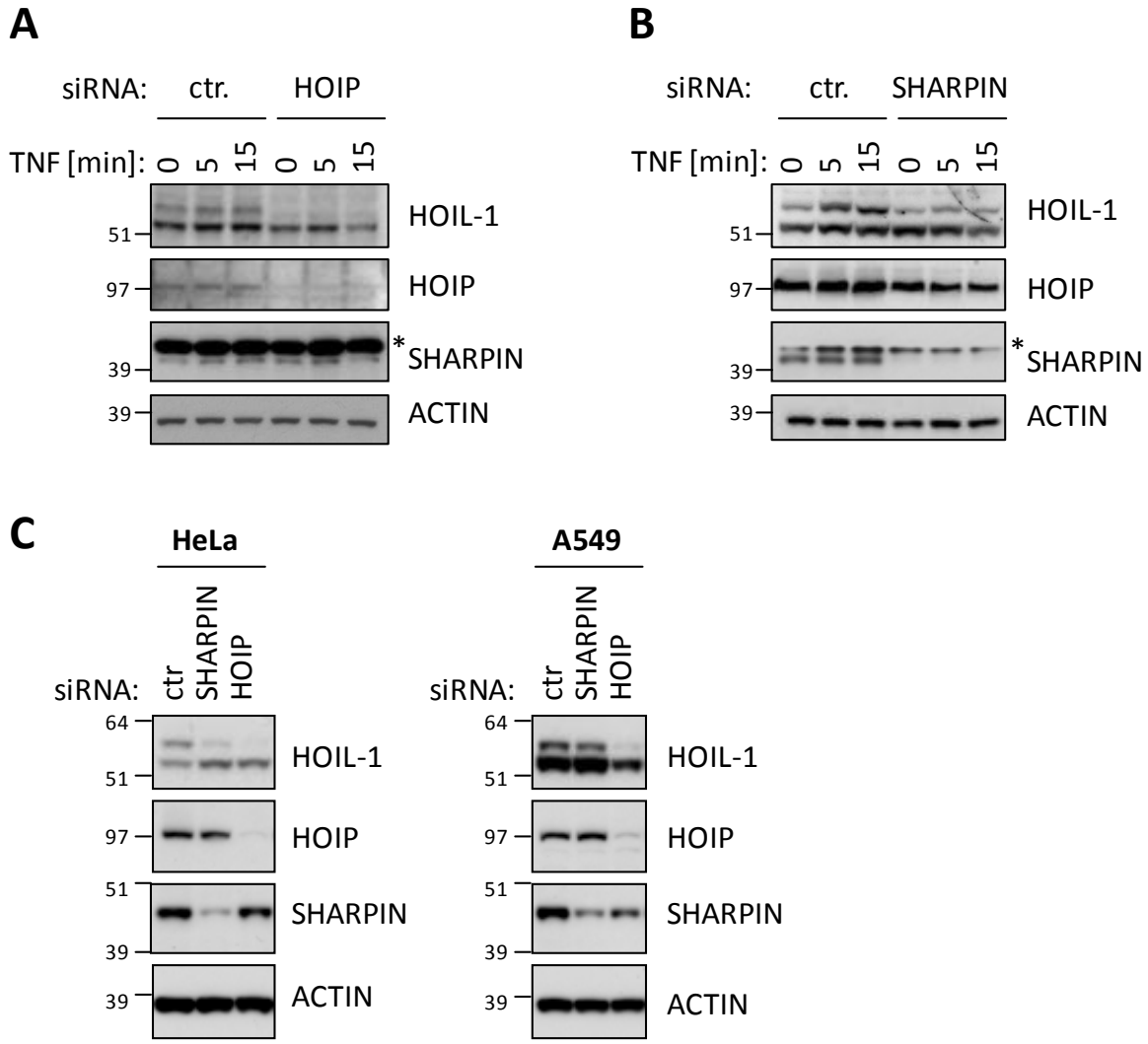


Figure 38: Modification of HOIL-1 depends on HOIP and potentially SHARPIN. A, B: Expression of HOIP (A) or SHARPIN (B) was transiently down-regulated in HeLa cells using siRNA. Cells were stimulated with TNF [1µg/mL] for five or fifteen minutes and the indicated proteins were analysed by western blotting. **C:** SHARPIN and HOIP were knocked down in HeLa (left panel) or A549 cells (right panel). HOIL-1 modification and knockdown efficiency were determined on western blot level.

To further clarify the type of the HOIL-1 modification and to assess the need for HOIP's activity in generating it, an in-vitro assay was performed. The fact that a modification of HOIL-1 can also be observed in this in-vitro system (Figure 39) provides further evidence that it is a ubiquitination, as other potential modifiers are not present here.

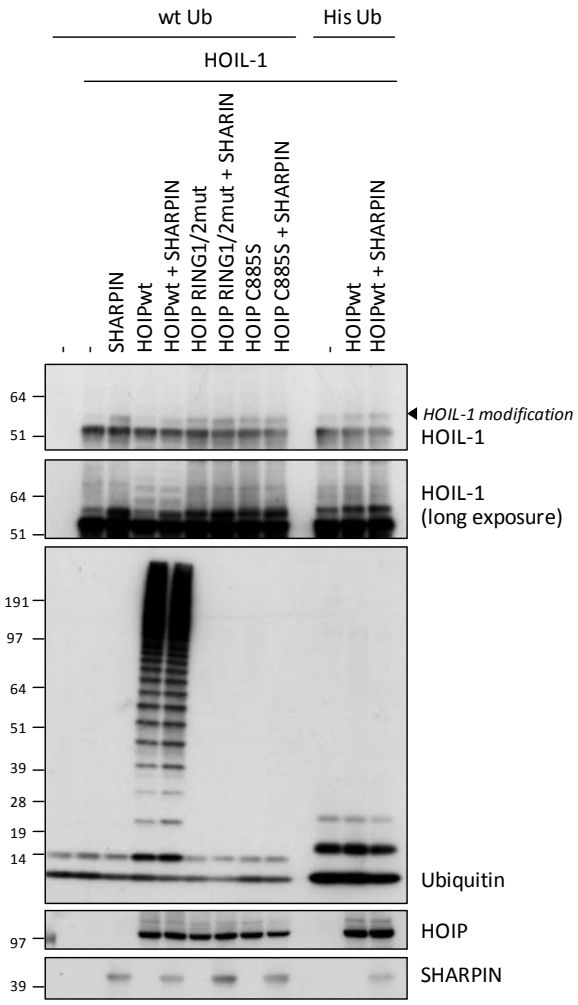


Figure 39: Modification of HOIL-1 can be observed in an in-vitro ubiquitination assay. Recombinant HOIL-1 was combined with the indicated forms of HOIP and SHARPIN, a ubiquitination assay was performed in presence of either wild-type or His-tagged ubiquitin and activity of LUBAC as well as HOIL-1 modification were assessed by western blotting.

In contrast to the observations made in cell lines (Figure 38) it could be noted that HOIL-1 was modified to some extent even in the absence of SHARPIN and HOIP (Figure 39, lane 2) and addition of SHARPIN alone was sufficient to increase the intensity of the band representing this modification (lane 3). A similar enhancement could be observed when inactive mutants of HOIP, alone or in combination with SHARPIN, were added to HOIL-1 (lanes 6-9) or when LUBAC's activity was blocked by His-tagged ubiquitin (lanes 11 and 12). In the last two lanes a slight increase in the distance between the bands representing the two forms of HOIL-1 could be observed which corresponds to the size difference between wild-type and His-tagged ubiquitin. This provides further evidence that HOIL-1 is indeed mono-ubiquitinated. When active LUBAC was created by adding wild-type HOIP to HOIL-1 in absence of presence of SHARPIN (lanes 4 and 5) a laddering pattern indicates that not only

one but several ubiquitin moieties are added to HOIL-1 in this case, which is in contrast to the observations made in cell lines where HOIL-1 modification seems to be restricted to mono-ubiquitination.

In spite of the differences between the in-vitro system and the events occurring in cell lines, the HOIL-1 modification was sufficiently similar between the two for the in-vitro assay to be deemed a suitable system for performing a first characterisation of this ubiquitination event. In order to locate the modification site within HOIL-1, I made use of the fact that Thrombin cleaves this protein at aminoacid 183. This cleavage creates an N-terminal fragment if around 21 kDa that contains the N-terminal aminogroup as well as the ε-amino-groups of eight lysine residues as potential ubiquitination sites. Another twelve lysines are contained in the C-terminal part which has a predicted molecular weight of approximately 39 kDa (Figure 40A). To determine which of the two parts comprises the modification sites a C-terminally V5-tagged recombinant version of HOIL-1 was subject to an in-vitro ubiquitination assay, resulting in modification of the protein. After completion of the reaction, each sample was split into two parts of which one was left untreated (lanes 1, 3, 5, 7, 9) whereas Thrombin was added to the second part (lanes 2, 4, 6, 8, 10). As the anti-HOIL-1 antibody was raised against a peptide in the N-terminal part of this protein, both the N-terminal and the C-terminal fragment could be detected after cleavage by western blotting using the anti-HOIL-1 and an anti-V5 antibody respectively. This analysis revealed that no band was detectable at a molecular weight that could have resulted from the addition of a 7kDa ubiquitin molecule to the N-terminal fragment. On the other hand a clear double-band was visible in the anti-V5 blot, demonstrating that HOIL-1 is modified within its C-terminal part *in vitro*.

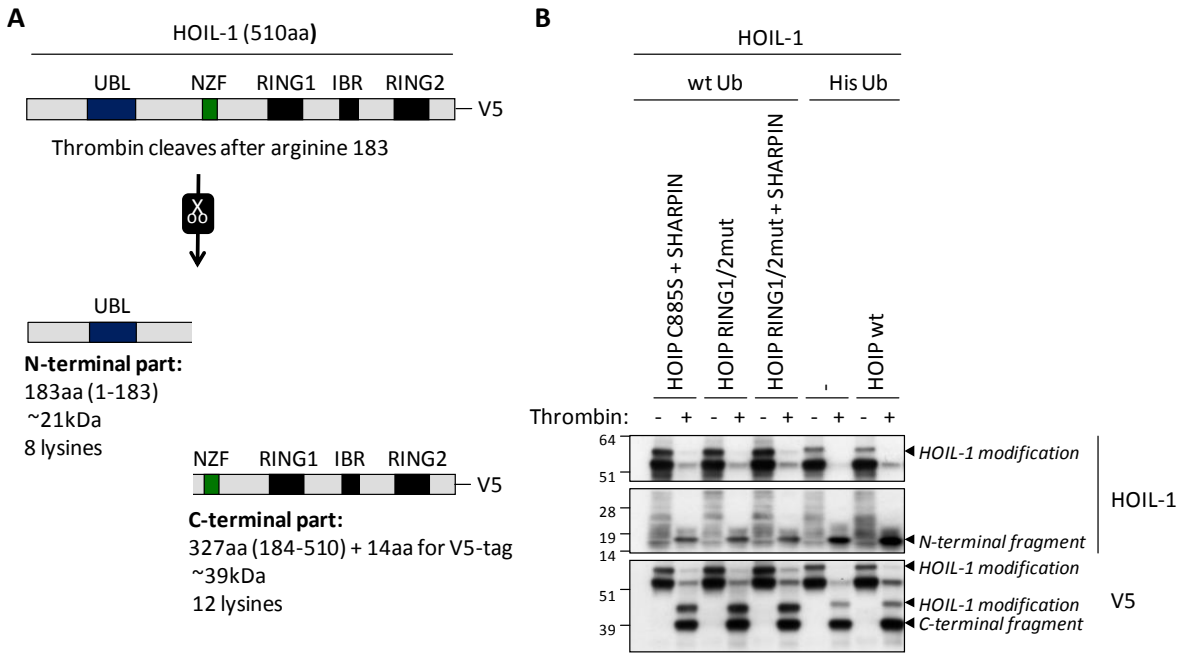


Figure 40: HOIL-1 is modified within its C-terminal part *in vitro*. A: Schematic representation of C-terminally V5-tagged HOIL-1 and the cleavage fragments generated by Thrombin. **B:** An in-vitro ubiquitination assay was performed as in Figure 36. After completion of the reaction, each sample was split into two parts of which one was treated with Thrombin for another two hours at 37˚C. Afterwards modification of HOIL-1 was assessed on western blot level using an anti-HOIL-1 antibody to detect the N-terminal and an anti-V5 antibody to show the C-terminal fragment. aa: aminoacid

Two recent studies reported on the mass-spectrometry based identification of ubiquitination sites on a proteome-wide level (Kim et al., 2011; Wagner et al., 2011). In combination the two reports found lysines 158, 174, 254 and 342 of HOIL-1 to be modified. Because K158 and K174 are N-terminal of the Thrombin cleavage site and because K342 was identified by only one of the two studies whereas K254 was reported to be modified by both publications, the latter residue seemed to be the most likely candidate for the residue to be targeted by mono-ubiquitination. Site-directed mutagenesis was therefore employed to generate a mutant of HOIL-1 (referred to as K254R) in which this lysine, that is located between its NZF- and RING1-domains, was replaced by an arginine. In an in-vitro assay comparing recombinant forms of wild-type and mutant HOIL-1 both variants showed equivalent levels of modification when ubiquitin was present in the reaction (Figure 41). The band representing this modification could be observed in both the anti-HOIL-1 and the anti-ubiquitin blot, further confirming that HOIL-1 is ubiquitinated. The lack of a difference between wild-type and mutant HOIL-1 in modifiability suggests that K254 is not the modification site of HOIL-1 required for its mono-ubiquitination.

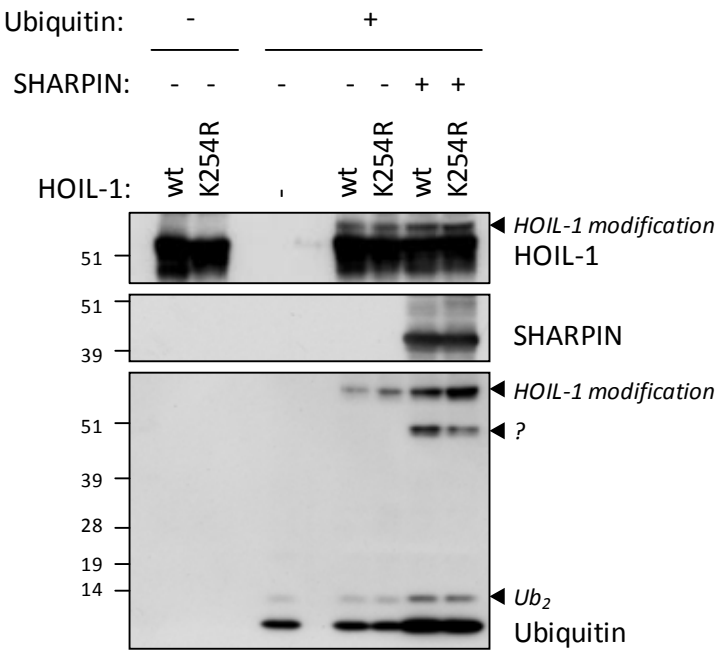


Figure 41: **Mutation of lysine 254 to arginine is insufficient to prevent modification of HOIL-1**. Recombinant forms wild-type HOIL-1 or a mutant in which lysine 254 had been replaced by arginine (K254R) were employed in an in-vitro ubiquitination assay in the presence or absence of ubiquitin and/or SHARPIN. Modification of HOIL-1 was assessed by probing the corresponding western blot with the indicated antibodies.

Another observation that can be made here is the presence of an additional band in the anti-ubiquitin blot that only becomes apparent when SHARPIN is added and that runs at an apparent molecular weight of just below 51 kDa. It could therefore correspond to a mono-ubiquitinated form of SHARPIN, which would be in line with a modification of SHARPIN present in the TNF-RSC that was shown to be sensitive to DUB-treatment (Figure 37).

Overall, these data indicate that all three LUBAC components are subject to modifications that due to their size and their DUB-sensitivity are likely to be ubiquitinations. Whereas modification of HOIP seems to be a stimulation-dependent event as seen by the appearance of additional bands following TNF-stimulation (Figure 10), ubiquitination of HOIL-1 is already present in an unstimulated state but depends on presence of HOIP and to a lesser degree of SHARPIN (Figure 38). The location of the HOIL-1 modification occurring *in vitro* could be mapped to its C-terminal part although the identification of the exact ubiquitination site will require further investigation (Figures 40 and 41).

5.7 LUBAC modifies NEMO in vitro and differs from other E3s in its linkage specificity

The ability of LUBAC to induce NF-κB activation when over-expressed and its role in TNF-induced signalling together with its activity as an E3 suggested that its effect on signal transduction is accomplished by modification of proteins relevant to the respective signalling cascades. To investigate which protein may be a target, different components of the TNF-RSC were ectopically expressed in HEK293T cells and precipitated via their respective tags or using specific antibodies. The immobilised proteins were then divided into five parts and incubated with the different LUBAC-combinations in an in-vitro ubiquitination assay (Figure 42).

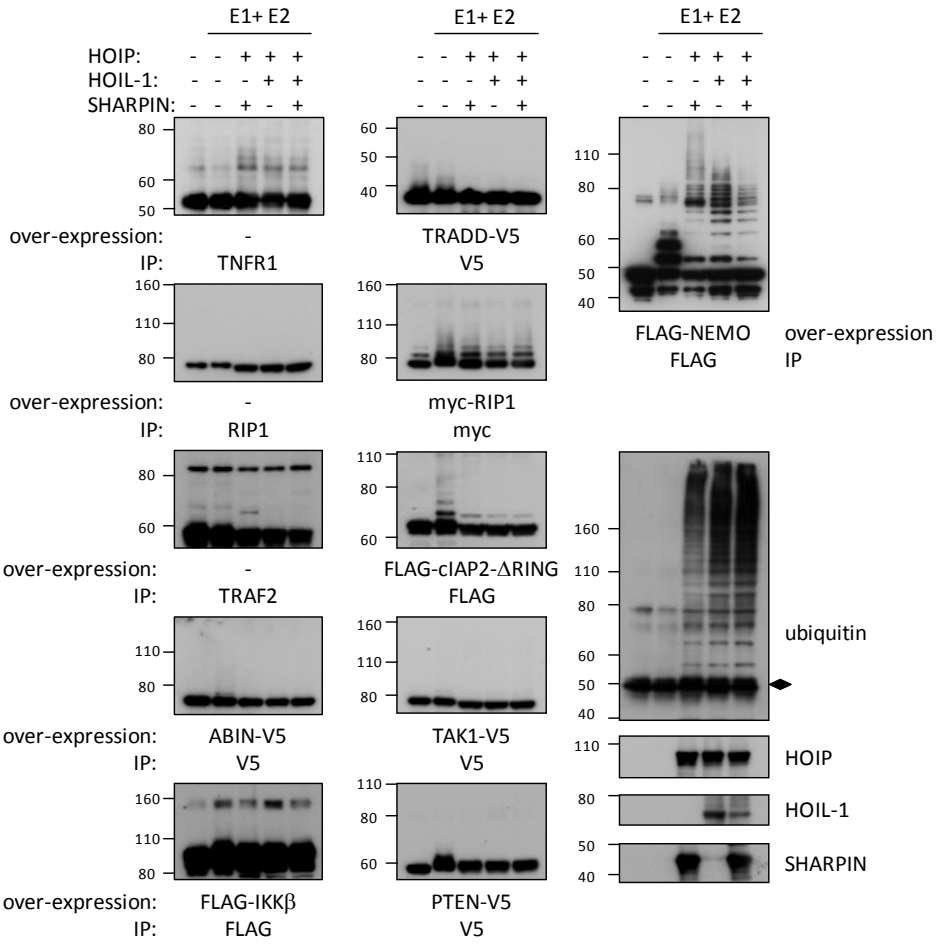


Figure 42: All three LUBAC-combinations modify NEMO *in vitro*. Different components of the TNF-RSC and PTEN, a protein that was described to interact with SHARPIN (He et al., 2010), were precipitated from transfected or untreated HEK293T cell. Precipitates were split into five samples that were either left untreated or employed as a substrate in an in-vitro ubiquitination assay using different combinations of untagged recombinant HOIP or SHARPIN and GST-tagged HOIL-1 as E3s. Modification was assessed on western blot level by probing the membrane with an antibody specific for the potential target. Activity of all three LUBAC combinations was demonstrated by probing with an anti-ubiquitin antibody.

Detection of bands at higher molecular weights by the antibody specific for the respective target protein in LUBAC-treated samples, that were not present in the immunoprecipitations left untreated (Figure 42, first lanes) or incubated with E1 and E2 only (second lanes) indicated that a modification mediated by LUBAC and not by an E3 co-precipitated with the target was taking place. This analysis revealed that all three LUBAC versions were capable of ubiquitinating NEMO *in vitro* whereas none of the other TNF-RSC components or PTEN, a protein that was described previously to be inhibited by SHARPIN (He et al., 2010), was modified to a significant extent. The identification of NEMO as a target of all LUBAC-combinations is in line with a report describing modification of this protein by HOIL-1 and HOIP (Tokunaga et al., 2009) and could also be corroborated by the mass-spectrometry based finding that linearly ubiquitinated forms of NEMO are present in the native TNF-RSC (Gerlach et al., 2011).

Because HOIL-1 and HOIP belong to the RING-in-between-RING (RBR)-family of E3s (Marin et al., 2004) I next investigated whether the property of exclusively generating linear ubiquitin chains is an intrinsic characteristic of this protein family. For this purpose, the RBR-ubiquitin ligases Parkin (PARK2), ARIH2 (ariadne homolog2, also known as TRIAD1), RNF19A (Dorfin), RNF144B (ring finger protein 144B or IBR domain-containing protein 2 (IBRDC2)), RNF216 (also known as triad domain-containing protein 3 (TRIAD3) or zinc finger protein inhibiting NF-kappa-B (ZIN)) and RNF14 (androgen receptor-associated protein 54 (ara54) or TRIAD2) (Figure 43) were produced in *E.coli*.

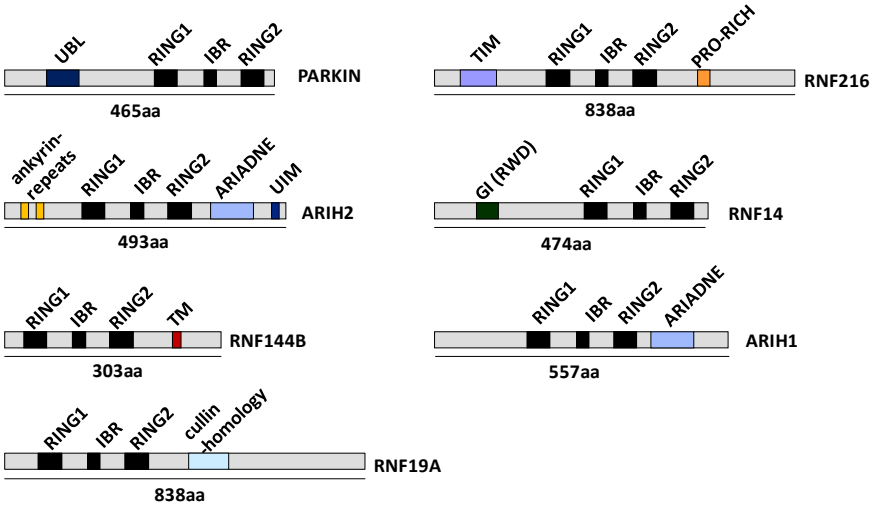


Figure 43: Schematic representation of RBR-family members. Proteins and their domains are not drawn to scale. UBL: ubiquitin-like; UIM: ubiquitin-interacting motif; TM: trans-membrane; TIM: TRAF-interaction motif; PRO-RICH: Proline-rich, Modified from (Marin et al., 2004).

The purified proteins were then tested for their ability to use wild-type or His-tagged ubiquitin as a substrate. Figure 44A shows that, in contrast to LUBAC, these E3s were not compromised in their activity by the N-terminal tag in ubiquitin, indicating that they are able to use lysine residues to conjugate ubiquitin moieties. However, this result does not exclude the possibility that linear linkage can be formed by these E3s when that option is available.

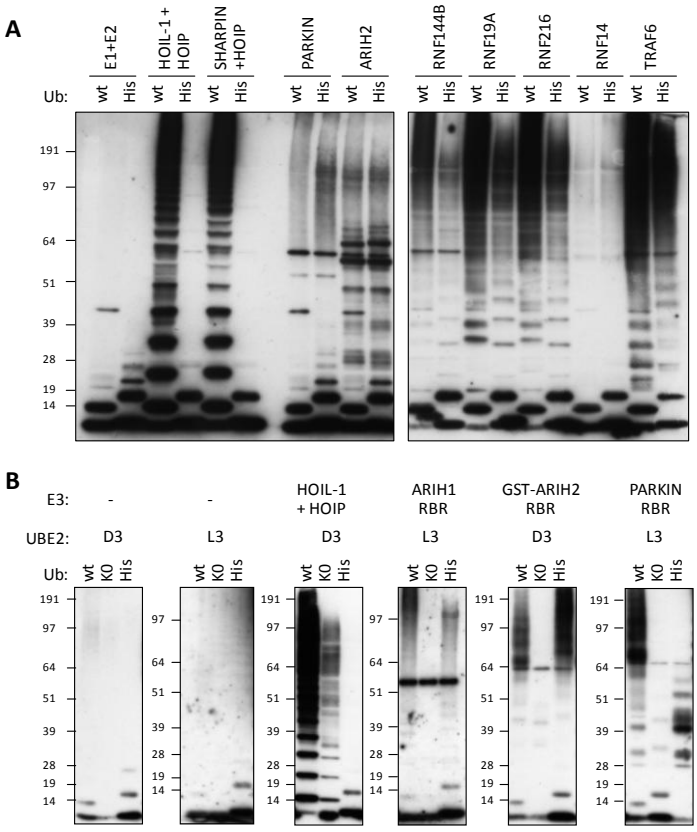


Figure 44: Other members of the RBR-family can employ His-tagged but not lysine-less ubiquitin as a substrate in in-vitro ubiquitination assays. A: Different members of the RBR-family were compared to HOIP in combination with SHARPIN or HOIL-1 in in-vitro ubiquitination assays preformed as described in Section x using wild-type (wt) or His-tagged (His) ubiquitin as a substrate as indicated. **B:** Recombinant forms of the RBR-domains of ARIH1, PARKIN (without a tag) or ARIH2 (GST-tagged) were compared to LUBAC in their capacity to generate ubiquitin chains from wild-type (wt), lysine-less (K0) or His-tagged (His) ubiquitin. The E2 employed in the different reaction is indicated. Combinations of E2 and E3 were chosen based on maximal activity in previous assays.

To investigate this aspect and to exclude effects like the auto-inhibitory influence of Parkin's UBL domain (Chaugule et al., 2011) the RBR-domains of ARIH1 (also known as human homolog of *Drosophila* ariadne-1 (HHARI)), ARIH2 and Parkin were purified and ubiquitination assays were performed using wild-type, lysine-less (K0) or His-tagged ubiquitin. As expected, the combination of HOIL-1 and HOIP was able to generate polyubiquitin from wild-type or K0- but not from tagged ubiquitin (Figure 44B). On the other

hand the RBR-domains of the other family members still showed activity in spite of the N-terminal tag on ubiquitin but were not able to generate ubiquitin-polymers from lysine-less ubiquitin to a significant degree. This does not exclude that formation of linear linkages can be catalysed by some members of the RBR-family, but it proves that not all proteins belonging to this group are capable of or even restricted to generating chains of this linkage type. To test whether the linear linkages identified in the native TNF-RSC by mass spectrometry (Gerlach et al., 2011) were definitely generated by LUBAC or whether a different E3 known to be present in this complex shows the same linkage specificity, recombinant forms of cIAP1, cIAP2 and TRAF2 were compared to LUBAC in an in-vitro assay.

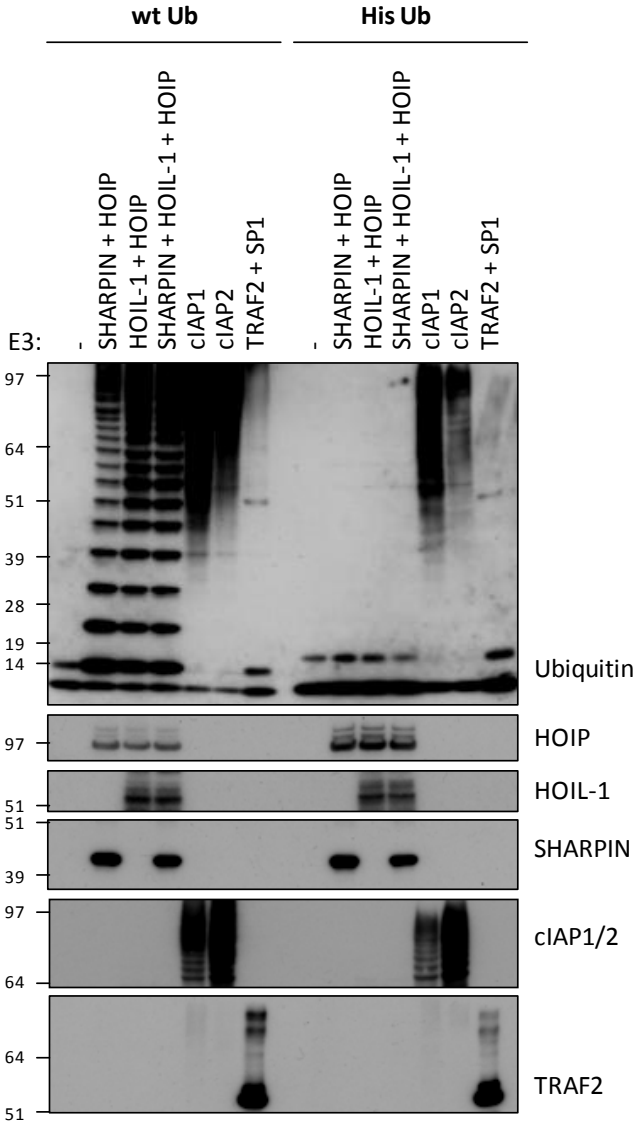
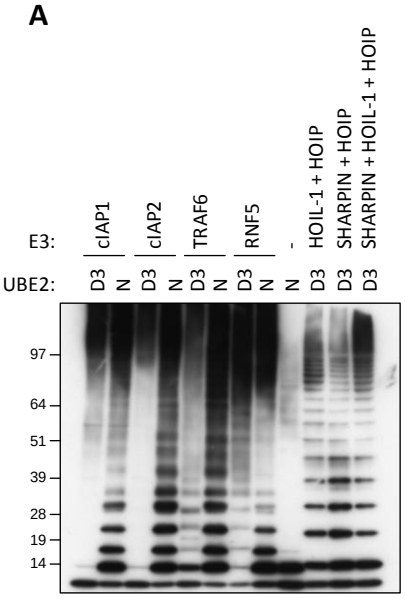


Figure 45: Other E3s present in the TNF-RSC are largely unaffected in their capacity to generate poly-ubiquitin by an N-terminal tag on ubiquitin. Recombinant forms of cIAP1, cIAP2 and TRAF2 were compared to the different LUBAC combinations in an in-vitro ubiquitination assay. Their capacity to generate ubiquitin conjugates from wild-type (wt) or N-terminally tagged (His) ubiquitin was assessed by western blotting. 100 nM sphingosine-1-phosphate was added to TRAF2 containing reactions as a cofactor.

Because it was reported that TRAF2 requires sphingosine-1-phosphate as a cofactor, this substance was added to the reactions containing this E3. In spite of this, only a faint smear representing the formation of higher-order ubiquitin conjugates by TRAF2 is visible on the western blot shown in Figure 45. Overall this blot shows that all LUBAC combinations are again unable to employ tagged ubiquitin as a substrate, whereas chain formation and auto-ubiquitination by the other E3s is largely unaffected.

To further corroborate the seemingly unique linkage specificity of LUBAC, another in-vitro ubiquitination assay using different E3s in combination with either UBE2D3 or the UBE2N-UBE2V1-complex was performed (Figure 46A). Mass-spectrometric analysis showed that the UBE2N-UBE2V1-complex indeed exclusively generates K63-linked chains when employed alone or in combination with any of the E3s investigated (Figure 46B). When combined with UBE2D3, cIAP1, cIAP2, TRAF6 and RNF5, of which the latter two are E3s so far not described to play a role in TNF-signalling that were used as a control in this assay, promoted the formation of K48- and K63- but not of linear chains. By contrast, the LUBAC-combinations exclusively generated M1-linkages further confirming the notion that among the E3s tested, LUBAC exhibits a special linkage specificity.

A



B

E3	E2	Linkages detected by MS		
		K48	K63	linear
cIAP1	UBE2D3	+	+	-
cIAP1	UBE2N/UBE2V2	-	+	-
cIAP2	UBE2D3	+	+	-
cIAP2	UBE2N/UBE2V2	-	+	-
TRAF6	UBE2D3	+	+	-
TRAF6	UBE2N/UBE2V2	-	+	-
RNF5	UBE2D3	+	+	-
RNF5	UBE2N/UBE2V2	-	+	-
-	UBE2N/UBE2V2	-	+	-
HOIL-1 + HOIP	UBE2D3	-	-	+
SHARPIN + HOIP	UBE2D3	-	-	+
SHARPIN + HOIL-1 + HOIP	UBE2D3	-	-	+

Figure 46: LUBAC has a unique linkage specificity. An in-vitro ubiquitination was performed using different E3s in combination with either UBE2D3 or the UBE2N/UBE2V2 complex. 10 % of each sample were used to control for ubiquitin-conjugation on western blot level (A). The rest was analysed by mass spectrometry for the presence of peptides specific for certain linkages types (B).

This result also draws attention to the fact that the E2 participating in the generation of ubiquitin chains by RING-class E3s is usually the critical factor in determining linkage specificity (Nagy and Dikic, 2010). In order to assess the influence of the E2 on ubiquitin conjugation mediated by HOIP in combination with SHARPIN or HOIL-1, in-vitro ubiquitination assays were performed with a panel of E2s, either alone or in combination with these two LUBAC-versions (Figure 47).

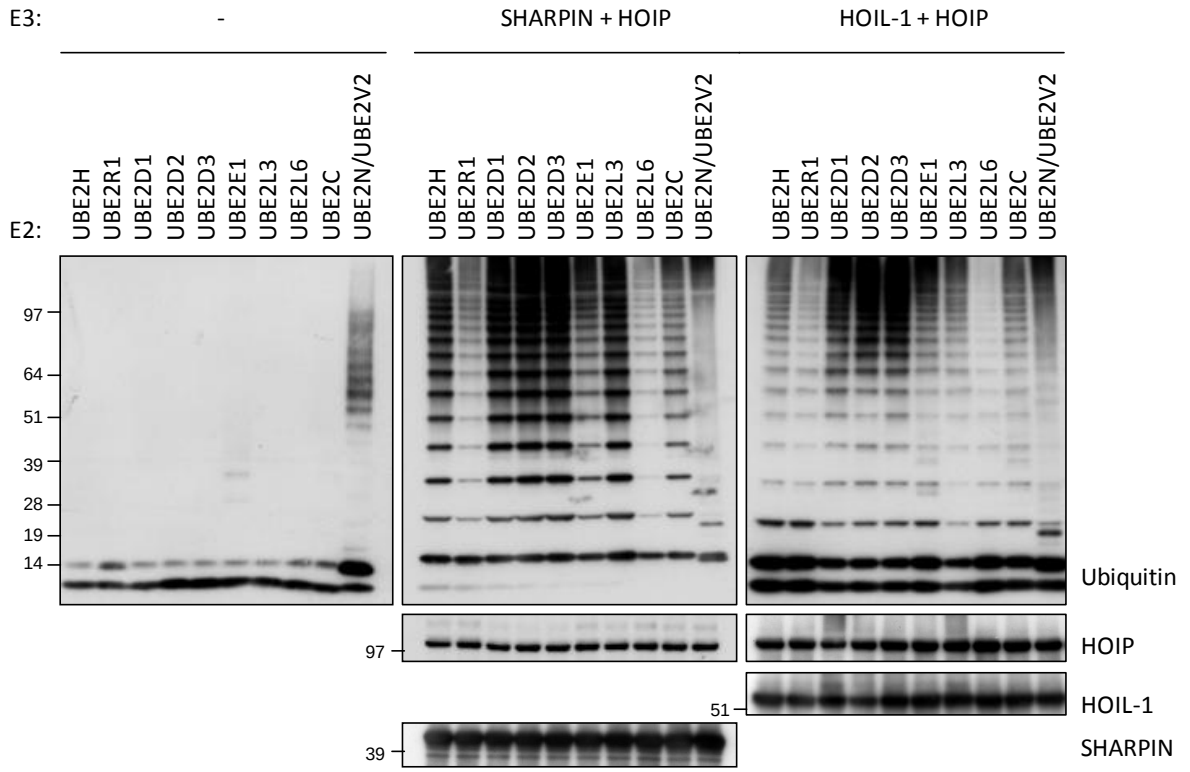


Figure 47: LUBAC is active with a panel of different E2s. The indicated E2s were employed in an in-vitro ubiquitination assay as described under section x either alone or in combination with complexes of HOIP and SHARPIN or HOIL-1. Conjugation of ubiquitin moieties was assessed by western blotting.

In accordance with the model that E3s mediate the interaction between substrate and E2 and substantially increase the rate of ubiquitin discharge from an E2's active site (Ye and Rape, 2009), most E2s were unable to generate ubiquitin-conjugates of more than two ubiquitin moieties on their own (left panel of Figure 47). The exception in this was the UBE2N-UBE2V1-complex, which in line with the literature (Hofmann and Pickart, 1999, 2001) and the results shown in Figure 46 indicates that this E2-complex mediates formation of K63-linked chains even in the absence of an E3. The other two panels of Figure 47 show that HOIP complexed with SHARPIN or HOIL-1 can employ all E2s tested in the generation of ubiquitin chains. Inferring from the position of the corresponding bands on the western

blot, all chains seem to be linear polyubiquitin conjugates irrespective of the E2 involved in their formation. Reactions including UBE2N-UBE2V1, where bands corresponding to both presumably linear and K63-linked chains can be observed constitute an exception to this observation. This result shows that linkage specificity is an intrinsic property of LUBAC that is unaffected by the E2 it collaborates with.

5.7 LUBAC works via a HECT-like mechanism and its activity is required for TNF-induced NF-κB activation

In addition, the data shown in Figure 47 provides evidence that LUBAC is active in combination with UBE2L3, an E2 that lacks lysine-reactivity (Wenzel et al., 2011a) and which therefore cannot transfer ubiquitin directly onto a substrate. Instead UBE2L3 needs to transfer ubiquitin moieties to a cysteine-residue within the catalytic centre of an E3, which in turn confers it onto the target protein. This limitation prevents UBE2L3 from working with RING-E3s as these do not usually have a catalytic cysteine that could accept the ubiquitin. However, a recent publication showed that apart from HECT-ligases also members of the RBR-family are capable of forming a thioester-intermediate between ubiquitin and a catalytic cysteine (Wenzel et al., 2011a). RBR-E3s that have this catalytic residue within their RING2-domain can therefore work via a HECT-like mechanism. To investigate whether LUBAC has similar properties, it was compared to different RING-class E3s in an in-vitro assay using either UBE2D3 or UBE2L3 as an E2 (Figure 48). In line with UBE2L3 exhibiting exclusively cysteine-reactivity, it was unable to act as an E2 for the RING-domain containing E3s cIAP1, cIAP2 and TRAF6 as seen by the lack of formation of polyubiquitin chains as well as the absence of auto-ubiquitination observed when the respective E3s were combined with UBE2D3. On the other hand, ubiquitin conjugation by all three LUBAC combinations was supported by both UBE2D3 and UBE2L3, suggesting that like ARIH1 and Parkin (Wenzel et al., 2011a) LUBAC also works via a HECT-like mechanism.

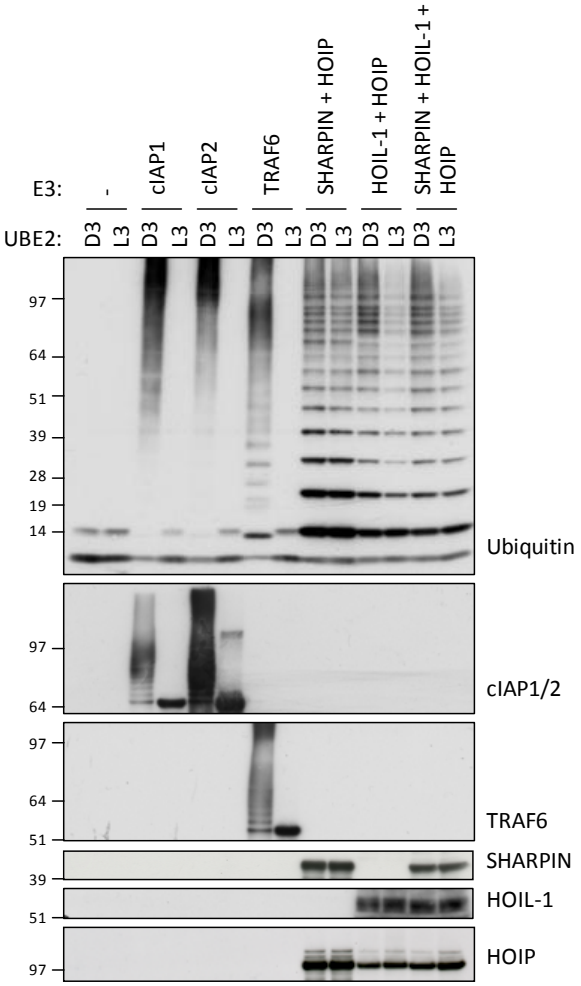


Figure 48: UBE2L3 is active with the different LUBAC combinations but not with RING-class E3s. UBE2D3 and UBE2L3 were compared in an in-vitro ubiquitination assay as described in section 4.2.3.16. Generation of ubiquitin chains by the two E2s in combination with the three LUBAC-versions or with different RING-class E3s (cIAP1, cIAP2, TRAF6) was analysed by western blotting.

Interaction studies (Figures 26A, 29 and 30) as well as functional assays (Figure 34 and (Figure 35B) indicate that HOIP is the central component of LUBAC both structurally and functionally. It was therefore hypothesised that a potential catalytic cysteine should be located in HOIP and more specifically within its RING2-domain. The consensus sequence surrounding the catalytic centre of RBR-family members as published by Wenzel et al. (Wenzel et al., 2011a) was therefore compared to the RING2-domain of HOIP (Figure 49A).

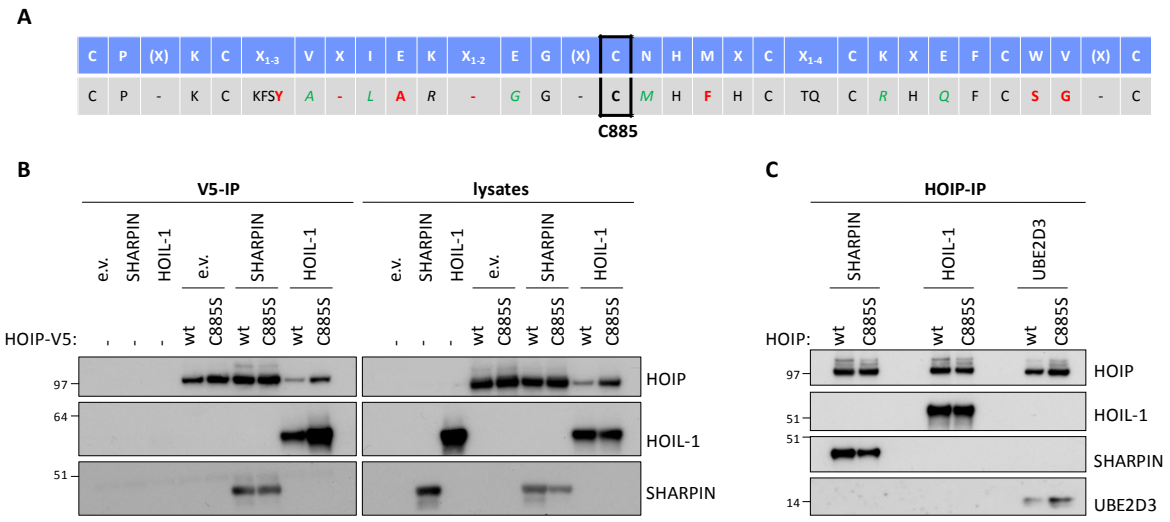


Figure 49: C885 of HOIP is required for LUBAC's activity. A: Alignment of the consensus sequence (in blue) surrounding the catalytic cysteine in RBR-family members as published by (Wenzel et al., 2011a) with the RING2-domain of HOIP (in grey). Identical residues are shown in black. Green letters in italics indicate that the aminoacids present in HOIP can also be found in at least one other family member at this position. Residues different from all other RBR-E3s analysed are shown in red bold print. **B:** Interaction of HOIP C885S with SHARPIN and HOIL-1 was compared to that of the wild-type (wt) protein. The indicated proteins were over-expressed in HEK293T cells followed by precipitation of HOIP variants via their V5-tag and western analysis for co-precipitation of SHARPIN and HOIL-1. **C:** Co-precipitation of SHARPIN, HOIL-1 and UBE2D3 with the two forms of HOIP in a pulldown using a specific HOIP antibody was investigated using recombinant proteins. e.v.: empty vector

Although seven of the thirty-six aminoacids aligned did not correspond to either the consensus sequence or the residues present in any of the other RBR-E3s, the overall similarity was sufficient for a potential catalytic cysteine to be identified as C885. A mutant of HOIP in which this residue was replaced by serine was therefore created. By over-expression of V5-tagged versions of wild-type HOIP or the C885S-mutant together with SHARPIN or HOIL-1 followed by precipitation using an anti-V5 antibody, it was found that mutation of cysteine 885 does not prevent HOIP from interacting with the other two proteins (Figure 49B). This result was corroborated by using recombinant proteins (Figure 49C). In this assay it was also established that mutated HOIP can still interact with UBE2D3, the E2 supplied in most ubiquitination-assays, *in vitro*. In spite of these intact interactions, HOIP C885S was unable to catalyse formation of ubiquitin chains when combined with either SHARPIN or HOIL-1 (Figure 50). In this inability it resembles another HOIP mutant, in which some of the zinc-coordinating cysteine residues of both RING1 (C699 and C702) and RING2 (C871 and C874) had been mutated. This shows that cysteine 885 within the RING2-domain of HOIP is essential for its activity. Together with LUBAC's capacity of generating poly-ubiquitin when combined with UBE2L3, this suggests that LUBAC works via a HECT-like

mechanism that probably involves the formation of a thioester between ubiquitin and cysteine 885 within HOIP.

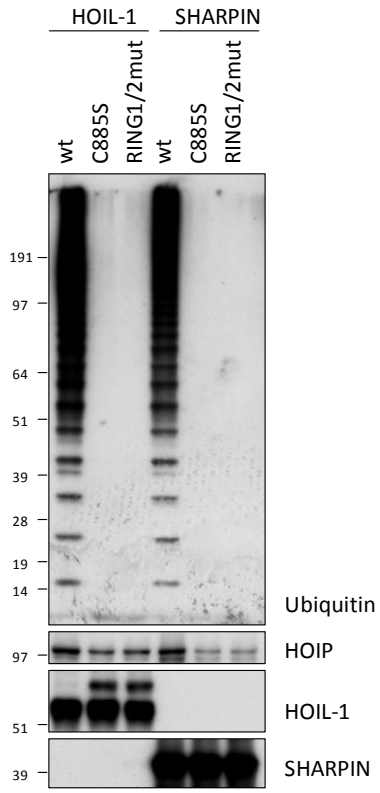


Figure 50: HOIP C885S is inactive in in-vitro ubiquitination assays when combined with SHARPIN or HOIL-1. HOIP C885S and HOIP C699,702,871,874S (RING1/2mut) were compared to wild-type HOIP in an in-vitro ubiquitination assay.

To address whether this activity of HOIP is required in TNF-induced NF-κB activation, V5-tagged versions of HOIP that were either unchanged or contained mutations in the RING-domains, were expressed in HEK293-NF-κB cells (Figure 51, right panel). NF-κB activation was assessed in a reporter-assay following TNF-stimulation (Figure 51, left panel). Forms of HOIP that contained mutations in the RING2-domain, in RING1 and RING2 or in cysteine 885 significantly reduced the luciferase-activity measured after TNF-stimulation, indicating that an intact RING2 domain and presence of C885 are required and that mutants with changes in these features exert a dominant-negative effect on TNF-induced signalling.

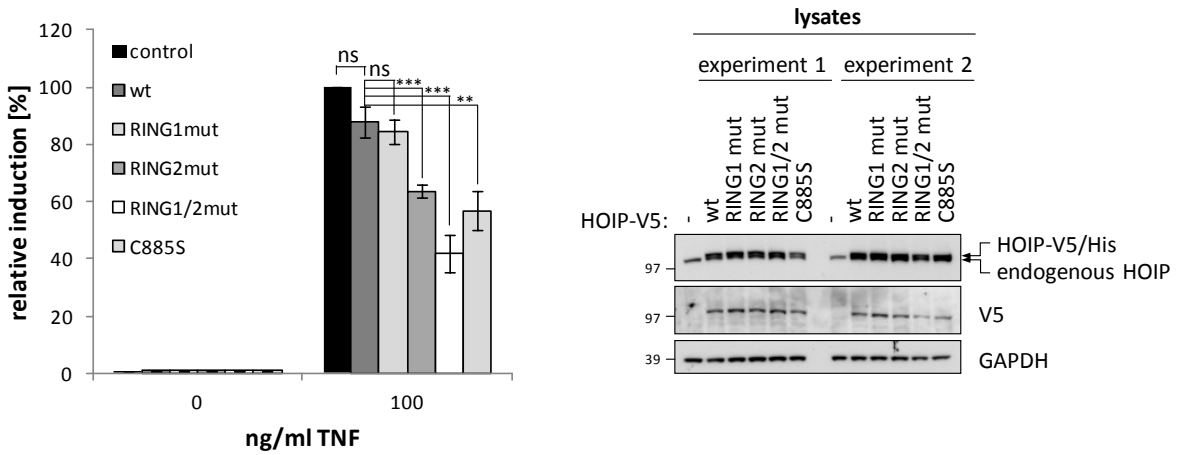


Figure 51: Mutants of HOIP containing alterations in the RING2-domain have a dominant-negative effect on TNF-induced NF-κB activation. V5-tagged mutants of HOIP were over-expressed in HEK293 NF-κB cells and luciferase-activity was analysed in a luciferase-assay following stimulation with TNF. Values were normalised to renilla-activity and the control was set to 100 %. Results are presented as mean +/- SEM; n=9 and significance was calculated using a student's t-test (left panel). Over-expression of the different HOIP mutants was verified by western blotting and the results of two representative experiments are shown (right panel). RING1mut: HOIP C699,702S; RING2mut: HOIP C871,874S; RING1/2mut: HOIP C699,702,871,874S.

6 Discussion and Outlook

TNF is a key inflammatory cytokine with great physiological relevance and diverse effects ranging from pro-inflammatory to immune-regulatory functions. On a cellular level, TNF can induce the production of pro-inflammatory cytokines, cell proliferation, differentiation or cell death (Chen and Goeddel, 2002; Hayden and Ghosh, 2008; Kovalenko and Wallach, 2006; Wajant et al., 2003). In initiating these diverse outcomes, TNF relies on two surface receptors, TNFR1 and TNFR2 (Wajant et al., 2003). Since both receptors belong to the TNFR-superfamily, a group of proteins that generally lack enzymatic activity within their intracellular domains (Aggarwal, 2003), they recruit cytoplasmic proteins upon stimulation, which in turn initiate downstream signalling events. The receptor-associated multi-protein complexes, referred to as receptor signalling complexes (RSCs), represent the apex of all signal transduction cascades emanating from the TNF-receptors. Events occurring within this assembly of proteins are therefore critical determinants of cellular fate and important regulators of diverse biological processes. Hence, it is essential to comprehend the composition of these complexes on a molecular level. For this purpose the TNF-RSC was isolated by a modified tandem affinity procedure and analysed by mass spectrometry (Haas et al., 2009). Next to almost all proteins previously described to form part of the TNF-RSC this analysis revealed the presence of three novel components, i.e. SHARPIN, HOIL-1 and HOIP, in this complex (Gerlach et al., 2011). Two of these three proteins had been described to form an E3 complex that mediates the formation of ubiquitin chains linked via the N-terminal methionine (M1) (Kirisako et al., 2006) and that is thus referred to as linear ubiquitin chain assembly complex (LUBAC). In this thesis, SHARPIN could be identified as a third structural and functional component of LUBAC. Furthermore, it was shown that this pre-formed complex is recruited to the TNF-RSC in a stimulation- and cIAP-dependent manner and that all potential LUBAC-variants exclusively generate linear ubiquitin chains via a HECT-like mechanism.

6.1 The role of SHARPIN, HOIL-1 and HOIP in the context of TNF-signalling

6.1.1 Isolation of the TNF-RSC verifies the stimulation-dependent recruitment of SHARPIN, HOIL-1 and HOIP to this complex and shows modification of different complex components

A kinetic study performed on the TNF-RSC of U937 cells showed that the three novel components identified by mass spectrometry are not present on unstimulated receptors but are recruited in a manner that depends on receptor crosslinking by its ligand. In this stimulation-dependency of their recruitment as well as the kinetics of their appearance and disappearance from the complex, SHARPIN, HOIL-1 and HOIP resemble each other and most other components of the RSC. Only TRADD seems to reach its maximal levels slightly earlier than the other proteins, which is in line with a direct interaction between the DD of this protein with those present in TNFR1 (Hsu et al., 1995) and with a role for the former as an adaptor for other complex constituents like TRAF2 and potentially RIP1 (Hsu et al., 1996a; Hsu et al., 1996b; Shu et al., 1996; Tsao et al., 2000). Apart from mere presence of proteins, the level of modifications they carry can be an indicator for stage and stability of the receptor-complex. The most obvious modifications observed in the TNF-RSC are the ubiquitination of RIP1 and cIAP, which like the levels of most components reach a maximum after five minutes of stimulation. Both modifications are most likely mediated mainly by the E3-activity of cIAP as indicated by the findings that the RING-domain of TRAF2 is not sufficient or required for RIP1 ubiquitination to occur and that reconstitution of cIAP1/2$^{-/-}$ MEFs with inactive forms of cIAP1 cannot restore this modification. This is in line with previous publications that cIAPs can directly target RIP1 *in vitro*, whereas TRAF2 can not. However, the identification of four different types of ubiquitin linkages on RIP1 by mass spectrometry (Gerlach et al., 2011) suggests that RIP1 may be modified by several E3s and that one of these ligases is LUBAC. The modification of cIAP is likely to be an auto-ubiquitination, as a similar alteration occurs in in-vitro assays in the absence of another E3. This would again be in accordance with literature (Bertrand et al., 2008; Dueber et al., 2011; Feltham et al., 2010) but as in the case of RIP1 the contribution of one or several other E3s cannot be excluded. Furthermore, the modifications present on RIP1 and cIAP do not seem to exclusively consist of ubiquitin-molecules. This is indicated by the finding that the catalytic domain of USP2, a DUB that cleaves a wide variety of ubiquitin chains and that is able to return all LUBAC components to their apparently unmodified state, cannot

completely remove the modifications from RIP1 and cIAP. This shows that USP2 is able to also remove the first ubiquitin unit from the target protein and that the remaining modification is hence unlikely to be a ubiquitination. The size difference of approximately 7 kDa of this remaining modification in relation to the unaltered proteins makes a modification with a UBL-molecule seem likely. However, no data are available to date to support this notion and further investigation, for example by treatment with deconjugating enzymes specific for other modifications or by a mass-spectrometry based approach will be required to clarify the identity and function of these modifications.

In addition, smaller shifts in the bands representing the non-ubiquitinated forms of RIP1 and TRAF2 can be observed in the TNF-RSC after 15 minutes of stimulation. Due to the fact that RIP1 has kinase activity (Hsu et al., 1996a) and that TRAF2 was previously described to be subject to that kind of modification (Blackwell et al., 2009; Li et al., 2009a; Thomas et al., 2009; Zhang et al., 2011), these additional bands might represent phosphorylations of the two proteins. Phosphorylation of TRAF2 by PKC was reported to promote its ubiquitination with K63-linked chains and to favour the recruitment of the TAB/TAK- and IKK-complexes (Li et al., 2009a). However, the upward shift in TRAF2 that could correspond to its phosphorylation is only observed after 15 minutes whereas the levels of NEMO, the subunit responsible for the recruitment of the IKK-complex (Ea et al., 2006; Wu et al., 2006), reach a maximum already after 5 minutes. This could be in line with another report describing the phosphorylation of TRAF2 as an event involved in the later stages of IKK-activation (Blackwell et al., 2009). Yet, in this context it was reported to form part of a secondary cytoplasmic complex, whereas the potentially modified form of TRAF2 observed here is still associated with the receptor.

In case of RIP1 an activity as a serine-threonine kinase that can mediate its auto-phosphorylation was described (Hsu et al., 1996a). However, it was also shown that this kinase activity is dispensable for the activation of the NF-κB- and p38-pathways in the context of TNF-signalling (Lee et al., 2004). On the other hand, activity of RIP1 is required in necroptosis-induction (Holler et al., 2000; Vandenabeele et al., 2010) and in this context phosphorylation of RIP1 by RIP3 or possibly another so far unidentified kinase was reported (Cho et al., 2009; He et al., 2009). However, modification of RIP1 observed in these studies occurs at much later times and again takes place in a secondary complex, suggesting that this event differs from the modification seen in the receptor-associated complex. Here, a

shift in the position of the bands representing RIP1 becomes apparent after 15 minutes and increases after 30 minutes. At the same time a decrease in the ubiquitination of RIP1 or a dissociation of this protein from the complex can be observed. It therefore seems possible that RIP1 phosphorylation favours its deubiquitination, a process considered important for the formation of secondary death-inducing complexes (O'Donnell et al., 2007). Overall, further investigation is required to unravel the identity and function of the non-ubiquitin modifications present on TNF-RSC components such as RIP1, TRAF2 and cIAP. It could be speculated that they have a role in the disassembly of the receptor-complex and are hence required in the termination of signals emanating from stimulated receptors. Another possibility is that they function in regulating and maintaining the balance between pro-survival and death-inducing signalling outputs.

6.1.2 TNFR1 versus TNFR2

The identification of SHARPIN, HOIL-1 and HOIP as proteins that are present in TNF-precipitations from U937 cells raised the question which of the two TNF-receptors expressed on the surface of these cells (Haridas et al., 1998; Shu et al., 1996) they are recruited to. Generally, most of the manifold effects induced by TNF are mediated via the more widely expressed TNFR1 (Wajant et al., 2003). Yet, independent and non-redundant roles for TNFR2 as well as cross-talk between the two receptors exist (Faustman and Davis, 2010; MacEwan, 2002). TNFR1 and TNFR2 recruit distinct but overlapping sets of intracellular proteins upon stimulation. Several lines of evidence presented here, including the isolation of the TNF-RSC from HeLa cells which do not express TNFR2 (Haridas et al., 1998; Shu et al., 1996); PhD thesis Dr. C.H. Emmerich) and the precipitation of TNFR1 and TNFR2 using specific antibodies, indicate that LUBAC components are recruited to TNFR1 rather than TNFR2, when soluble TNF is employed as a stimulus. It was shown previously that membrane-bound TNF is vastly superior to its soluble form in activating TNFR2 (Grell et al., 1998; Grell et al., 1995; Krippner-Heidenreich et al., 2002) and that it may therefore lead to not only quantitatively but qualitatively different responses. Based on this and on the finding that recruitment of LUBAC to TNFR1 depends on cIAPs which are also associated with TNFR2 (Rothe et al., 1995a), it seems possible that SHARPIN, HOIL-1 and HOIP can be recruited to TNFR2 when this receptor is efficiently activated. Additional studies using

membrane-bound TNF or a cross-linked version of this ligand that mimics membrane association, in both wild-type and TNFR1-deficient cells are required to test this hypothesis.

6.1.3 Mechanism of LUBAC's recruitment

In a setting where protein levels of cIAP1 and cIAP2 were down-regulated either genetically or by treatment with a Smac-mimetic compound, it could be shown that recruitment of LUBAC in HeLa cells or HOIL-1 in MEFs depended on the presence cIAP1/2. As it was demonstrated that SHARPIN, HOIL-1 and HOIP form a stimulation-independent complex and are therefore recruited in an interdependent-manner, the finding that the activity of cIAP is essential for the recruitment of HOIL-1 in MEFs can be extended to the other two LUBAC components. This requirement for cIAP's activity implied a role for ubiquitin in the recruitment process. Indeed, all three LUBAC-components can bind to ubiquitin, with HOIP displaying the strongest association with K63-linked tetraubiquitin in an in-vitro setting. Based on these results and published observations, the following mechanism of recruitment seems likely (Figure 52): Upon crosslinking of TNFR1 by trimeric TNF or higher order oligomers thereof, the adaptor protein TRADD is recruited via homotypic interaction of its DD with that of TNFR1 (Hsu et al., 1995). Although RIP1 can also directly interact with the receptor in a DD-dependent manner (Ermolaeva et al., 2008; Haas et al., 2009; Pobezinskaya et al., 2008) its recruitment may also be mediated or increased by TRADD (Hsu et al., 1996a). The latter protein also serves as an adaptor for TRAF2 (Hsu et al., 1996b), which in turn has a cIAP-interaction motif that is essential for the recruitment of cIAP1 and cIAP2 by binding to their BIR1-domains (Samuel et al., 2006; Varfolomeev et al., 2008; Vince et al., 2009). Structural studies have revealed that a TRAF2 trimer interacts with one cIAP2 molecule in an asymmetric manner (Zheng et al., 2010). Once recruited to the TNF-RSC cIAPs modify several components, including RIP1, with ubiquitin chains, the majority of which serve non-proteolytic functions at this stage as they mainly act as recruitment platforms for further components of the RSC (Bertrand et al., 2008; Ea et al., 2006; Varfolomeev et al., 2008; Wertz et al., 2004). This includes the TAB/TAK-complex which is recruited via TAB2 that specifically binds K63-linked ubiquitin-linkages via its NZF-domain (Kulathu et al., 2009) and the IKK-complex that can associate with K63- and possibly K11-linked chains via its regulatory subunit NEMO. The primary association of LUBAC with the receptor complex is likely to also be mediated by K63-linked chains, which are recognised by

the NZF1-domain of HOIP (Ikeda et al., 2011). It can be assumed that this initial recruitment is sufficient for LUBAC to modify its targets within the complex. These include but are not necessarily restricted to NEMO and RIP1 (Gerlach et al., 2011). The linear linkages could stabilise the presence of LUBAC in the complex as the NZF-domains of SHARPIN and especially HOIL-1 were shown to preferentially or even exclusively bind ubiquitin chains of this linkage type (Sato et al., 2011). The finding that the NZF-domains of these proteins are required for LUBAC's ability to induce NF-κB but not for the generation of ubiquitin chains (Ikeda et al., 2011; Sato et al., 2011; Tokunaga et al., 2009) suggests that this interaction with the complex or with potential target proteins is an important aspect of the role SHARPIN and HOIL-1 play in the context of LUBAC's function. Furthermore, linear ubiquitin increases the recruitment of IKK-complexes as NEMO's UBAN domain shows an affinity to this linkage type that is about 100-fold higher than that to K63-linked chains (Lo et al., 2009; Rahighi et al., 2009). Overall this suggests that LUBAC is recruited to the TNF-RSC by binding of its central component, HOIP, to cIAP-generated ubiquitin chains. This may not be restricted to K63-linked chains as a study based on a ubiquitin-replacement strategy reported that this linkage type is dispensable for TNF-signalling (Xu et al., 2009a) and by the finding that cIAPs can mediate the formation of different linkage types (Dynek et al., 2010; Feltham et al., 2012). The interaction of LUBAC-components and especially of HOIP with differentially linked ubiquitin will therefore have to be investigated. Once an interaction with the TNF-receptor complex has been established by HOIP, SHARPIN and HOIL-1 that are not essential to this initial recruitment may have a function in stabilising this association and in retaining LUBAC in the TNF-RSC at later stages. Finally, direct protein-protein interactions between LUBAC and other components of the TNF-RSC, such as the binding of HOIP to NEMO (Tokunaga et al., 2009) may contribute to LUBAC being recruited to and retained in the TNF-RSC although they do not seem to be essential or sufficient to mediate the recruitment process (Haas et al., 2009).

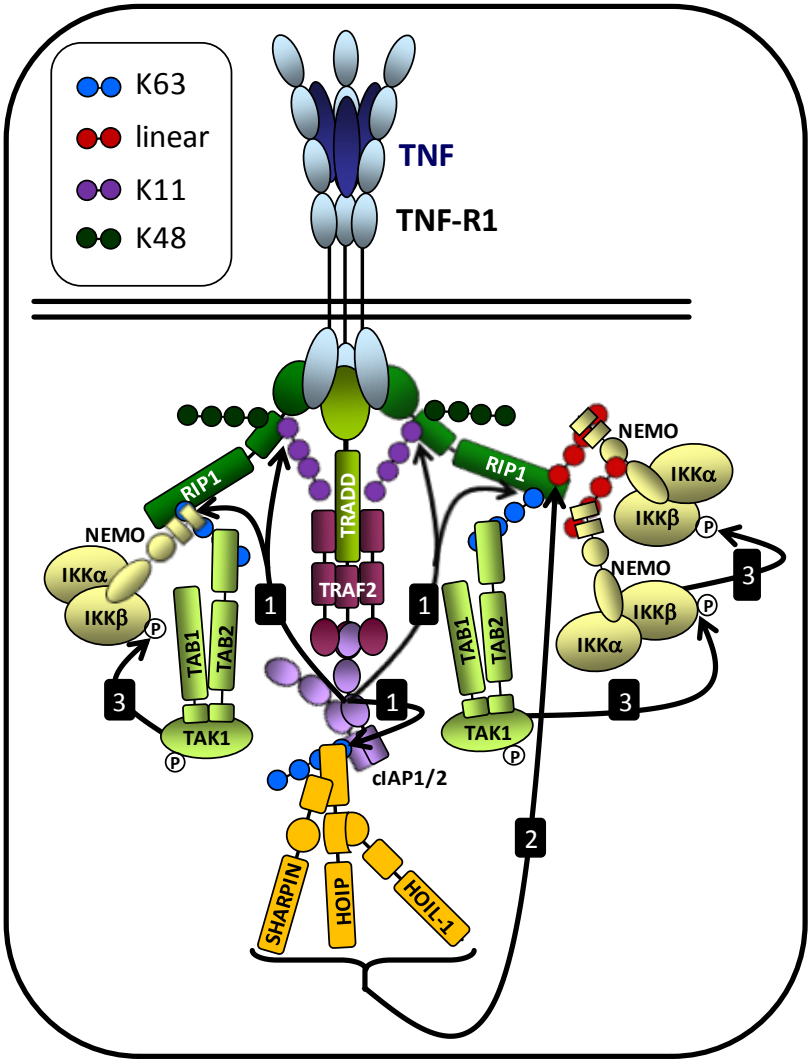


Figure 52: Schematic representation the process involved in recruiting LUBAC to the TNF-RSC. Upon crosslinking by TNF, TNFR1 recruits TRADD and RIP1 to its intracellular death-domain. TRADD serves as an adaptor of TRAF2 which in turn recruits cIAPs. Once recruited to the TNF-RSC cIAP attaches ubiquitin chains of different linkage types to components of the TNF-RSC including RIP1 and itself (1). These chains allow for the recruitment of the TAB-TAK- and IKK-complexes as well as LUBAC. LUBAC then attaches linear ubiquitin chains to RIP1, NEMO and possibly other targets (2). This increases the recruitment of IKK-complexes and allows for the activation of IKK to occur. This involves phosphorylation of IKKβ, an event that may be mediated by trans-auto-phosphorylation or by TAK1 (3).

6.1.4 The Role of LUBAC in TNF-induced signal transduction

Using both knockdown approaches and the over-expression of dominant-negative mutants of HOIP, it could be shown in this thesis that presence and activity of LUBAC are required for full activation of TNF-induced signalling. While depletion of HOIP led to a stronger reduction in the activation of signalling pathways initiated by TNF than down-regulation of SHARPIN or HOIL-1, it was insufficient to fully block signal transduction. The same is true for over-expression of inactive forms of HOIP which again reduced but did not abolish TNF-induced NF-κB activation. In both cases it is not possible to distinguish whether the remaining level

of active LUBAC is sufficient to mediate signalling or whether LUBAC-independent signalling pathways exist. The latter possibility is supported by the finding that TNF can still activate the NF-κB- and MAPK-pathways in cells derived from cpdm mice, which due to a spontaneous mutation lack SHARPIN expression (Seymour et al., 2007) as well as in HOIL-$1^{-/-}$ MEFs in which HOIL-1 is genetically deleted (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011; Tokunaga et al., 2009). Even in HOIP-deficient cells, signal transduction by CD40, a receptor that also requires LUBAC in its downstream signalling (Gerlach et al., 2011) is still possible to a certain extent (Hostager et al., 2011). Taken together this indicates that LUBAC is not essential for TNF-signalling but is required for signal transduction to occur at its full strength. Although investigation of TNF-induced signal transduction in HOIP-deficient cells is required to corroborate this notion, these results suggests a role for LUBAC in fine tuning signalling output rather than as an on/off-switch for signals emanating from the TNF-RSC. Because LUBAC acts as an E3 and influences TNF-induced signal transduction it is likely that LUBAC exerts its effect on signalling by modifying components of the respective pathways. Its recruitment to the TNF-RSC (Gerlach et al., 2011; Haas et al., 2009) as well as its effect not only on NF-κB activation but also on MAPK-cascades (Gerlach et al., 2011; Haas et al., 2009; Ikeda et al., 2011) suggests that LUBAC targets proteins within this complex that represents the apex of all TNF-induced signalling pathways rather than ubiquitinating downstream effectors. In an in-vitro ubiquitination assay testing nine TNF-RSC components, only NEMO could be identified as a target for all LUBAC-variants. However, this does not exclude that other proteins which were not modified by LUBAC *in vitro* may be targets *in vivo*, as conformations or previous modifications present in the context of the TNF-RSC may not be sufficiently mimicked in the in-vitro situation. The modification of NEMO with linear ubiquitin could be verified by mass spectrometry (Gerlach et al., 2011). In the same experimental setup M1-linkages were found together with RIP1 on a 2D-gel, indicating that this protein is modified with linear chains too. In both cases LUBAC is likely to be the relevant E3 as no other ubiquitin ligase could so far be shown to generate ubiquitin chains of this linkage type (Verhelst et al., 2011). There is a report on cIAPs being able to use lysine-less ubiquitin as a substrate (Bertrand et al., 2011). However, results presented in this thesis suggest that they do not make use of this ability if other possibilities in form of accessible internal lysine residues are available. TRAF2, the other E3 known to be present on the TNF-RSC showed very low if any

activity in an in-vitro ubiquitination assay even in the presence of S1P, which was reported to be an essential co-factor for this ligase (Alvarez et al., 2010). This lack of activity is in line with a publication that concluded, based on the structure of the RING- and first zinc-finger domains of TRAF2, that this E3 is unable to interact with UBE2N or related E2s and that it therefore cannot act as a functional ligase. Taken together these findings suggest that all linear linkages present in the TNF-RSC are generated by LUBAC and that NEMO and RIP1 are targets of this E3-complex. This could be fully clarified by investigating these modifications in the presence or absence of active LUBAC. For this purpose the native TNF-RSC isolated from cells in which expression of HOIP has been ablated by a knockout strategy or replaced by an inactive mutant in a knockin approach could be compared to that precipitated from wild-type cells by mass spectrometry. Absence of M1-linkages on NEMO and RIP1 in cells exclusively expressing an inactive form of HOIP would provide the final proof that not only LUBAC's presence but also its activity is required for the ubiquitination of these proteins. It has not been fully elucidated how linear ubiquitin chains favour the activation of TNF-induced signal transduction but several explanations for the connection between linear ubiquitination and the regulation of signalling are conceivable. Linear chains on RIP1 and NEMO may act as a recruitment platform for other ubiquitin-binding proteins which display a preference for this linkage type including NEMO itself. Indeed absence of LUBAC severely decreases the association between NEMO and the TNF-RSC (Haas et al., 2009). However, linear chains may not only increase the amount of IKK-complexes that are recruited to the TNF-RSC but binding of NEMO to ubiquitin attached to another NEMO molecule may also result in clustering of these kinase complexes (Rahighi et al., 2009) thereby creating a proximity between the kinase-subunits that allows trans-auto-phosphorylation, an event that has been implicated in the activation of IKKs (Tang et al., 2003a). Furthermore, it was reported that binding to linear ubiquitin induces a conformational change in NEMO (Rahighi et al., 2009) which could translate to similar changes in the kinase subunits of the IKK-complex, again favouring their activation. In addition, the activity of LUBAC leads to an overall stabilisation of the TNF-RSC (Haas et al., 2009). This effect that probably depends, at least partially, on the modification of RIP1 and NEMO, results in RIP1, TRAF2, cIAP and TAK1 being retained in the TNF-RSC for a longer period of time and consequently extends the half-life of this complex thereby allowing enhanced and prolonged activation of downstream signalling. This stabilisation is likely to be caused not only by the linkage

specificity of UBDs present in complex components but also by linear ubiquitin chains being more refractory to disassembly mediated by DUBs recruited to this complex (Komander et al., 2009b). DUBs associated with the TNF-RSC include A20, CYLD, Cezanne and USP21 (Harhaj and Dixit, 2011) and of these only CYLD and USP21 were shown to cleave linearly linked ubiquitin with considerable activity (Komander et al., 2009b; Ye et al., 2011). Binding of UBDs to linear chains could make them even more resistant to DUB-mediated cleavage. Further to the aforementioned possibilities, LUBAC could also influence signalling by its ubiquitination of NEMO. This modification was reported to occur at K285 and K309 and reconstitution of NEMO-deficient cells with a mutant in which these lysines had been replaced by arginines (K285,309R) was insufficient to rescue NF-κB activation induced by LUBAC over-expression or IL-1β-stimulation (Tokunaga et al., 2009). Mechanistically, this modification on the regulatory subunit of the IKK-complex may not only serve as a recruitment platform favouring clustering and trans-activation of kinase-complexes but might also itself induce a conformational change in NEMO and consequently in the associated kinases. Overall, LUBAC could regulate TNF-induced signalling by a tripartite mechanism. The first leg of this mechanism is constituted by provision of a binding platform that allows recruitment and possibly clustering of downstream components, the second aspect is the stabilisation of the TNF-RSC and thirdly LUBAC may induce activating conformational changes in the IKK-complex by directly ubiquitinating its regulatory subunit.

In addition to its positive regulatory effect on TNF-signalling a role for LUBAC in signal termination can be envisioned. In this context it is possible that linear chains also recruit ABIN-1 and Optineurin which, like NEMO, have a UBAN-domain that was shown to bind M1-linkages with much higher affinity than K63-linked chains (Lo et al., 2009; Rahighi et al., 2009). As these proteins were shown to act as adaptors for A20 and CYLD, two DUBs implicated in terminating TNF-signalling (Harhaj and Dixit, 2011; Heyninck et al., 1999; Mauro et al., 2006; Nagabhushana et al., 2011; Regamey et al., 2003), linear ubiquitin chains could also have a role in preventing prolonged signalling. The specific recognition of linear ubiquitin chains by particular UBDs will have to be analysed in more depth, additional LUBAC targets will have to be identified and the sensitivity of M1-linkages attached to certain targets towards cleavage by DUBs will have to be compared to that of chains linked via specific lysine residues, if the mechanism by which LUBAC regulates TNF signalling is to be fully elucidated.

6.2 Functional investigation of LUBAC as an E3-complex

6.2.1 LUBAC exists as a preformed complex

HOIL-1 and HOIP were previously reported to form a stimulation-independent protein complex of about 600 kDa that, due to its activity as an E3 ligase with exclusive linkage specificity, is referred to as LUBAC (Kirisako et al., 2006). Here it could be shown that SHARPIN co-elutes with HOIL-1 and HOIP in the high-molecular weight fractions of a gelfiltration chromatography and that it directly interacts with the original LUBAC components even in the absence of a stimulus. Further evidence for the association between the three proteins stems from the observation that down-regulation or absence of one of them decreases the protein expression levels of the other two (Gerlach et al., 2011; Tokunaga et al., 2011). As no such down-regulation can be observed on the mRNA-level it can be concluded that the interaction between SHARPIN, HOIL-1 and HOIP is required to maintain their stability on the protein level. The interaction between SHARPIN, HOIL-1 and HOIP does not seem to be tissue- or cell line-specific and even though a weak binding between SHARPIN and HOIL-1 could be detected, the association seems to be mediated mainly via HOIP. Although further data obtained from HOIP-deficient cells are required to verify the direct interaction between SHARPIN and HOIL-1, the existence of SHARPIN-HOIL-1 dimers cannot be excluded and a potential function for this dimeric complex will need to be investigated. This combination of proteins could either have an inhibitory role by preventing SHARPIN and HOIL-1 from forming active complexes with HOIP or it might itself act as an E3. In line with the latter idea HOIL-1 was reported to act as a ubiquitin-ligase conjugating K48-linked ubiquitin to IRP2 (Yamanaka et al., 2003) and possibly other substrates (Tian et al., 2007; Zenke-Kawasaki et al., 2007; Zhang et al., 2008). However, this is in is in contrast to data obtained in this thesis which show that no activity of HOIL-1 could be detected in in-vitro ubiquitination assays. On the other hand, Parkin, which is very similar to HOIL-1 in its domain structure, was described to be subject to an auto-inhibition. In this protein the auto-inhibitory back-folding of its UBL-domain needs to be relieved by binding of a cofactor or a substrate before Parkin can efficiently catalyse the formation of ubiquitin chains (Chaugule et al., 2011). It is possible that HOIL-1 is regulated in a similar fashion and that interaction with SHARPIN, or another yet to be identified cofactor, enables it to act as a ubiquitin ligase. If SHARPIN and HOIL-1 were to have E3-activity, an additional factor is likely to be required

as indicated by the observation that the combination of the two proteins can neither generate ubiquitin chains *in vitro* nor induce NF-κB upon over-expression.

However, data on the co-precipitation of SHARPIN and HOIL-1 in the presence or absence of HOIP clearly indicate that the latter protein increases the interaction between the former two. This demonstrates that tripartite complexes exist in which the central component HOIP interacts with both SHARPIN and HOIL-1 by binding their respective UBL-domains. The binding of SHARPIN could be mapped to the NZF2-domain of HOIP, which was reported to interact with HOIL-1 via its UBA-domain in an atypical manner (Kirisako et al., 2006; Yagi et al., 2012). The use of different domains of HOIP for interacting with its binding partners seems to be suitable to support simultaneous interactions with two different proteins. On the other hand, UBA-domains can serve as dimerisation- and as ubiquitin-binding motifs (Bayrer et al., 2005; Bertolaet et al., 2001; Kozlov et al., 2007; Raasi et al., 2005) and the UBA-domain of HOIP may therefore contribute to the interaction with SHARPIN even though is not sufficient to mediate it as can be concluded from the observation that deletion of this domain only slightly decreases the co-precipitation of HOIP with SHARPIN. A structural study of the UBA-domain present in XIAP suggested that different surfaces are involved in dimerisation and ubiquitin-binding thereby allowing the domain to fulfil both functions at the same time (Tse et al., 2011). Using a similar mechanism, two HOIP molecules could dimerise while each associating with the UBL-domains of SHARPIN or HOIL-1, respectively. The finding that LUBAC has a molecular weight of around 600 kDa (Kirisako et al., 2006), shows that several copies of SHARPIN, HOIL-1 and HOIP need to associate. Indeed it was reported that at least three molecules of HOIP are present in LUBAC (Tokunaga et al., 2011). Based on the respective molecular weights of approximately 40 kDa, 57 kDa and 120 kDa for SHARPIN, HOIL-1 and HOIP, several possibilities for the formation of the high-molecular weight complex exist and the stoichiometry has not been resolved. It can however be assumed that the ability to dimerise via their coiled-coil (Lim et al., 2001), RING- (Tatematsu et al., 2008) or UBA-domains allows SHARPIN, HOIL-1 and HOIP to mediate the association of the higher order protein assemblies that is required for the formation of LUBAC. The interactions between individual proteins within this E3-complex are displayed schematically in Figure 53.

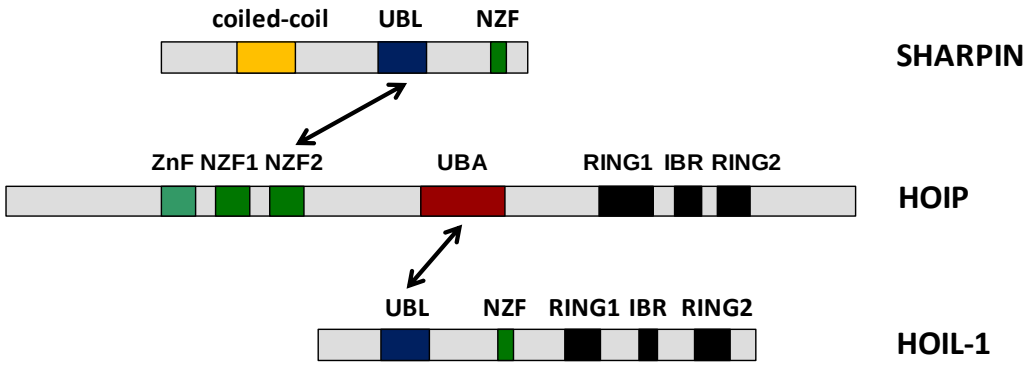


Figure 53: Schematic representation of the interactions between individual LUBAC-components. HOIP interacts with the UBL-domains of SHARPIN and HOIL via its NZF2- and UBA-domains, respectively. Arrows indicate interactions between the proteins. ZnF: Zinc Finger; NZF: Npl4 zinc finger; UBL: Ubiquitin-like domain; UBA: Ubiquitin-associated domain; IBR: in-between RING domain. RING: really interesting new gene.

6.2.2 SHARPIN, HOIL-1 and HOIP form an E3-complex with exclusive linkage specificity.

Beside the interaction studies that identified SHARPIN as a structural component of LUBAC, activity assays show that it is not only important for the stability of HOIL-1 and HOIP on the protein level (Gerlach et al., 2011; Tokunaga et al., 2011) but can also support HOIP in the formation of ubiquitin chains. In spite of the fact that SHARPIN lacks an RBR-domain no qualitative differences in the ubiquitin conjugation could be detected between SHARPIN-HOIP and HOIL-1-HOIP-complexes. This suggests that chain formation is mediated by HOIP whereas SHARPIN and HOIL-1 have an auxiliary or regulatory role. Indeed, both published data and results obtained in this study support this notion by showing that mutations in the RING-domains of HOIP but not in those of HOIL-1 abolish LUBAC's ability to generate ubiquitin chains and to activate NF-κB upon over-expression (Haas et al., 2009; Kirisako et al., 2006; Tokunaga et al., 2009). In contrast to HOIP's RING-domain and the UBL-domains of SHARPIN and HOIL-1, the NZF-domains of the latter two proteins are required for LUBAC ability to induce NF-κB but not for its ubiquitin chain generating activity (Ikeda et al., 2011; Sato et al., 2011; Tokunaga et al., 2009). This suggests that SHARPIN and HOIL-1 do not only need to interact with HOIP but that they may also fulfil additional functions in LUBAC's activity. Possible roles for SHARPIN and HOIL-1 might lie in the determination of linkage- or target specificity displayed by the different LUBAC variants. However, the chains generated by HOIP do not differ in their electrophoretic mobility independently of the binding partner involved in formation of the active LUBAC. In addition, assays based on the use of ubiquitin mutants or the mass-spectrometric analysis of LUBAC-generated polyubiquitin show that all

LUBAC-variants exclusively generate linear linkages even when other possibilities for conjugation of ubiquitin moieties, in the form of internal lysine residues, are available and an E2 without a strict linkage specificity such as UBE2D3 is used. In this preference LUBAC differs from all other E3s investigated so far (Verhelst et al., 2011), even if certain other ubiquitin ligases as for example cIAP seem to be able to use the N-terminal methionine as a conjugation point when forced to do so by the absence of alternative attachment points (Bertrand et al., 2011). Furthermore, comparison to other members of the RBR-family indicates that the linkage specificity is not an intrinsic property of this sequence of RING-domains. It is possible that certain members of this subclass of RING-E3s can mediate the formation of linear linkages in the presence of a suitable partner or cofactor, but in the assays performed here RBR-family members were able to employ N-terminally tagged but not K0-ubiquitin in the generation of ubiquitin conjugates. This is in line with publications that report that the RBR-family member Parkin generates K63- and K48-linked chains (Doss-Pepe et al., 2005; Lim et al., 2006) and that report on the generation of lysine-linked polyubiquitin by other proteins of this family (Marteijn et al., 2009; Nakhaei et al., 2009). Although HOIP is not very active as an E3 on its own, it seems that this central component of LUBAC is responsible for restricting the activity to the conjugation of ubiquitin molecules via their N- and C-terminus (Kirisako et al., 2006). A possible hypothesis is that by binding the nascending ubiquitin chain via their NZF-domains SHARPIN and HOIL-1 force it to adopt a conformation in which only the N-terminal methionine is accessible as an attachment point to HOIP, thereby determining linkage specificity. In case of HOIL-1 this does not seem to be the case as the electrophoretic mobility of chains generated by HOIP and HOIL-1 lacking their NZF-domains does not differ from those generated by the wild-type proteins. Similarly, deletion of SHARPIN's NZF-domain seems to decrease the overall ubiquitin chains generation by LUBAC but does not change the linkage type (Tokunaga et al., 2011).

Concerning target specificity, NEMO could be identified as a common target for all LUBAC variants and hence no data on different target specificities exhibited by SHARPIN- or HOIL-1 containing LUBAC-combinations exist so far. Yet, due to the differences between SHARPIN- and HOIL-1-deficient cells especially in the response to cell death induction by TNF (Ikeda et al., 2011), it seems likely that the two proteins might direct HOIP's activity to overlapping but distinct sets of target proteins. On the other hand, LUBAC-independent functions of monomeric SHARPIN or HOIL-1 might contribute to these differences as well. Thus, further

differentiation between LUBAC-dependent and potential LUBAC-independent effects of SHARPIN, HOIL-1 and HOIP will be necessary. Several reports on such complex-independent functions exist (Bayle et al., 2006; Ehrlund et al., 2009; He et al., 2010; Jung et al., 2010; Rantala et al., 2011; Tatematsu et al., 1998; Tian et al., 2007; Yamanaka et al., 2003; Zenke-Kawasaki et al., 2007) but in most cases the contribution of the respectively other two proteins to the observed effects was not investigated and the independence of LUBAC's linear ubiquitin generating activity is therefore not fully established. LUBAC-dependent and -independent roles of the three proteins in these contexts will have to be unravelled and the identification of additional LUBAC-targets and the characterisation of their role in signal transduction will be necessary to fully understand the role of LUBAC and of its potential variants. In this context it is interesting to note that upon knockdown of any of the LUBAC-components not only the modification pattern of RIP1, which was shown to be modified by M1-linked ubiquitin in the native TNF-RSC (Gerlach et al., 2011), but also that of cIAP1/2 upon recruitment to the TNF-RSC is changed, indicating that cIAP1 and/or cIAP2 could potentially be targeted by LUBAC.

6.2.3 LUBAC acts via a HECT-like mechanism

LUBAC's exclusive linkage specificity is not only surprising in that it does not seem to be shared by any other E3 investigated so far but also because linkage specificity is usually determined by the E2 rather than the E3, at least in reactions involving RING-class ubiquitin ligases (Nagy and Dikic, 2010). This is not the case for LUBAC as chains generated by this complex in combination of a panel of different E2s do not differ in their electrophoretic mobility, indicating that they are of the same linkage type. This intrinsic, E2-independent linkage specificity is a feature that can be observed in certain HECT-class E3s (Nagy and Dikic, 2010). Ubiquitin ligases of this class, in contrast to RING-E3s, form an obligatory thioester intermediate with ubiquitin. A recent study revealed that UBE2L3 requires an E3 that is able to form an E3~ubiquitin adduct on a catalytic cysteine as this E2 lacks the ability to transfer ubiquitin to lysine residues (Wenzel et al., 2011a). The same study also showed that ARIH1 and Parkin, members of the RBR-family, can catalyse ubiquitination together with UBE2L3 and that ARIH1 indeed forms a thioester intermediate. Comparison of UBE2D3 and UBE2L3 in in-vitro ubiquitination reactions mediated by LUBAC demonstrated that chain formation remains intact when LUBAC is provided as an E3 for UBE2L3 whereas ubiquitin

conjugation on monoubiquitin and auto-ubiquitination of the E3 cannot be supported by combinations of cIAP1, cIAP2 or TRAF6 with this E2. This suggests that LUBAC contains a catalytic cysteine that can accept activated ubiquitin from the E2. Because HOIP was identified as the central component of LUBAC both structurally and functionally and because the active site identified in ARIH1 was allocated to a highly conserved cysteine residues within its RING2 domain (Wenzel et al., 2011a) whereas E2 binding was mediated via its RING1-domain (Ardley et al., 2001), it was concluded that a cysteine involved in the catalytic activity of LUBAC was most likely to reside in the RING2-domain of HOIP. Mutation of cysteine 885 that was identified as a potential candidate by alignment of HOIP's RING2-domain with the published consensus sequence indeed abolished HOIP's ability to generate polyubiquitin when combined with SHARPIN or HOIL-1. It was reported that mutation of C357, the catalytic cysteine identified in ARIH1, leaves the structure of the RING2-domain intact as it is not a zinc-coordinating residue (Wenzel et al., 2011a). Co-precipitation studies showed that a cysteine-to-serine-mutation in residue 885 of HOIP does not prevent it from interacting with SHARPIN or HOIL-1 and that the interaction with the E2 UBE2D3 which, as typical for E2-E3 interactions, seems to be rather weak (Ye and Rape, 2009), was maintained. This strongly suggests that the overall structure of HOIP is unaffected by the mutation of C885 and therefore provides compelling evidence that LUBAC works via a HECT-like mechanism.

Thus the opportunity arises to inhibit LUBAC not only by interfering with its interaction with specific targets or competitively by the addition of N-terminally tagged ubiquitin, which might prove difficult in settings other than in-vitro assays, but also by inhibitors designed to prevent the obligatory formation of an E3~ubiquitin adduct. Such inhibitors targeting the active centre of LUBAC might not only be valuable as scientific tools to further investigate the role of linear ubiquitin in signalling but might also prove to have therapeutic potential in a context where LUBAC's activity is causing or aggravating a pathological condition. Whereas no such disease-causing role of elevated activity of this E3-complex was reported to date, increased expression levels of individual LUBAC components have been observed in certain types of cancer (Jung et al., 2010; Thompson et al., 2004) and it can be envisioned that inhibiting LUBAC and thereby shifting the balance between pro-survival signalling and cell death induction towards the latter outcome could prove beneficial in this situation. Additional approaches to modulating LUBAC's activity could be based on mechanisms

regulating this E3-complex *in vivo* as for example a potential regulation by post-translational modifications.

6.2.4 Post-translational modifications of LUBAC-components

The best studied post-translational modifications occurring in a signalling context are phosphorylations and ubiquitinations (Cohen, 2002; Pickart, 2004). Both ubiquitination, in the form of attachment of proteolytic or non-proteolytic ubiquitin chains to the E3 in reactions catalysed by auto-ubiquitination or by the action of another ubiquitin ligase, and phosphorylations were reported to play a role in the regulation of E3 ligases (de Bie and Ciechanover, 2011; Kee and Huibregtse, 2007). Although it cannot be excluded at this point that other types of modifications are involved in the regulation of LUBAC's activity, only the potential effects of phosphorylations and ubiquitinations will be discussed here. Treatment of the TNF-RSC with the catalytic domain of USP2 revealed that ubiquitin-based modifications are present on all three components of LUBAC. However, ubiquitination patterns differ between the three proteins. Whereas SHARPIN and HOIL-1 are subject to mono-ubiquitination, several ubiquitin-molecules seem to be attached to HOIP in the form of multi-mono- or polyubiquitination. In addition the modified form of HOIL-1 is already present in unstimulated lysates while modification of HOIP occurs in a stimulation-dependent manner. The ubiquitination of SHARPIN and HOIL-1 can be detected in in-vitro assays in the absence of active HOIP or another E3. This suggests that these modifications can be mediated by HOIL-1. However, the presence of HOIP is clearly required for HOIL-1 to be modified in a cellular context. Experiments based on reconstitution of HOIL-1- or HOIP-deficient cells with RING-mutants of the respective protein are therefore required to clarify which RING-domain is involved in the attachment of ubiquitin to HOIL-1.

One approach in functionally characterising the individual alterations could be to create non-modifiable mutants by site-directed mutagenesis and to test their effect on LUBAC's activity *in vitro* and their ability to replace the wild-type proteins in TNF-induced signal transduction by reconstituting cells deficient for one of the proteins by the respective mutant. Identification of the modification sites would be a prerequisite for this type of analysis. It could be shown here that the modification of HOIL-1 occurring *in vitro* takes place in its C-terminal part. In addition, mass-spectrometry based approaches aiming to identify ubiquitination sites on a proteome-wide scale found several sites in SHARPIN,

HOIL-1 and HOIP to be modified. Based on the data obtained in these studies lysines 189 and 318 of SHARPIN (Kim et al., 2011; Lee et al., 2011; Wagner et al., 2011), lysine 342 of HOIL-1 (Wagner et al., 2011) and lysines 458 and 640 in HOIP (Kim et al., 2011; Wagner et al., 2011) could serve as good candidates in identifying the respective modification sites. Additional lysines were found to be ubiquitinated on HOIL-1 and HOIP but their localisation within the N-terminal part of HOIL-1 or the fact that they were identified in only one of the studies makes them secondary candidates in comparison to the sites named above. In case of HOIP it also needs to be considered that its stimulation-dependent modification could occur at a site that is not ubiquitinated in unstimulated cells. Next to the mutagenesis-based investigation of modification sites suggested by previous studies a direct analysis of the modification status of LUBAC components in the TNF-RSC will be necessary to unambiguously identify the lysine residues that are or become modified in this context. The latter approach would also be suitable to find other, non-ubiquitin modifications on the three proteins. A prime candidate as an additional modification would be the phosphorylation of HOIL-1. It was reported that HOIL-1 is targeted by PKC and that phosphorylation by this kinase leads to cleavage of HOIL-1 between its NZF- and its RING1-domain resulting in two fragments of approximately 22 kDa and 42 kDa respectively, thereby negatively regulating LUBAC's activity (Nakamura et al., 2006). A different publication suggested S127, T151 and T191 of HOIL-1 as potential sites for phosphorylation by PKCβ (Tatematsu et al., 2008). On western blot level the anti-HOIL-1 antibody detects a clear double band in lysates whereas in the analysis of the TNF-RSC the upper of the two bands often appears blurry or as a double band in itself. The small distance between these bands indicates that they do not represent mono- and di-ubiquitinated forms of HOIL-1. On the other hand a phosphorylation event might be a possible explanation for the observed shift. Further analysis that could involve two-dimensional gel electrophoresis, potentially in combination with phosphatase treatment or mass-spectrometry is required to clarify whether it is indeed a phosphorylation event that is responsible for the observed banding pattern. If so, use of kinase inhibitors and knockdown of candidate proteins could be first steps in identifying the kinase catalysing this phosphorylation and in analysing the function of this modification. The observation that only the upper of the two bands of HOIL-1 that can be observed in lysates appears to be altered in the TNF-RSC suggests that only the ubiquitinated form of HOIL-1 can be further modified. This could have implications for

LUBAC's activity and its regulation. At the moment it cannot be predicted if phosphorylation would promote the full activation of LUBAC or limit its E3-activity. No thorough investigation of the phosphorylation status of SHARPIN and HOIP upon TNF-stimulation was undertaken to date but both proteins were found to be phosphorylated in proteome-wide mass-spectrometric studies. The residues in SHARPIN reported to be phosphorylated are serines 165 and 312 (Dephoure et al., 2008; Olsen et al., 2011), whereas HOIP is modified on serine 466 (Cantin et al., 2008; Chen et al., 2009; Dephoure et al., 2008). As in the case of HOIL-1 additional work is required to confirm these sites, to identify the responsible kinases and to unravel the functions of these phosphorylations. In these investigations it also has to be considered that phosphorylations occurring in response to a TNF-stimulus might differ from those present in an unstimulated state.

Although relatively little data is available concerning the potential regulation of LUBAC by post-translational modifications, data obtained in this thesis indicate that SHARPIN, HOIL-1 and HOIP may be ubiquitinated and possibly phosphorylated. Analysis of these modifications and unravelling their relevance to LUBAC's E3 activity and to TNF-induced signal transduction could prove to be a fascinating field of investigation in the future.

IV Abbreviations

A	Alanine
aa	aminoacid
ABIN	A20 binding inhibitor of NF-κB
AKT	V-akt murine thymoma viral oncogene homolog
AMPK	AMP-activated protein kinase
AP-1	Activator protein 1
APF-1	ATP-dependent proteolysis factor 1
APC/C	Anaphase-Promoting Complex
ARE	AU-rich element
ARIH	Ariadne Homolog
ASK1	Apoptosis signal-regulating kinase 1
ATG	Autophagy related gene
BAFF	B-cell-activating factor
BCA	Bicinchoninic acid
BIR	Baculoviral IAP repeat
C	Cysteine
cpdm	Chronic proliferative dermatitis mutation
cFLIP	Cellular FLICE-inhibitory protein
cIAP	Cellular inhibitor of apoptosis protein
CIM	cIAP1/2 interacting motif
CLP	Cecal ligation and puncture
CNS	Central nervous system
CRDs	Cysteine rich domains
CYLD	Familial Cylindromatosis Protein

DC	Dendritic cell
DD	Death domain
DDB	DNA damage binding
DISC	Death inducing signalling complex
DMEM	Dulbecco's modified Eagle's medium
DUB	Deubiquitinases
E	Glutamic acid
ER	Endoplasmic reticulum
ERα	Estrogen receptor alpha
ERAD	Endoplasmic-reticulum-associated degradation
ERK	Extracellular signal regulated kinase
EYA-1	Eyes absent 1
F	Phenylalanine
FADD	Fas-associated protein with a death domain
Fn14	Fibroblast growth factor inducible 14
G	Glycine
GCKs	Germinal centre kinases
H	Histidine
HECT	Homologous with E6-associated protein C-terminus
HEK	Human embryonic kidney
HF-TNF	His-FLAG-TNF
HHARI	Human homolog of Ariadne
HOIL-1	Heme-oxidized IRP2 ubiquitin ligase 1
HOIP	HOIL-1-interacting protein
HPIV	Human parainfluenza virus
HRP	Horseradish-peroxidase

HT	Hydroxy-tamoxifen
I	Isoleucine
IBR	In Between RING
IBRDC	IBR domain containing protein
ICAM	Intercellular adhesion molecule
ICC	Intestinal cells of Cajal
IFN	Interferon
IκB	Inhibitor of κB
IL	Interleukin
IPTG	Isopropyl-β-D-thio-galactoside
IRF	IFN regulatory factor
IRP	Iron regulatory protein
JNK	c-Jun N-terminal kinase
K	Lysine
KSHV	Kaposi's sarcoma associated herpes virus
L	Leucine
LB	Lysogeny Broth
LC3	Microtubule-associated protein1 light chain 3
LPS	Lipopolysaccharide
LT	Lymphotoxin
LTβR	Lymphotoxin β receptor
LUBAC	Linear ubiquitin chain assembly complex
M	Methionine
MAPK	Mitogen-activated protein kinase
MEFs	Mouse embryonic fibroblasts
MEKK	MAPK/ERK kinase kinase

moTAP	Modified tandem affinity purification
MS	Mass spectrometry
N	Asparagine
NEMO	NF-κB essential modulator
NES	Nuclear export sequence
NF-κB	Nuclear factor κB
NIK	NF-κB inducing kinase
NLR	NOD-like receptor
NLS	Nuclear localisation sequence
NOD	Nucleotide-binding Oligomerisation Domain
NZF	Npl4-type zinc finger
P	Proline
PAMP	Pathogen associated molecular pattern
PAUL	Putative Ariadne-like ubiquitin ligase
PDB	Pull-down buffer
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLAD	Pre-ligand-binding assembly domain
PRU	Pleckstrin like receptor for ubiquitin
Q	Glutamine
R	Arginine
RBCK1	RBCC protein interacting with PKC 1
RBR	RING-IBR-RING
RHD	Rel homology domain
RIG-I	Retinoic acid-inducible gene I

RING	Really Interesting New Gene
RIP1	Receptor interacting protein 1
RLR	RIG-like receptor
ROS	Reactive oxygen species
RSC	Receptor signalling complex
RTA	replication and transcription activator
S	Serine
SAPK	Stress-activated protein kinases
SCF	Skp1/cullin/F-box
SEM	Standard error of the mean
SF	Superfamily
SF1	Steroidogenic factor 1
SHANK	SH3 and multiple ankyrin repeat domains protein
SHARPIN	SHANK associated RH domain protein
SLE	Systemic lupus erythematosus
SM	Smac mimetic
STAT	Signal transducer and activator of transcription
SV	Simian virus
T	Threonine
TAB	TAK1 binding protein
TACE	TNF-α converting enzyme
TAD	Transactivation domain
TAK1	TGF-β-activated kinase 1
TIM	TRAF-interacting motif
TLR	Toll-like receptor
TNF	Tumour necrosis factor

TNF-RSC	TNF-R1 signalling complex
TRADD	TNF-R1-associated death domain
TRAF	TNF-receptor associated factor
TrCP	Transducin repeat-containing protein
Ub	Ubiquitin
UBA	Ubiquitin-associated
UBAN	Ubiquitin-binding domains found in ABINs and NEMO
Ubc	Ubiquitin conjugating
UBD	Ubiquitin-binding domains
UBE	Ubiquitin-conjugating enzyme
UBL	Ubiquitin-like
UIM	Ubiquitin-interacting motif
UIP28	UBCM4 interacting protein 28
UTR	Untranslated region
V	Valine
VEGI	Vascular endothelial growth inhibitor
W	Tryptophan
wt	Wild-type
XAP3	Hepatitis virus X-associated protein
XIAP	X-linked inhibitor of apoptosis
Y	Tyrosine
ZIBRA	Zinc IBR finger UBA domain containing protein
ZIN	Zinc finger protein
ZnF	Zinc finger
ZAP-70	Zeta-chain associated protein of 70 kDa

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7 References

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