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Recruitment of the Linear Ubiquitin Chain Assembly Complex Stabilizes the TNF-R1 Signaling Complex and Is Required for TNF-Mediated Gene Induction

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SUMMARY

TNF is a key inflammatory cytokine. Using a modified tandem affinity purification approach, we identified HOIL-1 and HOIP as functional components of the native TNF-R1 signaling complex (TNF-RSC). Together, they were shown to form a linear ubiquitin chain assembly complex (LUBAC) and to ubiquitylate NEMO. We show that LUBAC binds to ubiquitin chains of different linkage types and that its recruitment to the TNF-RSC is impaired in TRADD-, TRAF2-, and cIAP1/2- but not in RIP1- or NEMO-deficient MEFs. Furthermore, the E3 ligase activity of cIAPs, but not TRAF2, is required for HOIL-1 recruitment to the TNF-RSC. LUBAC enhances NEMO interaction with the TNF-RSC, stabilizes this protein complex, and is required for efficient TNF-induced activation of NF-κB and JNK, resulting in apoptosis inhibition. Finally, we demonstrate that sustained stability of the TNF-RSC requires LUBAC's enzymatic activity, thereby adding a third form of ubiquitin linkage to the triggering of TNF signaling by the TNF-RSC.

INTRODUCTION

Tumor necrosis factor (TNF) plays a critical role in inflammatory processes and is involved in the regulation of immune responses (Karin and Lin, 2002; Locksley et al., 2001). TNF initiates a complex cascade of signaling events that can lead to induction of proinflammatory cytokines, cell proliferation, differentiation, or cell death (Chen and Goeddel, 2002; Hayden and Ghosh, 2008; Kovalenko and Wallach, 2006; Wajant et al., 2003). TNF binds two cell-surface receptors, TNF-Receptors 1 and 2 (TNF-R1 and TNF-R2). However, most of the pleiotropic effects of TNF are mediated by the death domain (DD)-containing TNF-R1 (Wallach et al., 1999). Binding of TNF to TNF-R1 initiates several signaling pathways, including mitogen-activated protein (MAP) kinase cascades and NF-κB activation (Wajant et al., 2003).

Ligand-induced trimerization of TNF-R1 leads to the formation of an intracellular multiprotein complex, the TNF-R1 signaling complex (TNF-RSC). The adaptor protein TRADD and TNF-R1 bind to each other via homotypic interaction of their respective DDs (Hsu et al., 1995). TRADD serves as an assembly platform for binding of TRAF2 and RIP1 (Hsu et al., 1996; Shu et al., 1996; Tsao et al., 2000). RIP1 is also able to associate directly with the intracellular part of TNF-R1, presumably in a DD-dependent fashion (Ermolaeva et al., 2008; Pobezinskaya et al., 2008). TRAF2 and RIP1 play important roles in the activation of JNK and NF-κB, respectively (Devin et al., 2000; Kelliher et al., 1998; Yeh et al., 1997). In addition, cIAP1 and cIAP2 associate with TRAF2 to participate in TNF-induced signal transduction (Mahoney et al., 2008; Rothe et al., 1995; Shu et al., 1996; Varfolomeev et al., 2008; Vince et al., 2007).

It is well established that the activation of NF-κB is dependent on the cellular ubiquitin (Ub) system (Chen, 2005; Karin and Ben-Neriah, 2000). Ub is generally linked via isopeptide bonds between a lysine (K) residue and the C-terminal glycine. The transfer of Ub to a substrate or a pre-existing Ub chain is mediated by the sequential action of three enzymes: a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub ligase (E3). Ub contains seven K residues, and all mediate Ub chain formation (Ikeda and Dikic, 2008). The consequence of polyubiquitylation depends on the internal Ub K residue, which forms the isopeptide bond in the Ub chain. The best-characterized linkages are mediated via K48 and K63: K48-linked polyUb chains signal for proteasomal degradation, while K63-linked polyUb chains function in signal transduction and normally do not act as a degradation signal (Chen and Sun, 2009; Hoeller et al., 2006). Linear Ub chains in which Ub molecules are linked through their N and C termini in a head-to-tail-like fashion also exist (Ikeda and

Dikic, 2008; Kirisako et al., 2006; Pickart and Fushman, 2004); however, their function is not well characterized.

In the case of TNF-R1 signaling, TRAF2 and cIAP1/2 are believed to attach K63-linked Ub chains to RIP1 (Bertrand et al., 2008; Hoeller et al., 2006; Varfolomeev et al., 2008; Wertz et al., 2004). cIAPs can directly ubiquitylate RIP1 in vitro, but this has not been demonstrated for TRAF2 (Bertrand et al., 2008; Park et al., 2004), and it is possible that TRAF2 plays a structural role recruiting cIAPs to the vicinity of RIP1. Ubiquitylation of RIP1 leads to recruitment and activation of TAK1 and TAB1 together with TAB2 or TAB3. Recruitment and activation of the TAK/TAB complex is essential for activation of the Inhibitor of κB Kinase (IKK) complex, comprised of IKK1/IKKα, IKK2/IKKβ, and NEMO/IKKγ, and both complexes are believed to be recruited by K63-ubiquitylated RIP1 (Ea et al., 2006; Kanayama et al., 2004; Li et al., 2006; Wu et al., 2006). K63-linked ubiquitylation of NEMO is also thought to be crucial for the activation of IKK1 and 2 (Zhou et al., 2004). However, it was recently shown that linear Ub chains can be attached to NEMO and that this is important for NF-κB activation (Tokunaga et al., 2009). Once the IKK complex is active, it leads to the phosphorylation of IκB, a prerequisite for its K48-linked ubiquitylation and degradation. Subsequently, NF-κB translocates to the nucleus and induces transcription of target genes.

TNF stimulation can also lead to cell death. TNF-induced caspase activation is mediated by a second, intracellular complex (complex II), which is formed when the complex consisting of the intracellular proteins recruited to TNF-R1 (complex I) dissociates from the receptor. Subsequently, the adaptor protein FADD and caspase-8 are recruited to complex II, leading to TNF-induced apoptosis (Micheau and Tschopp, 2003).

Our understanding of the biochemical processes at the TNF-RSC remains incomplete. We hypothesized that not all components of this signaling complex are known and developed moTAP, a modification of the original tandem affinity purification (TAP) approach (Rigaut et al., 1999), to identify components of the TNF-RSC. By adding the modified TAP tag to the ligand, this technique enables the isolation of the native receptor signaling complex and its subsequent physical analysis by tandem mass spectrometry. In addition to known constituents of the native TNF-RSC, analysis of this protein complex by moTAP also revealed the presence of two previously unrecognized components: the heme-oxidized IRP2 Ub ligase-1 (HOIL-1) and the HOIL-1-interacting protein (HOIP). HOIL-1 and HOIP have been reported to form a linear Ub chain assembly complex (LUBAC), i.e., a Ub ligase (E3) that catalyzes head-to-tail ligation of Ub (Kirisako et al., 2006). LUBAC mediates ubiquitylation of NEMO with linear Ub chains, which is required for efficient NF-κB activation following TNF stimulation (Tokunaga et al., 2009). Furthermore, NEMO itself binds to linear polyUb chains with significantly higher affinity compared to K63-linked chains (Komander et al., 2009; Lo et al., 2009; Rahighi et al., 2009).

Here, we identify HOIL-1 and HOIP as components of the TNF-RSC and biochemically and functionally analyze their role in TNF signal transduction. We show that LUBAC recruitment does not require RIP1 and NEMO but is impaired in TRADD, TRAF2, and cIAP1/2 knockout cells and requires the catalytic activity of cIAPs. This result, together with our analysis of HOIL-1 and HOIP binding to polyUb chains of different linkage types, suggests that LUBAC is recruited to the TNF-RSC via cIAP-generated Ub chains. Once recruited to the TNF-RSC, LUBAC increases the efficiency of NF-κB and JNK activation, enhancing TNF-induced gene activation and inhibiting TNF-mediated apoptosis. Furthermore, NEMO recruitment to the TNF-RSC is more sustained in LUBAC-expressing than in LUBAC-deficient cells, and the enzymatic activity of LUBAC is required for overall stabilization of the TNF-RSC.

RESULTS

Biochemical Identification of Two RING Domain Proteins as Constituents of the Native TNF-RSC

To analyze whether known components of the TNF-RSC represent the whole spectrum of proteins recruited to this receptor, we developed a modified TAP (moTAP) procedure (Figures 1A and S1A). This technique is based on a modified TAP tag attached to the ligand. moTAP facilitates purification of native RSCs because of the small size of the moTAP tag and the PreScission (GE Healthcare; Fairfield, CT) protease site that enables protein complex purification at 4°C.

Like native untagged TNF, recombinant moTAP-tagged TNF (Figure 1A) induced IκBα phosphorylation and degradation (Figure 1B) and cell death in U937 cells (Figure S1B). Importantly, moTAP-TNF precipitated known components of the TNF-RSC in the same dynamic manner as described for TNF, with a single FLAG or Fc tag (Figure 1C) (Micheau and Tschopp, 2003). Reassured that moTAP-TNF was functional, we performed one-step and moTAP purifications of the TNF-RSC side by side. moTAP-purified TNF-RSC resulted in a striking reduction in binding of nonspecific proteins without loss of specific proteins like RIP1, TRAF2, and TRADD (Figure 1D). Compared to conventional immunoprecipitation procedures, moTAP therefore offers higher specificity without loss of sensitivity.

We next purified the TNF-RSC using moTAP-TNF and identified the components of the isolated protein complex by nanoscale liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS). In two independent experiments, we stimulated U937 cells with moTAP-TNF, purified the TNF-RSC, and analyzed a small percentage of both preparations by western blotting (Figure 1E) and silver staining (Figure 1F), revealing enrichment of specifically recruited proteins in both preparations. The remaining samples, corresponding to about 95% of isolated TNF-RSCs, were pooled and separated by SDS-PAGE. Twenty consecutive gel fragments were cut out without prior staining and subjected to nanoLC-MS/MS after in-gel tryptic digest. This analysis identified TNF-R1, TRADD, TRAF2, RIP1, Ub, cIAP2, ABIN1, IKK1, IKK2, NEMO, TAK1, TAB1, and TAB2 as components of the native TNF-RSC in U937 cells (Table S1). It also identified peptides distributed over the entire length of two proteins, HOIL-1 and HOIP, previously not known to form part of this protein complex (Figure 2A, Table S1). HOIL-1 and HOIP are heme-oxidized IRP2 Ub ligase-1 (HOIL-1/RBCK1/XAP3) and HOIL-1-interacting protein (HOIP/RNF31/Zibra), respectively. They belong to the In-Between-RING (IBR) subfamily of RING-finger Ub ligases and together form a complex with a Ub ligase activity that catalyzes the formation of linear polyUb chains. Due to this activity, the protein

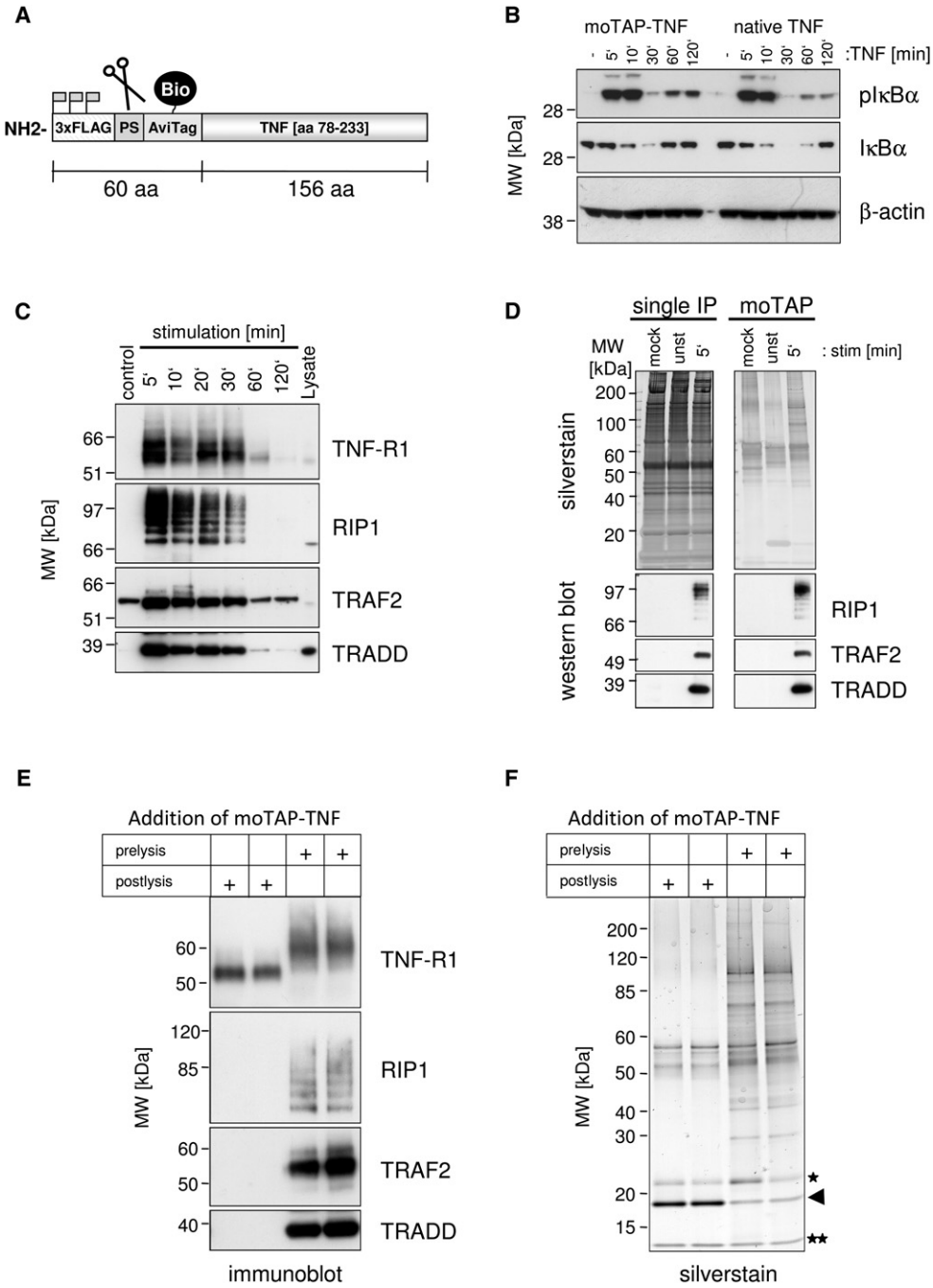


Figure 1. Identification of HOIL-1 and HOIP as Components of the Native TNF-RSC

(A) Schematic representation of moTAP-tagged TNF.

(B) Unmodified TNF and moTAP-TNF induce IκBα phosphorylation and degradation in U937 cells. U937 cells were treated with 50 ng/ml TNF for the indicated times and lysed, and the lysates were analyzed by western blotting using the indicated antibodies. β-actin served as loading control.

(C) Kinetic analysis of TNF-RSC formation induced by moTAP-TNF. U937 cells were stimulated with 1 μg/ml moTAP-TNF for the indicated times. The isolated protein complexes were analyzed by western blotting (control, beads only).

(D) moTAP enhances specificity of the TNF-RSC purification. A single FLAG tag IP was compared to moTAP by silver staining (whole protein) and western blotting (mock, beads only control; unst., unstimulated; 5′, stimulation for 5 min with 1 μg/ml moTAP-TNF).

(E) Two percent of the eluted samples from two independent preparative TNF-RSC isolations were examined by western blotting with the indicated antibodies. As a negative control, moTAP-TNF was added after lysis.

(F) Five percent of the RSB-eluted samples of two independent moTAP-isolated TNF-RSCs were analyzed by silver staining. ◀, moTAP-TNF; *, IgG1 light chain (M2); **, monomeric streptavidin.

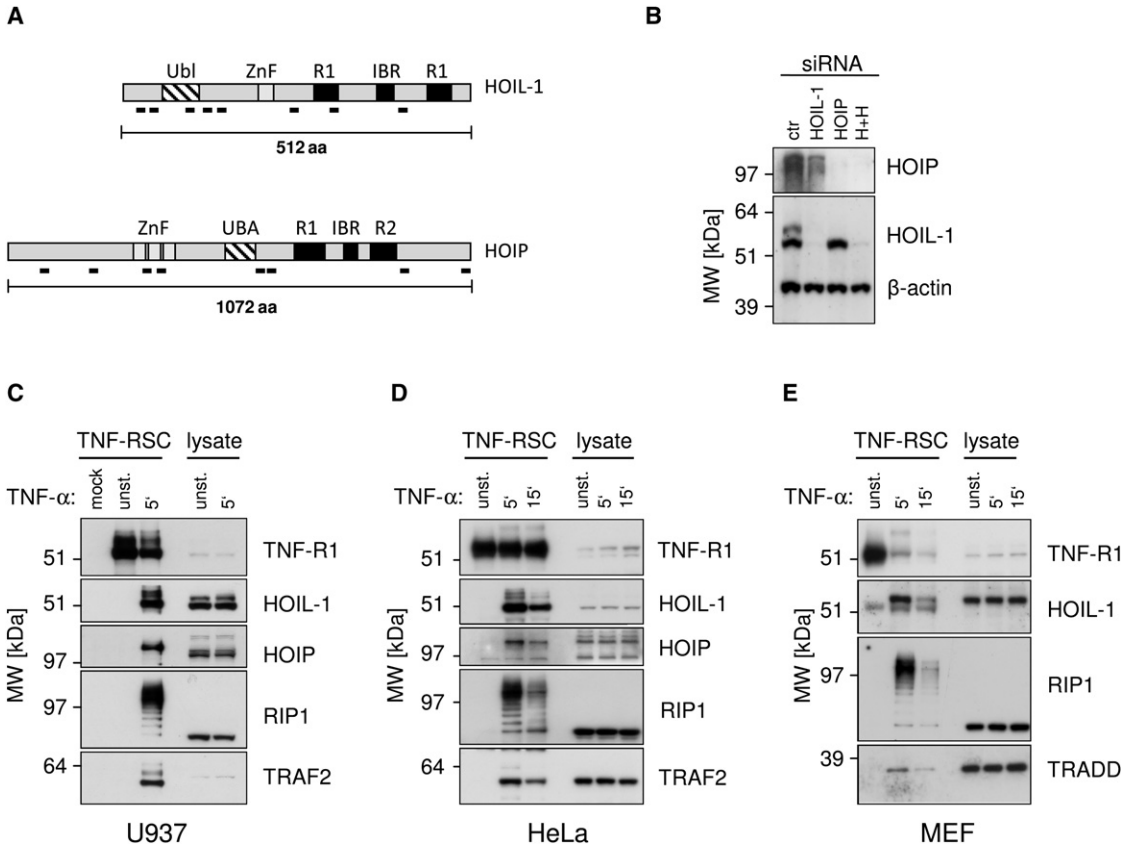


Figure 2. HOIL-1 and HOIP Are Recruited to the TNF-RSC in a Ligand-Dependent Manner

(A) Schematic representation of HOIL-1 and HOIP. The black bars indicate the position of peptides identified by nanoLC-MS/MS.

(B) HOIL-1- and HOIP-specific antibodies. HEK293-NF-κB cells were transiently transfected with the indicated siRNAs, and protein expression was analyzed by western blotting.

(C–E) HOIL-1 and HOIP are recruited to the TNF-RSC in a stimulation-dependent manner. HeLa cells, U937 cells, and MEFs were stimulated with TNF (1 μg/ml) prior to TNF-R precipitation, as described in the Experimental Procedures, and analyzed by western blotting.

complex formed by HOIL-1 and HOIP was named LUBAC (Kirisako et al., 2006). Consistent with these earlier findings, we also observed that HOIL-1 and HOIP interact with each other, and that the N terminus of HOIL-1 containing the Ubl domain is required for this interaction (Figure S2).

We next addressed whether recruitment of LUBAC to the TNF-RSC was stimulation dependent. Using newly developed HOIL-1- and HOIP-specific antibodies (Figure 2B), we found HOIL-1 and HOIP to be recruited to the native TNF-RSC in a stimulation-dependent manner in U937 and HeLa cells and HOIL-1 in MEF cell lines (Figures 2C–2E).

LUBAC Recruitment to the TNF-RSC Is Dependent on TRADD, TRAF2/5, and cIAP1/2 but Does Not Require RIP1 and NEMO

To dissect the importance of known components of the TNF-RSC in recruiting LUBAC to the TNF-RSC, we analyzed the composition of the TNF-RSC in wild-type (WT) MEFs and knockout MEFs. We were able to detect murine HOIL-1 with the monoclonal antibody raised against human HOIL-1 in the native TNF-RSC isolated from MEFs (Figure 2E), but murine HOIP was not detected by the antibody raised against human HOIP (data not shown). However, HOIL-1 and HOIP have been shown to form a stimulation-independent complex (Kirisako et al., 2006), and it is therefore likely that HOIP is recruited together with HOIL-1 in MEFs.

In cells lacking TRADD, RIP1 was still recruited to the TNF-RSC (Figure 3A). Consistent with previous observations (Ermolaeva et al., 2008; Pobezinskaya et al., 2008), ubiquitylation of RIP1 was completely abrogated, and TRAF2 could not be detected in the TNF-RSC of TRADD-deficient MEFs. HOIL-1 was also not precipitated with TNF-R1 in these cells (Figure 3A), underlining the pivotal role of TRADD in the formation of a functional TNF-RSC.

We addressed the role of RIP1 for HOIL-1 recruitment in a similar manner. Stimulated and unstimulated TNF-R1 was immunoprecipitated from WT or RIP1-deficient MEFs and analyzed for the presence of RIP1, TRAF2, TRADD, and HOIL-1. TNF-induced recruitment of TRAF2 and TRADD was also detected in RIP1-deficient cells, suggesting that their recruitment is independent of RIP1 (Figure 3B). Interestingly, the amount of TRADD and TRAF2 recruited in RIP1-deficient cells was increased when compared to WT MEFs. This is consistent with a model in which TRADD recruits TRAF2, but the DDs of RIP1 and TRADD compete with each other for TNF-R1 binding. However, we

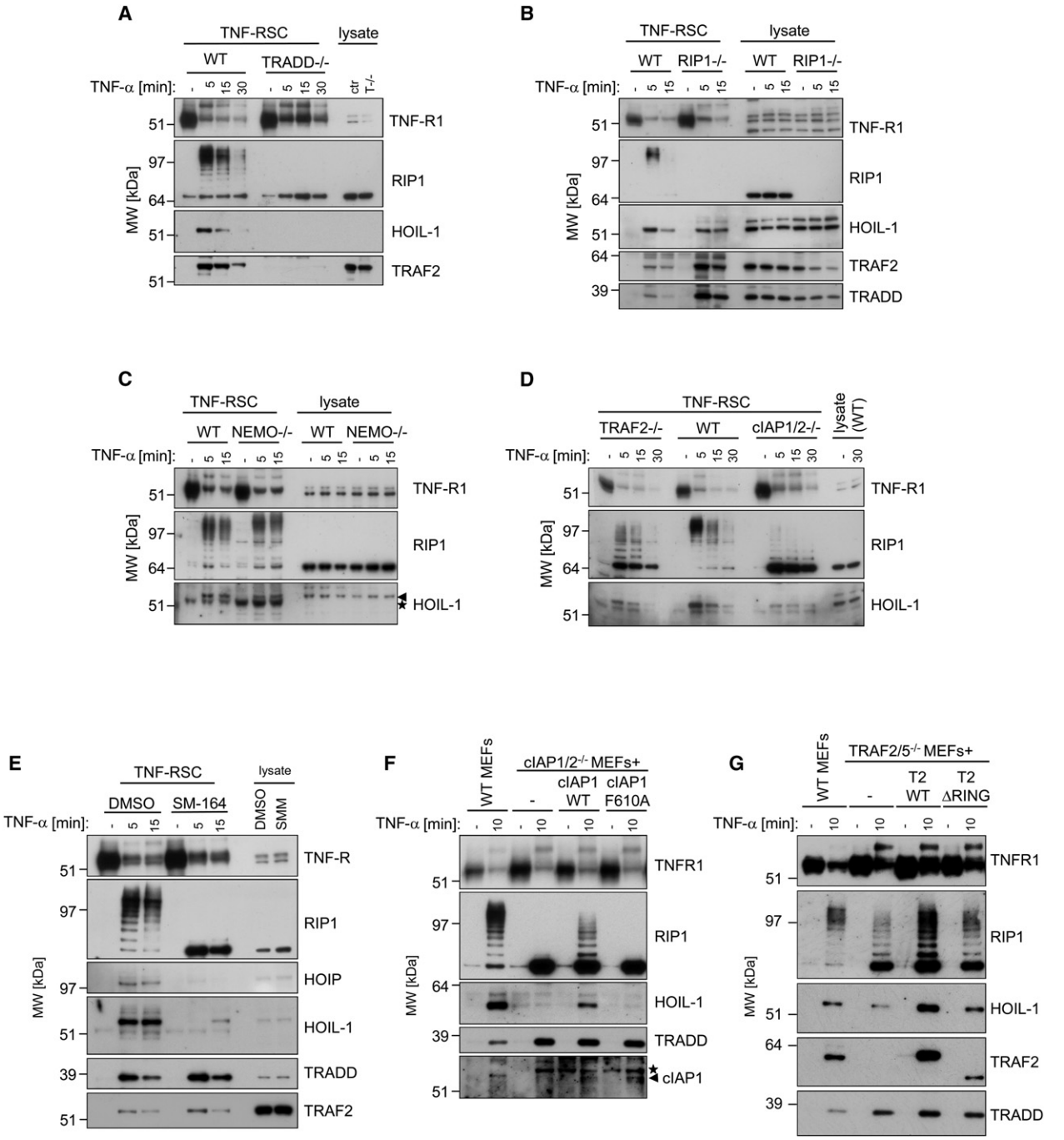


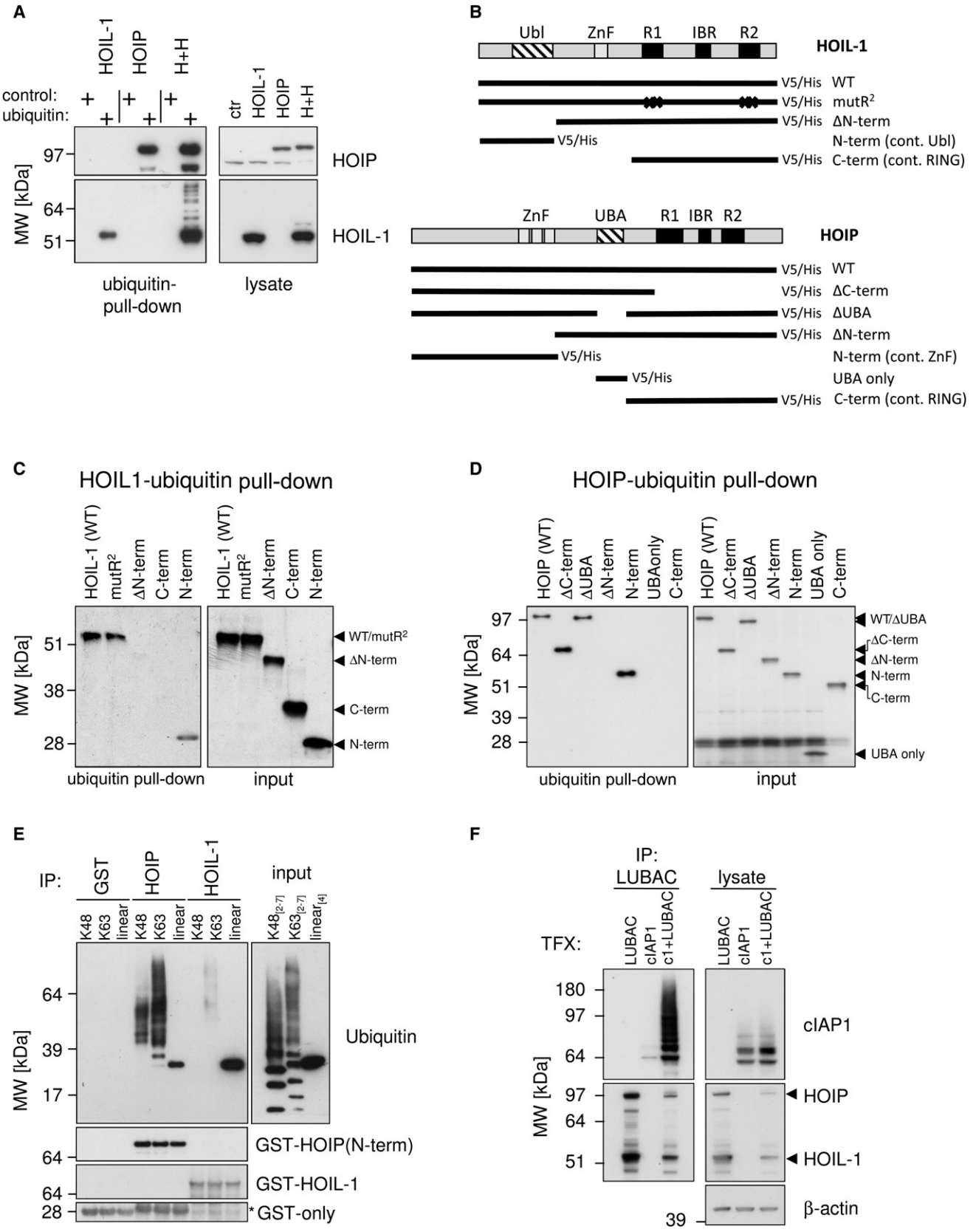
Figure 3. LUBAC Is Recruited to the TNF-RSC Independently of RIP1 and NEMO in a TRADD-, TRAF2-, and cIAP1/2-Dependent Manner and Requires the Ub Ligase Activity of cIAPs

(A–D) HOIL-1 is recruited to the TNF-RSC in a TRADD-/TRAF2-/cIAP1/2-dependent manner. The indicated WT and knockout MEFs were stimulated with 1 μg/ml TNF for the designated times. Cells were harvested, and the endogenous TNF-RSC was precipitated from cell lysates. *, nonspecific band.

(E) cIAP1/2 expression is essential for LUBAC recruitment in human cells. HeLa cells pretreated with SM-164 (100 nM) or DMSO for 2 hr at 37°C were stimulated for the indicated times with TNF (200 ng/ml), and the TNF-RSC was analyzed as in (A)–(D).

(F) cIAP1/2 catalytic activity is essential for HOIL-1 recruitment to the TNF-RSC. MEFs deficient for cIAP1/2 expression were reconstituted with inducible cIAP1 WT and catalytically inactive F610A mutant. Protein expression was induced as described in the Experimental Procedures. Cells were then treated as above (Figures 3A–3D), and endogenous TNF-RSC was isolated. *, nonspecific band.

(G) TRAF2 catalytic activity is not required for HOIL-1 recruitment to the TNF-RSC. TRAF2/5-deficient MEFs were reconstituted with WT TRAF2 (T2 WT) or RING-deficient TRAF2 (T2 ΔRING). Induction of protein expression was controlled by western blotting (Figure S3F). Cells were stimulated with 1 μg/ml TNF, and the isolated receptor complexes were analyzed by western blotting.


A
HOIL-1
HOIP
H+H
control:
ubiquitin:
ctr
97
64
51
MW [kDa]
HOIP
HOIL-1
ubiquitin-
pull-down
lysate
B
Ubl
ZnF
R1
IBR
R2
HOIL-1
V5/His WT
V5/His mutR2
V5/His ΔN-term
V5/His N-term (cont. Ubl)
V5/His C-term (cont. RING)
ZnF
UBA
R1 IBR R2
HOIP
V5/His WT
V5/His ΔC-term
V5/His ΔUBA
V5/His ΔN-term
V5/His N-term (cont. ZnF)
V5/His UBA only
V5/His C-term (cont. RING)
C
HOIL1-ubiquitin pull-down
HOIL-1 (WT)
mutR2
ΔN-term
C-term
N-term
51
38
28
WT/mutR2
ΔN-term
C-term
N-term
ubiquitin pull-down
input
D
HOIP-ubiquitin pull-down
HOIP (WT)
ΔC-term
ΔUBA
ΔN-term
N-term
UBAonly
C-term
UBA only
97
64
51
39
28
WT/ΔUBA
ΔC-term
ΔN-term
N-term
C-term
UBA only
ubiquitin pull-down
input
E
IP:
GST
HOIP
HOIL-1
input
K48
K63
linear
K48[2-7]
K63[2-7]
linear[4]
64
39
17
Ubiquitin
GST-HOIP(N-term)
GST-HOIL-1
*GST-only
F
IP:
LUBAC
lysate
TFX:
LUBAC
cIAP1
c1+LUBAC
180
97
64
51
39
cIAP1
HOIP
HOIL-1
β-actin

observed no differences in HOIL-1 recruitment in the absence of RIP1 (Figure 3B). Furthermore, we found that recruitment of HOIL-1 to TNF-stimulated TNF-R1 was still substantial, albeit reduced, in RIP1-knockdown HeLa cells when compared to control cells (Figure S3A). Hence, RIP1 may contribute to, but is not essential for, LUBAC recruitment to the TNF-RSC.

Recently, an interaction between LUBAC and NEMO was reported (Tokunaga et al., 2009). We therefore suspected that recruitment of HOIL-1 to the TNF-RSC might depend on NEMO. HOIL-1 was, however, recruited to the receptor complex in NEMO-deficient cells (Figure 3C), showing that, although LUBAC can bind to NEMO (Tokunaga et al., 2009), NEMO is not required for HOIL-1 recruitment to the TNF-RSC.

The analysis of TRAF2-deficient cells revealed that absence of TRAF2 resulted in reduction of RIP1 ubiquitylation and increased levels of unmodified RIP1 bound to the TNF-RSC (Figure 3D). This phenomenon was even more pronounced in cIAP1/2-deficient MEFs (Figure 3D), confirming that TRAF2 and cIAP1/2 play important roles in RIP1 ubiquitylation (Bertrand et al., 2008; Park et al., 2004; Varfolomeev et al., 2008; Wertz et al., 2004). Recruitment of HOIL-1 was significantly reduced in TRAF2-deficient and, even more severely, in cIAP1/2-deficient MEFs compared to WT cells, indicating that TRAF2 and cIAP1/2 are important for recruitment of LUBAC to the TNF-RSC (Figure 3D). To further investigate a role for the TRAF2/cIAP axis in recruiting HOIL-1, we treated TRAF2-deficient MEFs with the IAP antagonist SM-164, which, like other IAP inhibitors, promotes degradation of cIAP1 and 2 (Figure S3C) (Cossu et al., 2009; Lu et al., 2008; Sun et al., 2007; Vince et al., 2007). When TRAF2-deficient MEFs were treated with SM-164, association of HOIL-1 with the TNF-RSC was completely abrogated (Figure S3B), suggesting that TRAF2 and cIAPs cooperate to promote recruitment of LUBAC to the TNF-RSC.

To test whether recruitment of both LUBAC components is impaired in the absence of cIAP1/2 in human cells, HeLa cells were pretreated with SM-164. The association of HOIL-1 and HOIP with the TNF-RSC was only faintly detectable at later time points (Figure 3E), whereas the levels of TRADD and TRAF2 in the TNF-RSC were completely unaffected (Figure 3E). Consistent with previous observations (Bertrand et al., 2008; Varfolomeev et al., 2008), RIP1 modification was also severely attenuated in SM-164-treated cells. Using different experimental settings and species, these data confirm that cIAP1 and 2 play an important role in recruiting LUBAC to the TNF-RSC.

To test whether the catalytic activity of the cIAPs is required for LUBAC recruitment, we stably re-expressed a catalytically inactive mutant of cIAP1 (F610A) or, as a control, WT cIAP1 in MEFs knocked out for cIAP1 and 2 (Mace et al., 2008) (Figure S3E). Upon re-expression of WT cIAP1, both ubiquitylation of RIP1 and recruitment of HOIL-1 were restored (Figure 3F). Reconstitution with catalytically inactive cIAP1 (F610A) neither restored stimulation-dependent ubiquitylation of RIP1 nor association of HOIL-1 with the TNF-RSC (Figure 3F). This demonstrates that the E3 ligase function of cIAPs is required for recruitment of HOIL-1 to the TNF-RSC. A strikingly different result was obtained when we examined the importance of the catalytic activity of TRAF2: reconstitution of TRAF2/5-deficient MEFs with either WT or RING-deficient TRAF2 (ΔRING) reinstalls the ability to recruit HOIL-1 to the TNF-RSC (Figures 3G and S3F).

HOIL-1 and HOIP Directly Interact with PolyUb Chains of Different Linkage Types

The finding that loss of the E3 ligase activity of cIAP1/2 impairs HOIL-1 recruitment to the TNF-RSC led us to hypothesize that LUBAC recruitment to the TNF-RSC may be mediated by Ub chains. Consistent with our hypothesis, we found that HOIL-1 and HOIP were both capable of binding to Ub-conjugated beads. However, in the presence of HOIP, binding of HOIL-1 to Ub was strongly increased, suggesting that HOIP is the major contributor to the binding of LUBAC to Ub (Figure 4A). We used several in vitro translated point mutants and truncated forms of HOIL-1 and HOIP (Figure 4B) to map the Ub-binding domains of both proteins. Surprisingly, we found that the Ub-associated (UBA) domain of HOIP was not required for Ub binding but that HOIL-1 and HOIP bound to Ub-conjugated agarose via their N-terminal parts containing the Ubl and ZnF domains, respectively (Figures 4C and 4D). To investigate whether the type of Ub linkage influences binding of the LUBAC components to Ub chains, we expressed GST-tagged full-length HOIL-1 and the ZnF-containing N terminus of HOIP in *E. coli* and checked for their direct interaction with K48-, K63-, and linearly linked Ub chains. We found that HOIL-1 did not bind to K48-linked Ub chains but to linear and, albeit to a lesser extent, to K63-linked Ub chains, whereas the zinc finger domain of HOIP preferentially bound K63- over linearly and K48-linked Ub chains (Figure 4E). K63-linked Ub chains are thought to be present in the TNF-RSC and to be essential for recruitment and activation of both the TAK/TAB and IKK complex. Taken together, these data indicate that LUBAC is recruited to a polyUb chain platform generated by cIAP1/2 in the TNF-RSC and that the generation of this platform requires recruitment of TRADD, TRAF2, and catalytically active cIAPs to the TNF-RSC. Furthermore, we found that LUBAC can bind to ubiquitylated cIAP1 (Figure 4F). As cIAP1 has been shown

Figure 4. HOIL-1 and HOIP Directly Bind to PolyUb Chains

(A) HOIL-1 and HOIP bind to immobilized Ub. Lysates of HEK293T cells transfected with the indicated cDNAs were incubated with empty (control) or Ub-coupled agarose beads. Ub-bound proteins were examined by immunoblotting with anti-V5 antibody.

(B) Schematic representation of the HOIL-1 and HOIP mutants.

(C and D) HOIL-1 and HOIP bind Ub via their N-terminal parts containing the Ubl and ZnF domains, respectively. Mutants of HOIL-1 and HOIP described in Figure 4B were generated by in vitro transcription/translation and incubated with Ub-coupled agarose beads, and Ub-bound proteins were examined by autoradiography (HOIL-1) or immunoblotting with anti-V5 antibody (HOIP).

(E) Pull-down analysis with immobilized GST-tagged HOIL-1 and HOIP N-term. Purified recombinant GST-only, GST-tagged HOIL-1, and HOIP N-term (10 μg each) were immobilized on glutathione Sepharose resin. Two micrograms of the indicated K48-linked, K63-linked, or linear Ub chains were added, and a GST pull-down assay was performed. The input control represents 10% (0.2 μg) of the amount of Ub used for the pull-down assay (*, GST-containing HOIP fragment).

(F) LUBAC interacts with ubiquitylated cIAP1. HEK293T cells were transfected with V5-tagged HOIP/HOIL-1 and FLAG-tagged cIAP1. HOIP and HOIL-1 were immunoprecipitated with anti-V5.

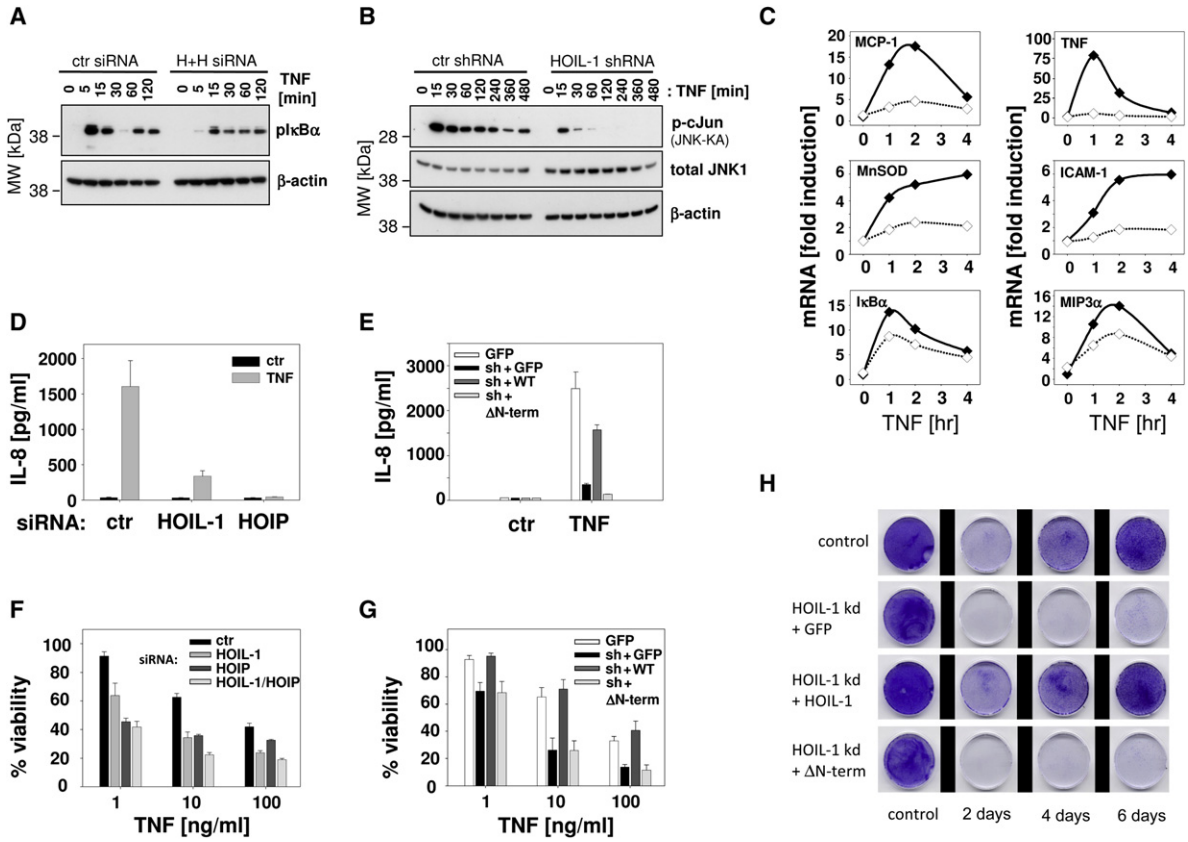


Figure 5. LUBAC Is Required for TNF-Induced NF-κB and JNK Activation and Gene Induction

(A) LUBAC mediates TNF-induced NF-κB activity. MCF-7 cells were transiently transfected with siRNA specific for HOIL-1 and HOIP and treated with TNF (50 ng/ml) for the indicated times. Cell lysates were analyzed by western blotting.

(B) HOIL-1 mediates TNF-induced JNK activity. Stable HOIL-1 knockdown MCF-7 cells were treated with TNF (50 ng/ml TNF) for the indicated times, and a nonradioactive JNK assay was performed.

(C) LUBAC is required for TNF-induced gene expression. HeLa cells, transfected with HOIL-1 and HOIP siRNA, were stimulated with TNF (10 ng/ml), and mRNA levels of TNF target genes were determined by qPCR (◆, control siRNA; ◇, HOIL-1/HOIP siRNA).

(D) HOIL-1 and HOIP knockdown cells (MCF-7) were treated with TNF (50 ng/ml) for 12 hr, and supernatants were analyzed by IL-8-specific ELISA. Average and SEM of three independent experiments done in triplicate are shown.

(E) The N-terminal part of HOIL-1 is essential for TNF-mediated IL-8 expression. Stable HOIL-1 knockdown MCF-7 cells were retransfected with the HOIL-1 mutants indicated, and an IL-8-specific ELISA was performed following stimulation with TNF (50 ng/ml). Average and SD of a representative experiment (n = 4) done in triplicate is shown.

(F) Knockdown of HOIL-1 and HOIP sensitizes cells to TNF-induced cell death. MCF-7 cells downregulated for HOIL-1 and HOIP by siRNA were treated with TNF, and cell viability was determined after 24 hr with CellTiter-Glo (Promega; Madison, WI). Average values of three independent experiments (±SEM) are shown.

(G) The N-terminal part of HOIL-1 is required for its prosurvival function. Retransfected cells (Figure 4E) were treated with TNF for 24 hr, and cell viability was measured. Average values of three independent experiments (±SEM) are shown.

(H) HOIL-1 expression is required for clonogenic survival of MCF-7 cells following treatment with TNF (50 ng/ml) for 24 hr.

to be able to autoubiquitylate with K63-linked chains in vitro (Blankenship et al., 2009), this suggests that the cIAPs not only generate the polyUb chains required for LUBAC recruitment but at the same time can be acceptors for these chains.

HOIL-1 and HOIP Mediate TNF-Induced Signaling Events and Gene Induction and Protect Cells from TNF-Induced Apoptosis

We next investigated the role of HOIL-1 and HOIP in downstream signaling processes. A recent report showed that HOIL-1 and HOIP attach linear Ub chains to NEMO and thereby influence NF-κB activation (Tokunaga et al., 2009). In agreement with Tokunaga et al., we found that overexpression of HOIL-1 and HOIP resulted in strong activation of NF-κB (Figure S4A). This was observed when HOIP was coexpressed with WT or double RING mutant (mutR^2) HOIL-1, but not with the noninteracting HOIL-1 ΔN-term or when either HOIP or HOIL-1 were expressed alone (Figures S4A and S4B). In addition, a mutant LUBAC complex composed of WT HOIL-1 and HOIP with mutations in the RING-finger domains (HOIP mutR) did not induce activation of NF-κB (Figures S4C and S4D). Conversely, the knockdown of HOIL-1 and/or HOIP reduced TNF-induced NF-κB activation in MCF-7 (Figure 5A) and HEK293 cells (Figures S5A and S5B).

However, in contrast to Tokunaga et al. (Tokunaga et al., 2009), we found that stable knockdown of HOIL-1 resulted in reduced TNF-induced JNK activity in all three cell lines tested; initial phosphorylation of JNK and cJun was lower and vanished more rapidly in HOIL-1 knockdown as compared to WT MCF-7 (Figures 5B and S5C), HEK293 (Figure S5E), and THP-1 cells (Figure S5F). This indicates that LUBAC is required for effective TNF-induced activation not only of NF-κB but also of JNK. Consistent with impairment of both of these gene-inducing pathways, concomitant knockdown of HOIL-1 and HOIP had a marked suppressive effect on TNF-induced expression of various TNF target genes in HeLa cells (Figure 5C). TNF-stimulated transcription of MnSOD, MCP-1, ICAM1, and TNF itself was almost completely abrogated by HOIL-1/HOIP double knockdown, and IκBα and MIP3α transcription was clearly decreased. Similar data were obtained in TNF-stimulated MCF-7 cells in which HOIL-1 was stably knocked down (Figure S6A). In addition, TNF-induced secretion of IL-8 was strongly reduced when HOIL-1 or HOIP were knocked down in MCF-7 (Figures 5D and S6B) and HeLa cells (Figure S6C). In accordance with the importance of the N-terminal part of HOIL-1 for the interaction between the two proteins (Figure S2), stable re-expression of WT HOIL-1 or HOIL-1 mutR[2] but not of HOIL-1 ΔN-term (Figure S5D) restored IL-8 secretion in stable MCF-7 HOIL-1 knockdown cells (Figure 5E). Together, this demonstrates that loss of HOIL-1 resulted in inefficient activation of NF-κB and JNK and that the N-terminal part of HOIL-1 was required for its function in TNF-mediated gene induction, but not its RING-finger domains. This suggests that HOIP must interact with HOIL-1, but that LUBAC's enzymatic activity resides in HOIP. Consistent with a reduction in NF-κB activation, HOIL-1 knockdown MCF-7 cells were sensitized to TNF-induced cell death (Figures 5F and S7A–S7C), an effect that could be reverted in stable MCF-7 HOIL-1 knockdown cells by re-expression of WT HOIL-1 but not HOIL-1 ΔN-term (Figure 5G). Loss of HOIL-1 dramatically reduced clonogenic survival of MCF-7 cells following TNF treatment (Figure 5H) and, as in the short-term survival assays, re-expression of WT HOIL-1 but not HOIL-1 ΔN-term rescued the cells from TNF-induced cell death (Figure 5H). Thus, HOIL-1 re-expression protected cells from TNF-induced apoptosis and did not merely delay its onset.

HOIL-1 and HOIP Are Required for Stable TNF-RSC Formation

The loss of TNF-induced JNK and NF-κB signaling in the absence of LUBAC, together with the discovery that LUBAC forms an integral component of the TNF-RSC, suggests that it functions within the membrane proximal receptor complex to activate these signaling pathways. We therefore investigated the composition of the TNF-RSC in cells stably overexpressing HOIL-1 and HOIP and also in cells lacking HOIL-1 and HOIP. Overexpression of WT LUBAC increased and prolonged overall ubiquitylation of TNF-RSC components (Figure 6A), and RIP1, TRAF2, and TAK1 were retained within the complex substantially longer than in control cells (Figure 6B), whereas TRADD retention was not greatly affected. Conversely, transient knockdown of HOIL-1 and HOIP severely reduced recruitment and/or retention of RIP1, TRAF2, and TAK1 in the complex (Figure 6C). TRADD recruitment was, however, not altered in the absence of LUBAC, suggesting that LUBAC acts downstream of TRADD to specifically stabilize the complex and to allow for full activation of downstream signaling pathways (Figure 6C). Because LUBAC generates linear Ub chains (Kirisako et al., 2006) and NEMO preferentially binds to them (Lo et al., 2009; Rahighi et al., 2009), we tested whether the reduced NF-κB activation caused by depletion of LUBAC might be due to impaired recruitment of the IKK complex to the TNF-RSC. In stable HOIL-1/HOIP double knockdown cells, much less IKK1 was detected in the TNF-RSC when the complex was immunoprecipitated using recombinant FLAG-tagged TNF (Figure 6D). In addition, NEMO immunoprecipitations following TNF stimulation contained substantially less RIP1, TRAF2, TRADD, and TNF-R1 in HOIL-1/HOIP double knockdown cells than in control cells (Figure 6E).

Finally, we asked whether the catalytic activity of LUBAC was essential for stabilization of the TNF-RSC (Figure 6B). We therefore expressed WT HOIL-1 together with WT or mutR HOIP, the catalytically inactive mutant of HOIP. Expression of HOIL-1/HOIP mutR resulted in normal initial recruitment and modification of RIP1 but rapid loss of RIP1 from the complex so that it was only weakly detectable at 30 min and undetectable at 60 min (Figure 6F). This contrasted with untransfected cells or with cells overexpressing WT LUBAC, which retained ubiquitylated RIP1 for at least 1 hr (Figure 6F), suggesting that overexpressed, catalytically inactive LUBAC may exert a dominant-negative effect. Together, these experiments show that recruitment of LUBAC to the TNF-RSC stabilizes the complex, that this stabilization requires LUBAC activity, and that LUBAC recruitment to the TNF-RSC and its activity are important for sustained interaction of the IKK complex with the TNF-RSC.

DISCUSSION

To elucidate the composition of the native TNF-RSC following ligand binding, we developed a modified TAP. The moTAP technique is a sensitive and selective method to purify multiprotein complexes formed under physiological conditions. It combines a first high-affinity purification with mild elution using a site-specific protease that works at low temperature and a second affinity purification step to obtain protein complexes with high efficiency and specificity, demonstrated here with TNF. A further advantage of moTAP is that all molecular interactions in the receptor signaling complex are native, as the tag is on the ligand. The moTAP tag can easily be swapped to other ligands and may therefore serve as a valuable tool to elucidate the composition of ligand-induced receptor signaling complexes in general.

Using this method, we found that a Ub ligase complex composed of two RING-finger proteins, HOIL-1 and HOIP, was recruited to the native TNF-RSC in a stimulation-dependent manner. Together, HOIL-1 and HOIP were described to form LUBAC, a protein complex that attaches head-to-tail-linked Ub chains to target proteins (Kirisako et al., 2006). LUBAC can bind to NEMO and conjugate linear polyUb chains onto specific lysine residues in NEMO's CC2-LZ domain in a Ubc13-independent manner (Tokunaga et al., 2009). Yet, how binding of TNF to its receptor and linear ubiquitylation of NEMO were linked was unknown. By demonstrating that LUBAC is recruited to the TNF-RSC in a stimulation-dependent manner, we now provide this link.

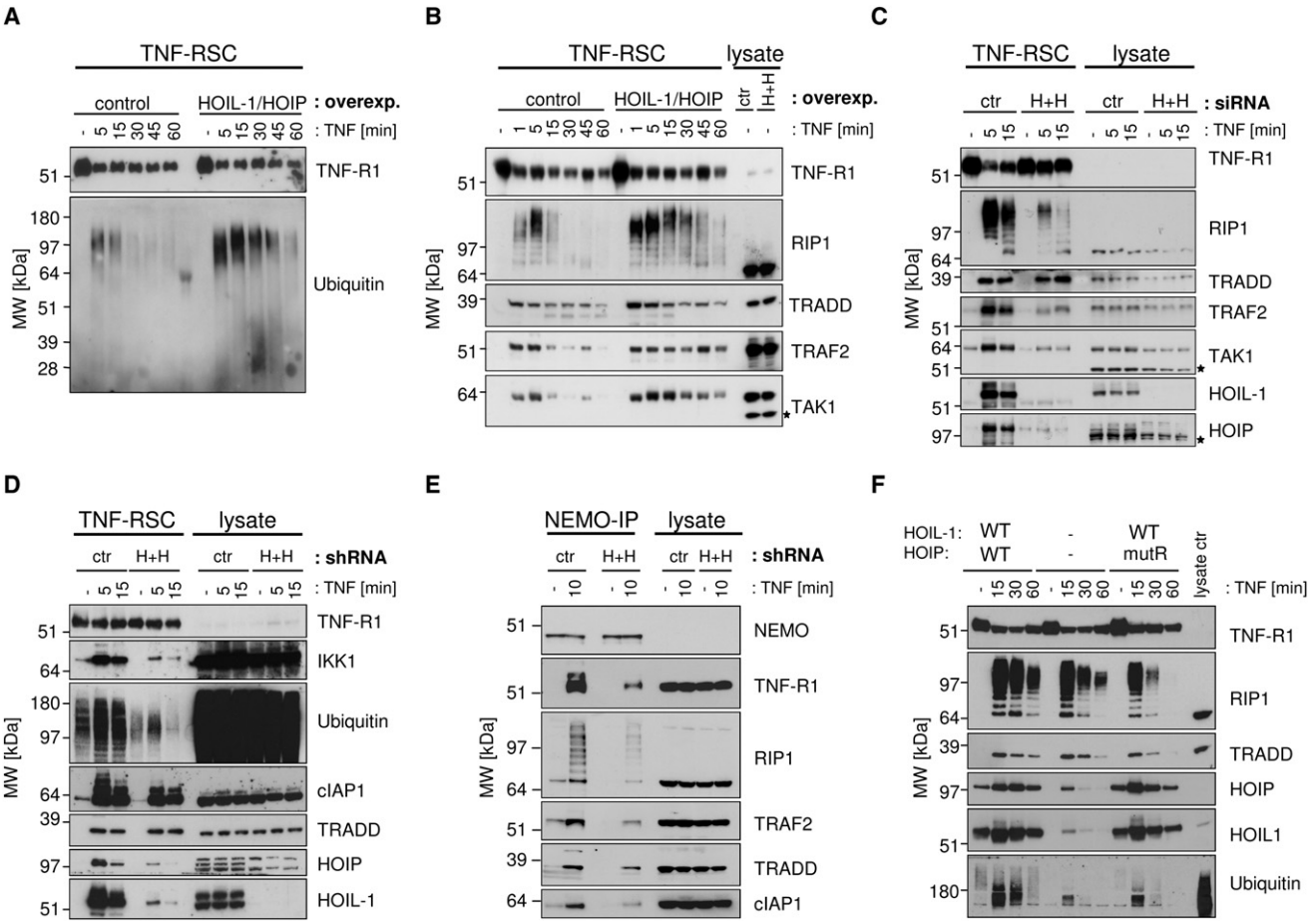


Figure 6. HOIL-1 and HOIP Enhance Ubiquitylation and Stabilization of the TNF-RSC

(A) HOIL-1 and HOIP overexpression increases Ubiquitylation in the native TNF-RSC. HeLa cells were stimulated with 1 μg/ml TNF, and the isolated receptor complexes were analyzed by western blotting.

(B) HOIL-1 and HOIP overexpression stabilizes the TNF-RSC. HeLa cells were treated as in (A), and the TNF-RSC was analyzed by western blotting (*, nonspecific signal).

(C) HOIL-1 and HOIP knockdown destabilizes the TNF-RSC. Expression of HOIL-1 and HOIP in HeLa cells was downregulated by siRNA transfection. Cells were stimulated with 1 μg/ml TNF 96 hr after transfection (*, nonspecific signal with the TAK1 and HOIP antibodies).

(D and E) LUBAC mediates the stimulation-dependent interaction of the IKK complex with the TNF-RSC. Expression of HOIL-1 and HOIP in HeLa cells was downregulated by lentiviral shRNA delivery. Five to seven days after transduction, the cells were stimulated with 1 μg/ml TNF for the times indicated, and the receptor complex (D) or NEMO (E) immunoprecipitates were analyzed.

(F) The E3 function of HOIP is essential for LUBAC-mediated stabilization of the TNF-RSC. HeLa cells or HeLa cells stably overexpressing WT HOIL-1 together with WT HOIP or catalytically inactive HOIP mutR were treated as in (A), and the TNF-RSC was analyzed by western blotting.

Using MEF cell lines deficient for different TNF-RSC components and an IAP antagonist, we showed that LUBAC recruitment is diminished in the absence of TRAF2 and almost undetectable in the absence of cIAP1 and 2. Reconstitution experiments with knockout MEFs suggest that the catalytic activity of the cIAPs, but not of TRAF2, is essential for LUBAC recruitment to the TNF-RSC. These results are consistent with recent work showing that TRAF2 is required to recruit cIAPs to the TNF-RSC in order to activate NF-κB in response to TNF, but that its RING domain is dispensable (Vince et al., 2009; Yin et al., 2009). Although we found that HOIL-1 and HOIP were capable of binding linear and/or K63-linked Ub chains, respectively, the interaction of HOIL-1 with Ub was strongly increased in the presence of HOIP, suggesting that HOIP is the major contributor of LUBAC binding to Ub chains. This suggests a model where HOIL-1 and HOIP are recruited to the TNF-RSC via polyUb chains that are attached to TNF-RSC components by cIAPs. Surprisingly, RIP1, despite being a prominent target of cIAP-mediated polyUb modifications (Bertrand et al., 2008; Mahoney et al., 2008; Varfolomeev et al., 2008; Vince et al., 2007; Wertz et al., 2004), was neither required for HOIL-1 recruitment in MEFs nor essential for it in HeLa cells. Thus, although ubiquitylated RIP1 may be capable of mediating LUBAC recruitment to the TNF-RSC, it is not necessary. This finding is in line with recent work demonstrating that RIP1 is not required in MEFs for TNF-induced activation of NF-κB (Wong et al., 2009).

Furthermore, NEMO is not required for LUBAC recruitment to the TNF-RSC, even though it can bind LUBAC (Tokunaga et al., 2009). Our data rather indicate the converse, because the stimulation-dependent interaction of the IKK complex with the TNF-RSC is reduced in the absence of LUBAC.

Given that LUBAC recruitment to the TNF-RSC is independent of NEMO, our findings shine light on recent evidence that NEMO selectively binds linear polyUb chains via a UBAN (Ub binding in ABIN and NEMO) motif (Komander et al., 2009; Lo et al., 2009; Rahighi et al., 2009). Two groups have shown that NEMO binding to linear Ub chains is important for efficient NF-κB activation (Lo et al., 2009; Rahighi et al., 2009). Our identification of LUBAC as a component of the native TNF-RSC now suggests the following explanation of these findings: NEMO recruitment to the TNF-RSC is significantly enhanced by LUBAC-mediated assembly of linear Ub chains. LUBAC attaches linear chains to NEMO and possibly other TNF-RSC components, thereby stabilizing the entire complex and increasing the retention times of NEMO and other signaling components. NEMO ubiquitylation with linear chains may also invoke a conformational change required to activate the IKK complex (Bloor et al., 2008; Rahighi et al., 2009). As a consequence, the stability of the TNF-RSC and the strength of gene-activatory signaling via NF-κB and JNK, emanating from this protein complex, are increased.

Our data also show that LUBAC, in addition to promoting retention of NEMO in the TNF-RSC, helps retain cIAP1, TAK1, RIP1, and TRAF2 in the TNF-RSC. In this context, it is interesting to note that, in addition to NEMO, other components of the TNF-RSC have been reported to bind to linear Ub chains, including ABINs via their UBAN domain (Komander et al., 2009; Rahighi et al., 2009) and cIAPs via their recently identified UBA domain (Gyrd-Hansen et al., 2008). Because cIAPs can bind linear Ub chains, LUBAC-mediated linear ubiquitylation might help retain cIAPs within the TNF-RSC. TRAF2 and RIP1 are known to bind to cIAPs and therefore might be retained in the TNF-RSC via their interaction with these cIAPs. In this context, it is interesting to note that RIP1 recruitment to the TNF-RSC was unaffected in NEMO-deficient MEFs. Thus, as in many other signaling pathways, there could be an amplifying feedback loop in TNF signaling such that cIAPs are required to recruit LUBAC, and LUBAC activity in turn helps retain cIAPs within the complex, thereby ultimately increasing the activation of NF-κB and JNK. Ubiquitylated RIP1 is important for TNF-induced activation of NF-κB (Devin et al., 2000; Ea et al., 2006; Lee et al., 1997; Yeh et al., 1997), and therefore, reduced levels of RIP1 in the TNF-RSC might contribute to the marked reduction in NF-κB signaling that we observed in HOIL-1/HOIP knockdown cells.

The effect of LUBAC on JNK signaling could be explained in a similar manner. TAK1 is recruited to the TNF-RSC via TAB2 and TAB3, which bind to K63-linked polyUb chains generated by cIAPs and/or TRAF2 and possibly also to linear Ub chains (Tokunaga et al., 2009), although this is still controversial (Iwai and Tokunaga, 2009; Komander et al., 2009). NEMO is also required for effective TAK1-mediated JNK activation following stimulation with LPS, IL-1β, or anti-CD40 (Matsuzawa et al., 2008; Yamamoto et al., 2006). Therefore, quicker loss of TAK1 from the TNF-RSC in HOIL-1/HOIP knockdown cells could be due to the reduced levels of cIAPs and TRAF2, resulting in fewer K63-linked Ub chains. Alternatively or additionally, it could be due to the loss of linear Ub chains in the absence of LUBAC, resulting in reduced retention of NEMO. Besides affecting JNK activation, quicker loss of TAK1 from the TNF-RSC would also impact NF-κB signal strength (Chen et al., 2006; Sato et al., 2005; Shim et al., 2005; Wang et al., 2001).

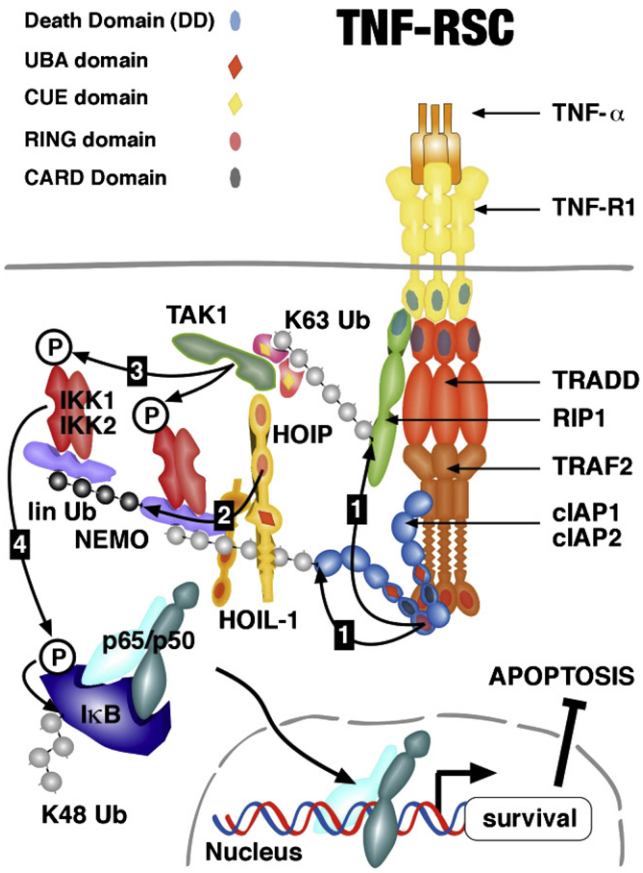


Figure 7. A Schematic of a Working Model for Recruitment of LUBAC into the TNF-RSC

Following crosslinking of TNF-R1 by TNF, TRADD and RIP1 are recruited to the death domain of TNF-R1. TRADD then facilitates recruitment of TRAF2, which in turn provides a binding and activation platform for cIAP1 and 2. cIAPs ubiquitylate components of the TNF-RSC, including RIP1 and the cIAPs themselves. These cIAP-generated polyUb chains serve as recruitment platforms for LUBAC as well as the TAK/TAB and NEMO/IKK complexes. Once recruited, LUBAC is able to linearly ubiquitylate NEMO and thereby facilitate the recruitment of additional NEMO/IKK complexes.

Based on our findings and other recent reports, we propose a working model for activation of signaling by the TNF-R1-associated protein complex (Figure 7), whereby cIAPs are recruited to the TNF-R1 complex via TRADD and TRAF2. cIAPs ubiquitylate several components in the complex, including RIP1 and themselves. cIAP-generated polyUb chains then allow the recruitment of LUBAC. LUBAC adds linear polyUb chains to NEMO and possibly other substrates in the complex, serving as an optimized scaffold to recruit more NEMO and potentially other components of the TNF-RSC. There has recently been a dramatic increase in the number of proteins that have been shown to bind

linear Ub, some with remarkable specificity (Komander et al., 2009; Rahighi et al., 2009). Several of these proteins have been known for some time to be recruited to the TNF-RSC. It is therefore likely that their ability to interact with linear Ub chains will play a role in stabilizing the TNF-RSC, resulting in greatly enhanced TNF-induced gene induction and prevention of apoptosis.

The identification of HOIL-1 and HOIP as functional constituents of the TNF-RSC provides evidence that LUBAC is an important regulator at the apex of TNF-induced signaling cascades. The recruitment of LUBAC not only increases the number of E3s directly associated with the TNF-RSC but also adds a third form of Ub linkage and Ub regulation to TNF signaling, thereby increasing the combinatorial complexity of Ub modifications within this receptor complex. Since Ub-mediated events are essential for signaling induced by antigen receptors, other TNF-R superfamily members, IL-1 receptor (IL-1R)/Toll-like receptor (TLR) 4, and NOD1 and NOD2 (Bertrand et al., 2008; Chen, 2005; Hitotsumatsu et al., 2008), it is possible that LUBAC also plays a role in the signal transduction triggered by other receptor-ligand systems.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins

The cDNA encoding human full-length TNF was a gift from Martin R. Sprick. The extracellular portion of TNF was cloned into a pQE32 expression vector (QIAGEN; Hilden, Germany) in which the NH_2-terminal moTAP tag, consisting of a 3× FLAG epitope, a PreScission cleavage site (LEVLFQ/GP), and the minimal peptide recognized by BirA (MAGGLNDIFEAQKEWHE) (Beckett et al., 1999), was integrated by oligonucleotide cloning. moTAP-TNF was expressed for 16 hr at 18°C using M15 bacteria (QIAGEN) and purified via standard Ni-NTA chromatography according to the manufacturer's protocol. After purification, protein integrity was determined by SDS-PAGE and Coomassie staining; biological activity of purified moTAP-TNF was compared to untagged TNF. Purified moTAP-TNF was biotinylated in vitro using recombinant BirA as described (Beckett et al., 1999). Biotinylation efficiency was determined using the HABA reagent (Pierce/Thermo Fisher; Waltham, MA) according to the manufacturer's protocol. His-FLAG-TNF (HF-TNF) was generated as described before (Diessenbacher et al., 2008). Unless stated otherwise, the TNF used in this study is HF-TNF. Recombinant GST-tagged HOIL-1 and HOIP N-term (residues 1–485) were expressed from a pET41a vector (Novagen, Merck; Nottingham, UK) in *E. coli* BL21(DE3) pLysS (Novagen, Merck) at 18°C overnight. The proteins were purified with GSTrap columns according to the manufacturer's protocol (GE Healthcare), and quality was controlled by SDS-PAGE and Coomassie staining.

Analytical Precipitation of the TNF-RSC

For analytical TNF-RSC preparation, 2×10^7 HeLa or MEF or 2×10^8 U937 cells were treated in the presence or absence of TNF at 1 μg/ml for the indicated times. Then cells were lysed in IP-lysis buffer (30 mM Tris-HCl [pH 7.4], 120 mM NaCl, 2 mM EDTA, 2 mM KCl, 1% Triton X-100, 1× COMPLETE protease-inhibitor cocktail) at 4°C for 30 min. The lysates were centrifuged at 15,000 × *g* for 30 min. TNF (1 μg) was added to the non-stimulated control, and the TNF-RSC was precipitated using M2 beads (SIGMA; Schnelldorf, Germany) for 16 hr. The beads were washed five times with 1 ml IP-lysis buffer and eluted with 2× LDS buffer (NuPAGE, Invitrogen; Carlsbad, CA). Proteins were separated by SDS-PAGE (NuPAGE) and analyzed by western blotting. Membranes were stripped with 50 mM glycine (pH 2.3) and reprobed with other antibodies.

Preparative moTAP of the TNF-RSC

U937 cells (5×10^9) were stimulated with 50 μg moTAP-TNF in 50 ml RPMI at 37°C for 5 min. The cells were washed twice in 100 ml ice-cold PBS and lysed in 1 ml IP-lysis buffer/10^8 cells at 4°C for 30 min. Benzonase (10 U/ml) (Novagen, Merck) was added to each sample, and the first IP was carried out using 5 μl M2 beads/10^8 cells for 10 hr. Beads were washed five times with 1 ml IP-lysis buffer without protease inhibitors. Lysis buffer (500 μl) containing 5 U/ml PreScission (GE Healthcare) and 50 μg/ml FLAG-peptide (SIGMA) was added for elution. Protein complexes were eluted from the beads for 12 hr, and the beads were rinsed once with 500 μl IP-lysis buffer. The second precipitation step was carried out using 25 μl streptavidin-coated beads (GE Healthcare) at 4°C for 6 hr. The beads were again washed, and bound proteins were eluted using 2× LDS buffer. The purified TNF-RSCs of two moTAP experiments were pooled and run on a NuPAGE gel. The lane of the gel containing the components of the TNF-RSC was cut into 20 pieces of equal size without prior staining. The proteins in the gel slices were subjected to tryptic digest and subsequent tandem mass spectrometric analysis.

Cell Lines

WT and knockout MEFs were generated from E15 embryos in accordance with standard procedures and were infected with SV40 large T antigen-expressing lentivirus. TRADD- and NEMO-deficient MEF cells were described previously (Ermolaeva et al., 2008; Schmidt-Supprian et al., 2000). HEK293-NF-κB cells were purchased from Panomics (Fremont, CA). MCF-7, HeLa, MEF, HEK293T, and HEK293-NF-κB cells were maintained in DMEM supplemented with 10% FBS and 10 mM sodium pyruvate. U937 and THP-1 cells were maintained in RPMI supplemented with 10% FBS.

Inducible Protein Expression

cIAP1/2-deficient MEFs were infected using an inducible lentiviral system as described before (Mace et al., 2008) to generate stable cell lines expressing WT mouse cIAP1 and F610A mouse cIAP1 mutant under the control of 4-HT. Expression of WT and F610A mouse cIAP1 was induced by the addition of 4-HT (20 nM) for 20 hr. Induction of WT mouse TRAF2 or mouse TRAF2 ΔRING in TRAF2/5-deficient MEFs was performed as described for cIAP1.

SUPPLEMENTAL DATA

Supplemental Data include one table and seven figures and can be found online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00778-3.

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