Ubiquitin chain formation by RBR E3-ligases

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List of abbreviations

Å	Angstrom (1 Angstrom = 0.1 nm)
AML	Acute myeloid leukemia
ATRA	All-trans retinoic acid
ATP	Adenosine triphosphate
βME	β-mercapto-ethanol
Cbl	Casitas B-cell lymphoma protein
Da	Dalton
DNA	Deoxyribonucleic acid
DRIL	Double-RING finger linked domain
DUB	De-ubiquitination enzyme
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
FACS	Fluorescence-activated cell sorting
FRET	Fluorescence resonance energy transfer
Gfi1	Growth factor independence 1
GFP	Green fluorescent protein
GST	Glutathione s-transferase
HECT	Homologous to the E6-AP carboxyl terminus
HHARI	Human homologue of Drosophila ariadne
HOIL-1L	Heme-oxidized IRP2 ubiquitin ligase-1
HOIP	HOIL-1L interacting protein
IBR	In-between RING domain
K_D	Dissociation constant
LDD	Linear ubiquitin chain determining domain
LUBAC	Linear ubiquitin chain assembly complex
MACS	Magnetic-activated cell sorting
MALLS	Multi-angle laser light scattering
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NMR	Nuclear magnetic resonance
NZF	NPL4 zinc-finger
NPL4	Nuclear protein localization 4
PD	Parkinson disease
PMA	Phorbol-12-myristate-13-acetate
RBR	RING-in-between-RING
RING	Really interesting new gene
RNF	Ring finger
SAXS	Small angle X-ray scattering

SCF	Skp1-Cul1-F-box-protein
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
TAMRA	Carboxytetramethylrhodamine
TRIAD	Two RING fingers and DRIL
UBA	Ubiquitin-associated domain
UBAN	Ubiquitin-binding domain in ABIN and NEMO
UBC	Ubiquitin-conjugating enzyme
UBL	Ubiquitin-like domain
UBZ	Ubiquitin-binding zinc-fingers
UIM	Ubiquitin-interaction motifs
WT	Wild type

Introduction

Posttranslational modifications

The activity of proteins is highly regulated to enable cells to respond accurately and rapidly to environmental and intracellular signals. The required dynamic responses of signalling pathways to stimuli are not regulated at the transcriptional level, but are mediated by a highly controlled system that activates, inactivates or even degrades the right proteins at the right time. This rapid modulation of protein activity is mediated by the attachment and removal of various types of signals to surface exposed amino acids[1]. These posttranslational modifications form a code on the protein surface that is read-out by recognition motifs within the same protein or other proteins, which leads to the required changes in constituents of molecular complexes and protein activity[2].

Many different types of posttranslational modifications are known, including phosphorylation, glycosylation, acetylation, methylation, hydroxylation and ubiquitination[3]. Ubiquitination forms one of the most versatile posttranslational modifications, since this small protein is used to signal on its own, as well as in a variety of different types of ubiquitin chains that function in many different cellular processes.

Ubiquitination

Ubiquitin is a 76 amino acid protein that forms a compact structure with a β-grasp fold and a flexible six-residue C-terminal tail (Figure 1)[4]. It is attached to its substrates by the formation of an isopeptide bond between its C-terminus and a target residue. In the early 1980's, ubiquitin was first linked to the proteasomal degradation of proteins[5]. However, now it is recognized that ubiquitin has many different signalling functions in cells[6,7]. It forms an inducible and reversible modification on proteins that leads to degradation, altered intracellular localization or changed interactions between proteins. Consequently, the misregulation of the ubiquitin signalling is associated with the initiation and progression of various human diseases including inflammation and cancer[8].

The important role of the ubiquitin system in many cellular pathways and the growing understanding of its regulation have lead to the development of anti-cancer therapies that target the ubiquitin system[9]. The most prominent clinical success is the proteasome inhibitor Bortezomib (Velcade®), which received FDA approval in 2003. By binding the proteasome active site with high affinity and specificity, it is successful in the treatment of multiple myeloma and mantle-cell lymphoma. In addition, to the development of general proteasome inhibitors, the increased understanding of the ubiquitination machinery has opened up possibilities for the development of more selective therapeutic interventions[10], as will be discussed below.

Ubiquitin signalling

Ubiquitin machinery

The ubiquitination of proteins is mediated by a dynamic system that regulates the specific assembly and disassembly of ubiquitin signals on targets. The formation of the ubiquitin signals is mediated by a cascade of E1, E2, and E3 enzymes[11-13]. First, an E1 enzyme activates a

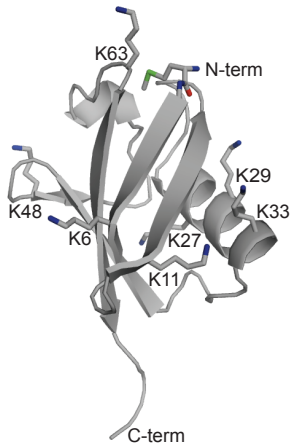

Figure 1. Crystal structure of ubiquitin The amine-groups of the seven lysines and the N-terminal amine-group that can be used in ubiquitin chain formation are shown as sticks.

donor ubiquitin in an ATP-dependent manner and forms a thioester bond with the ubiquitin C-terminal carboxyl group. Next, the thioester bond is transferred onto the active-site cysteine of an E2 (E2~ubiquitin), which interacts with E3-ligases to facilitate the final conjugation of the ubiquitin onto its target (Figure 2). The two main classes of E3-ligases mediate the formation of ubiquitin chains via different mechanisms. HECT E3-ligases take the ubiquitin from the E2 to form a thioester intermediate on an active-site cysteine before it is transferred onto its target. In contrast, RING E3-ligases facilitate the direct transfer of the ubiquitin from the E2 onto its target by bringing the E2~ubiquitin and the substrate together. Thus, HECT E3-ligases solitarily transfer the ubiquitin onto its target, while the ubiquitination of targets by RING E3-ligases is the result of the combined action of the E2 and the RING E3-ligase.

The vast majority of E3-ligases belong to the group of RING E3-ligases and RING-related E3s, such as the TRIM proteins with a RING-like B-box, members of the U-box family, and the PIAS proteins[14]. All these proteins are suggested to function via the classical RING E3-ligase mechanism, by bringing the substrate and the E2~ubiquitin in close proximity to mediate the direct transfer of the ubiquitin onto the substrate. Interestingly, the recently identified RING-subfamily of RING-in-between-RING (RBR) E3-ligases, was shown to function via a different mechanism (Chapter 4 of this thesis)[15-17]. The highly conserved catalytic unit of RBR E3-ligases contains two RING domains and an in-between RING (IBR) domain (Figure 3), which supposedly function via a combined RING/HECT-type mechanism.

In the past two years, the molecular mechanism underlying ubiquitin chain formation activity of RBR E3-ligases has been illustrated by studies on Parkin, HHARI, and HOIP (Chapter 4 of this thesis)[15-17]. In these E3-ligases the ubiquitin transfer is initiated by a classical RING-E2 interaction between the RING1 domain of the RBR and an E2~ubiquitin. In contrast to classical

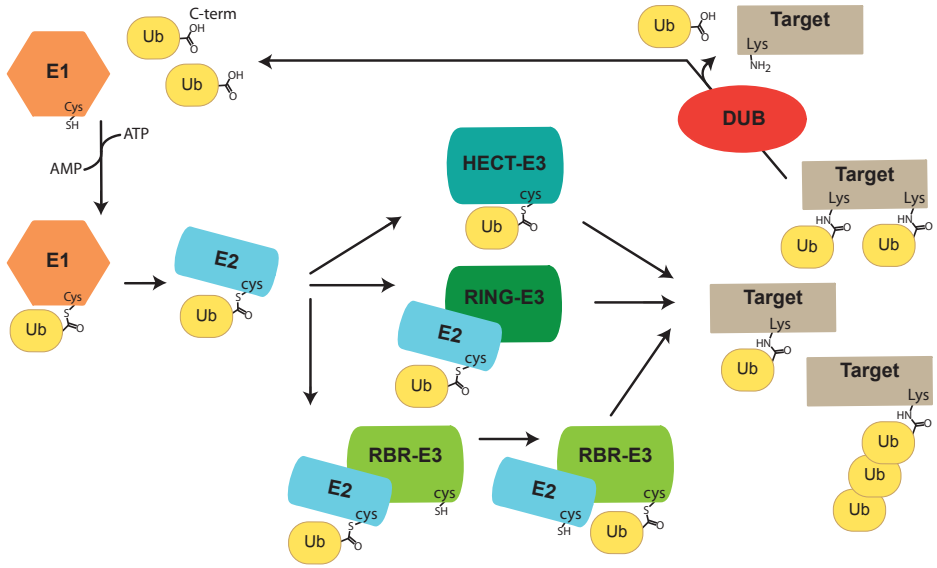

Figure 2. The ubiquitin system The ubiquitin (Ub) C-terminus is activated in an ATP-dependent manner by an E1 activating enzyme and is subsequently transferred to form a thioester intermediate on an E2 conjugase. The final transfer of ubiquitin onto its target is mediated by E3-ligases that form a thioester intermediate with the ubiquitin (HECT E3-ligases), mediate a direct transfer of the ubiquitin from the E2 onto its target (RING E3-ligases), or function as RING/HECT-type hybrids (RBR E3-ligases). Via this cascade of E1, E2 and E3 enzymes the ubiquitination machinery mediates the formation of mono-ubiquitination, multi-mono-ubiquitination, or ubiquitin chain formation on its targets. The ubiquitin signal can be removed by de-ubiquitination enzymes (DUBs).

RING E3-ligases, this interaction facilitates the formation of a HECT-type thioester intermediate on an active-site cysteine on RING2 before it is coupled to its substrate. Since multiple RBR E3-ligases have been shown to function via this combined RING/HECT-type mechanism, it is likely that it reflects a general mechanism for the functioning of the RBR class of E3-ligases.

The ubiquitination of proteins by the different mechanism of the ubiquitination machinery is counteracted by de-ubiquitination enzymes (DUBs), which selectively remove ubiquitin from its targets. More than 90 different DUBs have been identified to contribute to the regulation of the ubiquitination signal[20]. Consequently, the posttranslational modification of proteins with ubiquitin is a dynamic and tightly regulated process.

Ubiquitin signals

Ubiquitin is a versatile posttranslational modification that is used to form various signals on target proteins. It is attached as a single ubiquitin onto specific lysines on targets, which is referred to as mono-ubiquitination, but it is also used to form chains of ubiquitins on target proteins. The ubiquitin chains are formed by the attachment of the C-terminus of a donor ubiquitin to any

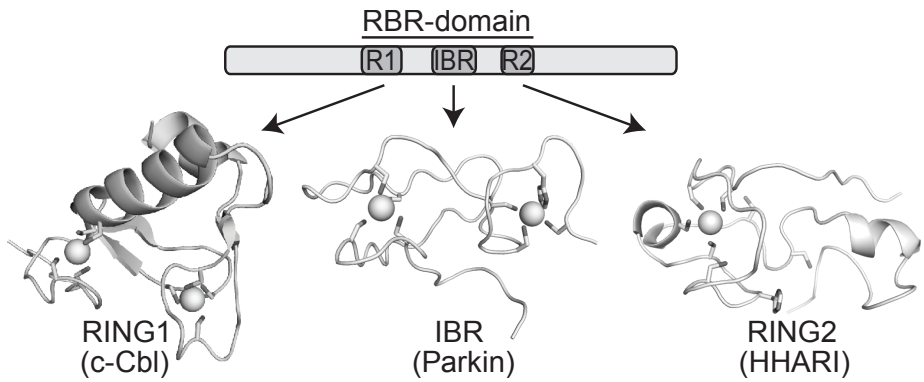

Figure 3. RBR E3-ligases contain two RING domains (R1 and R2) and an in-between RING (IBR) The crystal structure of the RING1 domain of the RBR-protein c-CBL (1wim) shows that it forms a classical RING-fold that coordinates two zinc ions (shown as spheres) by 8 cysteines (shown as sticks). Parkin coordinates 2 zinc ions by 7 cysteines and a histidine as shown in the solution structure of the Parkin IBR (2jmo)[18]. The RING2 domain in the solution structure of HHARI RING2 (1wd2) uses only 4 cysteines of its RING-motif to coordinate one zinc ion[19].

of the seven lysine residues, or the N-terminal amine-group of a target ubiquitin (Figure 1)[21]. Frequently, the ubiquitins in a chain are coupled via a specific ubiquitin residue to form K6, 11, 27, 29, 33, 48, 63, or "linear" M1-linked ubiquitin chains, which can all be found in cells[22-24]. However, also mixed chains with varying linkages within a single chain have been observed[25].

The ubiquitination patterns in cells are highly regulated and form cell-specific patterns that rely on the different stages of the cell cycle[22, 24, 26]. The separate ubiquitin signals influence cellular processes in different ways, because the structural features of the different ubiquitination events are selectively recognized by DUBs, and proteins with ubiquitin-interaction motifs (UIMs), nuclear protein localization 4 (NPL4) zinc fingers (NZFs), ubiquitin-binding zinc-fingers (UBZs), or other ubiquitin binding domains (UBDs), to change the cellular fate of the targeted proteins[20, 21, 27].

Mono-ubiquitin

The mono-ubiquitination of proteins signals to selectively recruit effector-proteins that recognize the ubiquitin in combination with its target. Therefore, the mono-ubiquitination of proteins is usually targeted towards a specific lysine or domain on a target[28, 29]. In addition to the attachment of a single ubiquitin, a protein can also be modified with multiple mono-ubiquitins that form one big signal for the recruitment of down-stream effectors[30].

The UBDs in effector-proteins interact with ubiquitin by making use of the slight flexibility in the ubiquitin β1/ β2-loop[31, 32]. Furthermore, they frequently recognize either of two hydrophobic patches on ubiquitin (Figure 4A). The hydrophobic surface that consists of Ile44, Leu8, Val70, and His68, is recognized by many UBDs and the proteasome (reviewed by Dikic et al.[33]). And

the ubiquitin hydrophobic patch, consisting of Ile36, Leu71, and Leu73, is recognised by UBDs[34], DUBs[35] and HECT E3-ligases[36]. In addition, binding proteins may recognize several other sites on ubiquitin, including a patch around Phe4[35, 37], the Thr12, Thr14, Glu34, Lys6, and Lys11 containing TEK-box[38], and possibly the negative charged residues Glu16 and Glu18 on the top of ubiquitin (Chapter 5 of this thesis). The recognition of these specific patches on ubiquitin by effector-proteins in combination with their binding to specific sites on the target protein, selectively determines the cellular fate of the mono-ubiquitinated proteins.

Depending on the proteins that recognize the ubiquitinated target, mono-ubiquitination can induce a variety of events. It can change the composition of functional complexes, such as the DNA replication-complex at sites of DNA damage. Upon DNA damage, the PCNA DNA sliding-clamp is mono-ubiquitinated and recruits the Y-family DNA polymerases[28, 39]. The combined recognition of PCNA and the ubiquitin by these polymerases causes a high-affinity interaction that facilitates a polymerase-switch that rescues stalled replication forks from collapsing[40, 41]. Furthermore, the mono-ubiquitination of targets can induce the translocation of the targeted proteins, such as the translocation of membrane-proteins to lysosomes via their mono-ubiquitin induced endocytosis[42], and the nuclear export of p53 upon its multi-mono-ubiquitination[43]. Even the in-frame fusion of a ubiquitin to a substrate can be sufficient for its translocation[29, 44]. Finally, the mono-ubiquitination of proteins can disrupt interactions between proteins, such as the interaction between Smad4 and the transcriptional cofactor Smad2[45]. Thus, the mono-ubiquitination of proteins signals to a variety of down-stream events.

Ubiquitin chains

Ubiquitin chains form more general signals on a protein than mono-ubiquitin, since ubiquitin chains can be recognized by effector-proteins, independent of the target to which they are attached. Different ubiquitin chains specifically recruit different binding partners, since the structural features of ubiquitin chains that are linked via different residues vary tremendously (Figure 4B and C)[21]. Out of the eight different ubiquitin chain types that can be formed, ubiquitin K48, K6, K11, K63, and linear chains are structurally and functionally best characterized.

Compact ubiquitin chains

Ubiquitin K6 and K48 linked ubiquitin chains form compact structures, as has been shown by crystallography, small angle X-ray scattering (SAXS), NMR and single-molecule fluorescence resonance energy transfer (FRET) (Figure 4B)[46-50]. The ubiquitin Ile44 hydrophobic patch is used for the interactions between the ubiquitins in the tightly packed structure, making it inaccessible for ubiquitin interaction motifs. However, the packing of the ubiquitins in K48-linked chains is proposed to be dynamic under physiological conditions, alternating between the compact conformation and a slightly more 'open' conformation that makes the ubiquitin surfaces available for its recognition by binding proteins[46, 47, 49, 51, 52].

The function and recognition by effector-proteins of K6-chains has not been extensively studied. The chains seem to function in proteasome independent pathways and might play a role in

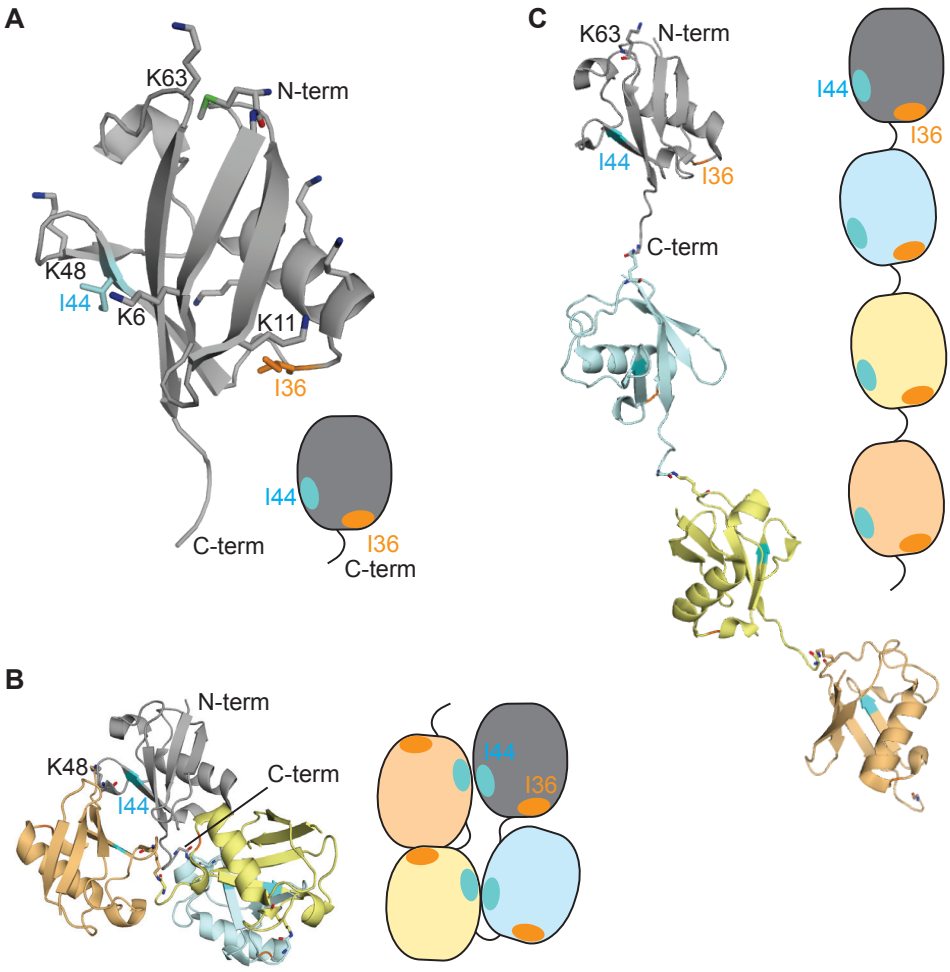

Figure 4. Ubiquitin signals (**A**) Crystal structure of mono-ubiquitin illustrating the position of Ile44 (blue) and Ile36 (orange). All lysines and the ubiquitin N-terminus are shown as sticks. In addition, the position of the hydrophobic patches is illustrated in a cartoon of ubiquitin. (**B**) Crystal structure of the compact conformation of a K48-linked tetra-ubiquitin chain (cyclic tetra ubiquitin, PDB: 3alb). (**C**) Crystal structure of a K63-linked ubiquitin chain (PDB: 3hm3), showing the extended conformation of these chains.

the DNA damage response, but further studies are needed to identify the exact nature of their signalling[53-56].

Many UBA domain-containing proteins bind with strong preference to K48-linked ubiquitin chains[57]. The binding of K48 chains by UBA domains is highly selective, because a single UBA molecule recognizes up to four ubiquitins within a K48-chain[58]. However, the chains are also recognized by proteins with multiple UIMs that are linked via linkers with a specific length that allow the binding of the UIMs to the packed K48-linked ubiquitin chain structure[59-61].

In this way, K48-linked ubiquitin chains are recognized by shuttle factors that deliver the targeted proteins to the 26S proteasome that recognizes the chains via its S5a/Rpn10 and Rpn13 subunits. Consequently, K48-linked ubiquitin chains mainly target their substrates for proteasomal degradation[5, 62, 63]. However, proteasomal targeting does not always lead to the complete degradation of proteins, as is seen for the NF-κB precursor, which is activated by the proteasome through the specific proteasomal cleavage of its inhibitory domains[64].

Like K48-linked ubiquitin chains, K11-linked ubiquitin chains form compact structures. However these chains are more flexible than K48-linked chains and have a solvent exposed Ile44 hydrophobic patch[26, 65, 66]. Nevertheless, K11-linked ubiquitin chains also induce the translocation of their substrates to the 26S proteasome[26, 38].

Extended ubiquitin chains

K63-linked and linear ubiquitin chains form extended structures in which the C-terminus at the bottom of the ubiquitin structure is attached to K63 or M1 that are positioned at the top of ubiquitin (Figure 4C). These ubiquitin chains do not form rigid structures, and can adopt extended 'open' structures with no interaction between the ubiquitin moieties in the chain[47-49, 52, 67], or more compact 'closed' conformations[49, 68]. These pre-existing conformations of K63 and linear ubiquitin chains are selectively recognized by ubiquitin-binding proteins and DUBs[49], which exploit the distance and flexibility of the chains for recognition[69]. Interestingly, even though K63-linked and linear ubiquitin chains are structurally similar, they have distinct signalling functions, since UBDs can distinguish between these chains[70-72].

K63-linked ubiquitin chains have various signalling functions in different pathways. They are recognized by the UIMs of Rap80 for the recruitment of proteins to DNA damage foci[73], and by the UIMs of the clathrin associated sorting protein Epsin1[74], which selectively interact with the specifically spaced Ile44 hydrophobic patches of the ubiquitins in K63-linked ubiquitin chains. Furthermore, the K63-linked ubiquitin chains induce the endocytosis and lysosomal degradation of proteins at the plasma-membrane via the recruitment of ESCRT-complexes[42, 75, 76], and they are essential for the activation of the NF-κB pathway where they are recognized by the TAB2 and TAB3 subunits of TAK1 to activate the kinase complex[77, 78].

Like K63-linked ubiquitin chains, linear ubiquitin chains have signalling functions that are crucial for the activation of the NF-κB pathway. However, linear ubiquitin chains are recognized by different UBDs in the NF-κB pathway than K63-linked ubiquitin chains, therefore they perform independent signalling functions. The NZF domain of HOIL-1L and the coiled-coil UBAN domain of NEMO specifically interact with the correctly spaced ubiquitins in linear chains to recruit more of these proteins to the receptor complexes and enhance the NF-κB signalling[37, 79, 80]. Furthermore, the chains are recognized by Traf3, which disrupts the MAVS-TRAF3 complex and prolongs the IFN induced NF-κB signalling[81]. The importance of these interactions with linear ubiquitin chains is illustrated by the fact that mutations of residues in NEMO that are involved in the interaction with linear ubiquitin chains are found in patients with X-linked ectodermal dysplasia and immunodeficiency[37].

Clinical implications for ubiquitin recognition

The various ubiquitin signals that are attached to target proteins signal for an enormous variety of down-stream effects, because the separate signals are selectively recognized by effector-proteins. Even ubiquitin chains that have similar structures are highly selectively recognized by UBDs that sense the precise spacing and orientation of the ubiquitin recognition patches of the ubiquitins in the chains. Consequently, multiple ubiquitin signals are used side-by-side in the same pathway to tightly regulate cellular functioning.

The insight in the conformation, function, and specific recognition of ubiquitin chains that has been generated in the past years has encouraged the development of compounds that possibly interfere with these interactions. As a result, the first molecules (Ubistatins) that can inhibit the binding of UBDs to ubiquitin have been developed, however since the first generation of these molecules cannot penetrate membranes, further research is needed to explore their application in molecular medicine[82].

Ubiquitin signalling in the NF-κB pathway

The nuclear factor-kappa B (NF-κB) pathway is one of the best-studied signalling pathways in which the ubiquitin system is involved with a variety of different ubiquitin chains[83, 84]. The pathway activates NF-κB transcription factors that belong to the Rel family, and was first described in 1986[85]. The NF-κB transcription factors are retained in the cytoplasm in resting cells by inhibitory proteins (inhibitors of NF-κB (IκBs)), and are released to translocate to the nucleus upon the activation of the pathway. The NF-κB pathway is activated by multiple stimuli, including cytokines, genotoxic agents, oxidative stress and pathogens, to induce the expression of numerous genes involved in innate and adaptive immune regulation, cell adhesion, osteogenesis, inflammatory responses, and anti-apoptosis (reviewed[86, 87]). The NF-κB transcription factors are activated via two different pathways that are involved in different biological processes: The canonical and the non-canonical pathway, which both are heavily regulated by posttranslational modifications, including ubiquitination (Figure 5)[86, 87].

The non-canonical NF-κB pathway

The non-canonical pathway is activated by TNF-receptor ligands like B-cell activating factor (BAFF), CD40 ligand, and lymphotoxin-β (LF-B) to induce B-lymphocyte survival and lymphoid organogenesis. The activation of the non-canonical pathway stabilizes the NF-κB-inducing kinase (NIK) via phosphorylation, to prevent its K48-linked ubiquitination and subsequent degradation (Figure 5). The stabilized NIK phosphorylates and activates homo-dimeric IKKα, which subsequently phosphorylates the NF-κB protein p100 to induce its ubiquitin and proteasome dependent processing to p52. Finally, the p52 protein forms a heterodimer with RelB that translocates to the nucleus to induce gene transcription. Thus, both phosphorylation and K48-linked ubiquitin chain formation that target proteins to the proteasome are essential for the activation of the non-canonical NF-κB pathway.

The canonical NF-κB pathway

The canonical NF-κB pathway is activated upon the receptor binding of proinflammatory factors, such as the cytokines TNFα and IL-β, Toll-like receptor ligands, and pathogen associated molecular patterns (PAMPs) like bacterial lipoprotein and lipopolysaccharide. The activation of the canonical pathway is involved in anti-apoptosis, immune and inflammatory responses, and leads to a more rapid activation of the NF-κB proteins than the non-canonical pathway. The activation of the receptors of the canonical NF-κB pathway induces the activation of the IKK (IκB kinase) complex, which consists of IKKα, IKKβ, and NF-κB essential modulator (NEMO, also called IKKγ), by phosphorylating IKKβ (Figure 5). The activated IKK complex then phosphorylates IκBα, which is subsequently ubiquitinated and degraded by the proteasome. The degradation of IκBα releases a heterodimer of NF-κB proteins p50 and p65/RelA to translocate to the nucleus and induce target gene transcription. Thus, also the canonical NF-κB pathway is dependent on phosphorylation and proteasomal targeting of proteins by ubiquitination.

In addition to signal for proteasomal targeting, the ubiquitin system is essential for the early steps in the activation of the canonical NF-κB pathway. Especially the activation of the pathway by TNFα has been extensively studied (Figure 6). TNFα is a critical proinflammatory cytokine for the activation of the canonical NF-κB pathway to induce cell survival. It binds to TNF receptor 1 and activates the TNF receptor signaling complex (TNF-R1), composed of receptor interacting kinase 1 (RIP1), TNF-R1-associated death domain (TRADD), TNF receptor-associated factor 2 (TRAF2), and cellular inhibitors of apoptosis protein 1 and 2 (c-IAP1/2)[88]. The c-IAPs and TRAF2 provide the first ubiquitination signals in the pathway upon receptor activation. In cooperation with Ube2N/Ube2V2 and Ube2D3, c-IAP1/2 and TRAF2 mediate the formation of K63 and K11-linked ubiquitin chains on RIP1[89, 90]. The ubiquitinated RIP1 recruits kinase-ubiquitin adaptor complexes, such as the TAK1 (TGFβ-activated kinase 1)-TAB1-TAB2/3 complex and the IKK complex that interact with the K63-linked chains via TAB2/3 and NEMO, respectively[91-93]. NEMO is ubiquitinated at the TNF-R1 with K63-linked ubiquitin chains by c-IAPs and TRAF proteins, which forms one of the requirements for the IKK-induced phosphorylation of IKKβ and subsequent NF-κB activation[94].

The E3-ligase activity of the TRADD, TRAF2 and the cIAPs also recruits the linear ubiquitin chain formation complex (LUBAC) to the TNF-R1 complex in an RIP1 and NEMO independent manner[95]. The LUBAC complex is an E3-ligase complex that contains at least three different proteins: Sharpin, HOIP and HOIL-1L[96-99]. The recruitment of LUBAC is essential for the activation of the NF-κB pathway, since the knock-down of this complex in cells inactivates the NF-κB signalling pathway and induces caspase 3 dependent apoptosis[98, 99]. At the receptor complex, LUBAC interacts via the ZF domains of HOIP and HOIL-1L with NEMO[99], and ubiquitinates NEMO and to a lesser extent RIP1 with linear ubiquitin chains to increase the efficiency of the phosphorylation of IKKβ[95, 96, 99].

In the past years the importance of the various ubiquitin chains in the activation of the canonical NF-κB pathway has become obvious; however the precise regulation of the different ubiquitin

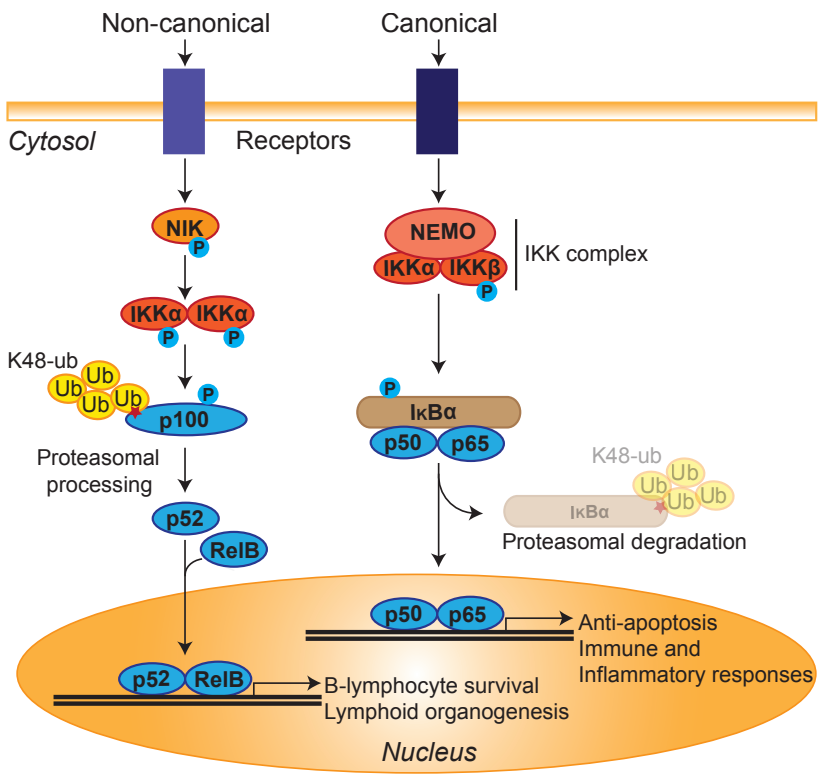

Figure 5. The non-canonical and canonical NF-κB pathways The activation of the non-canonical pathway induces the phosphorylation of NIK, which subsequently phosphorylates the IKKα homodimer. The activated IKKα causes the phosphorylation and subsequent ubiquitination of p100 to target it for proteasomal processing into p52 that forms a complex with RelB to induce gene expression. In contrast, the activation of the canonical pathway induces the phosphorylation of IKKβ in the IKK complex. Subsequently, IκBα is phosphorylated and targeted for proteasomal degradation to release p50 and p65 to translocate to the nucleus and induce gene expression.

signals remains to be revealed. Also, the mechanism by which the recruitment of NEMO to linear and K63-linked ubiquitin chains and its subsequent modification with these chains induces the phosphorylation of IKKβ is unknown. The chains may induce a conformational change in the IKK complex, or simply serve as recruitment platforms for many IKK complexes that activate each other via trans-autophosphorylation activity by the IKKβ subunits[100]. Further analysis is needed to clarify the balance between the different ubiquitination events and their distinct roles in the NF-κB activation.

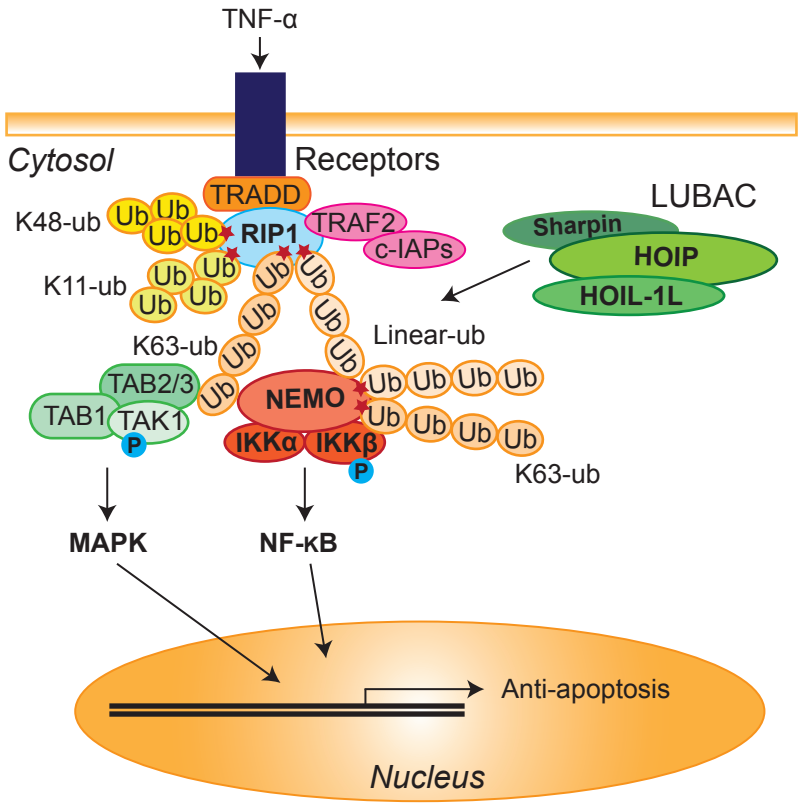

Figure 6. The activation of the canonical NF-κB pathway by TNFα is heavily regulated by the ubiquitin system The activation of the receptor induces multiple ubiquitination-events on RIP1 by the E3-ligases TRAF2 and the c-IAPs. The ubiquitin chains form a recruitment signal for TAK1 and NEMO. In addition, LUBAC is recruited to the receptor-complex to modify Rip1 and NEMO with linear ubiquitin chains. Al these ubiquitination events are critical for the correct activation of the pathway.

The ubiquitin signaling in the NF-κB is counteracted by DUBs that down-regulate the activation of the pathway[101, 102]. The DUB activity of CYLD is essential for its inhibitory role in the NF-κB pathway and may be important for the cleavage of both the K63-linked and linear ubiquitin chains, and Cezanne and USP21 remove ubiquitin chains from RIP1. Furthermore, A20, which is induced upon the activation of the pathway, forms a negative feedback-loop by inhibiting NF-κB activation in several ways[103]. The protein is likely to be recruited to the TNF-R1 complex by specifically binding to linear ubiquitin chains and thereby suppresses the formation of NEMO/LUBAC complexes that are required for NF-κB activation[104, 105]. In addition, A20 deubiquitinates K63-linked ubiquitin chains from RIP1 at the TNF-R1, and processes K48-linked ubiquitin chain formation activity in complex with the E3-ligases RNF11 and Itch that targets RIP1 for proteasomal degradation[106-108]. Finally, A20 disassembles E2-E3 complexes with TRAF proteins

and c-IAPs, and induced the proteasomal degradation of Ube2D3 and Ube2N[109]. Consequently, the activation of the NF-κB pathway is under the tight control of DUBs that prevent the hyper-activation of the pathway.

The extensive ubiquitination and deubiquitination events and their selective recognition by UBDs in activation of the NF-κB pathway illustrate the complexity of the ubiquitin system that is used in cells. The pathway is heavily regulated by the balanced activities of multiple ubiquitinating and deubiquitinating enzymes, of which most likely more will be identified in the future. Consequently, the tight regulation of assembly and disassembly of specific ubiquitin signals by the ubiquitin system is critical for cellular functioning.

Regulating target specificity

The cellular outcome of the ubiquitination of a target protein highly depends on the cellular location of the target, the site on the target that is modified and the ubiquitin signal with which it is modified. Therefore, the specificity of the ubiquitination machinery of E1, E2, and E3 enzymes towards its targets is crucial for the proper functioning of the system, and will be evaluated in the next section of this introduction.

The ubiquitination machinery targets a tremendous variety of substrates that are selectively modified with the different ubiquitin signals. Interestingly, only two E1 ubiquitin activating enzymes have been identified, illustrating the generality of the activating mechanism that is used to initiate the ubiquitination process. However, more that 35 E2 conjugases are known that cooperate with hundreds of E3-ligases to provide the required specificity to the ubiquitin system. The specific contributions of the E2s and E3s to the ubiquitin chain formation specificity and target selectivity are dependent on the specific E2 and E3 that are used in the reaction.

Ubiquitin chain formation specificity by E2's

E2 conjugases play a major role in the formation of ubiquitin chains, because many E2s have intrinsic ubiquitin chain formation capacities that selectively mediate the formation ubiquitin chains[11]. The E2s Ube2R1/2, Ube2G1/2 and Ube2K mediate the selective formation K48-linked ubiquitin chains[110-112]. While, Ube2C and Ube2S mediate the specific formation of K11-linked chains[38, 113-115], and the dimeric E2 Ube2N-Ube2V2 mediates the specific formation of K63-linked ubiquitin chains[111, 116]. Besides these highly selective E2s, more general E2s such as Ube2D, can mediate the formation of various different ubiquitin chains[117].

The E2-mediated ubiquitin chain formation requires the presence of a UBD adjacent to the E2 catalytic domain, which recognizes a target-ubiquitin to position the ubiquitin target-residue close to the E2 active-site. This mechanism has been best characterized for K63-linked ubiquitin chain formation by the E2-complex of Ube2N/Ube2V2[116, 118]. The E2 Ube2N does not mediate ubiquitin chain formation on its own, because it lacks the required internal UBD. Also Ube2V2 is inactive, because its E2-UBD does not contain an active-site cysteine. However, the formation of a complex between these two E2s enables Ube2V2 to specifically position the

K63 of the target ubiquitin to face the active-site of Ube2N to facilitate K63-linked ubiquitin chain formation[116, 119, 120]. Other E2s contain an internal ubiquitin interaction surface that non-covalently interacts with the target ubiquitin during chain formation. However, these interactions do not always lead to the formation of specific types of ubiquitin chains, as is seen e.g. for the highly promiscuous Ube2D3 that mediates the formation of all ubiquitin linkages[11, 121]. Nevertheless, ubiquitination reactions that use Ube2D3 as the E2 critically rely on the interaction of target ubiquitins with an interaction surface that is positioned on the back-site of Ube2D3[122]. Consequently, the positioning of a target ubiquitin close to the active site is essential for ubiquitin chain formation activity of E2s, but it does not always determine the ubiquitin chain formation specificity of the E2.

In addition to a ubiquitin interaction surface that docks target ubiquitins, E2s that target ubiquitins towards lysine residues require an internal acidic residue that can deprotonate the target lysine and turn it into an efficient nucleophile[17, 123]. Interestingly, the monomeric E2 Ube2S, which specifically mediates the formation of K11-linked ubiquitin chains, does not contain an acidic residue close to its active site. Instead, K11 on the target ubiquitin is activated internally by target ubiquitin Glu34, and Ube2S only positions K11 close to its active site by interacting with the ubiquitin TEK-box, to mediate the linkage formation[124]. Also Ube2C and Ube2D interact with the ubiquitin TEK-box of the target ubiquitin for K11-linked ubiquitin chain formation, suggesting that this is a general feature for the formation of these ubiquitin chains[125].

In sum, the E2 enzymes form the first level of ubiquitin chain formation specificity of the ubiquitination machinery by inducing the formation of different types of ubiquitin signals that have to be targeted towards their substrates by E3-ligases.

Ubiquitination specificity by HECT E3-ligases

HECT E3-ligases form a thioester intermediate with the C-terminus of ubiquitin to mediate its transfer onto targets. The N-terminal lobe of the HECT-E3 catalytic site interacts with the E2 to mediate the transfer of the ubiquitin onto the active-site cysteine in the HECT C-terminal lobe, after which the ubiquitin is specifically attached to a target lysine[126, 127].

Different HECT E3-ligases display different ubiquitin chain formation specificities that play a role in a large variety of physiological processes[128]. In cooperation with Ube2D, E6AP mediates the formation of K48-linked poly-ubiquitin chains, while Nedd4 (Rsp5 in yeast) assembles K63-linked ubiquitin chains[129, 130]. In addition, the swap of HECT domains between E3-ligases is sufficient to change the ubiquitin chain formation specificity of the HECT E3-ligases[129]. Thus, E2 conjugases do not contribute to the specificity of the final transfer of the ubiquitin from the HECT E3 onto its target.

The precise mechanism for the selective activities of HECT E3-ligases is poorly understood. The interaction of the Nedd4 N-terminal lobe with ubiquitin has been suggested to limit the ubiquitin chain length[131], but was also shown to facilitate ubiquitin chain elongation by stabilizing the E3-target interactions[132]. In addition, recent studies suggest that the N-terminal lobe localizes

and positions the target ubiquitin to promote ubiquitin chain elongation[130, 133, 134]. However, the exact role of these interactions in ubiquitination process is unknown. Nevertheless, it has become clear that the target recognition and ubiquitin chain formation specificity by HECT E3-ligases are embedded within the E3s. Therefore, HECT E3s are interesting potential pharmaceutical targets of which the molecular functioning requires further investigation.

Ubiquitination specificity by classical RING E3-ligases

RING E3-ligases function together with E2 conjugases to facilitate the transfer of the ubiquitin from the E2 onto the target, and thereby simultaneously interact with the E2~ubiquitin and the target to facilitate the ubiquitin transfer. (Reviewed by Deshaies et al.[14]).

The interaction between the RING domains and the loaded E2~ubiquitin accelerates the discharge of the ubiquitin from the E2[110, 135]. However, RING E3-ligases generally do not change the ubiquitin chain formation specificity that is embedded within some E2s. In this way, BRCA1/BARD1 and Chip mediate the formation of ubiquitin chains with Ube2D, but are limited to mono-ubiquitination of their substrates in cooperation with Ube2W[111, 121, 136]. In a similar way, the SCF and gp78, mediate the formation of K48-linked ubiquitin chains on their substrates[110, 137], and the APC/C mediates the formation of K11-linked poly-ubiquitin chains with Ube2C[38]. Different E3-ligases, such as Murf1, Mdm2, and Chip mediate the formation of mixed ubiquitin chains in cooperation with the less specific Ube2D3[121], but BRCA1-BARD1 use this E2 to predominantly form K6-linked ubiquitin chains[55, 121, 138].

Even though RING E3-ligases generally ubiquitinate their targets with the pre-specified ubiquitin chain-type of the E2 in the complex, they can use various E2 enzymes to specifically mono-ubiquitinate their targets. The RING E3-ligase Bmi1-Ring1b limits the activity of Ube2D to specifically mono-ubiquitinate H2A by exposing the Ube2D~ubiquitin active-site to H2A K119 and preventing ubiquitin chain elongation by steric hindrance[139, 140]. Also RING E3-ligase Rad18 limits the K48-linked chain formation activity of the E2 Rad6 to specifically mono-ubiquitinate PCNA. Rad18 prevents the binding of target ubiquitins to the non-covalent ubiquitin binding site on Rad6, which is needed for chain formation, and thereby restricts the activity of this complex to the attachment of a mono-ubiquitin on the substrate PCNA[141]. Thus, RING E3-ligases form specific ubiquitin chains with specific E2s, but are able to block the chain formation capacity of E2s, or push the equilibrium of more general E2s towards a preferred chain type.

Clinical implications for RING E3-ligases

RING E3-ligases mediate target ubiquitination by specifically bringing E2s and targets together. This central role of RING proteins in the ubiquitin pathway makes them potential interesting targets for molecular medicine, and the potency of several different strategies to target E3-ligases is currently being investigated.

In the past years significant advances have been made in the development of small molecules that rescue the E3-target interaction between the E3 MDM2 and the tumour suppressor p53[142].

The molecules were developed to specifically target the p53 binding-pocket on MDM2 to prevent the ubiquitin dependent degradation of p53. The initial developed small molecule MDM2 inhibitors Nutlins, which are in phase-one clinical trials for the treatment of retinoblastoma[143, 144], and RITA[145] possess some off-target effects. However, novel developed compounds such as the benzodiazepinediones are expected to target this complex more selectively[146, 147]. Also the modulation of the activity of E3-ligases is being explored as a potential therapeutic strategy. For example, inhibitors of the Nedd8 E1 activating-enzyme that reduce the activity of cullins by limiting their activating Neddylation, are tested in phase 1 clinical trials for the treatment of hematologic malignancies and melanoma[148]. Finally, several approaches aim to target the IAPs RING E3-ligases for degradation. The IAPs are important for the regulation of the Nf-κB pathway, and high expression levels of IAPs in tumours have been associated with poor treatment response. Therefore, several agents that aim to neutralize the IAP activity and sensitize tumour cells, such as SMAC mimetics, are in early clinical trials[149].

Ubiquitination by RBR E3-ligases

The novel identified RING-subfamily of RBR E3-ligases is a small E3-ligase family with only 15 human proteins. As discussed above, the proteins function via a combined RING/HECT-type mechanism. Therefore, these proteins do most likely not follow the features of the classical RING-domain E3-ligases that have been described above. However, we have only just begun to understand the functioning of RBR proteins, and reports on the contributions of the E2 and E3 in the target and chain-type determination are spares. Some RBRs, like Triad1, have been suggested to follow the chain formation specificity of the E2 (Chapter 2 of this thesis)[150], while HOIP determines its chain formation specificity independent on the E2 in the reaction (Chapter 4 and 5 of this thesis)[15, 98]. However, the different activities of separate RBRs have not yet been evaluated in the context of the complete RBR family in order to identify if there is a general mechanism for the RBR functioning.

It is important to develop our understanding of the functioning RBR proteins, because these proteins and their interaction partners are involved in the regulation of most major cellular events: transcription and RNA metabolism, translation, subcellular tethering, regulation of posttranslational modifications and protein stability, cellular and stress signalling, cell-cycle control, and the course of microbial infection[151]. In addition, the misregulation of the activity of RBR proteins has implications in various human diseases and may induce cancer[152-155], which makes them interesting potential drug targets. Therefore, in the next part of this introduction we compare the present knowledge of the different human RBRs to gain insight in the functioning of this subfamily of the ubiquitin E3-ligases.

RBR E3-ligases

The RBR class of proteins was first described in 1999 by two separate groups that identified the conservation of a triple-RING/zinc finger motif in eukaryotic species, including animals, plants, fungi and protists[156, 157]. Further analysis of the C_3HC_4-C_6HC-C_3HC_4 pattern indicated

that the RBR domain has arisen only once in evolution[158]. In 2004 an updated version of the phylogenetic tree (including all 237 available sequences) was reported, which describes a total of 15 separate human RBR genes that have been assigned to 8 distinct subfamilies[155].

The published table with human RBRs from 2004 contains 3 uncharacterized RBR genes: *KIAA1386* (Ariadne), *DJ1174N9.1* (Dorfin), *ARA54_2* (ARA54). We have updated the table with the protein information that is available to date (Table 1). The gene product of *KIAA1386* is now known as the RBR-containing ANKIB1 protein; however the protein has not been functionally characterized yet. Furthermore, we found that the gene product for the supposedly uncharacterized Dorfin paralog *DJ1174N9.1,* was already cloned in 1999 (now known as the protein: RNF19B, NKLAM, IBRDC3) when its expression was found to be associated with cytokine-induced processes[159]. However, its E3-ligase activity has not been described before 2006[160]. The complete updated set of human RBRs is shown in Table 1.

Table 1. The human RBR class of proteins ARA54_2 is thus far only found in the human genome and is likely to be a pseudogene (*).

Subfamily	Name	Alternative names	Uniprot
Ariadne	HHARI	ARI1, ARIH1	Q9Y4X5
Ariadne	Triad1	ARI2, ARIH2	O95376
Ariadne	Parc	Cul9, H7-AP1, KIAA0708	Q8IWT3
Ariadne	ANKIB1	KIAA1386	Q9P2G1
Parkin	Parkin	PRKN, PARK2	O60260
RNF144	RNF144A	KIAA0161, hUIP4, UBCE7IP4	P50876
RNF144	RNF144B	P53RFP, IBRDC2	Q7Z419
XAP3	HOIL-1L	RBCK1, RNF54, XAP3	Q9BYM8
Dorfin	RNF19A	Dorfin	Q9NV58
Dorfin	RNF19B	NKLAM, IBRDC3, DJ174N9.1	Q6ZMZ0
Paul	HOIP	RNF31, PAUL, ZIBRA	Q96EP0
TRIAD3	RNF216	ZIN, Triad3, UBCE7IP1	Q9NWF9
ARA54	RNF14	ARA54	Q9UBS8
ARA54		ARA54_2	*
Not assigned	RNF217	C6orf172, IBRDC1, FLJ16403	Q8TC41

Molecular functionality of RBR E3-ligases

The combined RING/HECT-type molecular mechanism underlying the RBR-mediated ubiquitination of proteins was first described just recently, in 2011[17]. Consequently, the exact mechanisms that dictate the ubiquitin chain formation specificity and target recognition of these proteins have not been studied extensively yet. In the next part, we will discuss the current knowledge about the targets and molecular mechanisms that underlie the functioning of the best-characterized RBRs (Parkin, HHARI, Triad1, and HOIP (Figure 7)), to gain a better understanding of the molecular mechanisms underlying the functioning of the RBR E3-ligases.

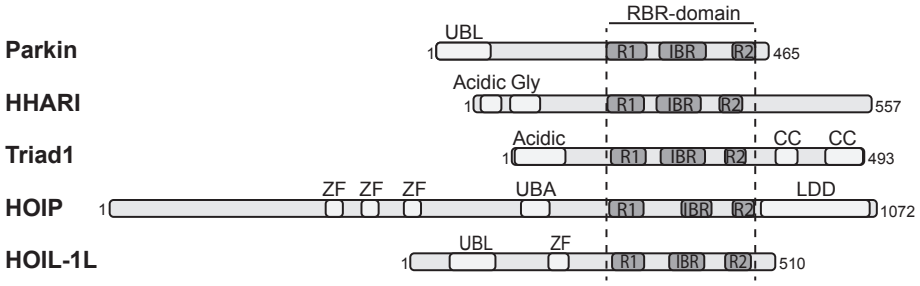

Figure 7. Domain organization of the RBR E3-ligases Parkin, HHARI, Triad1, HOIP and HOIL-1L
The domain borders are drawn to scale according to Uniprot definitions (www.uniprot.org), see table 1. The RBR domains are represented in dark grey. Ubiquitin like domain (UBL), acidic region (Acidic), glycine-rich region (Gly), coiled-coil (CC), zinc finger (ZF), ubiquitin-associated domain (UBA), linear ubiquitin determining domain (LDD).

Parkin

Parkin (PARK2) has been a major focus of research since 1998 when it was discovered that mutations in the *parkin* gene cause familial autosomal-recessive juvenile parkinsonism and form a frequent cause of sporadic early- and late-onset Parkinson disease (PD)[161-163]. Parkin is mainly expressed in several regions in the brain, including the substantia nigra, but it is also found in multiple other tissues[162]. Interestingly, the loss of dopaminergic neurons in the substantia nigra of the brain is a hallmark of PD, indicating a role for Parkin in long-term survival of the dopaminergic neurons. In addition to its role in PD, it is a putative tumour suppressor[164, 165].

The mechanism by which Parkin functions in cells is not well understood; however its E3-ligase activity is crucial for its function. The E3-ligase activity of Parkin is directed towards various substrates, including α-synuclein[166], CDCrel-1[167], Pael-R[168], and misfolded DJ1/PARK7[169], which accumulate in patients with PD. Therefore, the Parkin E3-ligase activity is thought to be crucial for the prevention of the formation of PD pathogenic Lewy bodies, which mainly contain ubiquitin and α-synuclein[170]. However, Parkin also plays a role in numerous other cellular processes as has become clear by the growing number of targets that are being identified, including mitofusin-1, mitofusin-2 and HKI that are involved in maintaining mitochondrial integrity[171-173]; the p38 subunit of aminoacyl-tRNA synthase[174]; RanBP2, which is part of the nucleocytoplasmic transport machinery[175]; transcription factor SIM2 [176]; anti-apoptotic and autophagy inhibitory protein Bcl-2[177]; and Parkin itself[17, 167]. Even though, the understanding of most Parkin interactions is still superficial, a major role for the Parkin E3-ligase function seems to be the protection of cells against the accumulation of misfolded and unfolded proteins.

The E3-ligase activity of Parkin is embedded within the RBR domain in its C-terminus (Figure 7). The catalytic activity of the RBR domain is tightly regulated in cells. Parkin is auto-inhibited by its N-terminal ubiquitin-like domain (UBL) that binds to a region between the IBR and RING2 of the RBR domain[178]. This interaction is crucial for the proper functioning of Parkin, since

multiple pathogenic mutations in the UBL have been found to disrupt the auto-inhibition[178]. At least three targets of Parkin contain domains that interact with the auto-inhibitory UBL domain, suggesting a target-induced Parkin activation[179, 180]. In addition, the posttranslational modification of Parkin with Nedd8 induces its E3-ligase activity[181]. Furthermore, the activity of Parkin is negatively regulated by the modification of multiple cysteines within its IBR with nitric oxide (s-nitrosylation)[182], which is possibly induced by increased levels of nitrosative stress in PD[183]. Thus, the Parkin E3-ligase activity is tightly controlled to regulate its functioning in cells.

Except for the release of the N-terminal inhibition, Parkin does not need cofactors for its ubiquitination activity[184]. The Parkin RBR domain is functionally active with the E2s Ube2D2[185], Ube2D3[17, 19, 167], Ube2L3[181, 186-188], and Ube2L6[167], with which it mediates the formation of various ubiquitin signals (Table 2). Parkin mediates the formation of K63-linked ubiquitin chains in cooperation with the K63-specific E2 Ube2N/Ube2V2[189, 190], to target misfolded DJ1 for dynein-mediated transport to aggresomes[169]. Furthermore, it catalyses the formation of K48-linked ubiquitin chains[189, 190], and works with Ube2L3 to target RanBP2 for degradation[181]. Finally, Parkin has been suggested to mediate the mono-ubiquitination of targets in an IBR-RING2 dependent manner in cooperation with Ube2L3 and Ube2N/Ube2V2[187], and thereby mono-ubiquitinates p38[186], Hsp70/Hsc70[191], and stabilizes Bcl-2[177]. The comparison of the Parkin mediated ubiquitin chain formation activities in the various studies, suggests that the E2s play a major role in the ubiquitin chain formation specificity. Consequently, the E2 enzyme that is used by Parkin, strongly determines the cellular-outcome of the proteins that are targeted.

HHARI

In PD with mutated Parkin, the E3-ligase activity of Parkin is abolished in all cells. Nevertheless, only the dopaminergic neurons in the brain seem to be sensitive to the loss of Parkin functioning. Thus, it is likely that other cells express a redundant E3-ligase to compensate for the loss of Parkin. HHARI is a likely candidate to fulfil such a function, since it shares a number of structural, functional, and biochemical properties with Parkin.

Parkin and HHARI share substantial sequence identity[192], and both interact with the E2s, Ube2L3 and Ube2L6[19, 182, 192-194]. Also, HHARI and Parkin bind many of the same protein partners, such as CDCrel-1, synphilin-1, and CASK[195], and both target synphilin-1 and SIM2 for degradation in cells[176]. Thus, Parkin and HHARI have many overlapping activities. Interestingly, HHARI is also found in Lewy bodies in dopaminergic neurons in PD, indicating that it is impaired to compensate the loss of Parkin activity in these cells[195]. Nevertheless, since HHARI is not mutated in patients with PD, it might be the E3-ligase that compensates the loss of Parkin activity in other cells.

In addition to the description of the possible overlapping interactions of HHARI and Parkin, only two studies present results on the HHARI mode of action in cells. First, HHARI is suggested to play a role in the regulation of protein translation by targeting the eukaryotic mRNA cap-binding protein 4EHP (translation initiation factor 4E homologous protein) for proteasomal

degradation[194]. Second, HHARI has been shown to positively regulate cellular proliferation, which functionally correlates with its over-expression in head and neck squamous cell carcinoma biopsies[196]. Thus, even though the cellular functions of HHARI remain to be identified, initial studies show that the proper functioning of the RBR protein is important for various cellular pathways.

The E3-ligase activity of HHARI is expected to be critical for its functioning, however only Ube2L3 and Ube2L6 have thus far been identified as interaction partners for the HHARI RING1/IBR domains (Table 2)[192-194]. The functionality of the E2/HHARI interaction has been validated for Ube2L3, and also Ube2D3, which was not identified as a binding partner in yeast-two-hybrid studies, has been shown to be functionally active with HHARI[17, 192, 194]. Unfortunately, there is no data available about the ubiquitin chain formation specificity and target selection by this E3, leaving the precise mechanism by which HHARI ubiquitinates its targets to be resolved.

Triad1

Triad1 is involved in the regulation of various cellular processes. The loss of Triad1 is associated with embryonic death and causes the degradation of IκBβ in the nucleus of cells, which leads to excessive NF-κB signalling in dendritic cells[152]. Furthermore, the depletion of Triad1 from cells causes a defect in membrane trafficking that leads to the accumulation of the growth hormone receptor and the epidermal growth factor receptor in intracellular vesicles and at the plasma membrane[197]. Finally, it plays a role in the regulation of the proliferation of myeloid progenitor cells, by modulating HoxA10 activity[198] and stabilizing proteins such as Gfi1B and p53[199, 200].

Even though Triad1 is an E3-ligase, thus far no *in vivo* targets have been identified for its E3-ligase activity. However, Triad1 regulates the stability of various proteins. It has been shown to inhibit the destabilizing ubiquitination of Gfi1B in myeloid progenitor cells, by interacting via the Triad1 RING2 domain with Gfi1B[200]. This interaction is suggested to compete for binding with E3-ligases that target Gfi1B, or to recruit DUBs that deubiquitinate Gfi1B. The direct target for the Triad1 E3-ligase activity in regulating Gfi1B has not been identified, but it has been shown to be essential for the inhibition of myeloid progenitor cell proliferation[150]. In addition, Triad1 has been reported to mediate the stabilization of p53, by preventing the ubiquitination of p53 by the E3-ligase Mdm2[201]. Triad1 binds via its N-terminus to p53 and thereby prevents the p53-Mdm2 interaction that is needed for the Mdm2-mediated degradative ubiquitination signal. Interestingly, initial *in vitro* ubiquitination assays with Triad1 and p53 show that p53 can be ubiquitinated with short ubiquitin signals by Triad1 (Chapter 3 of this thesis), however the relevance of this activity in the regulation of p53 needs to be validated in cells. Thus, Triad1 competes with other E3-ligases to regulate the stability of Gfi1 and p53 in cells, but the precise mechanisms underlying this function remain to be elucidated.

Besides Gfi1B and p53, Triad1 has been linked to the regulation of the activity of HoxA10. High expression levels of HoxA10 in cells correlate with a poor prognosis in Acute Myeloid Leukemia (AML)[202, 203], and induce Triad1 levels in myeloid progenitor cells, which increases

Table 2. E2-E3 interactions with human RBR E3-ligases E2 interaction-partners (Ube2 name (RBR interaction-site)) identified by yeast-two-hybrid (Y2H) and by other methods (e.g. pull down assays). Functional E2/E3 complexes mediate ubiquitin chain-formation, for some E2/E3 complexes the ubiquitin chain type has been defined.

		E2/E3 interactions		E2/E3 Chain formation		
Name	**E3 Activity**	**Y2H**	**Pull down/interactions**	**Functional**	**Ubiquitin Chain types**	**References**
HHARI	Yes	L3, L6	L3 (R1+20AA), L6 (R1,IBR)	D3, L3		17, 192-194, 204
Triad1	Yes	D1, D2, D3, D4, E1, E2, E3, L3, L6, R2, T, V1	L3 (R1), E1 (R1), N (R2)	D3, E1, E2, L3, N/V2	K48 (L3), K63 (N/V2), K* (D3)	150, 200, 205-207, chapter 2&3
Parc	Yes			L3		208
ANKIB1	nd					
Parkin	Yes		L3(R2), L6(R2)	D2, D3, L3, L6	K48, K63 (N/2V)	17, 167-169, 177, 185, 188, 189, 209
RNF144A	nd	H, L3, L6, V1, V2	L3(R1), L6(R1)			207, 210
RNF144B	Yes	I, L3, L6, T, U, Z	L3 (RBR), L6(RBR)			207, 211-213
HOIL-1L	Yes	D4, G1, L3, L6, N, S, U		D3		207, 214
RNF19A	Yes		L3(RBR), L6(RBR)			215-217
RNF19B	Yes		L3(FL), L6(FL)			160, 218
HOIP	Yes		L6 (FL)	D3, L3	K0 (all)	15, 219, chapter 4
RNF216	Yes		L3, L6		K48	220
RNF14	Yes	D1, D2, D3, D4, E1, E2, E3, U, V1, W	E1(R1), E2(R1), E3(R1)	E1, E2, E3		207, 213, 221, 222
RNF217	nd					
LUBAC	Yes			B, D1, D2, D3, K, L3	K0 (all)	95, 96, 98, 223

the total ubiquitination levels of proteins in cells[198, 224]. Interestingly, Triad1 antagonizes the HoxA10 induced cellular proliferation, but it remains to be resolved if HoxA10 is a direct target for Triad1[202, 203, 225]. Nevertheless, the inhibitory effect of Triad1 on the proliferation of myeloid progenitor cells critically relies on its RING domains, and is inhibited by proteasome inhibitors[150]. Therefore, the Triad1 E3-ligase activity is likely to target HoxA10 or regulators of HoxA10 for proteasomal degradation.

The E3-ligase activity of Triad1 in cells can potentially be mediated by a large variety of E2s that have been found as Triad1 interaction partners (Table 2). Of these E2s, Ube2L3 and Ube2E1 have been shown to interact with the Triad1 RING1 domain, and Ube2N binds to Triad1 RING2. The functionality of the interactions has been validated for several E2s, showing that Triad1 mediates the formation of K48-ubiquitin chains with Ube2L3, makes K63 ubiquitin chains in cooperation with Ube2N/Ube2V2, and mediates the formation of various different chains in cooperation with Ube2D3[200, 205] (Chapter 2 of this thesis). Thus, the E3-ligase activity of Triad1 follows the ubiquitin chain formation specificity of the E2 that is used in the reaction to determine the fate of its targets.

As shown above, the E3-ligase activity of Triad1 may have various roles in cells and therefore needs to be tightly regulated. Full length Triad1 is functionally active in ubiquitin chain formation assays *in vitro*, and its E3-ligase activity is only slightly auto-inhibited by its C-terminus (Chapter 3 of this thesis), showing that Triad1 does not critically rely on cofactors to release its E3-ligase activity. Instead, its concentration in cells is under control of various factors. Triad1 levels are up-regulated during granulocytic and monocytic differentiation, and are induced by HoxA10[205, 226]. In contrast, Triad1 protein levels are negatively controlled by its proteasomal degradation for which it is ubiquitinated by, for example, Mdm2[199, 205]. These studies show that the Triad1 activity in cells is tightly controlled via the modulation of its cellular protein levels.

HOIP

The RBR E3-ligase HOIP forms the catalytic centre of the LUBAC complex, which is essential for the activation of the NF-κB pathway (described above). The LUBAC complex comprises Sharpin, HOIP and HOIL-1L[96-99], of which HOIP and HOIL-1L belong to the class of RBR E3-ligases (Figure 7). Interestingly, HOIP contains all E3-ligase activity and linear ubiquitin chain specificity of the complex[98, 227]. The interaction between the HOIP UBA-domain and the HOIL-1L or Sharpin UBL-domains is needed to release the auto-inhibited E3-ligase activity of HOIP and direct its activity towards its targets (Chapter 4 of this thesis)[15, 16].

Thus far, HOIP is the only E3-ligase that is known to mediate the formation of linear ubiquitin chains. Its E3-ligase activity has been solely reported in the NF-κB pathway where LUBAC mainly targets its activity towards NEMO[95, 96, 99]. The conjugated linear ubiquitin chains on NEMO are selectively recognized by the UBAN domain (ubiquitin-binding domain in ABIN and NEMO) of NEMO, and the NZF (Npl4 zinc-finger) domains of HOIL-1L and Sharpin[37, 79, 96, 228, 229], which are believed to stabilize the TNF-R1/NEMO/LUBAC signalling complex, recruit additional

NEMO molecules, and facilitate NF-κB-dependent gene expression. However, also the DUB A20 is recruited to the linear ubiquitin chains and negatively regulates LUBAC-induced NF-κB activation by preventing the interaction between NEMO and LUBAC[104].

The E3-ligase activity of HOIP, which has been studied in the context of the full LUBAC complex, as well as in an N-terminal deletion construct that is constitutively active in the absence of LUBAC ($HOIP^{RBR\text{-}LDD}$), is induced by a variety of E2 enzymes. Interestingly, all E2s induce the selective formation of linear ubiquitin chains, showing that the E2s do not contribute to the chain formation specificity of HOIP (Table 2). Instead, the transfer of the ubiquitin from the E3 onto the N-terminal amine-group of the target ubiquitin is mediated by the specific positioning of the target ubiquitin by the C-terminal HOIP Linear ubiquitin chain-determining domain (LDD) (Chapter 4 of this thesis)[15, 16]. The LDD extension of the RBR domain is unique to HOIP and is not found in other RBR E3-ligases. Therefore, this mechanism for linear ubiquitin chain formation is likely to be unique to HOIP. Even though the full linear ubiquitin chain formation is embedded within the RBR-LDD domain of HOIP, the full LUBAC complex is needed to target it towards its targets (Chapter 5 of this thesis).

General molecular functioning of RBRs

RBR proteins are part of functional complexes

The activity of the different RBR E3-ligases that have been described is highly regulated. The proteins can be regulated at the transcriptional level, but are also activated and inactivated by posttranslational modifications. In addition, several RBRs are auto-inhibited by domains outside their catalytic domains, and need to be activated to mediate the formation of ubiquitin chains. Therefore the complex formation of RBRs with their target proteins or activating cofactors seems to be essential for the regulation of the RBR E3-ligase activity.

Many RBRs are part of functional complexes and even several cases of gene-fusions, which tend to occur between functionally related genes, have been described for RBR proteins. First, the RBR protein Parc originates from a gene fusion among an *ariadne* gene and a duplicate of the *Cullin7* gene[158], making it a true cullin protein that binds RBX1 and is covalently modified by NEDD8[230]. In addition, Parc is part of a large, still uncharacterized, cytoplasmic complex, which potentially ubiquitinates p53 to keep it in the cytoplasm[208]. Second, the very high similarity of *RBCK1* and *Sharpin* suggests that the mammalian gene that codes for RBCK1/HOIL-1L emerged from a fusion of a *Sharpin*-like gene, or a part of it, with an RBR family gene[158]. Interestingly, also this protein is part of large complexes, such as the LUBAC complex[99]. Third, even the RBR protein Parkin, which has been shown to be functionally active on its own, it is found in E3-ligase complexes with Cullin1 to target cyclin E for ubiquitination[188], and with Hsp70 to the ubiquitination of polyglutamine protein *and possibly* recruit misfolded proteins as substrates for its E3-ligase activity[180]. Consequently, the formation of functional complexes by RBR E3-complexes seems to be a common feature for many RBRs, which is needed to modulate their E3-ligase activity and recognize their targets.

Chain formation specificity by RBRs

RBRs mediate the transfer of ubiquitin from the E2 onto a target by a combined RING/HECT-type mechanism. The formation of the HECT-type ubiquitin~E3 intermediate would suggest that the target specificity of the RBRs resembles more the HECT E3-ligases in which the E2s do not play a role in the final transfer of the ubiquitin onto its target. However, the comparison of the RBR E3-ligases reveals that ubiquitin chain formation by the RBRs AriH2, Parkin, and RNF216 relies on the ubiquitin chain formation specificity of the E2 that is used in the reaction (Table 2). Therefore, the final transfer of the ubiquitin by these RBR E3-ligases is mediated by a mechanism that combines the classical RING-like E2-dependency for chain formation specificity with a HECT-type indirect transfer from the E2 onto the target. Apparently, the E2s stay in the complex to facilitate chain formation. Consequently, RBRs form a novel class of E3-ligases that combine RING and HECT-type features for the transfer of ubiquitins, as well as for the specification of the ubiquitin chain type that is attached to the targets.

Interestingly, not all RBRs determine the ubiquitin chain formation specificity via this RING/HECT-type mechanism, because the RBR E3-ligase HOIP does not follow the ubiquitin chain formation specificity of the E2s, and specifically mediates the formation of linear ubiquitin chains. HOIP contains all linear ubiquitin chain formation within its RBR-LDD domain; therefore it shows a higher resemblance to the HECT-type mechanism for chain-type determination than the other described RBR E3-ligases. The activity of HOIP is expected to be specific to this RBR, since its C-terminal LDD extension is not found in other RBRs. Nevertheless, the functional analysis of the RBRs that have not been extensively studied will reveal if yet to be identified ubiquitin interaction motifs that may determine the formation of specific ubiquitin chains, are present in other RBRs.

The comparison of the functioning of the different RBRs that have been evaluated thus far, suggests that most RBRs will follow the ubiquitin chain determination of the E2 that is used in the reaction. The detailed ubiquitin chain formation mechanism by which the RBR E3-ligases that follow the chain-type specificity of the E2 needs further investigation, because the mechanism by which the E2s contribute to the chain formation specificity remains to be resolved. In addition, the precise role of the RING domains in the RBR needs further analysis, since interaction studies identified that some RBRs interact with E2s via their RING2 domains, or bind possible targets via the RBR (Table 2). Consequently, the combined RING/HECT type mechanism might be blocked by some of these interactions and further studies are needed to elucidate the functionality of these interactions.

Outline of this thesis

The first insights in the molecular mechanisms underlying ubiquitin chain formation by RBR E3-ligases have been elucidated in the past years by the identification of the combined RING/HECT-type mechanism that is used by the RBRs Parkin and HHARI[17]. In this thesis we show in **chapter 4** that HOIP also functions as a RING/HECT-type hybrid, and thereby strengthen the

hypothesis that all RBRs mediate ubiquitin chain formation via a RING/HECT-type mechanism. In addition, we describe novel insights in the molecular functioning of RBR E3-ligases Triad1 and HOIP in cooperation with various E2s.

In **chapter 2**, we illustrate the importance of the Triad1 RBR domain for its inhibitory effect on the proliferation of myeloid cell proliferation. Furthermore, we show that Triad1 mediates the formation of K48 and K63-linked ubiquitin chains and that it can be functionally active with the E2s Ube2L3 and Ube2N/Ube2V2. The activity of Triad1 is further characterized in **chapter 3**, where we screen the *in vitro* activity of Triad1 in cooperation with eight different E2s. The functional analysis of Triad1 reveals that this E3 follows the ubiquitin chain formation specificity of the E2 that is used in the reaction and that it can target its activity towards p53.

In **chapter 4**, we describe the molecular mechanism by which HOIP mediates the specific formation of linear ubiquitin chains by studying an N-terminal deletion construct of HOIP ($HOIP^{RBR\text{-}LDD}$). We identify that $HOIP^{RBR\text{-}LDD}$ contains all linear ubiquitin chain formation specificity of the LUBAC complex by mediating the transfer of ubiquitin from the E2 onto its target by the RING/HECT-type mechanism and positioning the target ubiquitin through the unique HOIP C-terminal LDD domain.

The molecular requirements for the N-terminal ubiquitin modification by HOIP are further characterized in **chapter 5**. We show that the position of the ubiquitin N-terminal amine-group within the ubiquitin is crucial for its modification, illustrating the importance of its highly specific positioning by the HOIP LDD domain. Finally, we show that the HOIP-mediated linear ubiquitination of NEMO is also tightly regulated, and requires the presence of a minimal LUBAC complex of HOIP and HOIL-1L to target the HOIP ubiquitin chain-formation activity towards this target.

In **chapter 6** the novel insights gained in the work that is described in this thesis is discussed in the light of the general functioning of the RBR subfamily of RING E3-ligases.

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The ubiquitin-ligase Triad1 inhibits myelopoiesis through Ube2L3 and Ube2N interacting domains

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Abstract

Ubiquitination plays a major role in many aspects of hematopoiesis. Alterations in ubiquitination have been implicated in hematological cancer. The ubiquitin ligase Triad1 controls the proliferation of myeloid cells. Here, we show that two RING (really interesting new gene) domains in Triad1 differentially bind ubiquitin-conjugating enzymes (E2s), Ube2L3 (UbcH7) and Ube2N (Ubc13). Ube2L3 and Ube2N are known to catalyze the formation of different poly-ubiquitin chains. These chains mark proteins for proteasomal degradation or serve crucial non-proteolytic functions, respectively. In line with the dual E2 interactions, we observed that Triad1 catalyzes the formation of both types of ubiquitin chains. The biological relevance of this finding was studied by testing Triad1 mutants in myeloid clonogenic assays. Full-length Triad1 and three mutants lacking conserved domains inhibited myeloid colony formation by over 50%. Strikingly, deletion of either RING finger completely abrogated the inhibitory effect of Triad1 in clonogenic growth. We conclude that Triad1 exhibits dual ubiquitin ligase activity and that both of its RING domains are crucial to inhibit myeloid cell proliferation. The differential interaction of the RINGs with E2s strongly suggests that the ubiquitination mediated through Ube2L3 as well as Ube2N plays a major role in myelopoiesis.

Introduction

Ubiquitination plays a major role in many aspects of cell biology, including cell division, signaling, movement and apoptosis[1]. The conjugation of one or more ubiquitin peptides to a specific substrate protein can regulate the substrate's activity or may result in its recognition and degradation by the 26S proteasome. In normal hematopoiesis, the ubiquitin proteasome route regulates the fate of many proteins, including, for example, growth factor receptor c-kit, cyclin D, Bcl-2 (B-cell CLL/lymphoma 2) and transcription factors, such as GATA-2, acute myeloid leukemia-1 and Gfi1 (growth factor independence 1)[2]. Deregulation of the ubiquitin proteasome route is also implicated in malignant hematopoiesis[2]. For example, the von Hippel-Lindau (VHL) protein is responsible for poly-ubiquitination and subsequent degradation of the Hif1α (hypoxia-inducible factor 1, α-subunit) protein. Mutations in VHL that are found in polycythemia patients result in defective ubiquitination that causes stabilization of Hif1α. As a result, expression of hypoxia-induced genes, including erythropoietin, increases. High erythropoietin levels are believed to contribute to the high red blood cell counts that are found in congenital polycythemia[3]. In Fanconi anemia, mono-ubiquitination of the Fanconi anemia D2 protein is inhibited, resulting in a defect in the DNA repair mechanism. This results in genetic instability that predisposes Fanconi anemia patients to develop malignancies such as acute myelogenous leukemia[4]. Overexpression of MDM2, as found in many leukemia patients, results in increased poly-ubiquitination and degradation of the tumor suppressor protein, p53[5]. Recently, acquired mutations in Cbl (Casitas B-cell lymphoma) proteins were identified in acute myelogenous leukemia. Cbl is involved in the ubiquitination and subsequent internalization and degradation of the activated Flt3 (fms-related tyrosine kinase 3) receptor. As a result of mutations in Cbl, the Flt3 receptor is no longer ubiquitinated and thereby no longer inactivated, resulting in a constitutive active receptor[6, 7].

Although it is clear that ubiquitination plays a crucial role in regulating the activity of many important proteins that control hematopoiesis, little is known about the enzymes that facilitate ubiquitination. The covalent coupling of ubiquitin to a substrate protein requires subsequent action of three enzymes. First, the E1 enzyme activates ubiquitin, which is then transferred to an E2 ubiquitin-conjugating enzyme. In concert with E2s, ubiquitin E3-ligases catalyze the final step by facilitating the covalent attachment of ubiquitin to a substrate protein or the attachment of a ubiquitin to the preceding ubiquitin to form poly-ubiquitin chains. The E3-ligases are responsible for the specificity of the ubiquitination process as they recognize the substrates[8]. We have described earlier Triad1 as a ubiquitin ligase that regulates myelopoiesis through its ubiquitin ligase activity[9]. Expression of Triad1 is induced during myeloid differentiation and forced expression results in the inhibition of proliferation of immature murine bone marrow cells. Triad1 is the founding member of a subclass of ubiquitin ligases that contain two RING (really interesting new gene) finger domains. The RING finger containing E3-ligases represent the largest family of the group of proteins that have ubiquitin ligase activity. This family can be subdivided in E3-ligases that function asmulti-subunit complexes, like the APC/C (anaphase-promoting complex/cyclosome), SCF (Skp1-Cul1-F-box-protein) or the CBC (cullin-2/elongin BC) subfamilies, or as single protein ubiquitin ligases[1, 10]. The TRIAD (Two RING fingers and DRIL) protein family is characterized by a highly conserved TRIAD domain encompassing a conserved DRIL (double RING finger linked a.k.a. in between RINGs (IBR)) domain flanked by two RING fingers.

Importantly, the combination of the E2 and the ubiquitin E3-ligase determines the type of ubiquitin chain that is synthesized[11, 12]. These different types of ubiquitination regulate the fate of the ubiquitinated proteins. For example, proteins marked with poly-ubiquitin chains linked through lysine 48 (K48) of ubiquitin are, in general, recognized by the 26S proteasome providing the cell with a specific and efficient way to eliminate these proteins. The addition of ubiquitin chains linked through lysine 63 (K63) of the preceding ubiquitin, primarily results in an altered function and in general does not trigger proteasomal degradation of the modified protein[13]. K63- linked poly-ubiquitin chains may affect the subcellular localization of proteins, but can also function as scaffold to bind other proteins. K63 linked poly-ubiquitin chains are implicated in many important biological processes, including DNA repair[14] and NFκB (nuclear factor Kappa B) activation[15]. To date, it is generally believed that poly-ubiquitin chains are linked through a single iso-peptide bond (for example, in which ubiquitin moieties in the poly-ubiquitin chain are linked only through lysine 48). Interestingly, *in-vitro* studies have shown the formation of mixed and/or forked ubiquitin chains in which multiple different lysine residues are used for the formation of poly-ubiquitin chains[16, 17]. Although a single report has described the presence of these kinds of forked poly-ubiquitin chains *in vivo*[18], the physiological relevance of these chains remains to be determined.

We have shown earlier that the N-terminal RING domain in Triad1 interacts with Ube2L3, an E2 ubiquitin-conjugating enzyme implicated in lysine 48-linked ubiquitin chain formation. This interaction suggests that Triad1 may target substrate proteins for proteasomal degradation.

In line with this, we found that inhibition of the proteasome partially rescued the Triad1-induced growth repressive phenotype[9]. Recently, we showed that Triad1 interacts with Gfi1, a transcription factor essential to many developmental processes in hematopoiesis. Surprisingly, the ubiquitination and proteasomal degradation of Gfi1 was specifically inhibited by Triad1[19]. Thus, Triad1 does not seem to target Gfi1 for proteasomal degradation. To gain insight into the biological role of the different motifs present in Triad1 and their contribution to its E3-ligase activity, we tested their relevance in hematopoiesis. We show differential binding of the RING domains to different ubiquitin-conjugating enzymes and show that these interacting domains are essential for the biological function of Triad1 in hematopoiesis.

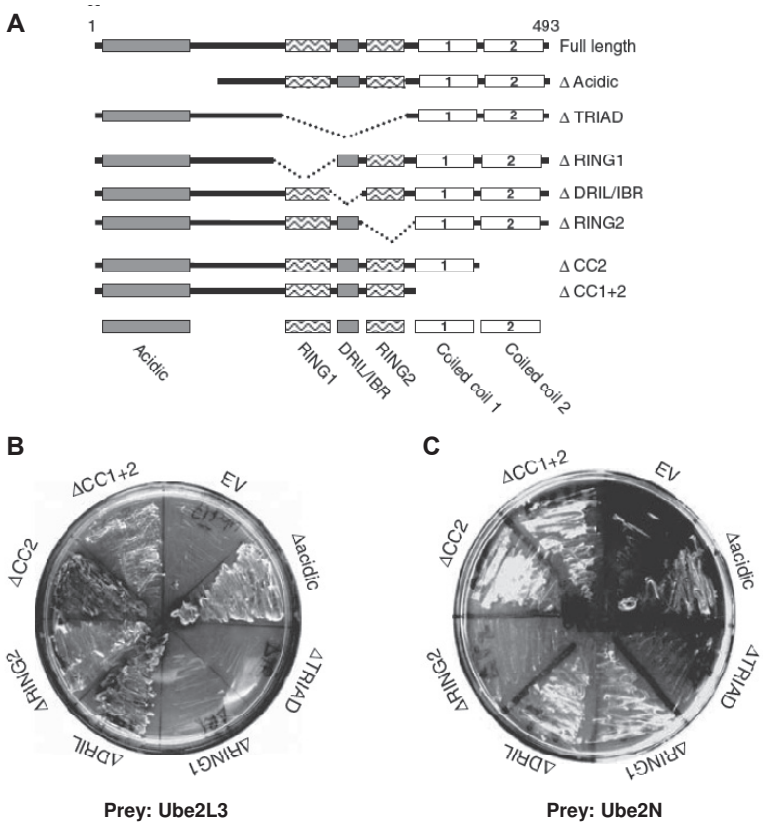

Figure 1. RING1 of Triad1 is essential for interaction with Ube2L3 and RING2 for Ube2N (**A**) Schematic representation of the generated Triad1 deletion constructs. The Triad1 deletion constructs were cloned into the pGAD and pLZRS vectors and sequence verified. (**B**) Ube2L3 interactions with empty vector (EV), Δacidic Triad1 and indicated Triad1 deletion constructs were studied in yeast-two-hybrid assays. AH109 yeast cells were transformed and assayed for interaction by growing on histidine selection and X-gal positivity. The complete TRIAD domain and RING1 were essential for the interaction with Ube2L3. The empty vector pGAD showed no interaction with the Triad1 deletion constructs (data not shown). (**C**) Ube2N interaction with EV and indicated Triad1 deletion constructs. Both the TRIAD domain and RING2 are essential for interaction with Ube2N.

Results

Triad1 inhibits the clonogenic growth of U937 cells

Triad1 is induced on differentiation towards monocytes and granulocytes and inhibits the clonogenic growth of primary murine bone marrow cells[9]. We tested whether Triad1 was also induced during PMA-induced monocytic differentiation of the myelo-monocytic cell line U937. U937 differentiation was confirmed by CD11c expression using FACS analysis. Triad1 became detectable after 24 h of PMA-induced monocytic differentiation and protein levels increased up to 72 h of differentiation (Figure 2A). In addition, ATRA-induced differentiation of U937 cells resulted in an increase of Triad1 protein levels (data not shown). The increase in protein expression during PMA-induced differentiation was confirmed by immunofluorescence microscopy. After 24 and 72 h of differentiation, Triad1 protein levels were increased and Triad1 protein was found to localize predominantly in the nucleus (Figure 2B).

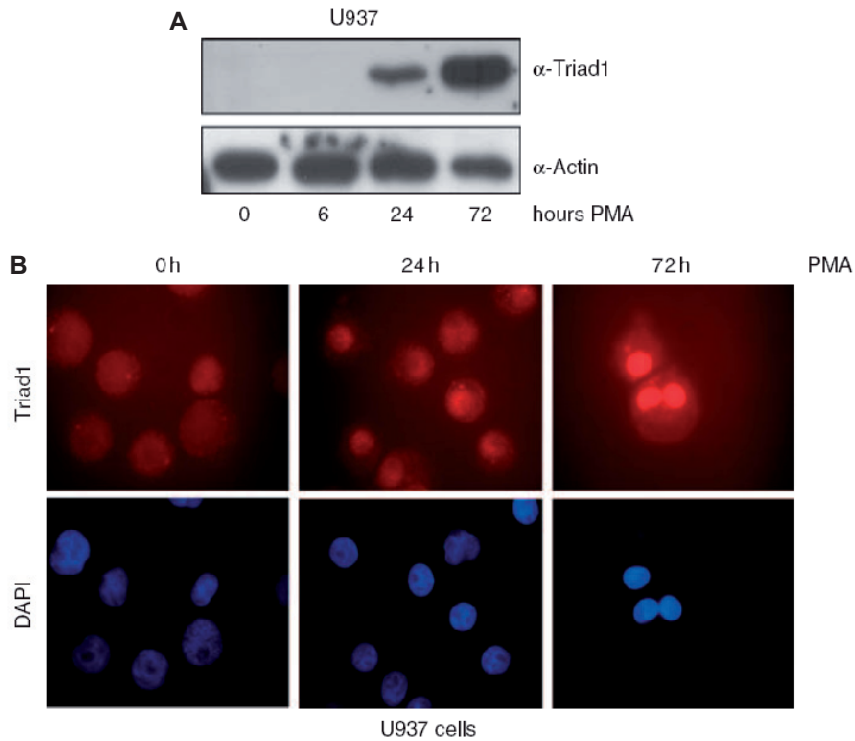

Figure 2. Triad1 is induced during monocytic differentiation of U937 cells (**A**) Cells were stimulated with PMA for the indicated time points and whole cell lysates were stained for Triad1. Triad1 protein levels were induced after 24 h on PMA-induced differentiation of U937 cells and were further upregulated after 72 h of differentiation. The same blot was reprobed with actin antibody to show equal loading. (**B**) Cytospins of unstimulated and PMA-treated U937 cells were stained with Triad1 antibody showing a clear induction of the nuclear Triad1 protein levels. Secondary antibody staining alone resulted in no signal (data not shown). The nucleus was stained with DAPI.

As Triad1 inhibits the proliferation of immature murine bone marrow cells[9], we next tested whether Triad1 can inhibit U937 cell growth. Triad1 coding sequences were cloned in front of an IRES-ΔNGFR cassette, which allowed us to MACS select transduced cells using an α-ΔNGFR antibody. Two independent batches of Triad1-expressing retroviruses were used (A and B), whereas empty vector (EV) and GFP-expressing viruses were used as control. U937 cells were retrovirally transduced and MACS sorted 24 h post-transduction resulting in more than 95% ΔNGFR positive cells. Stable expression of Triad1 in these cells after 2 weeks of culture was confirmed by western blotting (Figure 3A). Triad1 expression levels in transduced cells were comparable to endogenous protein levels observed after 24 to 72 h of PMA-induced differentiation, indicating that the experiments were performed with physiologically relevant

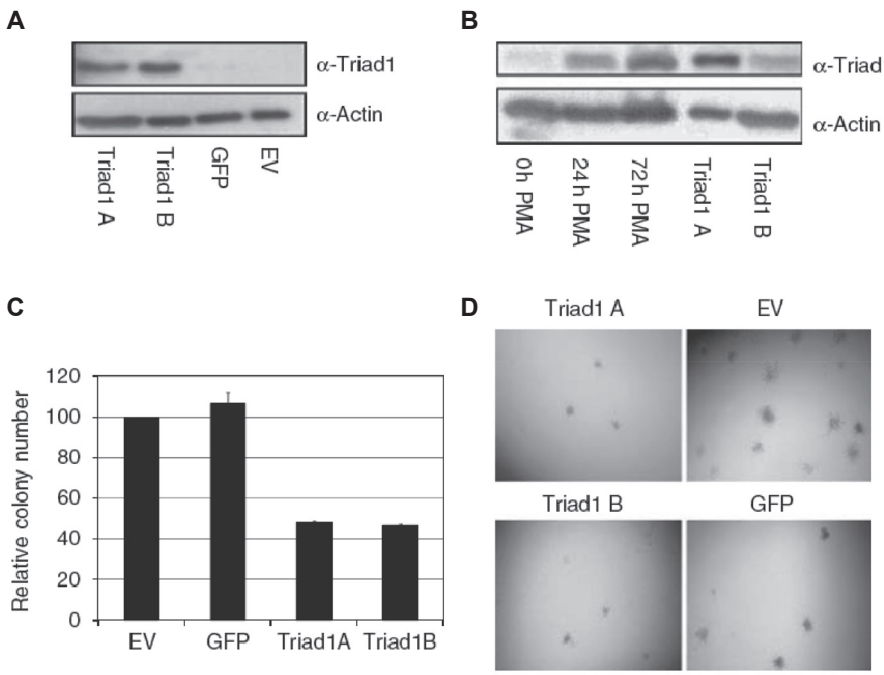

Figure 3. Triad1 inhibits clonogenic growth of U937 cells (**A**) Whole cell lysates of EV (empty vector), GFP, Triad1A and B transduced and MACSsorted U937 cells were stained for Triad1 to check for correct expression. Cells were harvested 14 days after transduction. Equal loading was confirmed by restaining the blot with actin antibody. (**B**) Whole cell lysates of PMA-treated untransduced U937 cells and Triad1 transduced and MACS-sorted U937 cells (24 h after transduction) were immunoblotted. Triad1 protein levels of transduced cells are comparable with the endogenous protein levels in 72 h PMA-treated U937 cells. Actin staining was used to check for equal loading. (**C**) Colony forming assays were performed on EV, GFP, Triad1A and B transduced and MACS-sorted U937 cells. 1000 cells were seeded in semi-solid medium. Colonies were counted 7 days after seeding. Empty vector transduced cells were set at 100. Triad1 transduction resulted in a 50% reduction of U937 colonies. (**D**) Pictures of the formed colonies after 7 days. Clearly visible is that the colonies are smaller when cells were transduced with Triad1, compared with EV or GFP transduced cells.

protein levels (Figure 3B). The effect of Triad1 on the clonogenic growth potential of U937 cells was studied by seeding U937 cells in semi-solid medium directly after MACS sorting. The expression of Triad1 in U937 cells resulted in a 50% decrease in colony numbers compared with the EV and GFP-transduced control cells (Figure 3C). Moreover, the colonies derived from the Triad1 transduced cells were significantly smaller compared with control transduced cells (Figure 3D).

Triad1 inhibits proliferation of U937 cells, without affecting their differentiation and apoptosis

The inhibitory effect of Triad1 expression on colony formation may be due to a decrease in proliferation or due to changes in differentiation and apoptosis. Therefore, we tested the sensitivity to PMA-induced differentiation of Triad1 transduced cells. In clonogenic assays, Triad1 transduced cells were equally sensitive to PMA as control transduced cells, indicating that Triad1 expression does not influence the sensitivity of U937 cells to differentiation by PMA (Figure 4A). We have shown earlier that Triad1 in U937 cells showed reduced proliferation as compared with untransduced cells in competitive growth experiments[9]. As Triad1 did not affect differentiation, we repeated the competitive growth experiments to investigate how Triad1 affects U937 proliferation. Triad1 and control transduced U937 cells were mixed in a 1:1 ratio with nontransduced cells tested and analyzed for the percentage of transduced cells over a period of 20 days. Although the percentage of EV and GFP transduced cells remained stable, the percentage of Triad1 transduced cells decreased to less than 15%, indicating that these cells have a growth disadvantage compared with non-transduced cells (Figure 4B). Differentiation, cell cycle status and apoptosis of transduced cells were measured in liquid medium. DNA histograms of the Triad1 transduced cells on day 3 showed a 10% accumulation of cells in G1/G0 phase compared with GFP and EV-transduced cells (Figure 4C). No effects of Triad1 on differentiation (CD11c) or apoptosis (Annexin V) were found (Supplementary Table S2). We conclude that Triad1 expression results in an accumulation of cells in G1/G0 but has no effect on differentiation, which is in line with the observed inhibitory effect of Triad1 on the growth rate of U937 cells.

RING1 is essential for Ube2L3 interaction, RING2 for Ube2N

The interaction between E2 conjugating enzymes and E3-ligases is essential for the ubiquitination of substrate proteins. The RING finger domain of E3-ligases is involved in the interaction with E2 conjugating enzymes[10]. In yeast-two-hybrid assays Triad1 specifically interacts with the E2 conjugating enzyme Ube2L3 and weakly with Ube2N. The binding between Triad1 and Ube2L3 was confirmed using GST-Triad1 captures using leukemia cell line lysates[20]. To study whether Triad1 interacts with Ube2N in mammalian cells, co-immunoprecipitation experiments were performed. HEK293 cells were transfected with Ube2N-flag and Triad1-GFP or Triad1-GFP alone. Ube2N-flag and interacting proteins were precipitated from the lysate using flag antibodies. Western blot analysis showed that Triad1-GFP only precipitates in the

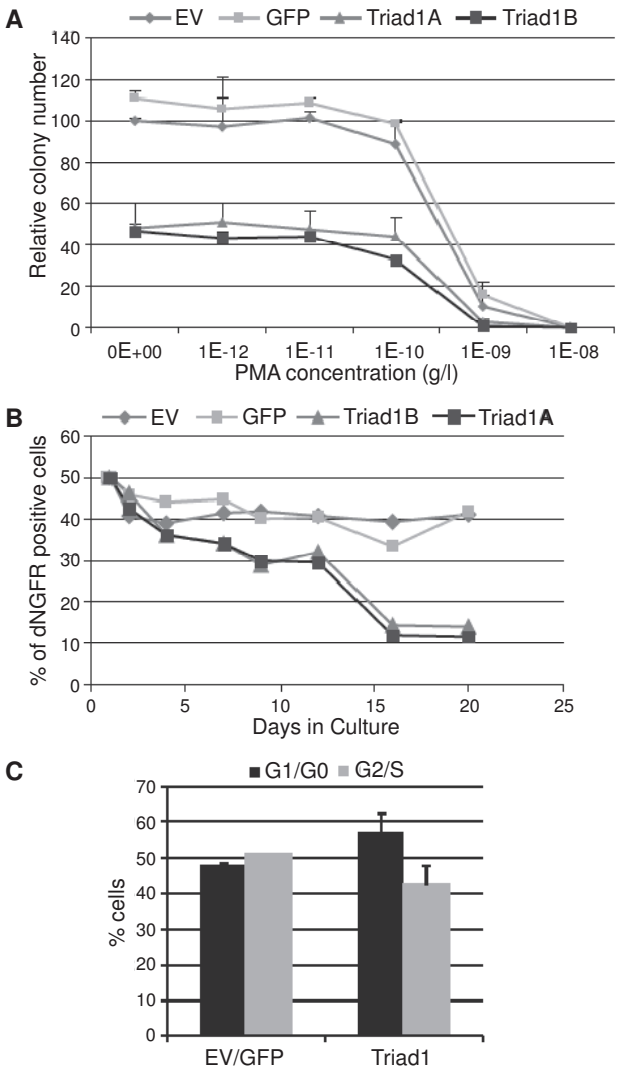

Figure 4. Triad1 inhibits proliferation of U937 cells (**A**) Colony forming assays were performed on transduced and MACS-sorted U937 cells with the indicated concentrations of PMA. We used EV and GFP as duplicate controls and two independent virus batches, Triad1A and B as duplicates to test Triad1 function. Colonies were counted 7 days after seeding. No clear difference was observed in sensitivity towards PMA of the transduced U937 cells. (**B**) EV, GFP, Triad1A and B transduced and MACS sorted cells were grown in a mixed culture with 50% untransduced U937 cells. The amount of DNGFR positive cells was analyzed by FACS at the indicated time points. The amount of Triad1A and B transduced cells was reduced to 11 and 14%, respectively, of the total amount of cells in culture after 20 days. The percentage of GFP and EV transduced cells remained stable, indicating that Triad1 inhibits proliferation of U937 cells. (**C**) DNA histograms of U937 transduced and MACS sorted cells showed a 10% increase of Triad1 transduced cells in G1/G0 phase of the cell cycle compared to the average of GFP and EV transduced cells.

presence of Ube2N-flag (Figure 5A). To confirm the interaction between Ube2N and Triad1 by other means, SPR was performed using the minimal functionally active Ube2N complex, Ube2N/Ube2V2 (Ubc13/Mms2). Triad1 was covalently bound to a CM5 chip and the Ube2N/Ube2V2 complex was flowed over the chip at different concentrations. Binding between the Ube2N/Ube2V2 complex and Triad1 could be clearly seen from the binding profiles at all concentrations. Dissociation rates were quite fast (T½=10±1.2 s) (Figure 5B). The equilibrium responses were fitted using a one-site binding model, resulting in a K_D of 0.85±0.5 μM. This direct interaction between Ube2N/Ube2V2 and Triad1 was confirmed using analytical gel filtration (Supplementary Figure S1). The elution profile shows a peak for the Triad1- Ube2N/Ube2V2 complex. The complex was not stochiometric, which is consistent with the fast off-rates as seen in the SPR data. It seems likely that it is the Ube2N component of the E2 that interacts with a Triad1 RING domain, analogous to the E2/E3 interaction observed in the crystal structure of the CHIP- Ube2N-Uev1A complex[21]. In contrast, Ube2L3 did not interact with Triad1 in the SPR experiments, nor did it co-elute with Triad1 in the analytical gel filtration. These results indicate that the observed Ube2L3-Triad1 interactions as observed in cell line lysates[20] may need other factors or the presence of a substrate.

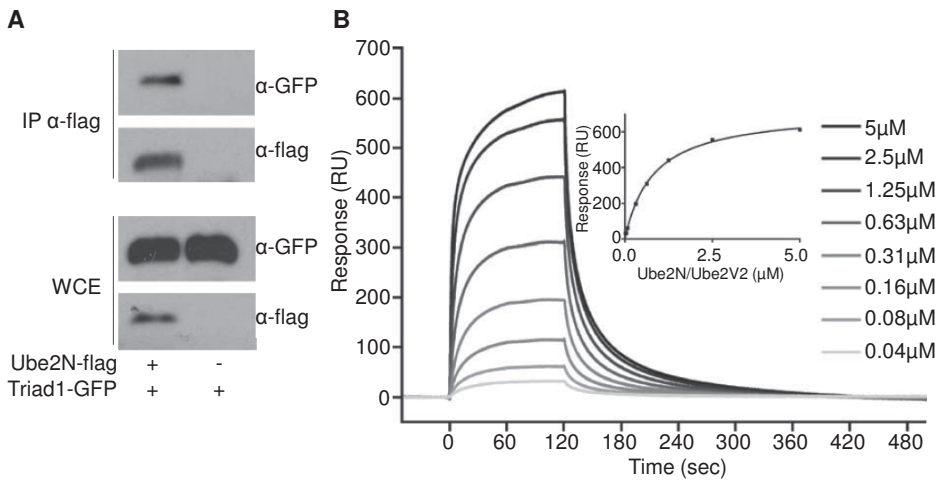

Figure 5. Triad1 shows a strong interaction with Ube2N (**A**) HEK293 cells were transfected with Ube2N-flag and Triad1-GFP or Triad1-GFP alone. Lysates were incubated with anti-flag antibodies to precipitate Ube2N-flag and interacting proteins. Equal amounts of whole cell extract (WCE) and IP sample were subjected to immunoblot analysis to detect the presence of Ube2N-flag and Triad1-GFP. Triad1-GFP was only precipitated on coexpression of Ube2N-flag. (**B**) Surface plasmon resonance analysis of the interaction of Triad1 with Ube2N. Triad1 was expressed in and isolated from Sf-9 cells. Ube2L3 and the Ube2N/Ube2V2 complex were expressed in and isolated from *E.coli*. Triad1 was immobilized onto a CM5 chip and Ube2N/Ube2V2 flowed over at a range of concentrations (0.04–5 μM). Binding could be detected at all concentrations. The K_D is determined at 0.8 μmM after fitting of a single component model (inset).

To test whether both ubiquitin-conjugating enzymes interact with the same domain of Triad1, we tested various Triad1 mutants for binding. Deletion constructs (Figure 1A) of Triad1 were cloned in frame in the pGAD vector and co-transformed with Ube2L3 or Ube2N to analyze the interactions in yeast-two-hybrid assays. Both Ube2L3 and Ube2N interacted with a Triad1 mutant lacking the N-terminal acidic domain (acidic) (Figures 1B and C). The deletion of the complete TRIAD domain abolished the interaction with both Ube2L3 and Ube2N, whereas both the coiled coil and DRIL domains were not necessary for E2 interaction. Earlier, we proved that introduction of a point mutation in RING1 abrogated binding to Ube2L3[9]. As expected, deletion of RING1 abrogated the interaction with Ube2L3, whereas deletion of RING2 did not disturb the Ube2L3 interaction. Conversely, yeast-two-hybrid assay showed that the other RING finger (RING2) was essential for Ube2N interaction. To further study the Triad1- E2 interaction, we transfected NIH3T3 cells with plasmids expressing Triad1-GFP and Ube2L3 or Ube2N-flag[22]. Similarly to Triad1, we found both Ube2L3 and Ube2N to be expressed in the nucleus and to a weaker extent in the cytoplasm (Supplementary Figure S2). We conclude that Triad1 binds two different ubiquitin-conjugating enzymes. The N-terminal RING domain interacts with Ube2L3. The C-terminal RING domain is essential for the interaction between Triad1 and Ube2N.

Triad1 catalyses both K48 and K63 linked poly-ubiquitination

To test the functionality of the interaction between Triad1 and Ube2N/Ube2V2 we performed *in-vitro* ubiquitination assays with purified Uba1 (E1), Ube2N/Ube2V2 (E2), Triad1 (E3) and ubiquitin. As expected, the Ube2N/Ube2V2 complex is capable of forming ubiquitin chains without the presence of an E3 ubiquitin ligase[23]. Four independent assays showed that Triad1 is enhancing free poly-ubiquitin chain formation above background at 150mM (Figure 6A, representative assay), indicating that the Ube2N/Ube2V2-Triad1 complex is a functionally active complex. Remarkably, at higher Triad1 concentrations we observed auto-inhibition of the ubiquitination reactions.

In addition to the direct *in-vitro* interaction with Ube2N/Ube2V2, Triad1 interacts with both Ube2L3 and Ube2N in cell line lysates. Ube2L3 has been implicated in K48 linked poly-ubiquitination and Ube2N in K63 linked poly-ubiquitination. Using *in-vitro* ubiquitination assays, we tested whether both types of ubiquitin modifications are catalyzed by Triad1. Increasing amounts of purified His-Triad1 were incubated with rabbit reticulocyte fraction IIA (enriched in E1 and E2 activity and devoid of ubiquitin and proteasome activity[8]) in the presence of different recombinant ubiquitin mutants. Reaction mixtures were resolved by western blotting and stained with an antibody that recognizes only poly-ubiquitinated proteins (Figure 6B). When incubated with wild-type ubiquitin, we observed a Triad1 dependent increase in poly-ubiquitinated proteins. Restaining of the same blot with an α-Triad1 antibody indicated that these poly-ubiquitinated proteins are not Triad1. As expected, no Triad1-mediated poly-ubiquitination could be detected using K0 ubiquitin, which is devoid of lysine residues. To test whether Triad1 can catalyze the formation of Ub chains linked through K48 or K63,

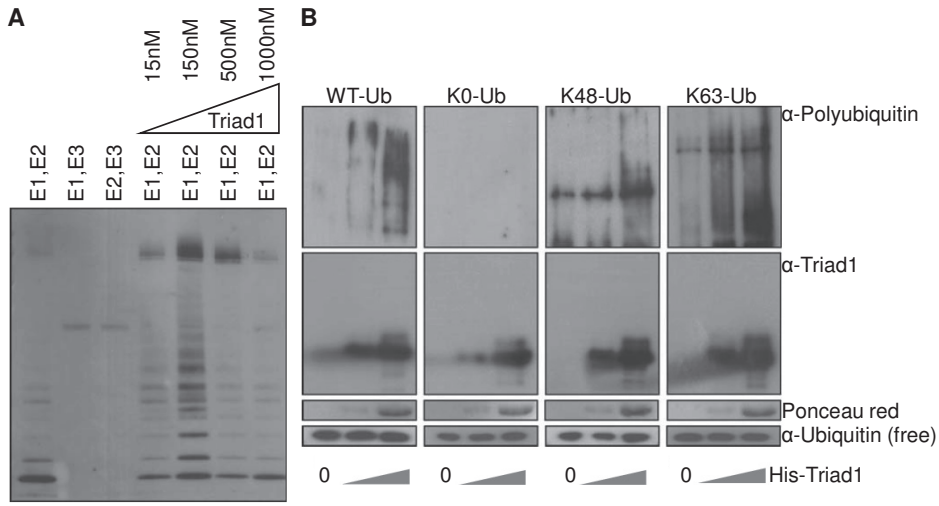

Figure 6. Triad1 catalyses the assembly of both K48 and K63 linked poly-ubiquitin chains (**A**) The effect of Triad1 on free ubiquitin chain formation by the Ube2N/Ube2V2 complex is tested at different concentrations of Triad1 using an α-ubiquitin antibody. The results show a catalytic effect of Triad1 on poly-ubiquitin chain formation. Note that at concentrations higher than 150 nM Triad1, Triad1 has an auto-inhibitory effect on the reactions. (**B**) The conjugation of poly-ubiquitin chains in rabbit reticulocyte fraction IIA was tested using recombinant wild-type ubiquitin and ubiquitin containing only lysine 48 (K48-Ub), only lysine 63 (K63-Ub) or no lysines at all (K0-Ub). Ubiquitin was added to Fraction IIA in the presence of increasing amounts of purified His-Triad1 and incubated for 1 h at 37°C. Western blot analysis of the samples, using an antibody that recognizes only poly-ubiquitinated proteins, indicated that Triad1 promotes the assembly of different poly-ubiquitin chains linked through both lysine 48 and 63 of ubiquitin. The band seen in the left lanes of the two right hand side panels are likely caused by E2/E3 contaminants present in the K48-Ub and K63-Ub mixtures because they are generated independently of Triad1 and are not seen in the lanes in which wild type and K0-Ub are used (see left lanes of the two left hand side panels). The same blot was restained with an α-Triad1 antibody and did not detect high molecular bands. This indicated that the detected poly-ubiquitinated proteins are not ubiquitinated forms of Triad1. Ponceau red staining was used to check the loading of His-Triad1. Staining with an antibody recognizing single ubiquitin moieties showed the presence of equal amounts of the wild type and mutant ubiquitin.

we used recombinant ubiquitin carrying only one lysine residue at position 48 (K48-Ub) or at position 63 (K63-Ub). Our results show that Triad1 can catalyze the formation of Ub chains linked through both K48 and K63. Remarkably, the patterns of modification differ between wildtype, K48 and K63 ubiquitin.

The RING fingers of Triad1 are essential for clonogenic inhibition whereas other domains are dispensable

Earlier, we showed that Triad1 inhibits hematopoietic cell growth. N-terminal RING finger point mutations in Triad1 that abrogate the Ube2L3 interaction did not inhibit cell growth. To further study the relevance of the conserved domains of Triad1 with regard to its growth

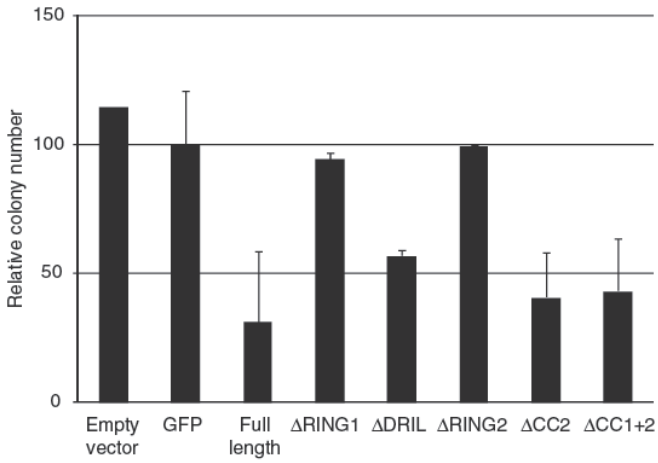

Figure 7. RING1 and RING2 of Triad1 are essential for clonogenic inhibition of U937 cells U937 cells were transduced with full-length Triad1 and indicated deletion constructs. The MACS-sorted cells were seeded in semi-solid medium and the number of colonies were analyzed after 7 days. Clearly visible is that deletion of the RING1 or RING2 domains abolishes the Triad1 induced inhibition of clonogenic growth. Deletion of the coiled coils or the DRIL domain had no effect on the clonogenic inhibition of Triad1.

inhibitory function, we tested various deletion mutants (Figure 1A) in clonogenic assays. U937 cells were transduced with viruses expressing the deletion mutants, subsequently, MACS sorted and seeded in semi-solid medium. Colonies were counted 5-7 days later (Figure 7). As expected, wild type Triad1 inhibited U937 colony formation by over 50% compared with GFP and EV-transduced cells. The deletion of one or both coiled coil domains had no effect on the Triad1-induced inhibition of clonogenic growth. In addition, the Triad1 mutant lacking the conserved DRIL domain inhibited the clonogenic growth equally well as wild-type Triad1. Deletion of the N-terminal RING finger completely abrogated the growth inhibitory effect of Triad1 in these colony assays. This was not unanticipated because earlier experiments showed that an intact N-terminal RING finger is required for the growth inhibition of Triad1[9]. Deletion of the Ube2N-interacting RING2 domain also completely abolished the observed clonogenic inhibition of Triad1. The latter finding is in line with data from earlier competitive liquid growth studies, in which the RING2 deletion mutant was used[19]. Together, these data show that both RING fingers of Triad1 are important for its function in the control of myeloid cell proliferation whereas other domains are not required. As the C-terminal RING finger is required to inhibit proliferation, interacts with Ube2N and because Triad1 functions as a dual ubiquitin ligase, (see Figure 6) these data strongly suggest that non-canonical ubiquitination is important for the role of Triad1 in myelopoiesis.

Discussion

Proteins can be modified in various ways with poly-ubiquitin chains. The type of chain determines the fate of the marked protein. Proteins that are conjugated with poly-ubiquitin chains linked through lysine 48 of ubiquitin are marked for rapid proteasomal degradation. In contrast, poly-ubiquitin chains linked through lysine 63 of ubiquitin do not mark proteins for degradation. Instead, they are often used to recruit other proteins to activate biological processes like for instance, signal transduction pathways[2].

Hematopoiesis is controlled by both lysine 48- and lysine 63-linked poly-ubiquitination. Alterations in both forms of ubiquitination disturb hematopoiesis and have been implicated in malignant transformation. Triad1 is an E3 ubiquitin ligase that controls the proliferation of myeloid cells[9]. Triad1 is the first identified member of a unique set of ubiquitin ligases that contain two RING finger domains[24, 25]. RING domains are known to mediate E2 ubiquitin-conjugating enzyme interactions.

We show here that the N-terminal RING finger (RING1) of Triad1 binds Ube2L3 and that the C-terminal RING finger (RING2) binds Ube2N (Figures 1B and C). In conjunction with E3-ligases, Ube2L3 is known to catalyze the formation of lysine 48-linked poly-ubiquitin chains and Ube2N can catalyze non-canonical lysine 63 poly-ubiquitin chains. In line with the interactions, Triad1 was found to catalyze the formation of both types of poly-ubiquitin chains in *in-vitro* ubiquitination experiments (Figure 6B). Our finding that different ubiquitin mutants result in distinct patterns of poly-ubiquitination, may suggest that Triad1 marks different substrates with different ubiquitin chains. The N-terminal RING finger domain in TRIAD proteins shows structural similarity with the classical RING domain as found in other, single RING ubiquitin ligases[26]. For many TRIAD proteins RING1 has been shown to interact with Ube2L3[9, 27-29]. The C-terminal RING finger (RING2) that binds Ube2N has a different topology as compared with the classical RING finger[26, 30]. RING2 is exclusively found in TRIAD proteins and the different topologies of the two RINGs may explain the differential binding to the E2 conjugating enzymes.

Earlier, we showed that the growth inhibitory function of Triad1 could be partially restored by proteasome inhibition. This suggests that Triad1 functions in part by marking proteins for proteasomal degradation. The functional interaction with Ube2N (Figures 5 and 6A) suggests that Triad1 may also mark proteins with ubiquitin in a non-proteolytic manner. Recent studies showed that the C-terminal RING domain of the TRIAD protein, Parkin also binds Ube2N[31]. In addition, Parkin can mark cellular proteins with lysine 63-linked ubiquitin chains that trigger their intracellular transport, but not proteasomal degradation[31-33]. Although lysine 63-linked ubiquitination of cellular proteins catalyzed by Triad1 needs to be identified, the Ube2N-interacting domain is crucial for the growth inhibitory function of Triad1. This is an interesting observation, as Ube2N is known to play very important roles in immune receptor signaling and inflammatory responses. The function of Ube2N in these processes mainly occurs in the cytoplasm. Yet, Ube2N plays also important roles in nuclear processes including DNA repair. In hematopoietic cells, Triad1 is a predominantly nuclear protein (Figure 2B)[9]. We show here

that Ube2L3 and Ube2N colocalize with Triad1 in the nucleus (Supplementary Figure S2). Thus, Triad1 may mark nuclear proteins with ubiquitin to control blood cell proliferation.

Unlike the RING fingers, other conserved domains in Triad1 like the DRIL and the coiled coil domains were not necessary to inhibit proliferation. This was somewhat unanticipated because we expected the coiled coils to be involved in recruiting substrates to be ubiquitinated by the RING domains of Triad1. Apparently, the coiled coil domains are dispensable for the function of Triad1 in myeloid cells. These data underscore the importance of the RING domains and suggest that the RINGs may recruit substrates themselves in addition to binding ubiquitin-conjugating enzymes.

Together with studies on Parkin[31, 32, 34, 35], our data suggest that members of the TRIAD family of ubiquitin E3-ligases may have the ability to catalyze multiple types of ubiquitin chains by interacting with different E2 conjugating enzymes. The fact that the TRIAD domain is heavily conserved and that RING2 is only found together with the other RING domain suggests that these modules act together and are required for an integrated function. Future studies should clarify whether the different ligase functions of Triad1 are used to differentially ubiquitinate substrates with different types of ubiquitin chains, whether one and the same substrate is modified with different types of ubiquitin chains, or whether they are used to mediate the conjugation of mixed ubiquitin chains[16, 17, 36].

Materials and methods

Cell culture

U937, HEK293, COS-1 and NIH3T3 cells were cultured as described[9, 37]. For monocytic differentiation, U937 cells were differentiated with 100 ng/ml PMA (Phorbol 12-myristate 13-acetate, Sigma, St Louis, MO, USA) or ATRA (all-trans retinoic acid)[19]. U937 cells were transduced as described[37] with retroviruses containing full-length Triad1[9] and the different mutants in the pLZRS-IRES-ΔNGFR vector. Transduced U937 cells were isolated by magnetic cell sorting (MACS) on the basis of ΔNGFR (truncated nerve growth factor receptor) positivity[37]. Flowcytometric analysis of ΔNGFR expression of the MACS-sorted cells showed a purity of more than 95% in all experiments.

Western blotting and immunofluorescence microscopy

Whole cell lysates were made in radio-immunoprecipitation assay buffer as described[37], and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Blots were probed with rabbit antibodies to Triad1[9], Flag (M2, Sigma), GFP (Roche, Basel, Switzerland), Poly-ubiquitin (FK1, Affinity, Golden, CO, USA), ubiquitin (6C1, Sigma) or actin (I-19, Sigma). Cytospins of U937 cells were fixed, permeabilized and stained with anti- Triad1 antibody followed by donkey anti-rabbit PE (Jackson, Suffolk, UK), washed three times with phosphate buffered saline and embedded in Vectashield with DAPI (4',6-diamidino-2- phenylindole, Vector Lab, Burlingame, CA, USA). NIH3T3 cells were grown on coverslips and transfected using lipofectamine 2000

(Invitrogen, Carlsbad, CA, USA). Cells were stained using α-Flag (M2, Sigma) or α-Ube2L3 (Transduction Laboratories, Lexington, KY, USA) followed by donkey-α-mouse TexasRed (Jackson) staining. Pictures were obtained using a Leica DMRBE microscope and Leica FW4000 software (Leica Microsystems, Rijswijk, The Netherlands).

Generation of Triad1 expression vectors

A full-length Triad1 construct as described[9] was used to generate Triad1 mutants ΔRING1, ΔDRIL, ΔRING2 and ΔTRIAD (Figure 1A) using sexual PCR as described[38] using Pfu and Taq polymerase (Invitrogen). The ΔCC2 and ΔCC1+2 mutants were made by introducing a stop codon using PCR primers. The sequences of used primers can be found in Supplementary Table S1. All fragments were verified by sequence analysis. Triad1 full length and deletion mutants were cloned into the pLZRS-IRES-ΔNGFR retroviral expression vector[37] and in pGAD-C1 for yeast-two-hybrid assays. Full-length Triad was cloned in frame with the His-tag in pFastBacT A to generate a bacmid for baculoviral transduction of insect cells.

Yeast-two-hybrid assays

Ube2L3 and Ube2N bait[39] and Triad1 prey deletion constructs were transfected to the yeast strain AH109 (Clontech, Palo Alto, CA, USA) for yeast-two-hybrid assays. This strain contains the selectable marker histidine, adenine and a lacZ reporter. A positive interaction in yeast was defined by growth of blue colonies after plating cells on leucine, tryptophane, histidine and adenine deficient SD medium containing X-α-gal.

Protein expression and purification

For *in-vitro* ubiquitination assays with reticulocyte fraction IIA, His-tagged Triad1 was produced using the baculovirus system (Baculovirus Bac-to-Bac Expression System, Invitrogen) in Hi-5 cells[9]. Three days after infection cells were harvested and lysed in radio-immunoprecipitation assay buffer with complete protease inhibitor cocktail (Roche). After centrifugation, His-Triad1 was isolated from Hi-5 cells using His-select spin columns followed by elution with 250 mM imidazole. Imidazole was removed from His-Triad1 protein eluates using a Microcon YM-10 filter (Amicon, Beverly, MA, USA) and protein concentrations were determined with the Bradford assay. For surface plasmon resonance (SPR) studies, analytical gel filtration and *in-vitro* assays with purified proteins, His-tagged Triad1 was expressed from pFastBac, using the baculovirus expression system in Sf9 cells. Cells were harvested 3 days after infection and lysed by sonification. The cleared lysate was incubated with Talon beads and Triad1 was eluted from the beads in buffer A (50 mM Tris-HCl pH 8, 200 mM NaCl, 2 μM ZnCl2) +250 mM imidazole. Further purification was achieved by anion exchange chromatography on a Resource Q column using buffer A, eluting with a linear NaCl gradient to 0.5 M NaCl. Proteins were eluted at 350 mM, followed by size exclusion chromatography (Superdex 200) in 20 mM Hepes pH 7.5, 150 mM NaCl, 2 μM ZnCl2, 15 mM β-mercaptoethanol (β-ME).

The Ube2N/Ube2V2 complex was produced in *Escherichia coli* BL21 (DE3) cells by co-expression of GST (glutathione S-transferase)-tagged Mms2 and His-tagged Ube2N from the pGEX4T-1 vector with both genes on a single promoter. Cells were induced with 0.4 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) at an OD 600 of 0.8 in Luria-Bertani medium supplemented by 50 μg/ml carbenicillin and further cultivated at 15°C. Cells were harvested after 15-20 h and lysed by sonification. The cleared lysate was incubated with Talon beads and washed with 20 column volumes of buffer C (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM β-ME, 5 mM imidazole). The complex was eluted in buffer C+500 mM imidazole. Subsequently, the complex was incubated with glutathione beads and washed with buffer C+3 mM CaCl2. The GST-tag was cleaved overnight on beads by Thrombin protease. Ube2L3 was expressed in *E.coli* Rosetta2 (DE3) cells from a pGEX2 vector. Cells were induced by autoinduction and cultivated overnight at 15°C. The cells were lysed by a high-pressure EmulsiFlex-C5 device (Avestin, Mannheim, Germany) and cleared lysates were incubated with glutathione sepharose beads. GST-tagged Ube2L3 was eluted in 20 mM Tris-HCl pH 8.0, 250 mM NaCl, 15 mM β-ME and 50 mM glutathione. The GST-tag was digested with thrombin for 2 h at 37°C and the reaction was terminated by the addition of 1 mM phenylmethanesulfonyl fluoride. Further purification of Ube2L3 was achieved through gel filtration (Superdex 200) using 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 μM ZnCl2, 15 mM β-ME. Final purification was achieved through gel filtration (Superdex 75) using buffer C without imidazole.

Co-immunoprecipitation

For immunoprecipitations, transfected HEK293 cells were lysed in TNI (Tris-NaCl-Igepal) buffer containing 0.1% Nonidet P-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl and complete protease inhibitor cocktail and incubated with indicated antibodies for 15 h at 4°C. Protein G sepharose beads (GE Healthcare, Zeist, The Netherlands) were washed three times in TNI buffer, added to the lysate and incubated for 2 h at 4°C. Bound proteins were washed, eluted in loading buffer by heating for 5 min at 95°C, separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis, immunoblotted and stained with relevant antibodies.

Analytical gel filtration

Triad1-E2 interaction studies by analytical gel filtration were performed on a Superdex 75 column on the SMART system (GE Healthcare) in a buffer containing 20 mM Hepes pH 7.5, 150 nM NaCl, 2 μM ZnCl2 and 5 mM β-ME. A total of 50 μl of a mixture of Triad1 and Ube2N/Ube2V2 at 40 μM each was incubated for 10 min at 4°C before loading on the column. Eluted fractions were run onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Coomassie blue staining.

Surface plasmon resonance

Triad1-E2 interactions were analyzed quantitatively using SPR experiments. The SPR experiments were performed at 10°C on a Biacore T-100 instrument (GE Healthcare, WI,

USA). The experiments were performed in duplicate and Triad1 was immobilized at densities of 6000RU and 1000RU through amine coupling on a CM5 chip. The Ube2N/Ube2V2 complex was injected over the sensor chip at 30 µl/min with a 120 s association phase followed by a 10 min dissociation phase. Standard double referencing data subtraction methods were used before data analysis. Curve fitting and other data analyses were performed using Biaevaluation (GE Healthcare) and GraphPad software (GraphPad Software Inc., San Diego, CA, USA).

In-vitro ubiquitination assays

For the *in-vitro* assays using purified E1, E2 and E3, 30 µl reactions were performed in a buffer containing 25 mM Tris pH 8, 150 mM NaCl, 10 mM MgCl2, 2 µM ZnCl2, 5 mM β-ME, 0.5 mM DTT and 3 mM ATP at 30°C. Wild-type ubiquitin (3 µM) was used together with 15 nM Uba1, 150 nM Ube2N/Ube2V2 and 15 nM-1.5 µM Triad1. The samples were run on 4-12% NuPage gels in MOPS buffer (Invitrogen) and were analyzed by western blotting with an α-ubiquitin antibody (P4D1, Santa Cruz Biotechnology, Santa Cruz CA, USA).

In-vitro ubiquitination assays in lysates were performed as described earlier[9, 31] using 1.6 µg reticulocyte fraction IIA (Boston Biochem, Cambridge, MA, USA) as a source of E1 and E2 activity. Although this fraction may not contain high levels of Ube2L3 and Ube2N, it contains sufficient E2 activity to support the formation of K48 and K63 linked poly-ubiquitin chains[31]. To test chain formation, recombinant wild-type Ub, K0-Ub, K48-Ub or K63-Ub (Boston Biochem) was incubated together with reticulocyte fraction IIA and freshly prepared His-Triad1 for 60 min at 37°C followed by staining of ubiquitinated proteins in western blot analysis using a poly-ubiquitin specific antibody (FK1).

Colony-forming assays, differentiation and cell cycle characteristics

Clonogenic growth of transduced and MACS-sorted U937 cells was measured by plating 1000 cells/ml in semi-solid (0.75% agar) culture medium. Colonies were counted after 6 days. Differentiation of U937 cells was assessed measuring CD11c expression (Immunotech, Marseille, France). Apoptosis and DNA histograms were determined using AnnexinV (Molecular Probes, Eugene, OR, USA) and propidium iodide staining, respectively[9], and the percentage of ΔNGFR positive cells was monitored measured using the α-ΔNGFR antibody and goat-α-mouse PE by flowcytometry.

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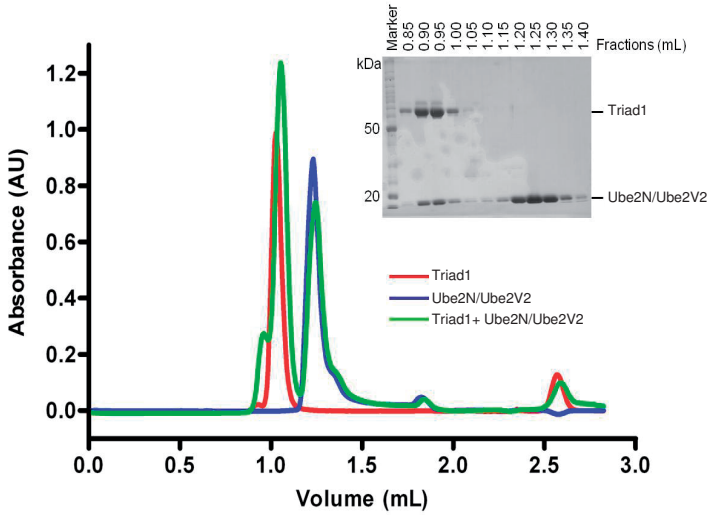
Supplementary information

Supplementary Table S1. Primers used for making the deletion mutants of Triad1 as depicted in figure 4A.

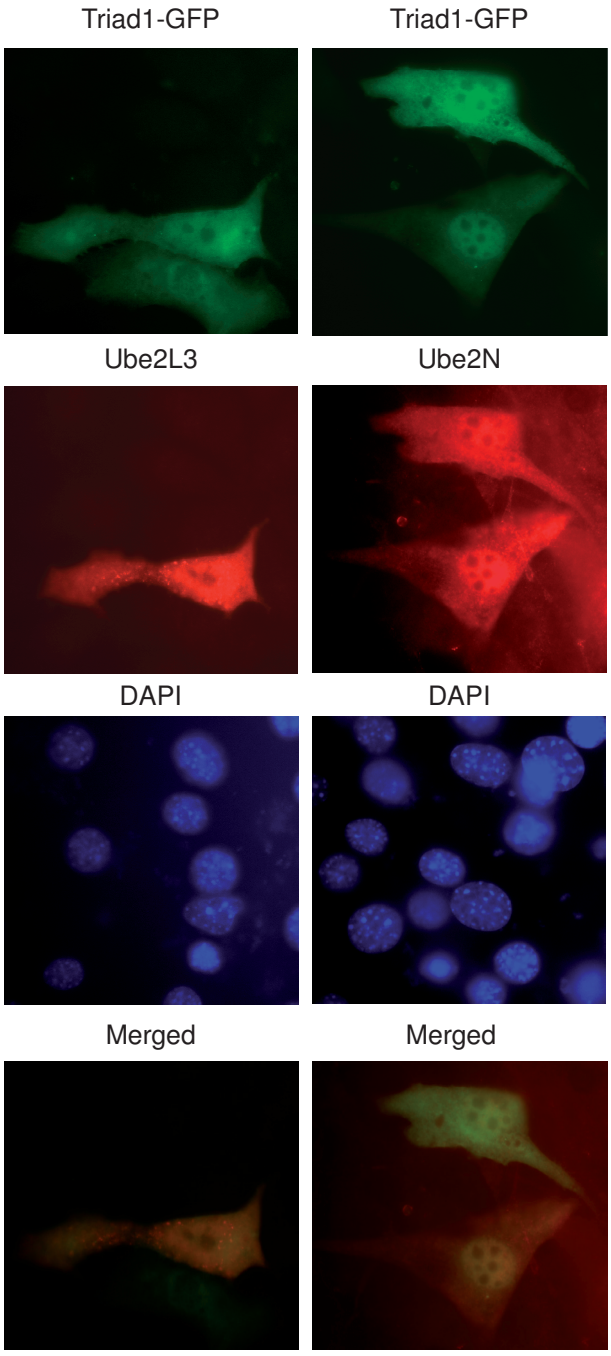
DTRIAD Fw	ACATCCCATCCCCCTCACCACCTAGGAGATTGGAAGACTCATGGCAGT
DTRIAD Rev	ATGAGTCTTCCAATCTCCTAGGTGGTGAGGGGGATGGGATGTGGGAAC
DRING 1 Fw	ACATCCCATCCCCCTCACCACCCACTCCGTACACCAGAGGACTTTGTG
DRING 1 Rev	GTCCTCTGGTGTACGGAGTGGGTGGTGAGGGGGATGGGATGTGGGAAC
DDRIL Fw	AGTCATTACCAGCTCCAGCTGGCCACAATCCGGAAATGGCTCACGAAG
DDRIL Rev	GAGCCATTTCCGGATTGTGGCCAGCTGGAGCTGGTAATGACTCTCCAC
DRING 2 Fw	ATTAGTGCTCACACTAAAGACCTAGGAGATTGGAAGACTCATGGCAGT
DRING 2 Rev	ATGAGTCTTCCAATCTCCTAGGTCTTTAGTGTGAGCACTAATGTAGTT
DCC 2 Rev	TCGCGAATTCTTATCCGGACTCCATGTAATATGCATA
DCC 1+2 Rev	TCGCGAATTCTTACACGATGTCAGGATTCTCCTTGTA

Supplementary Table S2. Annexin-V and CD11c staining of transduced and sorted U937 cells showed no clear differences in the amount of apoptotic cells when Triad1 transduced cells were compared to EV of GFP transduced control cells.

Culture Days	**2**	**3**	**4**	**5**
% annexin V positive cells				
EV/GFP	1,4	0,9	1,4	3,9
Triad1	2,9	1,0	1,4	4,8
% CD11c positive cells				
EV/GFP	2,4	3,3	4,0	2,6
Triad1	4,3	4,7	4,5	3,0



Supplementary Figure S1. Analytical gel filtration chromatogram shows that Triad1 interacts with Ube2N/Ube2V2 Single protein runs are compared with the complex. Each run contained 40 μM Triad1 and 40 μM Ube2N/Ube2V2 and followed by coommassie staining. Twelve consecutive fractions ranging in elution volume from 0.85 to 1.4 mL are loaded on gel (inset).



Supplementary Figure S2. Localization of with Triad1-GFP and Ube2L3 or Ube2N-flag in co-transfected NIH3T3 cells Triad1 is present in the same cellular compartments as Ube2L3 and FLAG-Ube2N. DAPI staining was used to stain the Nucleus.

Characterization of the E3-ligase activity of Triad1

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Manuscript in preparation

Abstract

Triad1 is induced during granulocytic and monocytic differentiation. Its ubiquitin E3-ligase catalytic activity inhibits the proliferation of these committed myeloid progenitors. Triad1 is the first RBR E3-ligase that has been identified. Nevertheless, the biochemistry of the Triad1 mediated ubiquitin chain formation has not been extensively studied. Here we characterize the *in-vitro* activity of Triad1 in combination with various E2s. We show that Triad1 is functionally active with a subset of the E2s, and reveal that the chain type specificity and target recognition of the E3-ligase strongly depend on the E2 that is used in the reaction.

Introduction

Ubiquitination is a key posttranslational modification for many cellular processes[1]. The modification is most commonly formed on a lysine of the target substrate on which an isopeptide-bond is formed with the C-terminus of the 76 amino acid ubiquitin. This initial ubiquitination of a target can be extended by the formation of ubiquitin chains that are linked via any of the 7 lysines on ubiquitin, or the ubiquitin N-terminus. Depending on the site on ubiquitin that is used in the chains and the number of ubiquitins that is attached to a substrate, the ubiquitination of proteins induces a variety of responses, such as proteasomal degradation and nonprotealytic signaling like the translocation of the target protein or the recruitment of downstream factors[2].

The ubiquitination of proteins is mediated by the sequential action of an E1 activator, E2 conjugase and E3-ligase. First, ubiquitin is activated by the E1 in an ATP-dependent manner to form a thioester intermediate between its C-terminus and a cysteine residue on the E1. Next, the thioester bond is transferred onto the active site cysteine of an E2, after which the ubiquitin can be covalently attached to its target via the mediation of an E3. The two major classes of E3-ligases mediate the transfer of ubiquitin onto its target via different mechanisms. RING E3-ligases mediate the final transfer of ubiquitin in an indirect manner by bringing the E2~ubiquitin and the target in close proximity, while HECT E3-ligases mediate the transfer of ubiquitin by forming a thioester intermediate with the ubiquitin during the transfer[3, 4].

In 1999 a novel class of RING-in-between-RING (RBR) E3-ligases (also known as TRIAD (two RINGs and a double RING-linked domain) was identified[5, 6]. The RBR E3-ligases are specified by their highly conserved catalytic unit that consists of two RING domains and an in-between-RING zinc finger. Recently, it became clear that RBRs function via a combined RING/HECT-type mechanism to transfer the ubiquitin from the E2 onto its target[7-9]. First the E2~ubiquitin is recruited to RING1 after which the ubiquitin is transferred to form a HECT-type thioester intermediate on a cysteine in RING2. Interestingly, the chain type specificity of RBR E3-ligases is regulated in various ways. Some RBR E3-ligases, such as Parkin, depend on the E2s for the ubiquitin chain formation specificity, while the RBR HOIP specifies the formation of its N-terminally linked ubiquitin chains in an E2-independent manner via its unique C-terminal LDD extension[7, 8].

Triad1 is the first identified member of the RBR E3-ligase family[6]. It is induced during granulocytic and monocytic differentiation[10], and is involved in regulating the proliferation of cells by stabilizing proteins such as Gfi1 and p53[11, 12]. The Triad1-mediated stabilization of these proteins is done in an E3-ligase activity-independent manner, with Triad1 competing against the binding of E3-ligases that target these proteins for degradation. Nevertheless, the Triad1 inhibitory effect on the proliferation of cells does also rely on its RING domains and can be inhibited by proteasome inhibitors[13]. Thus, the E3-ligase activity of Triad1 is important for the regulation of the proliferation of cells, but the targets for Triad1-mediated ubiquitination remain to be resolved.

The E3-ligase activity of Triad1 has not been extensively studied. It is known that Triad1 can mediate the formation of different ubiquitin chain types *in vitro*, however only the formation of K63 ubiquitin chains has been characterized to be mediated by Triad1 in cooperation with Ube2N/Ube2V2 (Ubc13/Mms2)[13]. Here, we performed an *in-vitro* analysis of the E3-ligase activity of Triad1 to gain more insight in its E3-ligase function and to identify new E2-Triad1 combinations that lead to the formation of ubiquitin chains.

Results and discussion

Triad1 functions as a monomer

For the analysis of Triad1, we purified full-length Triad1 and two Triad1 deletion-constructs using baculovirus infection in insect cells (Figure 1A). The optimal buffer conditions for Triad1 stabilization were identified by thermal-shift assays, which revealed that full length Triad1 is more stable in buffers with NaCl and a high pH-value (Figure 1B, C). The protein was most stable (Tm= 57.5Co) in buffer with pH 9, 150 mM NaCl, 10% glycerol and 5 mM βMe. However, for the *in-vitro* analysis of Triad1, we used buffers at the more physiological pH 8.0 (Tm= 56.5C°).

For the *in-vitro* analysis of Triad1, we first established the oligomeric state in which the protein is active. The 57.8 kDa Triad1 contains two RING domains, which are domains that often function as homo- or hetero-dimerization domains[3], indicating that Triad1 could potentially function as a multimer. We analyzed the oligomeric state of Triad1 by gel filtration in line with Multi-angle Laser Light Scattering (MALLS) (Figure 1D). Full length Triad1 eluted as a single peak form the analytical gel filtration column that corresponded to a calculated mass from the MALLS of 58.7 kDa, showing that Triad1 is a monomer in solution. Consequently, Triad1 is unlikely to form homo-dimers in cells and functions as a monomer in the *in-vitro* ubiquitin chain formation assays, which contain low concentrations of Triad1[13, this study].

Triad1 is active with a subset of the E2s

Triad1 interacts in cells with various E2s, amongst which Ube2N (Ubc13), Ube2L3 (UbcH7), UbE2E1 (UbcH6), Ube2E2 (UbcH8) and possibly Ube2Z (Use1)[10, 12, 14-16]. However, only the functionality of the interaction with Ube2N has been validated[13]. In this study we tested the *in-vitro* activity of Triad1 in cooperation with the known Triad1-interacting E2s Ube2N,

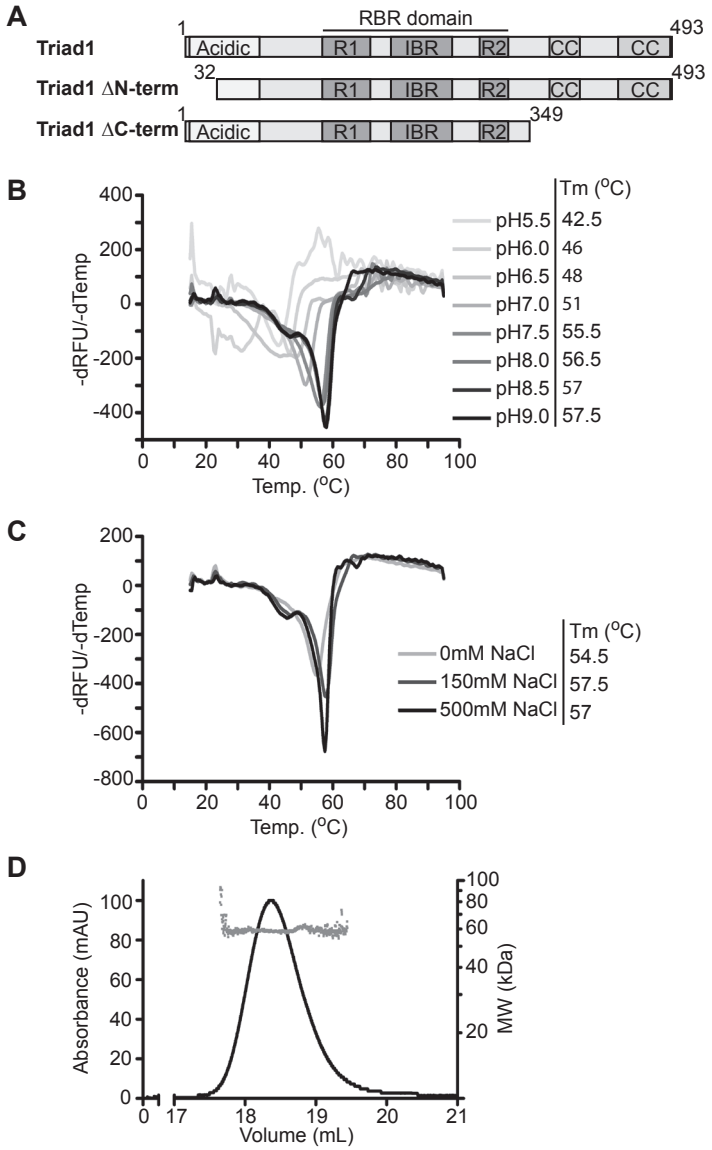

Figure 1. Protein properties of Triad1 (**A**) Triad1 constructs used in this study. Asp/Glu-rich domain (Acidic), Coiled coil (CC), RING-in-Between-RING domain (RBR) consisting of two RING domains (R1 and R2) and an in-between RING domain (IBR). The domain borders are drawn to scale according to Uniprot definitions (www.uniprot.org). (**B**) First derivative of the melting curves of Triad1 in 20 mM MMT-buffer (Malic acid, MES, Tris) with various pH-values, 150 mM NaCl, 10% glycerol and 5 mM βME. Melting temperature (Tm). (**C**) First derivative of Triad1 melting curves in the presence of 0 mM, 150 mM or 500 mM NaCl in buffer containing: 20 mM MMT pH 8.5, 10% glycerol and 5 mM βME. Melting temperature (Tm). (**D**) S200 10/300 gel filtration elution profile (280nM) of Triad1 (black line). The MALLS signal is plotted against the elution volume (grey dots). The single elution peak and calculated mass from the MALLS (58.7 kDa) show that Triad1 is a monomer in solution.

Ube3L3, UbE2E1, Ube2E2 and Ube2Z, as well as with purified Ube2D3 (UbcH5c), Ube2W and Ube2K (E2-25K) (Figure 2A). Triad1 mediated the formation of free ubiquitin chains in cooperation with the interaction partners Ube2E1, Ube2E2 and Ube2N, and was functionally active with the newly tested Ube2D3.

In agreement with an earlier study, Triad1 did not mediate the formation of free ubiquitin chains with the interaction partner Ube2L3[13] (Figure 2A). Also the interaction of Triad1 with Ube2Z was not validated by the *in-vitro* assay (Figure 2A). However, Ube2Z mediates the transfer of ubiquitin and Fat10[17], indicating that further research is needed to exclude this as a functional E2/E3-couple for Fat10. In sum, free ubiquitin chain formation by Triad1 is mediated by a subset of the E2s, including Ube2E1, Ube2E2, Ube2N and Ube2D3.

Triad1 mediated ubiquitin chain types are specified by the E2

The specific combination of RING E3-ligases with different E2s is important for the determination of the ubiquitin chain-type that is formed. For RBR E3-ligases the precise mechanism for determining chain type specificity is organized in different manners. The activity of some RBR E3-ligases seems to be dependent on the E2[9, 13], while the RBR E3-ligase HOIP mediates specific linear ubiquitin chain formation without contributions of the E2[7]. Triad1 has been indicated to mediate the formation of various ubiquitin chain types, but in cooperation with Ube2N/Ube2V2 it only forms K63-linked ubiquitin chains. Therefore, the formation of different ubiquitin chain-types by Triad1 was suggested to be E2-dependent[13].

To verify that Triad1 mediated ubiquitin chain formation specificity is dependent on the E2 conjugase, we compared the *in-vitro* ubiquitin chain formation activity of Triad1 in cooperation with Ube2D3, which catalyzes mixed-linkage ubiquitin chains, and the K63-linked ubiquitin chain forming Ube2N/Ube2V2. The Triad1-Ube2D3 complex mediated the formation of a high molecular weight smear of ubiquitin chains and each of the single lysine ubiquitin point mutants (K*R), as well as HIS-ubiquitin, were effectively used in ubiquitin chain formation (Figure 2B). Therefore, ubiquitin chain formation by Triad1-Ube2D3 is not selective for a single lysine on ubiquitin. In contrast, the Triad1-Ube2D3 complex was strongly impaired for chain formation with ubiquitins that lack all lysines (K0), and also the activity with each of the single lysine containing ubiquitins (K* only) was less than with wild-type ubiquitin. This indicates that Ube2D3-Triad1 does not mediate N-terminally linked ubiquitin chains and normally uses more than one ubiquitin lysine for chain formation. Consequently, Ube2D3 mediated ubiquitin chain formation with Triad1 targets multiple different lysines, possibly including mixed lysine-linked ubiquitin chains.

The complex of Triad1-Ube2N/Ube2V2 formed a specific pattern of poly-ubiquitin bands on SDS-PAGE-gel, corresponding to the formation of a single type of ubiquitin chains (Figure 2B). The ubiquitin chains were formed with all ubiquitin single lysine point-mutants (K*R), except with ubiquitin K63R. Furthermore, the K63-only ubiquitin could be used in ubiquitin chain formation by Triad1-Ube2N/Ube2V2. We hereby confirm that the chain formation activity

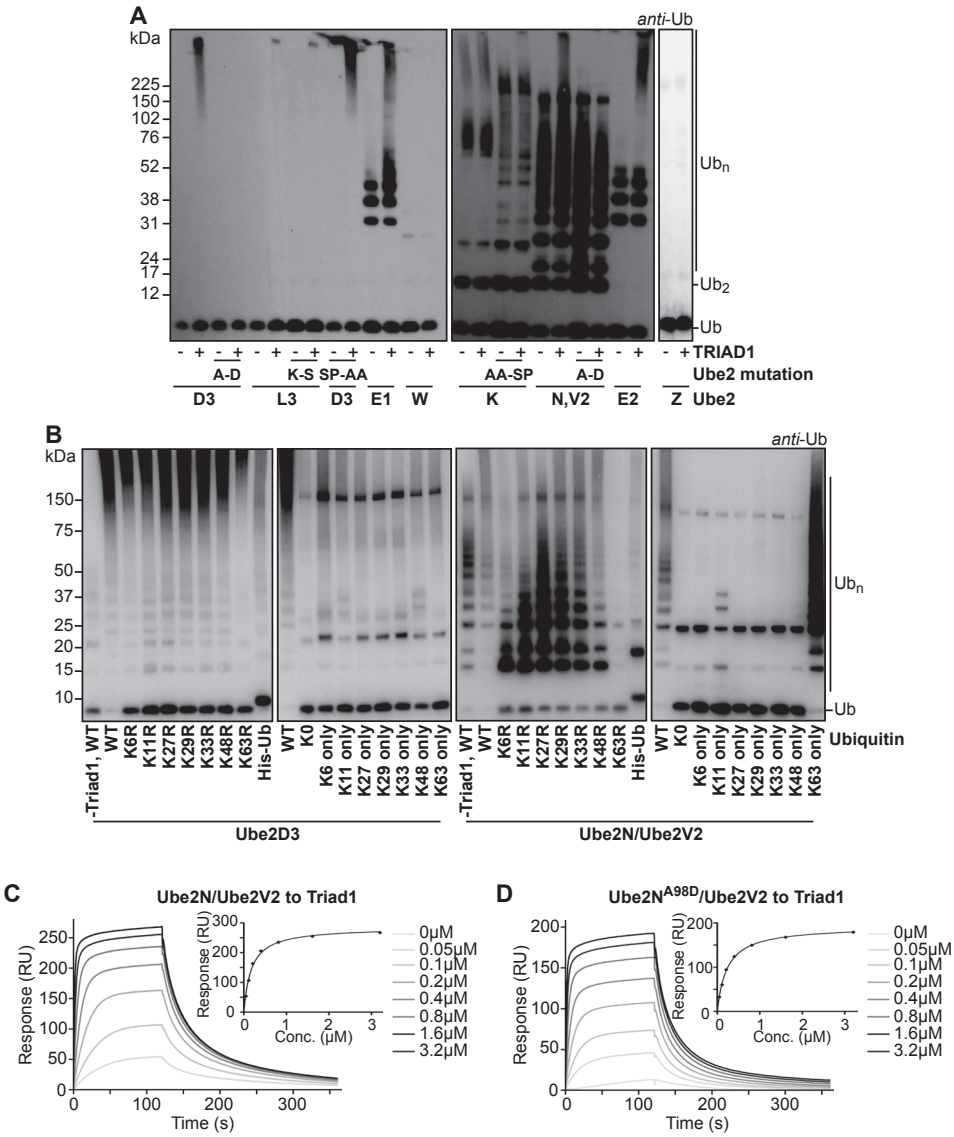

Figure 2. Triad1 is functionally active with a subset of the E2 ubiquitin conjugating enzymes (**A**) *In-vitro* free ubiquitin chain formation assay with Triad1 in the presence of different E2s and E2 point-mutants. (**B**) Ubiquitin chain formation assay with Triad1 in the presence of Ube2D3 or Ube2N/Ube2V2, and different ubiquitin mutants. Ubiquitin single lysine point mutants (K*R), ubiquitin with all lysines mutated into arginines (K0), single lysine containing ubiquitins (K* only). (**C**) Surface plasmon resonance analysis of the interaction between Triad1 and the E2 Ube2N/Ube2V2. A range of E2 concentrations (0-3.2 μM) was flown over a CM5-chip with Triad1. The K_D was determined at 174 nM (standard error 0.014) after fitting a single component model (inset). (**D**) Suface plasmon resonance analysis of the interaction between Triad1 and Ube2N$^{SPA\text{-}SPD}$/Ube2N2, similar to figure 2C. The K_D was determined at 229 nM (standard error 0.009) (inset).

of Triad1 in cooperation with Ube2N/Ub2V2 leads to specific K63-linked ubiquitin chains. Together, these results show that linkage-type of the ubiquitin chains that are formed by Triad1 is determined by the E2 that it cooperates with.

Functional Triad1-E2 pairs rely on the E2 SPA-motif

The Triad1 interacting E2s Ube2E1, Ube2E2 and Ube2N, as well as the promiscuous Ube2D3, are functionally active with Triad1 in *in-vitro* ubiquitin chain formation assays. All these E2s contain the SPA-motif that is essential for the formation of a functional complex with the RING E3-ligases CHIP and BRCA1[18, 19], while the non-functional Ube2L3 and Ube2K lack this motif (Supplementary Figure S1). Therefore, we tested if the SPA-motif is important for Triad1 mediated ubiquitin chain formation.

We introduced the SPA-motif in the non-functional E2s Ube2L3 and Ube2K, but the introduction of the SPA-motif did not enable chain formation with Triad1 (Figure 2A). Next we mutated the Ube2D3 SPA-motif into SPD and AAA. The Ube2D3$^{SPA\text{-}AAA}$ mutation did not change the activity with Triad1 (Figure 2A), however the Ube2D3$^{SPA\text{-}SPD}$ point mutation abolished all activity of the E2-E3 pair. In addition, the Ube2N$^{SPA\text{-}SPD}$ point mutant was also impaired in Triad1 mediated ubiquitin chain formation. These results indicate that the alanine in the SPA-motif of E2s is involved in Triad1 mediated activity.

Interestingly, the SPA-containing E2 Ube2W is not functionally active with Triad1 and also the introduction of the SPA-motif into Ube2L3 or Ube2K did not lead to the formation of ubiquitin chains with Triad1. Although, the E2 SPA-motif is used for the Triad1 mediated ubiquitin chain formation, other regions in the E2 are also needed for the formation of a functional E2-E3 pair with Triad1.

To gain further insight in the role of the SPA-motif in the formation of a functional E2/E3-complex with Triad1, we performed surface plasmon resonance experiments to measure the affinity between Triad1 and the different E2 variants. In these experiments, Triad1 was immobilized on a biacore-chip and the different E2s were flown over the chip. We could only determine binding curves for Ube2N/Ube2V2, because the other E2s introduced artifacts in the measurements by binding nonspecifically to the chip. Wild type Ube2N/Ube2V2 bound with an affinity of 174nM to Triad1 and the non-functional Ube2N$^{SPA\text{-}SPD}$/Ube2V2 mutant with an affinity of 229nM (Figure 2C, D). These data reveal that the affinities of wild type Ube2N/Ube2V2 and Ube2N$^{SPA\text{-}SPD}$/Ube2V2 for Triad1 do not differ significantly, which indicates that the E2-Triad1 interaction does not critically depend on the SPA-motif of the E2. Consequently, the E2 SPA-motif is important for the catalysis of Triad1-mediated ubiquitin chain formation, but other regions in the E2 are needed as well for the formation of a functional E2/Triad1 complex.

Triad1 mediated p53 ubiquitination is E2-dependent

The E3-ligase activity of Triad1 is essential for the clonogenic growth of cells; however targets for the ubiquitin chain formation activity of Triad1 have not been identified yet. Triad1 regulates

p53 and Gfi1 stability in cells by competing with other E3-ligases for the binding to these proteins[11, 13]. Nevertheless, unpublished data indicate that Triad1 might ubiquitinate p53 in cells as well (communication with Sylvie M. Noordermeer and Bert A. van der Reijden). Therefore we tested the *in-vitro* ubiquitination activity of Triad1 towards purified p53.

Triad1 was not highly active towards p53, but it did ubiquitinate p53 in cooperation with Ube2D3 over time (Figure 3A, B). Interestingly, even though the amount of mono-ubiquitinated p53 increased with increasing concentrations of Triad1, the ubiquitin chain-formation on p53 reached an optimum at a concentration of 1 μM Triad1 in conditions with 2 μM Ube2D3. The Ube2D3/Triad1 mediated poly-ubiquitination of p53 was reversed by the p53 specific de-ubiquitinating enzyme HAUSP (USP7)[20] (Figure 3B). These results show that p53 is a target for *in-vitro* Triad1 mediated ubiquitination.

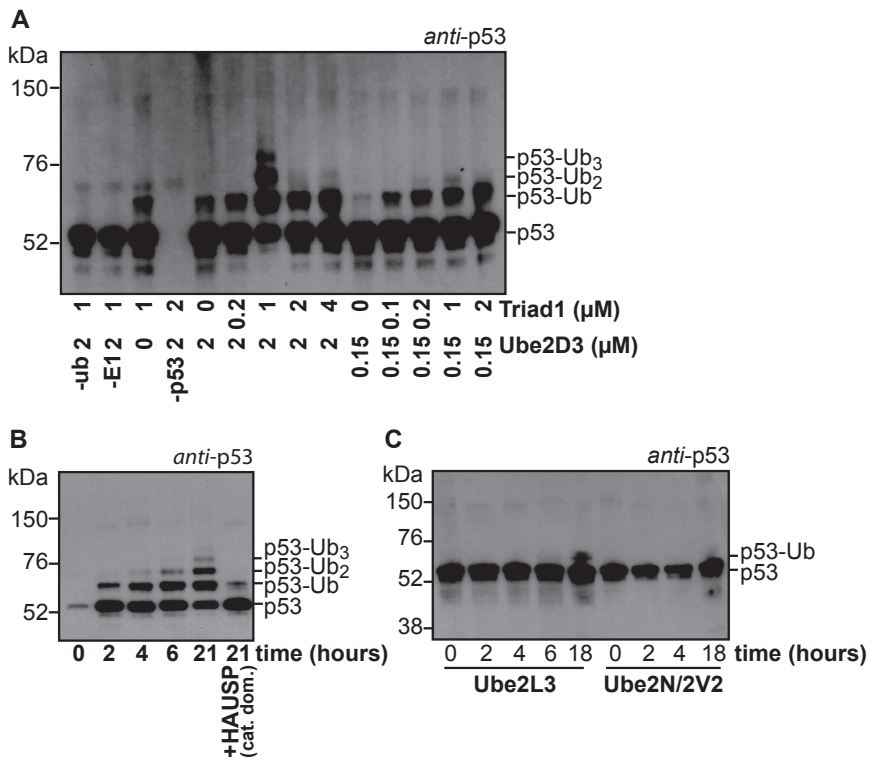

Figure 3. Triad1 mediates p53 ubiquitination (**A**) *In-vitro* ubiquitination of p53 by Triad1 in combination with Ube2D3. Reactions were stopped after 21 hours. Control reactions wereperformed in the absence of ubiquitin (-ub), E1 (-E1), E2 (0 μM Ube2D3) and p53 (-p53). (**B**) *In-vitro* p53 ubiquitination by Triad1 in cooperation with Ube2D3. After 21 hours, 14.5 uM HAUSP208-560 catalytic domain was added to one of the reactions and incubated for 2 hours at room temperature. (**C**) Triad1 mediated p53 ubiquitination with the E2s Ube2L3 and Ube2N/Ube2V2.

Next we tested the Triad1 mediate p53 ubiquitination in combination with Ube2L3, which does not mediate free ubiquitin chain formation, and Ube2N/Ube2V2 that is highly active for free ubiquitin chain formation. Surprisingly, the complex of Triad1 with Ube2L3 did mono-ubiquitinate p53 *in vitro*, while the Triad1/Ube2N/Ube2V2 complex did not modify p53 (Figure 3C). We conclude that Triad1/Ube2L3 forms an active E2/E3-pair that solely mono-ubiquitinates its targets, while the chain formation activity of Triad1/Ube2N/Ube2V cannot be directed towards p53. These results show that Triad1 dependent p53 ubiquitination is reliant on the E2 in the reaction.

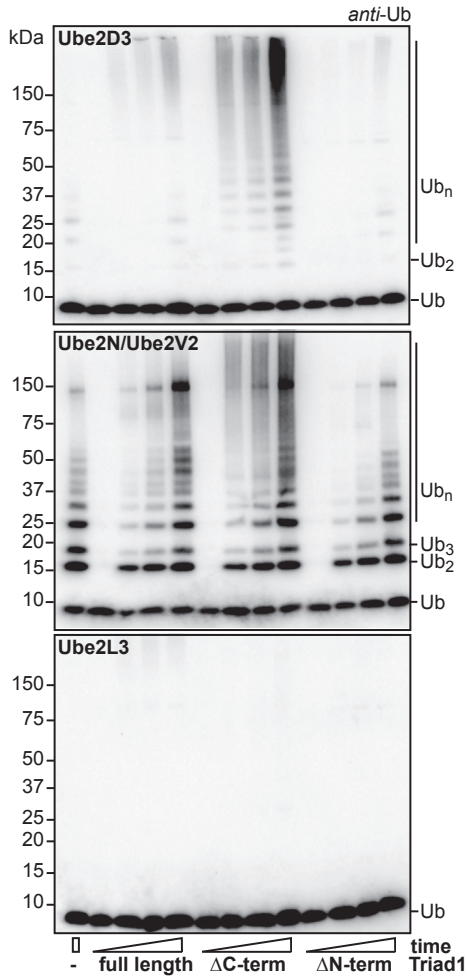

Figure 4. Triad1 is auto-inhibited by its C-terminus *In-vitro* ubiquitin chain formation activity of Triad1, Triad1ΔNterm and Triad1ΔCterm in cooperation with Ube2D3, Ube2N/Ube2V2 and Ube2L3, shows a higher activity of Triad1ΔCterm in cooperation with Ube2D3 and Ube2N/Ube2V2. Reactions were stopped after 0, 1, 2, 4 hours.

The Triad1 C-terminus auto-inhibits the E3-ligase activity

Triad1 mediates the *in-vitro* formation of free ubiquitin chains and ubiquitination of p53 in the presence of various E2s. However, other RBR E3-ligases such as Parkin and HOIP need additional factors to release their E3-ligase activity, because they are auto-inhibited by their N-termini[7, 8, 21]. Even though full length Triad1 is active *in vitro*, we tested if the E3-ligase activity of Triad1 is affected by its accessory domains. We purified N-terminal and C-terminal deletion constructs of Triad1 using baculovirus in insect cells (Figure 1A), and tested their activity in chain-formation assays.

Interestingly, the purified Triad1$^{\Delta N\text{-term}}$ was almost inactive in catalyzing free ubiquitin chain formation, indicating that the N-terminus of Triad1 is important for its RBR activity (Figure 4). In contrast, Triad1$^{\Delta C\text{-term}}$ was more active with the E2s Ube2D3 and Ube2N/Ube2V2 than full length Triad1 (Figure 4). However, the deletion of the C-terminus did not activate free ubiquitin chain formation with Ube2L3, indicating that Triad1 can not form ubiquitin chains with this E2 (Figure 4). Therefore, the C-terminus of Triad1 has a partial auto-inhibitory effect on the catalytic activity of Triad1.

Conclusions and outlook

The analysis of Triad1 revealed that it is functionally active as a monomer in solution, showing that the two RING domains in the Triad1 RBR do not function as multimerization domains for Triad1 molecules. We identified that a C-terminal region outside the Triad1 RBR domain, which restrains the *in-vitro* ubiquitin chain formation activity, internally modulates the catalytic activity of Triad1. A similar inhibition of the RBR domain by regions outside the catalytic center is seen in the RBRs Parkin and HOIP[7, 8, 21]. However, in contrast to these proteins, which require the binding of cofactors to release their catalytic activity, Triad1 is not completely inactivated by its C-terminus. Nevertheless, the Triad1 C-terminal auto-inhibition suggests that Triad1 may use specific binding-partners to release its full catalytic activity.

Interestingly, we found that the Triad1 mediated *in-vitro* ubiquitin chain formation activity towards p53 reached a Triad1 concentration-dependent optimum. In a previous study, we also observed such a concentration-dependent optimum for Triad1 in free ubiquitin chain formation assays[13]. The optimal Triad1 concentration only applies to ubiquitin chain formation, and not to the mono-ubiquitination of p53, indicating that the priming of p53 with the first ubiquitin and the subsequent ubiquitin chain formation are mediated via a different set of requirements. Intriguingly, this *in-vitro* concentration dependent auto-inhibition of Triad1 is only seen in very specific conditions, which are difficult to reproduce. Consequently, we could not systematically study this feature of Triad1, leaving the molecular mechanism underlying the concentration dependent auto-inhibition of Triad1 to be revealed. Nevertheless, these results show that, like for other RBR E3-ligases, some level of auto-regulation of the catalytic activity Triad1 is present.

The E3-ligase activity of Triad1 strongly depends on the cooperating E2 enzyme in the reaction. It forms free ubiquitin chains with a subset of the E2s that contain the SPA-motif, which is

known to be essential for the formation of various E2/E3 complexes. For example, the SPA-motif is critical for the functional interaction of E2s with E3-ligases in complexes of Ube2L3/c-Cbl[22], Ube2D3/Ring1b[23], Ube2D3/CHIP[19], Ube2N/CHIP[19], and it is important for the interaction of BRCA1 with several E2s[18]. The alanine in the SPA-motif, which is important for the BRCA1 interaction, is also essential for the functionality of the E2/Triad1 complex. However, the affinity of Triad1 for the Ube2N was not changed when the SPA-motif was mutated, indicating that other sites on the Ube2N are also needed for the formation of the Ube2N/Triad1 complex. We conclude that Triad1 activity critically relies on the E2 SPA-motif, but additional sites on the E2 are required for the E2/E3 interaction.

Even though the ubiquitin chain formation by Triad1 critically relies on E2s that contain the SPA-motif, Triad1 RING1 also interacts with Ube2L3 that lacks the SPA-motif[13]. The complex does not mediate the formation of ubiquitin chain, but we show that Triad1 mono-ubiquitinates p53 *in vitro* with Ube2L3. Ube2L3 is also functionally active with a wide range of HECT-type E3s[24], and with the RBR E3-ligases HHARI, Parkin and HOIP[7-9], to which it can donate its ubiquitin to an active-site cystein on the E3. The mono-ubiquitination of p53 by the Ube2L3/Triad1 complex reveals that Triad1 can be functionally active with Ube2L3, which does not contain the SPA-motif, and it adds a fifth RBR E3-ligase to the list of RBRs that are functionally active with Ube2L3 (HHARI[9], Parc[25], HOIP[7], Parkin[9], Triad1).

In this study we identified multiple E2s that form functional complexes with Triad1. The importance of the E2s in determining the target and chain type specificity varies between different RBRs and is not well understood[9, 26]. Our results show that in the case of Triad1, the *in-vitro* ubiquitin chain types that are formed and the Triad1 mediated p53 ubiquitination strongly depend on the E2 in the reaction.

The *in-vivo* targets for the Triad1 mediated ubiquitination are currently unknown. However, even though it has been shown that Triad1 regulates p53 stability in an -ligase activity independent manner[11], initial results from unpublished data show that p53 can also be ubiquitinated by Triad1 in cells. The *in-vitro* ubiquitination of p53 by Triad1 in our assays was very slow; consequently it is unlikely that the *in-vitro* reactions in this study directly resemble an *in vivo* situation. Nevertheless, the *in-vitro* p53 ubiquitination by Triad1 in cooperation with a subset of the E2s provides new evidence that the E3-ligase activity of Triad1 can be directed towards p53. Further research is needed to identify if p53 is a true target for Triad1 mediated ubiquitination *in vivo* and what the specific requirements for this reaction are.

In sum, the *in-vitro* analysis of Triad1 provided new insights in the functioning of the E3-ligase, however the molecular details by which the RBR-domain of Triad1 in specific and the whole RBR family in general mediates ubiquitin chain formation remains to be resolved. The structure solution of crystal structures of RBR domains (in complex with an E2) of different RBR E3-ligases will be of great value to shed light on the precise mechanism by which RBR domains form functional units. We crystallized Triad1 in various conditions (Supplementary figure S2), but unfortunately the crystals did not diffract to high enough resolution to solve the

structure. Therefore, the precise mechanism by which RBR E3-ligases in general, and Triad1 in specific, form functional units remains to be solved.

Materials and Methods

Plasmid construction

Full-length Triad1[13], Triad1$^{\Delta Cterm}$ (AA1-349) were cloned in frame with the His-tag in pFastBac-HTa to generate a bacmid for baculoviral transduction of insect cells. Triad1$^{\Delta Nterm}$ (AA32-493) was made by using the pFastBac-HTa full-length Triad1 as a template to introduce a 3C-protease site in front of residue 32. pGEX-6P-1 Ube2D3, pGEX-6P-1 Ube2L3, pGEX Ube2K and pGEX-4T-1 Ube2N/Ube2V2 point mutations of the SPA-motif were introduced by using the Quick-Change Mutagenesis Kit from Stratagene (La Jolla, CA, USA). The pET24-Ube2E2 and pET24-Ube2W were a gift from Rachel Klevit[18]. The pET24a-hITEV p53 superstable quadruple mutant M133L/V203A/N239Y/N268D expression construct was kindly provided by Caroline Blair and Alan R. Fersht[27].

Protein expression and purification

Triad1 and Triad1 constructs were expressed and purified as described previously[13]. Ubiquitin, hUba1, Ube2D3, Ube2L3, Ube2W, Ube2N/Ube2V2 were expressed and purified as described previously[13, 18, 28-30]. The hUba6 and Ube2Z proteins were kindly provided by Prakash Rucktooa. HAUSP208-560 catalytic domain was a gift from Alex Faesen[31]. p53 was expressed in E. coli Bl21 (DE3) pLysS cells in 2x TY medium by induction with 0.42 mM isopropyl-1-thio-b-D-galacto-pyranoside (IPTG) and 0.01 mM ZnSO4 overnight at 16°C. Cells were resuspended in 25 mM kPi pH 8, 300 mM NaCl, 5 mM Imidazole, 15 mM β-mercapto-ethanol (βME), Complete EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland), DNase I (Roche), 1 mM MgCl2 and RNase (Sigma). Cells were lysed by a high-pressure EmulsiFlex-C5 device (Avestin, Mannheim, Germany). Initial purification was achieved by binding the protein to a 5 mL HisTrap HP column (GE Healthcare), and elution over a gradient up to 300 mM Imidazole. The HIS-tag was cleaved from the protein with TEV-protease over night at 4°C while dialyzing against buffer containing 5 mM Imidazole. The sample was reverse-purified over a HisTrap HP column, followed by a 5 mL HiTrap Heparin HP column (GE Healthcare) from which p53 was eluted with a 0-600 mM NaCl gradient. Final purification was performed by gel filtration (Superdex 200) in 25 mM Kpi pH 7.5, 300 mM NaCl, 10% glycerol, 5 mM βME.

Thermal shift assays

Thermal shift assays were performed in 25 uL reactions in a Biorad PCR 96well-plate. Each well contained 4 μg protein and 0.03 μL Sypro Orange protein gel stain (Sigma). The thermal stability of the tested proteins was measured over a temperature range from 15°C to 95°C, by increasing the temperature every 2 minutes by 0.5°C on a Thermal Cycler with a MyIQ single color real-time PCR detection system (Bio-Rad) and using the MyIQ5 Bio-Rad software. Data processing was performed in Ms Excel.

Gel filtration in line with multi-angle laser light scattering (MALLS)

The MALLS experiment was performed on a Mini-Dawn light scattering detector (Wyatt Technology) in line with a Superdex S200 10/30 column at 4°C in buffer containing 2 5mM Tris/HCl pH 8, 150 mM NaCl, 1 mM ZnCl2 and 5 mM βME. Refractive index and light scattering detectors were calibrated against toluene and BSA. Data were analyzed based on the UV-signal by using the Astra software.

In-vitro ubiquitin chain formation assays

In-vitro ubiquitin chain formation reactions were performed in standard conditions, unless specified otherwise. Standard conditions for ubiquitin chain formation were 100 nM hUba1, 600 nM of the indicated E2, 1 μM E3, 15 μM ubiquitin and 10 mM ATP in buffer containing 20 mM Hepes/HCl pH 8, 150 mM NaCl, 10 mM MgCl2, 5 mM βME. Reactions with the E2 Ube2Z were performed in the presence of the E1 Uba6. p53 ubiquitination reactions contained 1 μM p53, 250 nM hUba1, 2 μM E2 and 2 μM Triad1. Standard reactions were performed at 32°C and stopped by the addition of protein loading buffer containing SDS and βME after two hours. Samples for anti-ubiquitin western blots were separated on 4–12% Nu-PAGE gels (Invitrogen) in MES buffer. Samples for anti-p53 western blots were separated on 10% Nu-PAGE gels (Invitrogen) in MOPS buffer. Western blot analysis was done by using anti-ubiquitin antibody (P4D1, Santa Cruz biotechnology), anti-p53 antibody (Pab240, Thermo scientific) and HRP conjugated anti-Mouse antibody (Bio-Rad, Hercules, CA, USA).

Surface plasmon resonance (SPR)

Triad1-E2 interactions were analyzed quantitatively using SPR experiments. The SPR experiments were performed as described previously, using lysine-coupling to a CM5-chip for the immobilization of Triad1[13]. Standard double referencing data subtraction methods were used before data analysis. Curve fitting and other data analyses were performed using Biaevaluation (GE Healthcare) and GraphPad software (GraphPad Software Inc., San Diego, CA, USA).

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Author contribution

JJS designed and performed the *in-vitro* experiments and wrote the manuscript. TKS supervised experiments and manuscript writing.

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Supplementary information

Materials and methods

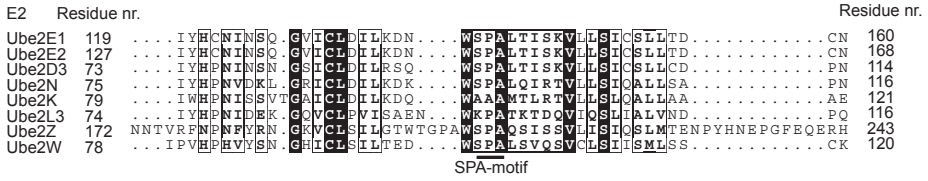
Multi-sequence alignment

Multi-sequence alignments were made with the online ClustalW server (*www.ebi.ac.uk/Tools/msa/clustalw2)* and the figure was produced with ESPript (*http://espript.ibcp.fr*).

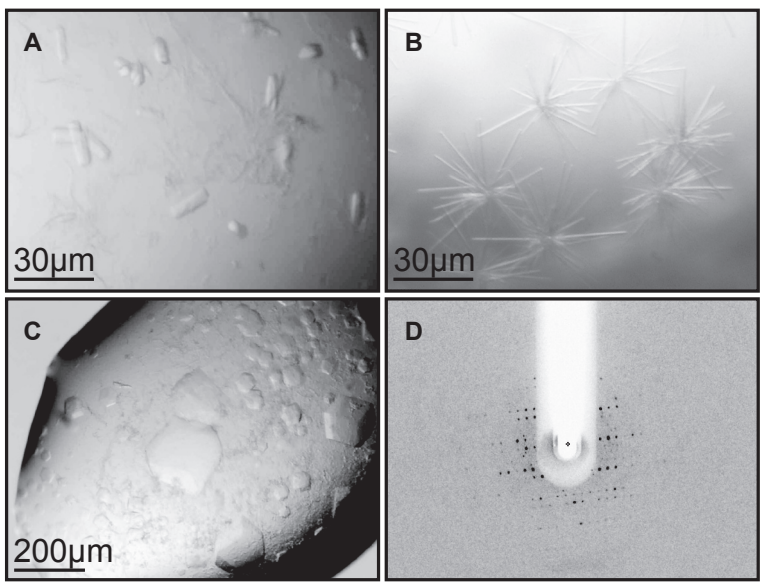
Crystallization and data collection

Triad1 crystallizations were performed at room temperature with a 6 mg/mL stock of Triad1 (cleaved HIS-tag).

The crystals were cryoprotected in mother liquor supplemented with 20% (w/v) ethylene glycol and the diffraction pattern was collected at the ESRF microfocus beamline ID23-2.



Supplementary figure S1. Multi-sequence alignment of the E2s that have been tested in the present study The sequence around the SPA-motif is shown. UniprotID Ube2E1 (P51965), Ube2E2 (Q96LR5), Ube2D3 (P61077), Ube2N (P61088), Ube2W (Q96FI0), Ube2Z (Q9H832), Ube2L3 (P68036), Ube2K (P61086). The SPA-motif is underlined by a black line.



Supplementary figure S2. Crystallization of Triad1 (**A**) Crystals of Triad1 in 100nL protein + 100nL precipitant sitting drops. Precipitant: 0.1M Tris pH8, 5% isopropanol (pH-clear screen). (**B**) Crystals of Triad1 in sitting drops containing 100nL protein + 100nL precipitant. Precipitant: 0.1M Hepes pH7.0, 10% (w/v) isopropanol (pH-clear screen). (**C**) Triad1 crystals in hanging drops containing 1uL protein and 0.1uL precipitant: 0.1M Tris pH9.0, 25% (w/v) PEG3350, 6mM Phenol. (**D**) Diffraction pattern of a crystal from the condition as shown in supplementary figure 2C, showing diffraction up to 20Å.

The E3-ligase HOIP specifies linear ubiquitin chain assembly through its RING-IBR-RING domain and the unique LDD extension

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Abstract

Activation of the NF-κB pathway requires the formation of Met1-linked 'linear' ubiquitin chains on NEMO, which is catalysed by the Linear Ubiquitin Chain Assembly Complex (LUBAC) E3 consisting of HOIP, HOIL-1L and Sharpin. Here, we show that both LUBAC catalytic activity and LUBAC specificity for linear ubiquitin chain formation are embedded within the RING-IBR-RING (RBR) ubiquitin ligase subunit HOIP. Linear ubiquitin chain formation by HOIP proceeds via a two-step mechanism involving both RING and HECT E3-type activities. RING1-IBR catalyses the transfer of ubiquitin from the E2 onto RING2, to transiently form a HECT-like covalent thioester intermediate. Next, the ubiquitin is transferred from HOIP onto the N-terminus of a target ubiquitin. This transfer is facilitated by a unique region in the C-terminus of HOIP that we termed 'Linear ubiquitin chain Determining Domain' (LDD), which may coordinate the acceptor ubiquitin. Consistent with this mechanism, the RING2-LDD region was found to be important for NF-κB activation in cellular assays. These data show how HOIP combines a general RBR ubiquitin ligase mechanism with unique, LDD-dependent specificity for producing linear ubiquitin chains.

Introduction

Ubiquitin conjugation is a highly versatile system for conferring post-translational modifications, since this 76-amino acid protein can make a variety of chains that signal to different downstream effectors. The ubiquitins in these chains are usually linked between the ubiquitin C-terminus of the donor ubiquitin and any of the seven lysines in the acceptor ubiquitin, but the donor ubiquitin can also link to the amino group in the N-terminal methionine of the acceptor ubiquitin, leading to the formation of linear ubiquitin chains.

Linear ubiquitin chains are assembled by the Linear Ubiquitin Chain Assembly Complex (LUBAC), which plays a critical role in the activation of the NF-κB pathway that is involved in various functions, including cell survival and inflammation. NF-κB activation can be induced by e.g. cytokines or DNA-damage, which lead to LUBAC-mediated ubiquitination of NEMO with linear ubiquitin chains[1, 2]. This linear ubiquitination of NEMO causes IKKβ phosphorylation and activation. Subsequently, IκBα is degraded and free NF-κB translocates to the nucleus to activate the transcription of target genes[3-6].

The LUBAC complex consists of at least three different proteins, HOIP (RNF31), HOIL-1L (RBCK1) and Sharpin[5, 7-9]. HOIP and HOIL-1L belong to the RING-in-between-RING (RBR) class of E3-ligases. However, only the RBR domain of HOIP and not HOIL-1L is required for linear ubiquitin chain formation by LUBAC and subsequent IKKβ phosphorylation[5, 10]. Nevertheless, a combination of HOIP with either HOIL-1L or Sharpin is the minimal requirement for linear ubiquitin chain catalysis[5, 7].

The RBR class of E3-ligases, also known as the TRIAD class (two RING fingers and DRIL (double RING linked)), was first described in 1999[11, 12]. The structures of the separate RING

domains and the in-between RING (IBR) have been solved (PDB entry 1WIM)[13, 14], however it remains unclear how the RBR forms a functional unit. RING1 has a classical RING fold, which is typically used for E2-E3 interactions[15, 16]. Also RING2 interacts with different E2s in yeast-two-hybrid studies and the cysteine and histidine distribution of RING2 suggests that it forms a RING domain[17-20]. However, even though Zn^{2+} stochiometry analysis indicates that all RING domains in Parkin coordinate two zinc ions[20], the solution structure of HHARI RING2 does not have a classical RING fold and coordinates only one zinc ion per monomer. Furthermore, the HHARI RING2 domain was recently shown to form a thioester adduct with ubiquitin (HHARI~ubiquitin) on a free cysteine as an intermediate step in the ubiquitin transfer[21], similar to that found in HECT domains. Although the thioester adduct could not be visualized on the RBR protein Parkin in the same study, mechanistic analysis indicated that both RBR proteins include a HECT-like step in the ubiquitin transfer.

An intact RBR domain is necessary for efficient E3-ligase functioning, however Parkin IBR-RING2 can mediate the formation of ubiquitin linkages in the absence of RING1[22, 23]. In addition to the interaction of both RING domains with E2 enzymes, the RBR of Parkin also interacts non-covalently with ubiquitin during chain formation[24].

The specificity for ubiquitin chain types is regulated completely at the level of the E3-ligase in HECT domains[25, 26]. In contrast, with RING domain E3-ligases the E2 enzymes contribute to the chain types that are formed. Some E2s directly mediate the formation of specific ubiquitin chains via the non-covalent binding of an acceptor ubiquitin, positioning a particular lysine residue to attack the thioester bond between the E2 and the donor ubiquitin[27-29]. A single RING E3 can recruit several of these E2s and makes different chains dependent on the E2 specificity[30, 31]. Occasionally the chain type that is being formed by a combination of a RING E3 and a less specific E2, such as Ube2D3 (UbcH5c), is determined by the specific E2-E3 combination[32, 33].

So far LUBAC is the only E3-ligase complex that is known to promote linear ubiquitin chain formation. Although it contains RING domains, its ubiquitin chain forming specificity overrides that of the collaborating E2 enzymes. Thus even highly specific E2s that are known to catalyze the formation of very specific chain types, such as Ube2K (E2-25K) that forms K48-linked chains[34], will form linear ubiquitin chains in the presence of LUBAC[5]. Therefore, chain type specificity is thought to be embedded within the LUBAC complex, but it is unclear how this specificity is organized.

We performed an *in-vitro* analysis of HOIP ubiquitin chain assembly activity to investigate the mechanism underlying linear ubiquitin chain formation by the LUBAC complex. We show that a truncated form of HOIP is active in *in-vitro* linear chain formation in the absence of HOIL-1L and Sharpin. The catalytic activity and specificity for linear ubiquitin chain assembly of the LUBAC complex is completely embedded within HOIP RING2 and a newly identified Linear ubiquitin chain Determining Domain (LDD) in the C-terminus of HOIP. Furthermore, we show that the ubiquitin thioester is first transferred from the E2

onto HOIP and is subsequently linked to a target ubiquitin that is docked on the LDD. This study strengthens the knowledge on the general mechanism for RBR mediated ubiquitin chain formation and provides novel mechanistic insights in linear ubiquitin chain assembly by HOIP.

Results

Linear ubiquitin chain formation specificity is embedded within HOIP

To study linear chain formation, we expressed full-length human HOIL-1L, full-length HOIP and a series of HOIP deletion constructs in *E. coli*. We used synthetic genes that are optimized for bacterial expression (Figure 1A) and used the purified proteins for *in-vitro* reactions, analysing free ubiquitin chain formation. As expected, full-length HOIP alone was not active in forming ubiquitin chains, but in the presence of HOIL-1L robust chain formation was observed (Figure 1B).

Since previous published data were derived from assays performed in the absence of sodium chloride[5, 7] under conditions that are far from physiological (~150 mM NaCl), we tested the influence of NaCl and pH on the *in-vitro* reactions. In the absence of salt the reactions were more active and it was easier to visualize detailed chains (Supplementary figure S1A, B), but the overall pattern of the bands on gel remained the same. Furthermore, the proteins were only active in conditions above pH 7 and raising the pH up to pH 9.5 caused a minor extra activation of the reactions (Supplementary figure S1C). We mainly used reaction conditions with 150 mM NaCl at pH 8; however, conditions without NaCl are used in some of our experiments as a tool to improve visualization of the activity of the LUBAC proteins.

Next, we used an N-terminally truncated form of HOIP, which includes only the RBR domain and the C-terminal region that we have named Linear ubiquitin chain Determining Domain (HOIP$^{RBR\text{-}LDD}$, Figure 1A). The sequence of the LDD is not conserved between RBR proteins, and Psi-BLAST searches and a Phyre threading analysis on this region reveal that it is exclusive to HOIP. Nevertheless, between HOIP orthologues the LDD is highly conserved (Supplementary figure S1D), which suggests that the LDD functions specifically in the context of the upstream RBR domain in HOIP. When we tested HOIP$^{RBR\text{-}LDD}$ for *in-vitro* activity we found, surprisingly, that this construct does not require HOIL-1L and Sharpin for *in-vitro* activity (Figure 1B, C). HOIP$^{RBR\text{-}LDD}$ does not contain the UBA domain that is needed for the interaction with HOIL-1L and Sharpin[2, 5, 9], explaining why the activity of HOIP$^{RBR\text{-}LDD}$ is hardly increased by the addition of HOIL-1L in the reactions (Figure 1D).

As HOIL-1L and Sharpin have been shown to be important for HOIP activity, we wondered whether the short RBR-LDD construct of HOIP retained the specificity for making N-terminally linked ubiquitin chains. We tested chain formation, using either Ube2D3 (UbcH5c) or Ube2L3 (UbcH7) as E2 enzymes. In both cases, HOIP$^{RBR\text{-}LDD}$ forms ubiquitin chains with lysine-less ubiquitin (K0) and mutated ubiquitins that contain either a single lysine or a lysine point mutation (Supplementary figure S1E). In addition, when the

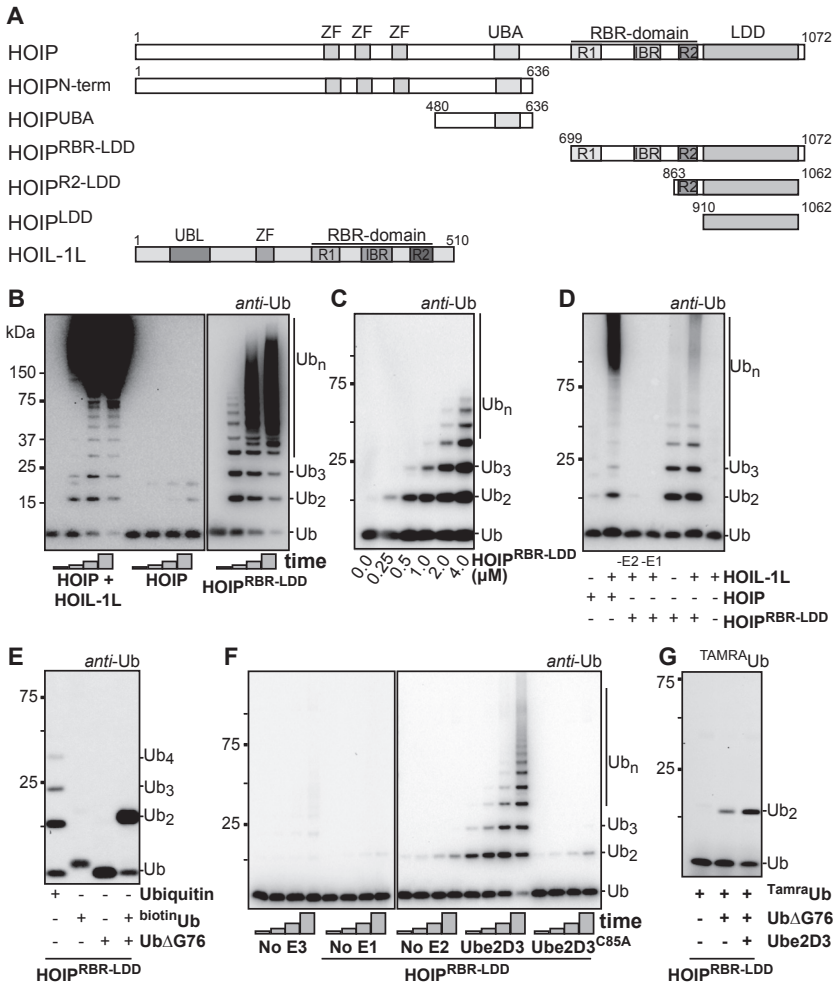

Figure 1. HOIP mediates linear ubiquitin chain formation (**A**) HOIP and HOIL-1L constructs used in this study. Ubiquitin Like domain (UBL), Npl4 Zinc Finger (ZF), Ubiquitin Associated domain (UBA), Linear chain Determining Domain (LDD) and a RING-in-Between-RING domain (RBR) consisting of two RING domains (R1, R2) and an in-between RING domain (IBR). The domain borders of the UBL, ZF, UBA and RBR-domains are drawn to scale according to Uniprot definitions (www.uniprot.org). (**B**) Ubiquitin chain formation with Ube2L3 in combination with 2 μM of the different E3s after 0, 10, 20 and 40 min. Standard reaction conditions are described in the materials and methods. Reactions were performed without NaCl. (**C**) Increasing amounts of ubiquitin chain formation with increasing concentrations of HOIP$^{RBR\text{-}LDD}$. Reactions were performed in the presence of Ube2D3 and were stopped after 1 hour. (**D**) HOIP$^{RBR\text{-}LDD}$ can not be further activated by HOIL-1L in a 1 hour reaction with Ube2L3. Reactions were performed without NaCl. (**E**) Ubiquitin chain formation by HOIP$^{RBR\text{-}LDD}$ with N-terminally blocked ubiquitin (^{biotin}ubiquitin) and C-terminally truncated ubiquitin (UbiquitinΔGly76). Reactions were stopped after 90 min at 32°C. (**F**) HOIP$^{RBR\text{-}LDD}$ activity with and without Ube2D3 and the Ube2D3 active site mutant (Ube2D3^{C85A}). Reactions were stopped after 15, 30, 60 and 120 min. (**G**) Di-ubiquitin linkage formation with ^{TAMRA}ubiquitin in the presence and absence of ubiquitinΔGly76 and Ube2D3 after 2 hours.

ubiquitin N-terminus is blocked with a His-tag, a biotin or a TAMRA-label, the ubiquitin chain formation is eliminated (Figure 1E, Supplementary figure S1F, G), indicating that the accessibility of the N-terminus is critical for this reaction. Combinations of any of the N-terminally blocked ubiquitins with ubiquitinΔGly76, which can only function as an acceptor ubiquitin, produces solely di-ubiquitins (Figure 1E, Supplementary figure S1F, G), confirming that a free ubiquitin N-terminus is essential for ubiquitin chain formation by HOIP. Consequently, the RBR-LDD in the C-terminus of HOIP is sufficient for the linear ubiquitin chain formation specificity of the LUBAC complex and does not require the presence of other LUBAC-proteins.

Since HOIL-1L and Sharpin are essential for full-length HOIP activity, but not for the $HOIP^{RBR\text{-}LDD}$, it seems that the catalytic centre is not available for catalysis in the full-length protein. The UBL domains of either HOIL-1L or Sharpin have to bind to the UBA domain of HOIP, which lies N-terminally of the catalytic RBR-LDD, to activate the proteins in the NF-κB pathway[35, 36]. This could suggest some level of auto-inhibition within HOIP, similar to that seen in the RBR protein Parkin, where the N-terminal UBL is binding to the C-terminal ubiquitin binding domain to block the catalytic center[24]. Therefore, we tested if the N-terminus of HOIP can inhibit $HOIP^{RBR\text{-}LDD}$ *in trans*. Full-length HOIP, $HOIP^{N\text{-}term}$ or $HOIP^{UBA}$ were added to the reaction with $HOIP^{RBR\text{-}LDD}$, but the constructs did not inhibit the $HOIP^{RBR\text{-}LDD}$ mediated chain formation (Supplementary figure S1H). Apparently the covalent linkage of the N-terminal domains to the RBR is required for the inhibition, either by increasing the local concentration or by arranging some position specific conformational change that can be released by the Sharpin or HOIL-1L interaction. Consequently, the exact mechanism by which the catalytic domain is kept in an inactive state in full-length HOIP remains to be resolved.

The active LUBAC complex mediates the specific formation of linear ubiquitin chains in cooperation with many different E2 enzymes that are normally highly specific in the formation of different types of ubiquitin chains[5]. This ability to override the E2 specificity is retained in $HOIP^{RBR\text{-}LDD}$. It specifically catalyses the formation of linear ubiquitin chains in the presence of the E2s Ube2D3, which can mediate the formation of many different types of lysine-linked ubiquitin chains[31], and Ube2L3, which targets to cysteines[21], indicating that the E2s are important to deliver the activated ubiquitin to the complex, but do not contribute to the chain type specificity.

HOIP has E2-independent linear chain forming activity

Interestingly, we observed very weak chain formation activity with $HOIP^{RBR\text{-}LDD}$ even with an inactive Ube2D3 mutant (C85A) (Figure 1F). Therefore, we analysed the $HOIP^{RBR\text{-}LDD}$ activity in the absence of E2 enzymes and still observed HOIP dependent activity (Figure 1F), confirming that the E3 does not require an E2 for activity. However, in the absence of the E1 no activity is observed (Figure 1F). The chains formed in the E2-independent reaction are exclusively linear ubiquitin chains (Figure 1G). A similar E2-independent activity was

recently described for the RBR-protein Parkin[22], indicating that this may be a general feature of RBR proteins. Nevertheless, E2-independent activity is unlikely to reflect a physiological activity, since the reaction is much more efficient in the presence of an E2~Ub thioester. However, these data emphasize that linear chain specificity does not rely on E2s, but is completely embedded within HOIP.

HOIP RING1 and IBR are involved in E2 mediated activity

We next examined how HOIP promotes linear ubiquitin chain formation. To address this point, we made a series of point mutations and deletion constructs to unravel the contributions of the various domains within HOIP$^{RBR\text{-}LDD}$ (Supplementary figure S2). The activities of all point mutants that are used in this study are shown in figure 2 and are summarized in supplementary figure S2. The effect of the mutations in the different domains of HOIP will be discussed throughout this article.

First, the importance of RING1 and the IBR were analysed. RING and IBR domains coordinate two zinc-ions via 8 Cys/His residues, whereby each zinc-ion is coordinated by 4 Cys/His residues. Cysteine mutations in RING1 that disrupt the coordination of the zinc-ions caused reduced E2 dependent activity with both Ube2D3 and Ube2L3 (Figure 2). Also HOIP$^{RBR\text{-}LDD}$ V701A, which was designed to interfere directly with the E2-E3 interaction but not to disrupt the RING fold[37], inhibited the ubiquitin chain formation. Interestingly, the C171,179A mutant solely disrupted Ube2L3 dependent activity and not Ube2D3 mediated chain formation, revealing a difference in the binding-interface between HOIP and different E2s. Nevertheless, the complete set of mutants reveals that RING1 is essential for the activity with both E2s. The E2-independent activity of HOIP$^{RBR\text{-}LDD}$ was not affected by the RING1 mutations, indicating a classical RING-type role for RING1 where the RING domain catalyzes the transfer from the E2 onto a target site. The IBR cysteine mutants also influenced the E2 dependent ubiquitin chain assembly, but not for E2-independent activity (Figure 2). Therefore, both RING1 and the IBR are important for E2 mediated ubiquitin chain formation by HOIP.

HOIP$^{R2\text{-}LDD}$ forms the minimal domain for linear ubiquitin chain formation

The linear ubiquitin chain assembly specificity of HOIP is preserved in HOIP RING1 mutants (Figure 3A) and RING1/IBR mutations do not affect the E2-independent activity (Figure 2), indicating that these domains are not used in the actual linkage formation between two ubiquitins. Accordingly, when the RING1 and IBR domains are deleted (HOIP$^{R2\text{-}LDD}$, Figure 1A), the ability to form ubiquitin linkages in an E2 independent manner is retained (Figure 3B). HOIP$^{R2\text{-}LDD}$ cannot be further activated by Ube2D3 (Figure 3B, Supplementary figure S3), showing the importance for RING1-IBR in E2 dependent activity. Thus a completely intact HOIP$^{RBR\text{-}LDD}$ is needed for efficient ubiquitin chain formation that is facilitated by the E2, but the intrinsic ubiquitin chain assembly activity is located more C-terminally in the RING2 and the LDD.

We aimed at mapping the regions in HOIP$^{R2\text{-}LDD}$ that are essential for ubiquitin chain catalysis. The importance of RING2 was explored by comparing the activity of HOIP$^{R2\text{-}LDD}$ and a construct that lacks all of the RBR-domain (HOIP^{LDD}) (Figure 1A). Although HOIP$^{R2\text{-}LDD}$ can still form E2 independent di-ubiquitin linkages, HOIP^{LDD} is catalytically inactive even at high concentrations (Figure 3C). In addition, single cysteine to alanine mutants of HOIP$^{RBR\text{-}LDD}$ RING2 are catalytically inactive (Figure 2). Therefore, RING2 is essential for ubiquitin chain assembly. Next, the relevance of the LDD in ubiquitin chain formation was investigated. We were unable to express constructs of HOIP that lack the LDD and all LDD mutants are catalytically inactive (Figure 2). Nevertheless, the LDD alone is not sufficient for catalysis. Consequently, the integrity of both RING2 and the LDD are needed for linear ubiquitin chain assembly by HOIP.

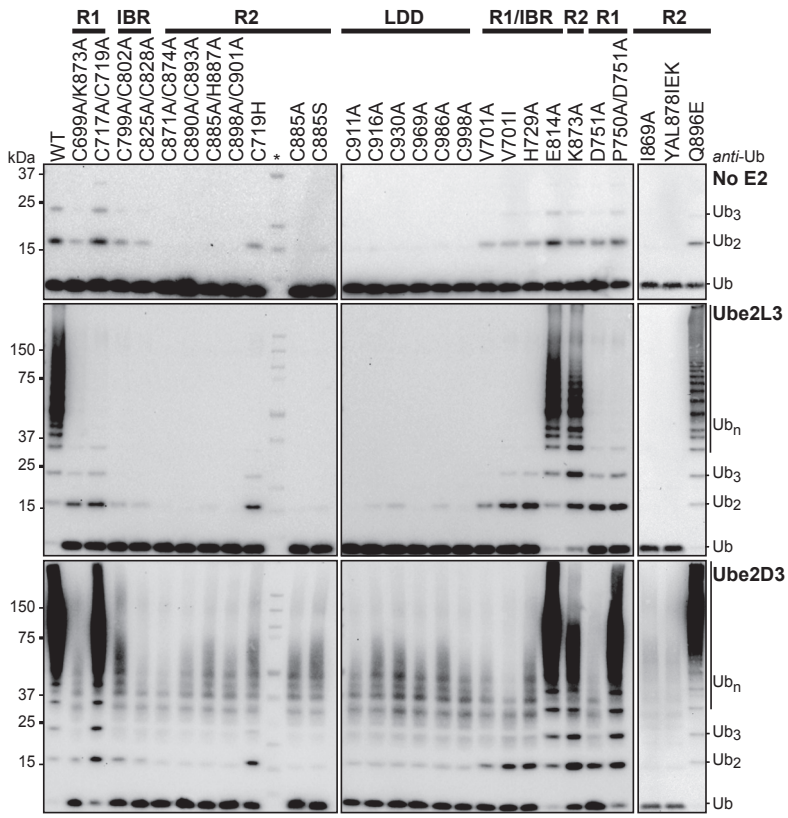

Figure 2. All domains in HOIP$^{RBR\text{-}LDD}$ are involved in ubiquitin chain formation Ubiquitin chain formation with HOIP$^{RBR\text{-}LDD}$ mutants in the presence and absence of the E2 Ube2L3 or Ube2D3. Reactions were stopped after 6 hours. The molecular weight marker is indicated by the asterisk (*). The solid bars indicate the location (RING1 (R1), IBR, RING2 (R2), LDD) of the mutations within HOIP$^{RBR\text{-}LDD}$.

HOIP mediates ubiquitin chain formation *in cis*

The presence of multiple copies of HOIP within the LUBAC complex[5] suggests that HOIP might assemble ubiquitin chains *in trans*. Therefore we next examined if the ubiquitin chain formation reaction is catalyzed by single HOIP molecules (*in cis*) or by the cooperation of multiple copies of HOIP (*in trans*). The gel filtration profile and multi angle laser light scattering (MALLS) of HOIP$^{RBR\text{-}LDD}$ show that the protein is purified as a mixture of monomers, dimers and multimers (Figure 3D). Nevertheless, the different fractions of the gel filtration profile show equal activity in free ubiquitin chain formation assays (Figure 3E), implying that the multimerization of HOIP$^{RBR\text{-}LDD}$ is not a requirement for activity.

To confirm these data, we combined inactive HOIP$^{RBR\text{-}LDD}$ mutants in chain formation assays to test whether they would collaborate to rescue the ability to form ubiquitin linkages. RING1 mutants are affected in E2 dependent chain formation and LDD mutants cannot support the formation of the isopeptide bond between two ubiquitins. Consequently, a combination of a RING1 mutant and a LDD mutant is expected to be effective in chain formation if the reaction occurs *in trans*. The combination of a RING1 and a LDD mutant did not lead to effective ubiquitin chain assembly, showing that the mutants do not complement each other (Figure 3F). Furthermore, a combination of a RING1 mutant and a RING2 mutant, or a RING2 mutant and a LDD mutant did not result in chain formation (Figure 3F), suggesting again that HOIP$^{RBR\text{-}LDD}$ proteins act individually and do not collaborate in ubiquitin chain formation. Finally, HOIP$^{R2\text{-}LDD}$ is purified mainly as a monomer (Figure 3D) and is still active in E2-independent chain formation. Therefore we conclude that multimerization of HOIP is not a requirement for activity and linear ubiquitin chain formation is catalyzed within single HOIP molecules.

HOIP forms a reversible covalent intermediate with ubiquitin

In light of the HECT-like character of RING2 in other RBR proteins[21], we tested whether HOIP could make a covalent thioester intermediate. We used single-cycle turnover assays, with pre-charged E2~^{TAMRA}ubiquitin thioester and were able to trap an E3-ubiquitin intermediate with HOIP$^{RBR\text{-}LDD}$. A covalent E3~^{TAMRA}ubiquitin complex could be visualized on non-reducing gels using anti-HOIP western blotting or, more clearly, by the TAMRA signal of ubiquitin (Figure 4A, B, Supplementary figure S4A (Ube2D3), S4B (Ube2L3)). The HOIP$^{RBR\text{-}LDD}$~ubiquitin intermediate could be disrupted by the addition of reducing loading buffer, which illustrates that HOIP$^{RBR\text{-}LDD}$ forms a reversible covalent bond with ubiquitin in cooperation with both Ube2D3 and Ube2L3.

Here we show for a second RBR protein the presence of an E3~ubiquitin thioester bond. The covalent HOIP~ubiquitin is transient, as indicated by the low signals, however the bond could be detected in the RING1 mutants as well as in the C916A LDD mutant (Figure 4A, Supplementary figure S4B). The RING2 mutants were completely impaired in forming this intermediate (Figure 4A, Supplementary figure S4B). RING2 has been suggested in literature as the actual site for the E3~ubiquitin thioester in RBR proteins, although

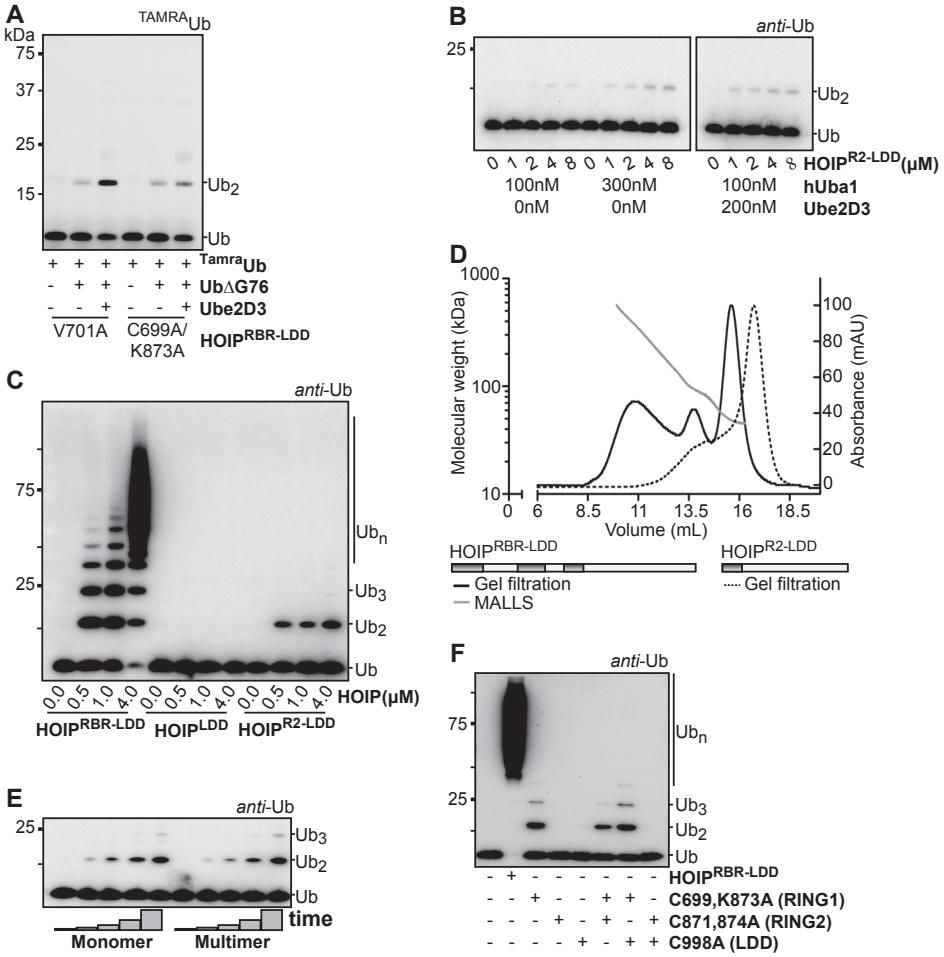

Figure 3. HOIP$^{R2\text{-}LDD}$ is the catalytic centre for *in cis* linear ubiquitin chain assembly (**A**) Di-ubiquitin formation with HOIP$^{RBR\text{-}LDD}$ RING1 mutants with ^{TAMRA}ubiquitin in the presence and absence of ubiquitinΔGly76 and Ube2D3. Reactions were stopped after 60min (**B**) HOIP$^{R2\text{-}LDD}$ mediated ubiquitin chain assembly after 2 hours cannot be activated by Ube2D3. (**C**) Activity of different HOIP constructs after 1 hour in the presence of Ube2D3 reveals that HOIP^{LDD} is not active. (**D**) S200 10/300 gel filtration elution profile of HOIP$^{RBR\text{-}LDD}$ (black line) and HOIP$^{R2\text{-}LDD}$ (dotted line). Maximum absorbance has been set at 100 mAU. The according calculated molar mass from the MALLS for HOIP$^{RBR\text{-}LDD}$ (gray dots) is plotted against the elution volume. Three peaks are identified, correlating to HOIP$^{RBR\text{-}LDD}$ monomers (42.9 kDa), dimers (85.8 kDa) and multimers (> 85.8 kDa). (**E**) Ubiquitin chain formation by HOIP$^{RBR\text{-}LDD}$ monomers and multimers. Monomers and multimers were taken from corresponding single fractions from the middle of the different peaks of the gel filtration profile and used immediately (without concentrating the samples) in ubiquitin chain formation reactions. HOIP$^{RBR\text{-}LDD}$ was assayed at 0.5 μM in the presence of Ube2D3. Time points were taken after 0, 10, 20, 40, 60 min. (**F**) Combinations of HOIP mutants do not rescue ubiquitin chain formation activity with Ube2D3 (assay performed in absence of NaCl). Mutations were introduced in RING1 (C699A, K873A), RING2 (C871,874A), and LDD (C998A).

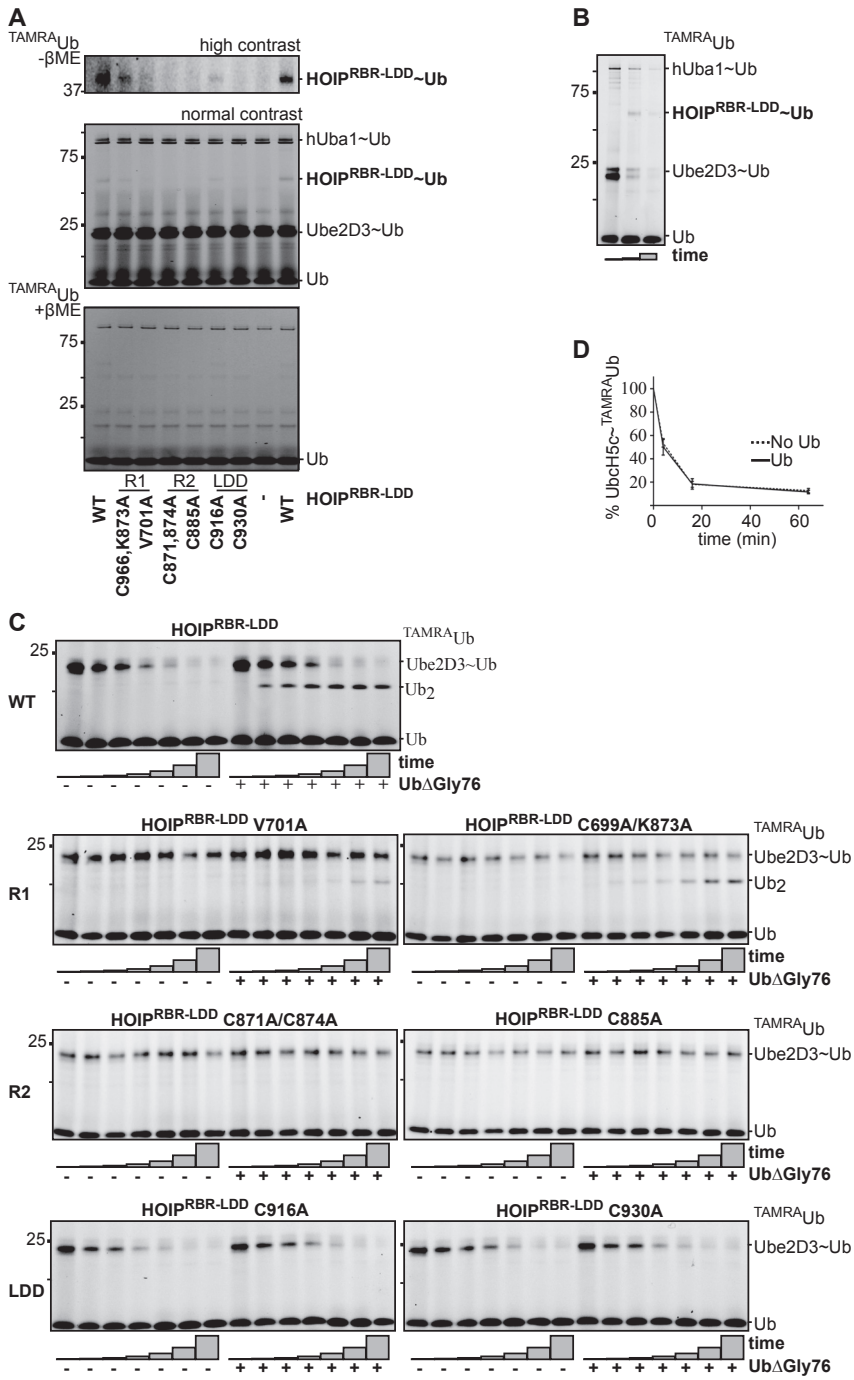

Figure 4. E2~ubiquitin discharge and chain formation are two separate events (**A**) Formation of a reversible covalent intermediate between HOIP and TAMRAubiquitin with different HOIP$^{RBR-LDD}$ mutants. Ube2D3 was used as the E2 enzyme. The TAMRA-signal is visualized on a reduced ►

visualization of the E3~thioester has only been successful for HHARI[21]. We could not assign the thioester forming cysteine, since several cysteines in RING2 are impaired in thioester formation. The HOIP Cys885 that aligns with the thioester-forming Cys357 in HHARI, could not form an oxyester HOIP~ubiquitin intermediate, when mutated to serine (Supplementary figure S4C). However, this could be due to detection limits of the assay, since the reaction is less favorable. Unlike the LDD, RING2 is conserved between RBR proteins (Supplementary figure S4D) and it is essential for E2~ubiquitin discharge and E3~ubiquitin formation. Therefore, it seems likely that RING2 provides the actual site on which the E3~ubiquitin is formed.

HOIP mediated ubiquitin transfer from the E2 onto a target is a two-step mechanism

To understand how the different domains within HOIP$^{RBR\text{-}LDD}$ contribute to the assembly of ubiquitin chains, we monitored the *in-vitro* E2~ubiquitin discharge and di-ubiquitin formation in single-cycle turnover assays with ^{TAMRA}ubiquitin and the selected purified HOIP$^{RBR\text{-}LDD}$ mutants. The amino-terminus of ^{TAMRA}ubiquitin is not available for linear ubiquitin chain formation and can only be linked to a ubiquitin with a free N-terminus by HOIP$^{RBR\text{-}LDD}$. This feature allowed us to uncouple the discharge of ubiquitin from the E2 active site cysteine (in the absence of ubiquitinΔGly76) and the formation of the isopeptide bond between the N- and C-terminus of two ubiquitins (in the presence of ubiquitinΔGly76).

HOIP$^{RBR\text{-}LDD}$ completely discharged ^{TAMRA}ubiquitin from the E2 over time in the single-cycle turnover assays and formed di-ubiquitins when ubiquitinΔGly76 was added to the reaction (Figure 4C, Supplementary figure S4E). The E2~ubiquitin discharge is less efficient when RING1 mutants are used in the reaction and also the amount of di-ubiquitin that is formed declines (Figure 4C, Supplementary figure S4E). This confirms the role of RING1 in E2 mediated activity. Nevertheless, the E2-independent activity of the RING1 mutants is hardly affected (Figure 2), showing that RING1 is less important for the E2 independent driven activity and the ubiquitin linkage formation.

The discharge of the ubiquitin from the E2 on HOIP and the linkage of the ubiquitin to a target ubiquitin by RING2-LDD were uncoupled in the single-cycle turnover assays. Although LDD mutants do not have any ubiquitin linkage formation activity in ubiquitin chain formation reactions, they are capable of efficiently discharging ubiquitin from the E2

► gel and at two contrast levels on a non-reduced gel. (**B**) Single-cycle turnover assay monitoring Ube2D3~^{TAMRA}ubiquitin discharge and HOIP~^{TAMRA}ubiquitin formation after 0, 4, 16 min at 37°C. (**C**) Single-cycle turnover assays showing Ube2D3~^{TAMRA}ubiquitin discharge by HOIP mutants (left half of each gel), and di-ubiquitin formation upon the addition ubiquitinΔGly76 (right half of each gel). Discharge reactions were stopped after 0, 2, 4, 8, 16, 32, 64 min. RING1 (R1), RING2 (R2). (**D**) Ube2D3~^{TAMRA}ubiquitin discharge rates in the presence and absence of an acceptor ubiquitin. Standard error for the Ube2D3~^{TAMRA}ubiquitin were calculated over three independent experiments.

(Figure 4C, Supplementary figure S4E). This indicates that the LDD is not involved in the destabilization of the E2~ubiquitin thioester, but is critical for ubiquitin chain assembly. Apparently the trans-thiolation of the ubiquitin from the E2 onto HOIP is independent of ubiquitin chain assembly. Accordingly, the E2~ubiquitin discharge efficiency is not dependent on the presence of an acceptor ubiquitin to which the donor ubiquitin can be transferred (Figure 4D). Interestingly, RING2 mutants are impaired in both the E2~ubiquitin discharge and the ubiquitin linkage formation (Figure 4C, Supplementary figure S4E), suggesting that RING2 is required for both steps of the ubiquitin transfer. This central role for RING2 in the transfer of the ubiquitin further supports its role as acceptor site for the E3~ubiquitin intermediate. Consequently, efficient ubiquitin chain formation is initiated by the E2-dependent delivery of ubiquitin to HOIP RING2 and is completed by subsequent LDD mediated ubiquitin chain assembly.

HOIP LDD catalyzes the final step of the ubiquitin transfer

The binding of ubiquitin to ubiquitin docking sites in E3-ligases is suggested to play a role in ubiquitin chain formation specificity by bringing a specific ubiquitin lysine residue in close proximity of the ubiquitin thioester bond[15]. HOIP has several known ubiquitin interaction motifs (UBA and the NFZs), but since these are not present in HOIP$^{RBR\text{-}LDD}$ they cannot explain the linear ubiquitin chain formation. We found that the LDD is important for linking ubiquitins together, but not for E2~ubiquitin discharge and HOIP~ubiquitin intermediate formation. Therefore we wondered whether the LDD could function as a ubiquitin docking site.

We measured the affinity of ubiquitin for HOIP$^{RBR\text{-}LDD}$ and HOIP^{LDD} by fluorescent polarization (FP) with TAMRA labelled ubiquitin (^{TAMRA}ubiquitin and ubiquitin^{TAMRA}). Both HOIP$^{RBR\text{-}LDD}$ and HOIP^{LDD} interacted with ubiquitin^{TAMRA} with an affinity of approximately 100 μM, and do not bind to the free TAMRA-dye, showing that the LDD does interact with ubiquitin (Figure 5A, Supplementary figure S5A, B). Then we analysed the effect of the C930A mutant of the LDD, which is impaired in ubiquitin chain formation, on the affinity for ubiquitin in the FP assays. Unexpectedly, in the context of HOIP$^{RBR\text{-}LDD}$, the C930A mutation did not affect affinity for ubiquitin^{TAMRA}. In contrast, in the LDD alone, the HOIP^{LDD} C930A has a greatly reduced affinity for ubiquitin^{TAMRA} (Figure 5A, Supplementary figure S5A). The loss of activity of the HOIP$^{RBR\text{-}LDD}$ C930A was not caused by unfolding of the protein as was shown by the gel filtration profile (Supplementary figure S2D). Therefore, the loss of binding of the LDD C930A mutant indicates interference with ubiquitin binding. In the longer construct, the mutation is possibly not strong enough to disrupt the complete interaction and just interferes with the proper ubiquitin orientation for chain formation or a second ubiquitin interaction site may be present elsewhere outside the LDD.

The interaction between the LDD domain and ubiquitin was verified in *in-vitro* ubiquitin chain formation assays. First, HOIP^{LDD} was titrated into the ubiquitin chain reaction with HOIP$^{RBR\text{-}LDD}$. The increasing amounts of HOIP^{LDD} inhibited HOIP$^{RBR\text{-}LDD}$ mediated ubiquitin

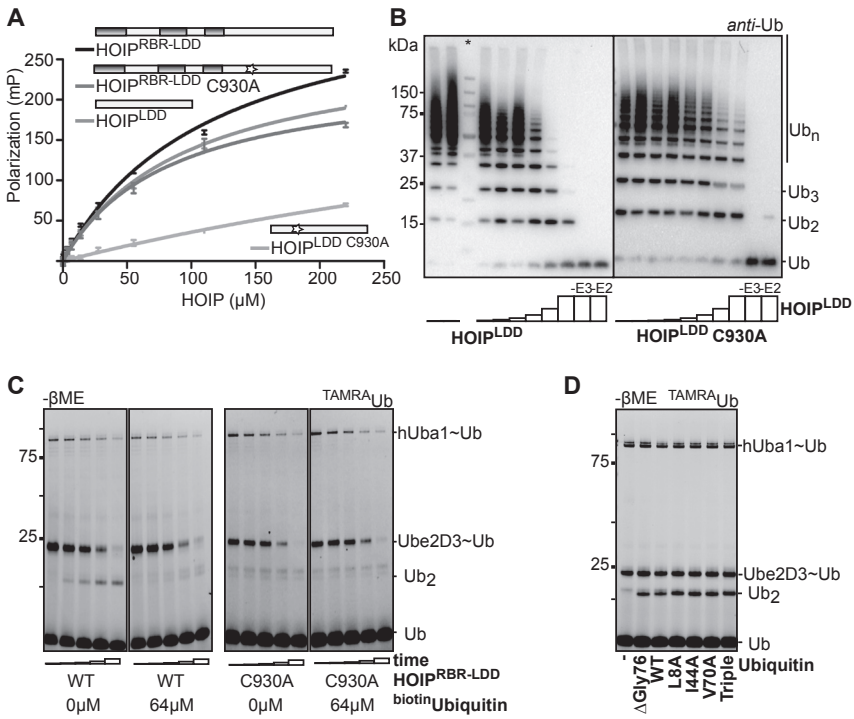

Figure 5. HOIP LDD interacts with the acceptor ubiquitin (**A**) FP assay of ubiquitin^{TAMRA} binding to HOIP, showing increase in FP as a function of [HOIP], HOIP$^{RBR\text{-}LDD}$ K_D= 118 ± 8 μM, HOIP$^{RBR\text{-}LDD}$ C930A K_D= 83 ± 9.2 μM, HOIP^{LDD} K_D= 97 ± 7 μM, HOIP^{LDD} C930A K_D= 734 ± 395 μM. Standard deviations were calculated over three repeats. (**B**) HOIP^{LDD} and HOIP^{LDD} C930A inhibition on ubiquitin chain formation by HOIP$^{RBR\text{-}LDD}$ in a concentration series of 0, 1, 2, 4, 8, 16, 32, 64 μM. Control reactions at the highest concentration of HOIP^{LDD} do not contain either HOIP$^{RBR\text{-}LDD}$ (-E3) or Ube2D3 (-E2). The molecular weight marker is indicated by the asterisk (*). (**C**) Single-cycle turnover assays in the presence and absence of the acceptor ubiquitin-competitor: ^{biotin}ubiquitin. The TAMRA-signal visualizes di-ubiquitin formation by HOIP$^{RBR\text{-}LDD}$ and Ube2D3~ubiquitin discharge by HOIP$^{RBR\text{-}LDD}$ C930A after 0, 2, 4, 8, 16 minutes. (**D**) Di-ubiquitin formation between ^{TAMRA}ubiquitin and different ubiquitin mutants visualized by the TAMRA-signal on a non-reduced gel. T= 10 minutes. Ubiquitin^{Triple} = L8A, I44A, V70A triple mutant.

chain formation, presumably by competing away the freely available ubiquitin (Figure 5B). Importantly, addition of the HOIP^{LDD} C930A to linear ubiquitin chain formation assays did not inhibit HOIP$^{RBR\text{-}LDD}$ mediated ubiquitin chain formation (Figure 5B). The loss of inhibition by the LDD C930A mutant indicates that this site is indeed important for ubiquitin interaction.

We then tested if the interaction between the acceptor ubiquitin and HOIP is needed for di-ubiquitin formation in a single-cycle turnover assay. Ube2D3 was loaded with ^{TAMRA}ubiquitin, after which the HOIP$^{RBR\text{-}LDD}$ dependent discharge of the E2~^{TAMRA}ubiquitin, and the linkage of ^{TAMRA}ubiquitin to wild type ubiquitin was monitored. To test if the acceptor ubiquitin

interacts with HOIP during the di-ubiquitin formation, a ^{biotin}ubiquitin, which cannot act as an acceptor, was added during the discharge reaction to compete with the wild type ubiquitin (Figure 5C). Under these conditions the E2~^{TAMRA}ubiquitin discharge and HOIP~ubiquitin intermediate formation were unaffected by the presence of ^{biotin}ubiquitin, showing that the transfer of the donor ubiquitin from the E2 onto HOIP was not affected (Figure 5C, Supplementary figure S5C). In contrast, the di-ubiquitin formation was inhibited by the ^{biotin}ubiquitin, suggesting that the N-terminally blocked ubiquitin competes with the wild type ubiquitin for binding to HOIP in the final step of the ubiquitin transfer.

These results are in line with the fact that the LDD mutants are impaired in ubiquitin-ubiquitin linkage formation but not in E2~ubiquitin discharge (Figure 4C, Supplementary figure S4E), showing that the LDD does not interact with the donor ubiquitin, but rather with the acceptor ubiquitin. Interestingly, the LDD/ubiquitin interaction does not require the ubiquitin hydrophobic patch, which is used by many ubiquitin interaction motifs[38], since point mutants of the hydrophobic patch still accept ^{TAMRA}ubiquitin via HOIP$^{RBR\text{-}LDD}$ (Figure 5D, Supplementary figure S5D). Consequently, the interaction between the LDD and ubiquitin is specific to HOIP, which is in line with the unique linear ubiquitin specificity that is evoked by HOIP.

The HOIP RING2 and LDD domains are crucial for NF-κB activation

Both RING2 and the LDD of HOIP are essential for linear ubiquitin chain formation *in vitro*. To determine the biological relevance of this finding, we tested wild type HOIP and the RING2 C885A, LDD C916A, LDD C930A, single and LDD C916,930A double mutants in HEK293FT cells. First, we verified the expression levels of the mutants and their capacity to interact with HOIL-1L in pull-down assays. All of the mutants retained the capacity to bind to HOIL-1L (Supplementary figure S6A), showing that the mutations did not cause major deficiencies in the folding of the full-length protein. Next, we tested the activity of the mutants in NF-κB luciferase reporter assays. In line with published data[2], the combined expression of HOIP and HOIL-1L resulted in NF-κB activation to levels approaching those when using MEK-kinase 1 (Figure 6)[39]. However, the combined expression of HOIL-1L with any of the HOIP cysteine mutants did not activate NF-κB. As expected, the observed NF-κB signalling activity was dependent on the presence of the presence of the HOIL-1L interaction domain, the ubiquitin-associated-domain (UBA domain) in HOIP, as well as on the co-expression with HOIL-1L, since neither wild-type nor HOIP mutants were active without it (Supplementary figure S6B). These data show that RING2 and the LDD are essential for linear ubiquitin chain formation and LUBAC mediated NF-κB activation in cells.

Discussion

NF-κB activation is an important signal during immune and DNA-damage responses. Upon stimulation, the formation of linear ubiquitin chains on NEMO forms an essential early event in the activation of the pathway. The linear ubiquitin chains are assembled by the

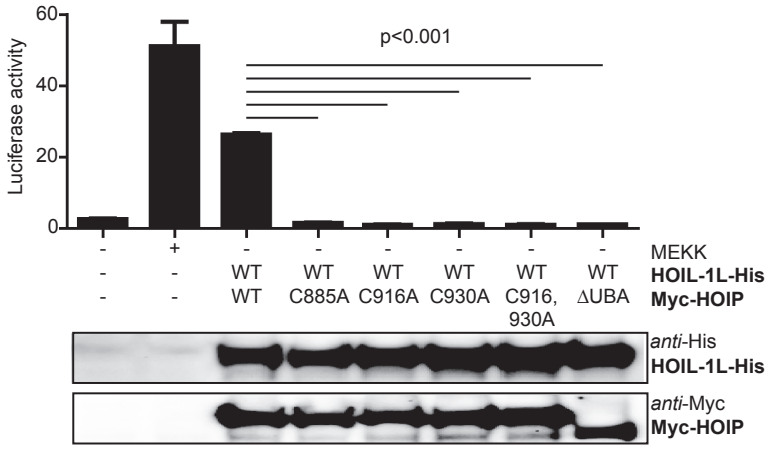

Figure 6. HOIP RING2 and LDD are essential for NF-κB pathway activation Dual Luciferase™ reporter assay for NF-κB activation. Full-length HOIP wild type or HOIP cysteine mutants were co-expressed together with HOIL-1L (see lower panel) and a luciferase reporter construct, containing 5 NF-κB binding sites. A luciferase renilla construct was used as transfection control. Firefly luciferase values were normalized to renilla luciferase values. Normalized luciferase activity of 5x NF-κB reporter vector (upper panel) is shown as mean ± s.e.m. (*P < 0.001, Student's t-test, representative experiment of n=4).

LUBAC complex, which contains the two RBR E3-ligases HOIP and HOIL-1L. It is poorly understood how RBR proteins form functional units to mediate the assembly of ubiquitin chains or how linear ubiquitin chain specificity is determined by the LUBAC complex.

Our results give a detailed insight in the molecular mechanism by which linear ubiquitin chains are formed by HOIP (Figure 7). We show that removing the N-terminal 698 residues of HOIP unmasks the full linear ubiquitin chain formation activity and bypasses the need for HOIL-1L and Sharpin. Within this active region of HOIP, the first two domains (RING1-IBR) are needed for the catalysis of the E2-mediated delivery of ubiquitin to the E3, but the mechanism that directs the linkage to the N-terminus of a target ubiquitin is embedded within HOIP RING2-LDD. The relevance of this activity in the context of the full-length protein within the LUBAC complex was verified by experiments in HEK293FT cells, showing that single point mutations in either RING2 or the LDD impair NF-κB signalling.

The transfer of ubiquitin from the E2 onto an acceptor ubiquitin is mediated by HOIP in a two-step mechanism. First, the ubiquitin thioester is transferred from the E2 onto HOIP, most likely on RING2, to form a reversible covalent intermediate (Figure 7C). This step can be catalyzed by the RING1-IBR mediated interaction with an E2~Ub thioester. Second, the ubiquitin is transferred from HOIP onto the N-terminus of the target ubiquitin to form an isopeptide bond. This uncoupling of the E2 catalyzed step from the transfer step to the acceptor ubiquitin explains why E2 enzymes do not affect the chain type specificity of HOIP.

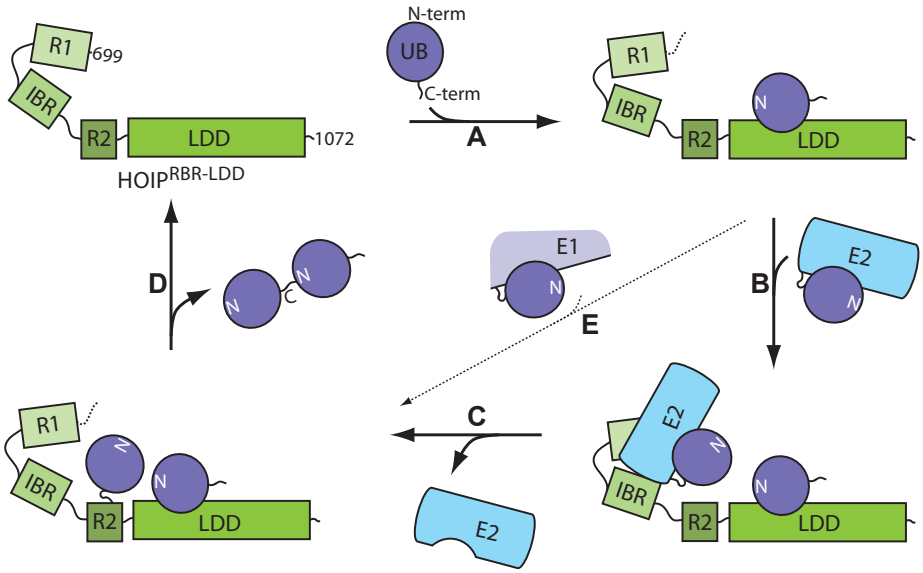

Figure 7. Model for HOIP$^{RBR-LDD}$ mediated ubiquitination Linear ubiquitin chain assembly requires both (**A**) the binding and correct orientation of an acceptor ubiquitin by the LDD and (**B**) the recruitment of an E2~ubiquitin to RING1-IBR. The ubiquitin is transferred from the E2 onto the acceptor ubiquitin in two independent steps. (**C**) First the ubiquitin thioester is transferred form the E2 onto RING2 and (**D**) second the ubiquitin is covalently linked to the N-terminus of the acceptor ubiquitin that is oriented by the LDD. (**E**) The E2 dependent activity can be bypassed by a less pronounced E2-independent activity.

The LDD is essential for the specific transfer of the donor ubiquitin from HOIP onto the acceptor ubiquitin (Figure 7). We have shown that the interaction between the LDD and the acceptor ubiquitin is important during this process, suggesting that it functions as a ubiquitin docking domain for the acceptor ubiquitin. The need for a C-terminal ubiquitin interaction-domain within HOIP is likely to reflect a general feature for ubiquitin chain catalysis of RBR proteins, since Parkin also contains a recently identified ubiquitin interaction domain, which is located just before RING2 of the RBR domain that is used in ubiquitin chain formation[24].

Among RBRs, the RING2-LDD uniquely promotes linear ubiquitin chain formation. This selectivity for the amino terminus is exquisite since the ubiquitin N-terminus and K63 are located close to each other, indicating that precise positioning of the acceptor ubiquitin by the LDD is very important. It seems plausible that RING2-LDD provides additional contributions to selective targeting, possibly by preferring the chemical constellation of the ubiquitin amino-terminus over a lysine amino group.

Materials and methods

Construction of plasmids

Codon optimized cDNA for *E. coli* expression of HOIP and HOIL-1L was obtained from Genscript. The cDNA was subcloned into pGEX-6P-1 vectors (GE Healthcare) with an N-terminal GST-tag for E. coli expression. $HOIP^{RBR\text{-}LDD}$, $HOIP^{R2\text{-}LDD}$ and $HOIP^{LDD}$ were cloned into a pETNKI-His-3C-LIC-amp vector for *E. coli* expression[40]. Mammalian expression constructs pcDNA3.1-HOIL-1L-His, pcDNA3.1-Myc-HOIP and pcDNA3.1-Myc-HOIP-$\Delta UBA^{563\text{-}616}$ were kindly provided by Dr. K. Iwai (Osaka University, Japan). UbiquitinΔGly76, Ubiquitin single and triple point-mutations and point-mutations in HOIP were introduced by using the QuikChange Mutagenesis Kit from Stratagene (La Jolla, CA, USA). The luciferase NF-κB reporter construct, pNF-κB-Luc, and the positive control pFC-MEKK were obtained from Agilent Technologies. Renilla luciferase vector, pRL-null, was obtained from Promega.

General, proteins and antibodies

Ubiquitin, hUba1, Ube2D3 and Ube2L3 were expressed and purified as described previously[19, 41-43]. ^{TAMRA}Ubiquitin, ubiquitin^{TAMRA}, ^{His6}ubiquitin and ^{biotin}ubiquitin (inhibition assay) were generously provided by Remco Merkx, Dharjath Hameed and Huib Ovaa[42]. ^{Biotin}Ubiquitin (di-ubiquitin formation assays) and ubiquitin lysine mutants were obtained from Boston Biochem.

Protein expression and purification

Full-length HOIP and HOIL-1L were expressed in E. coli Bl21 (DE3) pLysS cells by induction with 0.8 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 0.2 mM $ZnSO_4$ over night at 18°C. Cells were resuspended in 20 mM Tris/HCl pH 8.0, 100 mM NaCl, 5 mM βME and Complete EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). Cells were lysed by a high-pressure EmulsiFlex-C5 device (Avestin, Mannheim, Germany). Initial purification was achieved by binding the proteins to glutathione beads, washing the beads with buffer supplemented with 0.5 M NaCl and elution in buffer containing 50 mM GSH. The GST-tag was cleaved by incubation with 3C protease, followed by gel filtration (Superose6) online with a GST-column in 20 mM Hepes/HCl pH 8, 100 mM NaCl, 1 μM $ZnCl_2$ and 5 mM βME.

$HOIP^{RBR\text{-}LDD}$ (699-1072) was produced in E. coli Bl21 (DE3) pLysS cells. Expression was induced by the addition of 0.4 mM IPTG and 10 μM $ZnCl_2$ at an OD600 of 0.8 in LB medium supplemented with 50 μg/ml carbenicillin and chloramphenicol. Expressions were further cultivated over night at 16°C. The cells were lysed in 50 mM Tris/HCl pH 8, 150 mM NaCl, 2 mM Imidazole, 1 μM $ZnCl_2$, 5 mM β-mercapto-ethanol (βME) in the presence of complete EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland), DNAse and 1 mM $MgCl_2$ by a high-pressure EmulsiFlex-C5 device (Avestin, Mannheim, Germany). Cleared lysate was incubated with Talon beads. The protein was eluted from the beads

in buffer containing 200 mM imidazole and was subsequently loaded on a Resource-Q column. The His-tag was cleaved in solution with 3C protease. Further purification was achieved by a Heparin column followed by size-exclusion chromatography (Superdex 200) in buffer containing 25 mM Hepes/HCl pH 7.0, 150 mM NaCl, 1 μM $ZnCl_2$ and 5 mM βME.

$HOIP^{RBR\text{-}LDD}$ point-mutants, $HOIP^{R2\text{-}LDD}$ and $HOIP^{LDD}$ were expressed and purified as described for $HOIP^{RBR\text{-}LDD}$, excluding the cleavage of the His-tag and size-exclusion chromatography. For comparison with the $HOIP^{RBR\text{-}LDD}$ point-mutants, wild type $HOIP^{RBR\text{-}LDD}$ was prepared following the same protocol.

In-vitro ubiquitin chain formation

In-vitro ubiquitin chain formation reactions were performed in standard conditions, unless specified otherwise. Standard conditions for ubiquitin chain formation were 100 nM hUba1, 600 nM of the indicated E2, 1 μM E3, 15 μM ubiquitin and 10 mM ATP in buffer containing 20 mM Hepes/HCl pH 8, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT. Reactions were performed at 37°C and stopped by the addition of protein loading buffer containing βME. Samples were separated on 4-12% NuPAGE gels (Invitrogen) in MES-buffer and analysed by western blot using ubiquitin antibody (P4D1, Santa Cruz biotechnology) and HRP conjugated anti-Mouse antibody (BioRad).

Single-cycle turnover assays

Single-cycle turnover assays were performed in the same buffer conditions as described for the ubiquitin chain formation. ^{TAMRA}ubiquitin (500 nM) was loaded onto E2 (600 nM) in an ATP (1 mM) dependent manner via hUba1 (100 nM) in 120 μL final reaction volume for 20 min at 37°C. The charging reaction was terminated by depleting the ATP with 2U apyrase. After 5 min incubation at room temperature, the sample was divided into smaller aliquots to compare the effects of the addition of $HOIP^{RBR\text{-}LDD}$ (1 uM) and ubiquitinΔGly76 (500 nM). ^{Biotin}Ubiquitin was added simultaneously with $HOIP^{RBR\text{-}LDD}$ and wtUbiquitin in the acceptor ubiquitin competition assays. Reactions were performed at 37°C and stopped by the addition of non-reducing loading buffer on ice. Samples were analysed on 4-12% NU-PAGE gels (Invitrogen) in MES buffer and the TAMRA signal was visualized on a ChemiDoc XRS (BioRad). Band quantification of Ube2D3~^{TAMRA}ubiquitin was done with the ImageJ program (http://imagej.nih.gov/ij). Loading differences were accounted for by measuring the total amount of TAMRA signal per lane. Percentages normalized for the total amount of Ube2D3~^{TAMRA}ubiquitin at T=0. Western blot analysis was performed using anti-HOIP (ab85294, Abcam) and HRP-conjugated anti-Rabbit (BioRad) antibodies.

Covalent HOIP~ubiquitin intermediate formation

E2~ubiquitin was prepared as described for the single-cycle turnover assays in buffer containing 20 mM Hepes pH 8.5 and 5 mM βME. After the addition of Apyrase, HOIP (2 uM) was added to the mixture. Reactions were performed for 5 min on ice. The TAMRA

signal was visualized on a ChemiDoc XRS (BioRad) and $HOIP^{RBR\text{-}LDD}$ was visualized on non-reducing western blots with anti-HOIP antibody (ab85294, Abcam). Sample loading buffer was supplemented with 1 M Urea to partially unfold the proteins.

Fluorescence polarization assays

The fluorescence anisotropy of the C-terminal TAMRA-labeled ubiquitin (1nM) in binding buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 5 mM βME and 1 g/l chicken ovalbumin) was measured on a PerkinElmer EnVision 2010 Multilabel Reader. The binding was measured in 75 µL reactions. Serial 1:1-dilutions, starting at 220 µM HOIP, were performed in three repeats. Reactions were incubated for 20 min at 4°C before the measurements. The samples were excited at 531 nm and emission was measured at 579 nm, with correction for both the buffer background and G-factor of the instrument. The assays were performed in "non binding surface flat bottom" black 96-well plates (Corning) at room temperature. The resulting binding isotherms (anisotropy vs. HOIP concentration) were fit to a 1:1 non-linear binding model ($Y=Bmax*X/(K_D+X)$). All experimental data were processed using Ms Excel and Prism 4.03 (GraphPad Software, Inc.).

Multi-angle laser light-scattering (MALLS)

MALLS experiments were performed on a Mini-Dawn light scattering detector (Wyatt Technology) inline with a Superdex S200 10/30 column at 4°C in buffer containing 25 mM Hepes/HCl pH 7, 150 mM NaCl, 1 µM $ZnCl_2$ and 5 mM βME. Refractive index and light scattering detectors were calibrated against toluene and BSA. Data were analysed using the Astra software.

Cell culture and transient transfection

HEK293FT cells were cultured in Dulbecco modified Eagle medium (DMEM; GIBCO) supplemented with 10% non-heat-inactivated fetal calf serum (GIBCO), 1% penicillin/streptomycin (MP Biomedical), 1% non essential amino acids (GIBCO) and 1% L-glutamine (MP Biomedical). Cells were cultured in 24-wells plates at 37°C supplied with 5% CO_2.

For transient expression, 400 ng plasmid DNA (pcDNA3.1-HOIL-1L-His, pcDNA3.1-Myc-HOIP, pcDNA3.1-Myc-HOIP-mutants, pFC-MEKK) was used. Empty vector pcDNA3.1 was used to compensate for differences in DNA amounts. Furthermore, 400 ng of luciferase NF-κB reporter construct and 200 ng of Renilla luciferase vector were added to the transfection medium. In total 2 µg of DNA was transfected in each condition. Transfection was performed at 60% confluence with lipofectamine 2000 (Invitrogen). Each condition was experimentally tested in triplicate.

NF-kB transactivation assay

As readout for NF-kB activation we performed a Dual luciferase™ reporter assay (Promega). 48 Hours after transfection cells were washed with PBS and lysed in 100µL of passive

lysis buffer (Promega) for 1 hour. Luciferase assays were performed according to the protocol provided by the manufacturer (Promega). Luciferase signals were measured on the Lumat LB 9507 (EG&G Berthold). Western blot analysis was performed to confirm protein expression. Proteins of total lysates generated as described above were separated on 10% polyacrylamide gels and transferred to polyvinylidenefluoride (PDVF) membranes (Bio-Rad, Hercules, CA). PVDF membranes were probed with anti-Myc (Santa Cruz) and anti-His (Abcam) primary antibodies, followed by probing with HRP-conjugated secondary antibodies. Antibody signal was visualized by chemiluminescence using the Biorad ChemiDox XRS+.

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We thank Rick Hibbert for contributing to the cloning and purification of ubiquitin mutants, and suggesting the basis for the ^{biotin}ubiquitin inhibition experiment. Francesca Mattiroli for contributing to the E2 purifications. Dr. K. Iwai for mammalian expression constructs of HOIP and HOIL-1L, Remco Merkx for TAMRA-labeled ubiquitin and Dharjath Hameed for ^{biotin}ubiquitin. Members of the Sixma laboratory for discussions on the project. Rick Hibbert, Francesca Mattiroli, Danny Sahtoe, Flora Groothuizen and Fred van Leeuwen for critical reading of the manuscript. These studies were funded by an ERC advanced grant and EU Spine2C FP6 grant.

Author contribution

J.J.S designed and performed the *in-vitro* experiments and wrote the manuscript. W.J.v.D. contributed to protein expression, purification and western blots. D.M and S.M.N. designed and performed the cell culture based experiments. B.A.v.d.R. designed and supervised the cell culture based experiments. T.K.S. designed and supervised experiments and wrote the manuscript. All authors critically read the manuscript.

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Supplementary information

Materials and methods

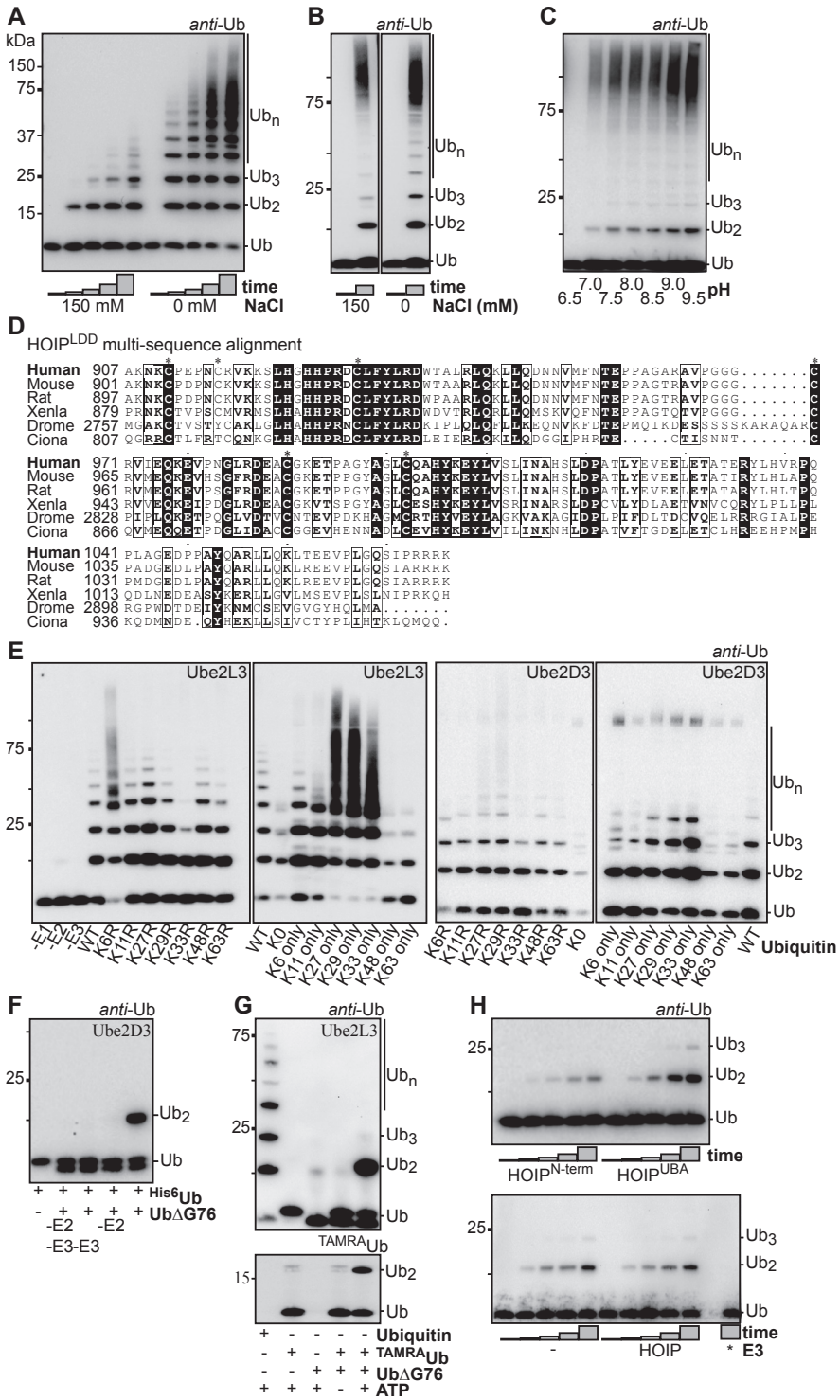
Multi-sequence alignment

Multi-sequence alignments were made in ClustalW and the figures were produced with ESPript (http://espript.ibcp.fr).

Co-immunoprecipitation (CO-IP)

For co-immunoprecipitation experiments, approximately 2.5 million cells were plated in standard round 9 cm culture petri dishes 24 hours prior to transfection. 16 hours post transfection the culture medium was refreshed and 40 hours post transfection, cells were harvested and lysed using RIPA buffer supplemented with complete protease inhibitors cocktail (Roche diagnostic) and benzonase (Novagen). Cell lysates were incubated with mouse anti Myc-antibody overnight at 4°C. The remaining cell lysates were used as control for protein expression on immunoblot. The immunoprecipitation was performed using Protein G Sepharose beads (GE Health care). Beads were washed with RIPA buffer and incubated with the antibody-containing-lysates for 2.5 hours at 4°C. After incubation, beads were washed with RIPA buffer to remove all non-specifically bound proteins. Proteins bound to the beads were released by adding SDS loading buffer and boiling at 95°C for five minutes. Western blot analysis was run to confirm protein expression. Proteins were separated on 10% polyacrylamide gels and transferred to polyvinylidenefluoride (PDVF) membranes (Bio-Rad, Hercules, CA). PVDF membranes were probed with anti-Myc (Santa Cruz) and anti-His (Abcam) primary antibodies, followed by probing with HRP-conjugated secondary antibodies. Antibody signal was visualized by chemiluminescence using the Biorad ChemiDox XRS+.

Supplementary Figure S1. (**A**) Ubiquitin chain formation by $HOIP^{RBR\text{-}LDD}$ and Ube2D3 in the presence and absence of 150 mM NaCl. Time points were taken after 0, 10, 20, 40, 80 min. (**B**) Ubiquitin chain formation activity of HOIP and HOIL-1L in the presence and absence of 150 mM NaCl. Ube2D3 was used as the E2 enzyme. Reactions were stopped after 0 and 30 min. (**C**) Ubiquitin chain formation by HOIP and HOIL-1L with Ube2D3 at increasing pH-values in 50 mM MMT-buffer. Reactions were stopped after 40 min. (**D**) Multi-sequence alignment of the LDD-domain of different HOIP orthologues. UniprotID: Human (Q96EP0), Mouse (Q924T7), Rat (E9PU29), Xenla (A0JMU3), Drome (Q8IPJ3), Ciona (Q1RL47). The asterisks indicate the sites of the cysteine mutations that have been introduced in this study. (**E**) Ubiquitin chain formation by $HOIP^{RBR\text{-}LDD}$, Ube2L3 or Ube2D3 and ubiquitin mutants. Single lysine ubiquitin point-mutants (K*R), all lysines mutated to arginine except for one lysine (K* only), all lysines mutated to arginine (K0). Control reactions lack either hUba1 (-E1); Ube2L3 and Ube2D3 (-E2); or $HOIP^{RBR\text{-}LDD}$ (-E3). Reactions were stopped after 1 hour at 32°C. (**F, G**) Ubiquitin chain formation by $HOIP^{RBR\text{-}LDD}$ with N-terminally blocked ubiquitin (^{His6}Ubiquitin or ^{TAMRA}ubiquitin) and C-terminally truncated ubiquitin (ubiquitinΔGly76). 2 Hour reactions contained hUba1 (E1), Ube2D3 or Ube2L3 (E2) and $HOIP^{RBR\text{-}LDD}$ (E3) unless indicated otherwise. (**H**) Ubiquitin chain formation with 600 nM $HOIP^{RBR\text{-}LDD}$ in the presence of 10 μM $HOIP^{N\text{-}term}$, 100 μM $HOIP^{UBA}$ or 2 μM HOIP. The lane with the asterisk shows the reaction with HOIP in the absence of $HOIP^{RBR\text{-}LDD}$. Reactions were stopped after 0, 10, 20, 40, 80 min at 37°C. ►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6.5 7.5 8.5 9.5
pH
D
HOIP^{LDD} multi-sequence alignment
Human 907 AKNKCPEPNCRVKKSLHSHHPRDCLFYLRDWTALRLQKLLQDNNVMFNTEPPAGARAVPGGG
Mouse 901 AKNKCPDPNCKVKKSLHSHHPRDCLFYLRDWTAARLQKLLQDNNVMFNTEPPAGTRAVPGGG
Rat 897 AKNKCPDPNCKVKKSLHSHHPRDCLFYLRDWPAARLQKLLQDNNVMFNTEPPAGTRAVPGGG
Xenla 879 PRNKCTVPSCMVRMSLHSHHPRDCLFYLRDWDVTRLQRLLQMSKVQFNTEPPAGTQTVPGGG
Drome 2757 MGAKCTVSTYCAKLGLHAHHPRNCLFYLRDKIPLQLQFLLKSQNVKFDTEPMQIKDESSSSKARAQAR
Ciona 807 QGRRCTLFRTCQNKGLHAHHPRDCLFYLRDLEIERLQKILQDGGIPHRTE.....CTISNNT.....
Human 971 RVIEQKEVPNGLRDEACGKETPAGYAGLCQAHYKEYLVSLINAHSLDPATLYEVEELETATERYLHVRPQ
Mouse 965 RVMEQKEVHSGFRDEACGKETPPGYAGLCQAHYKEYLVSLINAHSLDPATLYEVEELETATIRYLHLAPQ
Rat 961 RVMEQKEVPSGFRDEACGKETPPGYAGLCQAHYKEYLVSLINAHSLDPATLYEVEELETATARYLHLTPQ
Xenla 943 RVVEQKEIPDGLRDEACGKVTSPGYAGLCESHYKEYLVSRINARSLDPCVLYDLAETVNVCQRYLPLLPL
Drome 2828 PIPLQKETPQGLVDTVCNTEVPDKHAGMCRTHYVEYLAGKVAKAGIDPLPIFDLTDCVQELRRGIALPE
Ciona 866 QVMEQQETPDGLIDACGGEVHENNADLCEVHYKEYLVILINKNHLDPATVFTGDELETCLHREEHPMPH
Human 1041 PLAGEDPFAVQARLLQKLTEEVPLGQSIPRRRK
Mouse 1035 PADGEDLPAVQARLLQKLREEVPLGQSIARRRK
Rat 1031 PMDGEDLPAVQARLLQKLREEVPLGQSIARRRK
Xenla 1013 QDLNEDEASYKERLLGVLMSEVPLSLNIPRKQH
Drome 2898 RGPWDTDEIYKNMCSEVGVGYHQLMA.....
Ciona 936 KQDMNDE.QYHEKLLSIVCTYPLIHTKLQMQQ.
E
anti-Ub
Ube2L3
Ube2L3
Ube2D3
Ube2D3
75
25
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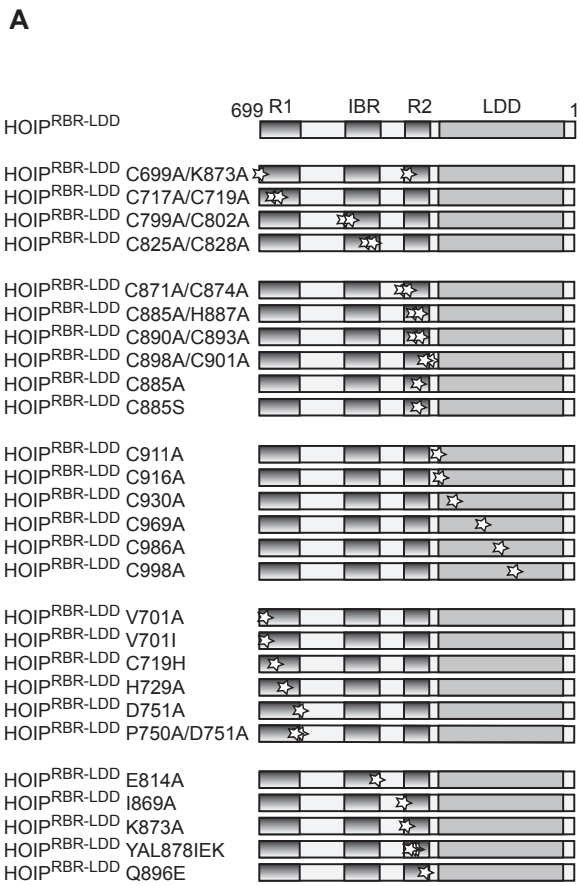
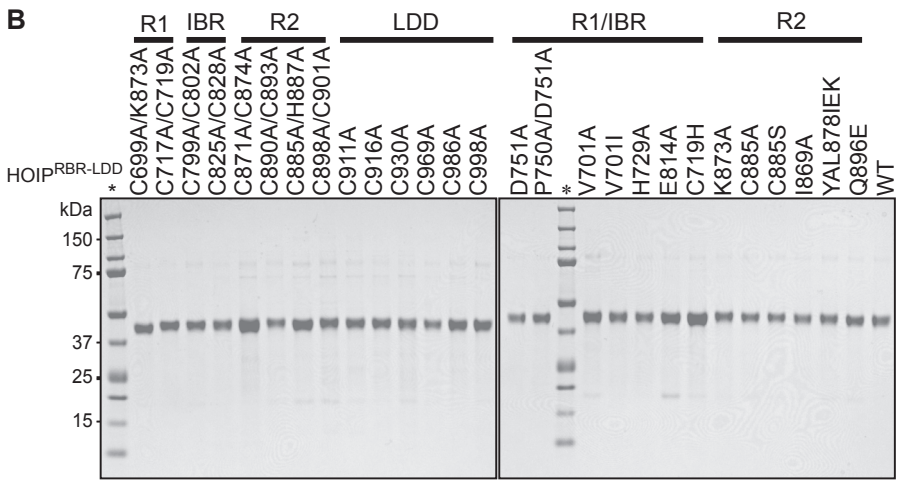
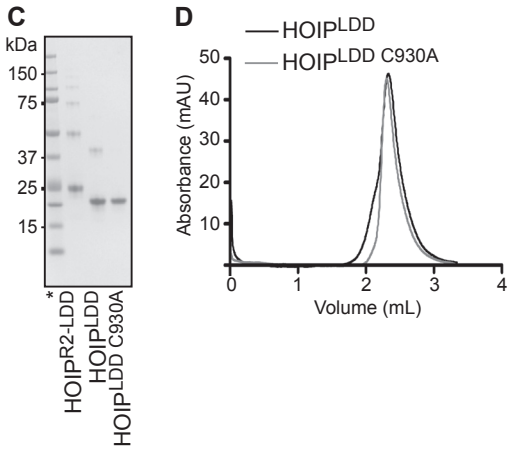


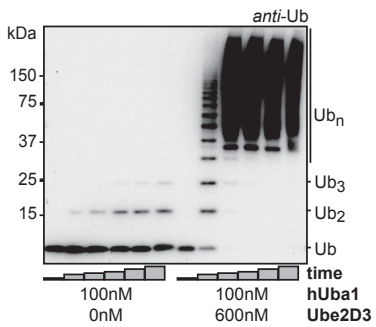
	Chain formation		E2 discharge	E3 thioester
	E2 dep.	E2 indep.		
$HOIP^{RBR-LDD}$	+++	+	++	+
$HOIP^{RBR-LDD}$ C699A/K873A	+	+	+	+
$HOIP^{RBR-LDD}$ C717A/C719A	+	+	nd	nd
$HOIP^{RBR-LDD}$ C799A/C802A	-	+	nd	nd
$HOIP^{RBR-LDD}$ C825A/C828A	-	+	nd	nd
$HOIP^{RBR-LDD}$ C871A/C874A	-	-	-	-
$HOIP^{RBR-LDD}$ C885A/H887A	-	-	nd	-
$HOIP^{RBR-LDD}$ C890A/C893A	-	-	nd	-
$HOIP^{RBR-LDD}$ C898A/C901A	-	-	nd	-
$HOIP^{RBR-LDD}$ C885A	-	-	-	-
$HOIP^{RBR-LDD}$ C885S	-	-	nd	-
$HOIP^{RBR-LDD}$ C911A	-	-	nd	+
$HOIP^{RBR-LDD}$ C916A	-	-	++	+
$HOIP^{RBR-LDD}$ C930A	-	-	++	-
$HOIP^{RBR-LDD}$ C969A	-	-	nd	nd
$HOIP^{RBR-LDD}$ C986A	-	-	nd	nd
$HOIP^{RBR-LDD}$ C998A	-	-	nd	nd
$HOIP^{RBR-LDD}$ V701A	+	+	+	+
$HOIP^{RBR-LDD}$ V701I	++	+	nd	nd
$HOIP^{RBR-LDD}$ C719H	+	+	nd	nd
$HOIP^{RBR-LDD}$ H729A	+++	+	nd	nd
$HOIP^{RBR-LDD}$ D751A	++	+	nd	nd
$HOIP^{RBR-LDD}$ P750A/D751A	++	+	nd	nd
$HOIP^{RBR-LDD}$ E814A	+++	+	nd	nd
$HOIP^{RBR-LDD}$ I869A	-	-	nd	nd
$HOIP^{RBR-LDD}$ K873A	+++	+	nd	nd
$HOIP^{RBR-LDD}$ YAL878IEK	-	-	nd	nd
$HOIP^{RBR-LDD}$ Q896E	+++	+	nd	nd

B

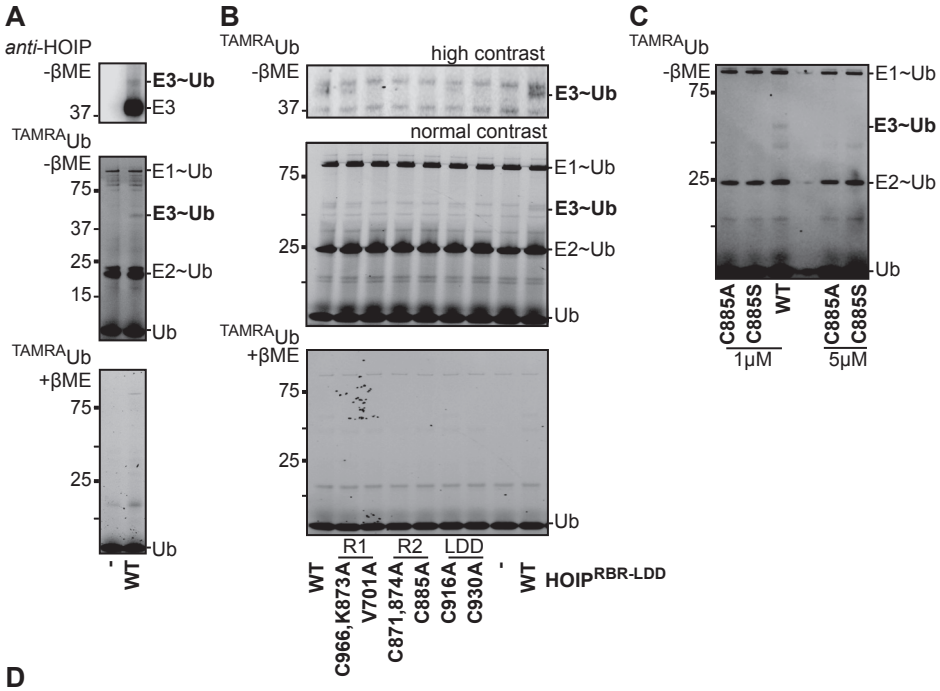




Supplementary Figure S2. (**A**) Schematic representation of the HOIP$^{RBR\text{-}LDD}$ point-mutants used in this study. Cysteine to alanine mutations in the RING domains and the IBR were designed to disrupt proper folding of the separate domains. The additional mutations were designed to explore RBR functioning without disrupting the fold of the domains, by interfering with possible E2-E3 interaction sites in the RING domains (V701A/I, K873A), changing residues that are likely to be surface exposed in RING1 (H729A) and the IBR (E814A), or residues that are located just outside the RING domains (P750A, D751A, I869A). All other RBR mutations were introduced at random throughout the protein to enhance our changes to identify the importance of the separate domains in linear ubiquitin chain formation. The cysteine mutations in the highly conserved cysteines of the LDD were introduced to cause local changes in the LDD to help unravel the importance of this domain in linear ubiquitin chain formation. The table lists the E2 dependent activity (E2 dep.), E2 independent activity (E2 indep.), discharge of ubiquitin from an E2 (E2 discharge) and E3~ubiquitin thioester formation (E3 thioester) of the mutants in the different experiments in this study. (**B**) Final purification product of the HOIP$^{RBR\text{-}LDD}$ mutants shown on SDS-PAGE gel. Solid bars indicate the location (RING1 (R1), IBR, RING2 (R2), LDD) of the mutations within HOIP$^{RBR\text{-}LDD}$. The molecular weight marker is marked with the asterisk (*). (**C**) Final purification product of HOIP$^{R2\text{-}LDD}$, HOIP^{LDD} and HOIP$^{LDD\ C930A}$ shown on SDS-PAGE gel. The molecular weight marker is indicated by the asterisk. (**D**) Analytical gel filtration profiles of HOIP^{LDD} and HOIP^{LDD} C930A on a S200 5/150 column.



Supplementary Figure S3. Control reactions with HOIP$^{RBR\text{-}LDD}$ for E2 dependent activity in figure 3B. The E2 increases the amount of ubiquitin chain formation with HOIP$^{RBR\text{-}LDD}$, but not with HOIP$^{R2\text{-}LDD}$. Reactions were stopped after 0, 1, 1.5, 2, 3, 4 hours.



D

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HOIP      681 AVRHSQDRAFLRRLLAQECAVCGWALPHNRMQALTSCECTICPDCFRQHFTIALKEKHITDMVCPACGRPDLTDD
HOIL-1L   271 .......RSLVLNTEPAECCPVCYSVLAPGEAVVLRECLHTFCRECLQGTIRN.......SQEAEVSCPFIDNTYS
ARIH1     171 RTRQMNTRSSAQDMP...CQICYLNYPN.SYFTGLECGHKFCMQCWSEYLTT......KIMEEGMGQTISCPAHG
Parkin    221 .ETSVALHLIATNSRNITCILCTDVRSP.VLVFQCNSRHVICLDCFHLYCVTRLNDRQFVHDPQLGYSLPCVAGC
Triad1    121 RVQPNPSKHVPTSHPPHHCAVCMQFVRK.ENLLSLACQHQECRSCWEQHCS.......VLVKDGVGVGVSCMAQD

HOIP      756 TQLLSYFSTLDIQLRESLEPDAYALFHKKLTEGVLMRDPK..FLWCAQCSFGFIYE.REQLEATCP.....QCHQ
HOIL-1L   332 CSGKLLEREIKALLTPED....KYQRFLDLGISIAENRSAFSYHCKTPDCKGWCFFE.DDVNEFTCP.....VCFH
ARIH1     236 CDILVDDNTVMRLITDSKVKLKYYQHLITNSFVECNRLLK...WCPAPDCHHVVKVQYPDAKPVRC......KCGR
Parkin    294 PNSLIKELHHFRILGEE....QYNRYQQYGAEECVLQMGG.VLCPRPGCGAGLLPE.PDQRKVTCEGGNGLGCGF
Triad1    188 CPLRTPEDFVFPLLPNEELREKYRRYLFRDYVESHYQLQ...LCPGADCPMVIRVQEPRARRVQCN.....RCNE

HOIP      823 TFCVFCKRQWEEQHR.......GRSCEDFQNWKRMNDPEYQAQGLAMYLQENGIDCPKCKFSYALARGCMHFHC
HOIL-1L   397 VNCLICKAIHEQMN.....CKEYQEDLALRAQNDVAARQTTEMLKVMLQQGEAMRCPQCQIVVQKK.DGCDWIRC
ARIH1     302 QFCFNCGENWHD.............PVKCKWLKKWIKKCDDDSETSNWIAANTKECPKCHVTIEKD.GGCNHMVC
Parkin    363 AFCRRCKEAYHEGECSAVFEASGTTTQAYRVDERAAEQARWEAASKETIKKTTKPCPRCHVPVEKN.GGCMHMKC
Triad1    255 VFCFKCRQMYHA..............PTDCATIRKWLTKCADDSETANYISAHTKDCPKCNICIEKN.GGCNHMQC

HOIP      891 T..QCRHQFCSGCYNAFYAKNKCP..........EPNCRVKKSLHGHHPRDCLFYLRDWTALRLQKLLQDNNVMF
HOIL-1L   466 T..VCHTEICWVTKGPRWGPGG.....................PGDTSGGCRCRVNGIPCHPSCQNCH......
ARIH1     363 RNQNCKAEFCWVCLGPWEPHGSAWYNCNRYNEDDAKAARDAQERSRAALQRYLFYCNRYMNHMQSLRFEHKLYAQ
Parkin    437 PQPQCRLEWCWNCGCEWN.........................RVCMGDHWFDV.....................
Triad1    316 S..KCKHDFCWMCLGDWKTHGSEYYECSRYKENPDIVNQSQQAQAREALKKYLFYFERWENHNKSLQLEAQTYQR

HOIP      954 NTEPPAGARAVPGGGCRVIEQKEVPNGLRDEACGKETPAGYAGLCQAHYKEYLVSLINAHSLDPATLYEVEELET
HOIL-1L   ....................................................................................
ARIH1     438 VKQKMEEMQQHNMS.WIEVQFLKKAVDVLCQCRATLMYTYVFAFYLKKNNQSIIFENNQADLENATEVLSGYLER
Parkin    ....................................................................................
Triad1    389 IHEKIQERVMNNLGTWIDWQYLQNAAKLLAKCRYTLQYTYPYAYYMESGPRKKLFEYQQAQLEAEIENLSWKVER

HOIP      1029 ATERYLHVRPQPLAGEDPPAYQARLLQKLTEEVPLGQSIPRRRK..
HOIL-1L   .....................................................
ARIH1     512 DISQDSLQDIKQKVQDKYRYCESRRRVLLQHVHEGYEKDLWEYIED
Parkin    .....................................................
Triad1    464 ADSYD.....RGDLENQMHIAEQRRRTLLKDFHDT...........
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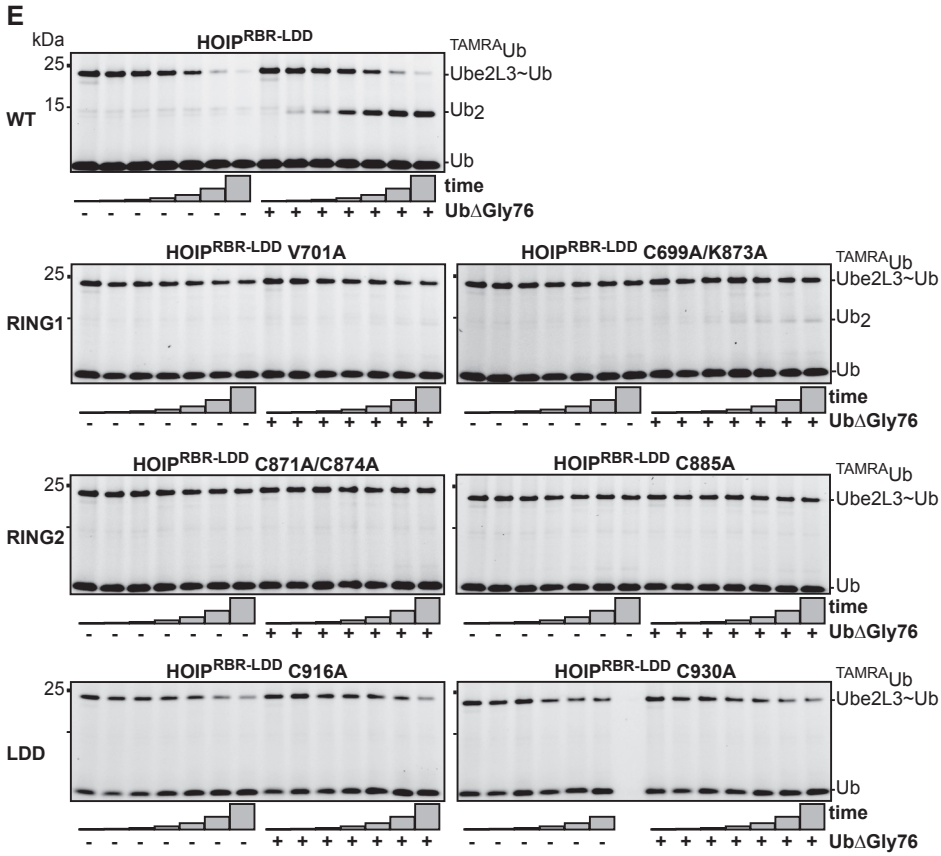
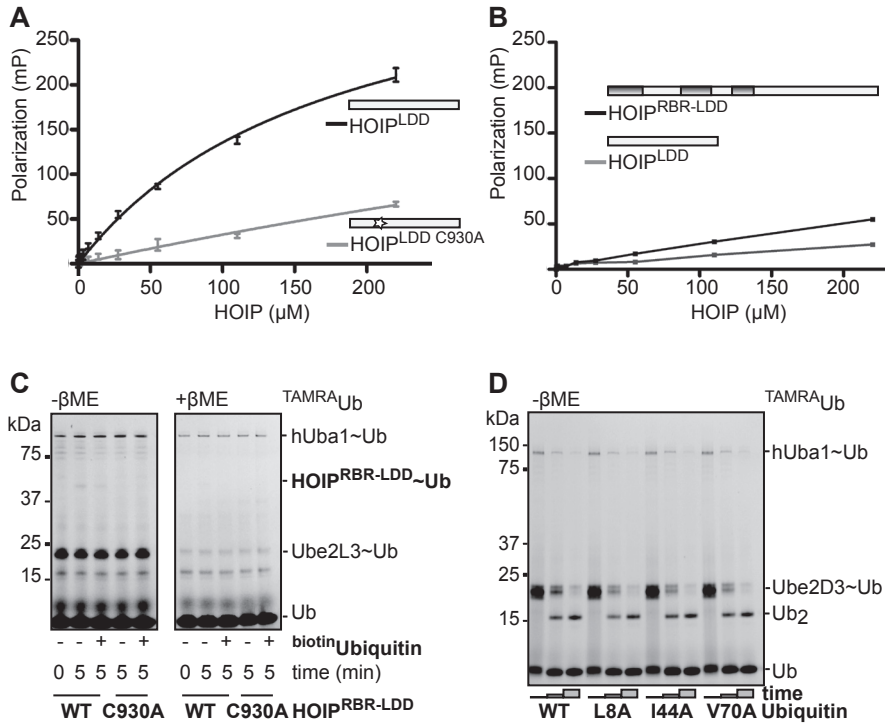
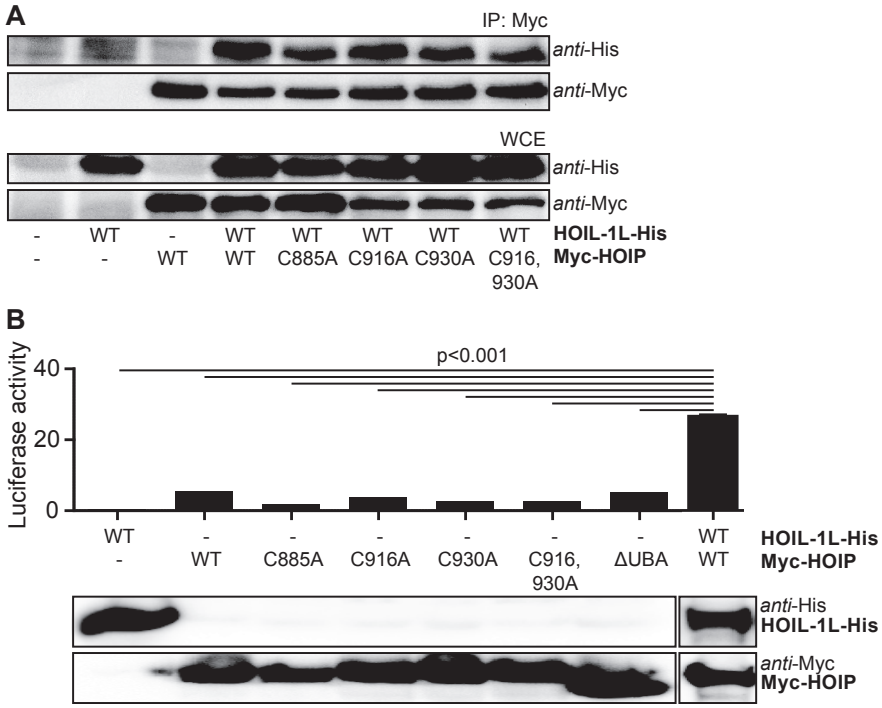


Supplementary Figure S4. (**A**) Formation of a reversible covalent intermediate between HOIP$^{RBR\text{-}LDD}$ and ^{TAMRA}ubiquitin. An anti-HOIP western blot and the according reduced and non-reduced gels for the TAMRA-signal are shown. The reversible covalent bonds are indicated as E1~Ub (hUba1), E2~Ub (Ube2D3) and E3~Ub (HOIP$^{RBR\text{-}LDD}$). (**B**) Formation of a reversible covalent intermediate between HOIP$^{RBR\text{-}LDD}$ and ^{TAMRA}ubiquitin with different HOIP$^{RBR\text{-}LDD}$ mutants. Same assay as in Figure 4A, but with UBE2L3 as the E2 enzyme. The TAMRA-signal is visualized on a reduced gel and at two contrast levels on a non-reduced gel. The reversible covalent bonds are indicated as E1~Ub (hUba1), E2~Ub (Ube2L3) and E3~Ub (HOIP$^{RBR\text{-}LDD}$). (**C**) Formation of a reversible covalent intermediate between HOIP$^{RBR\text{-}LDD}$ and ^{TAMRA}ubiquitin with RING2 mutants in the presence of Ube2D3. The reversible covalent bonds are indicated as E1~Ub (hUba1), E2~Ub (Ube2D3) and E3~Ub (HOIP$^{RBR\text{-}LDD}$). (**D**) Multi-sequence alignment of the C-terminus of human RBR protein sequences, including the RBR domain. UniprotID: HOIP (Q96EP0), HOIL-1L (Q9BYM8), ARIH1 (Q9Y4X5), Parkin (O60260), Triad1 (O95376). RING domains are underlined with the black line, the IBR is underlined with the dotted line and the LDD of HOIP is indicated by the gray line. Asterisks show the positions of the mutations in HOIP$^{RBR\text{-}LDD}$ that have been introduced in this study. (**E**) Single-cycle turnover assay with the E2 Ube2L3 corresponds to the assay with Ube2D3 in figure 4C (main text). Assay shows Ube2L3~^{TAMRA}ubiquitin discharge by HOIP mutants (left half of each gel) and di-ubiquitin formation upon the addition ubiquitinΔGly76 (right half of each gel). Discharge reactions were stopped after 0, 2, 4, 8, 16, 32, 64 min.



Supplementary Figure S5. (**A**) FP-assay for ^{TAMRA}ubiquitin binding to HOIP^{LDD}, as a control for the ubiquitin^{TAMRA} binding to HOIP in figure 5A. The increase in FP is shown as a function of [HOIP]. HOIP^{LDD} K_D= 170 ± 21 μM, HOIP^{LDD} C930A K_D= 1218 ± 1437 μM. Standard deviations were calculated over three repeats. (**B**) FP-assay for free TAMRA-dye binding to HOIP$^{RBR-LDD}$ and HOIP^{LDD}. The increase in FP is shown as a function of [HOIP]. (**C**) Formation of a reversible covalent intermediate between HOIP$^{RBR-LDD}$ and ^{TAMRA}ubiquitin. Wild type HOIP$^{RBR-LDD}$ and HOIP$^{RBR-LDD}$ C930A were tested in the absence and presence of ^{biotin}Ubiquitin. (**D**) Single-cycle turnover assay with ^{TAMRA}ubiquitin. The formation of di-ubiquitin between the ^{TAMRA}ubiquitin and different ubiquitin mutants was followed over time. Reactions were stopped after 0, 5 and 15 min at 37°C.



Supplementary Figure S6. (A) Co-immunoprecipitation of full-length HOIP, HOIP mutants and HOIL-1L. Western blots are shown for the immunoprecipitated protein (IP) and whole cell extracts (WCE). (**B**) Activity of HOIP and HOIP mutants in the absence of HOIL-1L in a Dual Luciferase™ reporter assay for NF-κB activation (control conditions for Figure 6). Full-length HOIL-1L, HOIP or HOIP cysteine mutants (see lower panel) were expressed separately in the presence of a luciferase reporter construct, containing 5 NF-κB binding sites. A luciferase renilla construct was used as transfection control. Firefly luciferase values were normalized to renilla luciferase values. Normalized luciferase activity of 5x NF-κB reporter vector (upper panel) is shown as mean ± s.e.m. (**P* < 0.001, Student's *t*-test, representative experiment of n=4).

Requirements for LUBAC-mediated NEMO priming and linear ubiquitin chain elongation

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Manuscript submitted

Abstract

The ubiquitination of NEMO with linear ubiquitin chains by the E3-ligase LUBAC is essential for the activation of the canonical NF-κB pathway. NEMO ubiquitination requires separate target specificities of LUBAC for NEMO and ubiquitin respectively. Here we show that multiple LUBAC-components are required to target the HOIP mediated priming ubiquitination towards NEMO. In contrast, N-terminal ubiquitin chain elongation is controlled by HOIP alone and requires specific positioning of the 'top' of ubiquitin and its N-terminus. Thus, the complete LUBAC complex is required to select NEMO as a target for HOIP mediated priming ubiquitination and subsequent linear ubiquitin chain extension.

Introduction

Nuclear factor of kappa-B (NF-κB) is a transcription factor that plays a central role in inflammatory and immune responses[1, 2]. Its activation is regulated by a variety of post-translational modifications, including phosphorylation and ubiquitination. Recently, the formation of the novel type of Met1-linked 'linear' ubiquitin chains on the NF-κB essential modulator (NEMO, also known as IKKγ) was shown to be crucial for the activation of the canonical NF-κB pathway[3-5]. Upon TNF-receptor activation by cytokines, a complex of NEMO, IKKα and IKKβ is recruited to the receptor. At the receptor, NEMO is ubiquitinated with linear ubiquitin chains, which increases the efficiency of the activating phosphorylation of IKKβ[6]. The activation of IKKβ leads to the phosphorylation and subsequent degradation of the inhibitor of NF-κB, IκBα, which enables the NF-κB proteins p50 and p65 to translocate to the nucleus and induce anti-apoptosis and inflammatory responses[4, 6-8]. Consequently, the linear ubiquitin chain formation on NEMO is a key early event in the activation of the pathway.

Ubiquitin chains consist of multiple ubiquitins that are typically linked via the donor ubiquitin C-terminus to any of the seven lysine residues on the target ubiquitin, but in linear ubiquitin chains the N-terminal amine-group of the target ubiquitin is used[8, 9]. Depending on which target site is used in a ubiquitin chain, the ubiquitination of proteins leads to different cellular outcomes, such as proteasomal degradation and intracellular translocation. Linear ubiquitin chains are essential for the activation of the NF-κB pathway by acting as interaction sites for NEMO and HOIL-1L[10-12]. However, they also recruit the negative regulator of NF-κB, A20, illustrating the dual role of this posttranslational modification[13, 14].

The formation of ubiquitin chains is mediated by a cascade of E1-E2-E3 enzymes[15-17]. A donor ubiquitin is activated in an ATP-dependent manner by an E1, after which the thioester bond that is formed between the ubiquitin C-terminus and a cysteine on the E1 is transferred onto the active site cysteine of an E2. The final conjugation of the ubiquitin C-terminus onto its target is mediated by E3-ligases. Two major classes of E3-ligases are the RING- and HECT-type E3-ligases. RING E3-ligases indirectly mediate the transfer of the ubiquitin by interacting with the E2 and the target, while HECT E3-ligases form a thioester intermediate with the ubiquitin during the transfer onto a target.

The linear ubiquitination on NEMO is performed by the E3-ligase Linear Ubiquitin Chain Assembly Complex (LUBAC)[4]. LUBAC consists of the proteins HOIP, HOIL-1L and Sharpin[4, 8, 18-20], of which HOIP and HOIL-1L belong to the novel class of RING-between-RING (RBR) E3-ligases[21, 22]. RBRs contain two RING domains in a conserved unit[23, 24], which function as RING/HECT-hybrids[21, 22, 25]. The first RING-domain of the RBR interacts with the E2 to facilitate the formation of a HECT-type intermediate between the ubiquitin and a reactive cysteine in the RING2-domain[21, 25]. Even though both HOIP and HOIL-1L have an RBR domain, HOIP is the catalytic subunit of the complex[8, 26]. Interestingly, even though the LUBAC complex may contain all three proteins, HOIP is fully active in the presence of either HOIL-1L or Sharpin[18-20].

The linear ubiquitin chain forming activity and specificity of LUBAC is completely embedded within HOIP, which is the only E3-ligase that is known to build linear ubiquitin chains[21, 22]. It catalyzes the specific linear ubiquitin chain formation by transferring the ubiquitin from the active site cysteine on RING2 to the N-terminus of the target ubiquitin that is positioned by its unique C-terminal linear ubiquitin chain determining domain (LDD)[21]. The isolated HOIP RBR-LDD domain efficiently mediates the formation of free linear ubiquitin chains *in vitro*[21, 22]. However, full-length HOIP needs to form a complex via its UBA domain with the UBL domain of either HOIL-1L or Sharpin to release this N-terminally inhibited HOIP catalytic activity[22, 27].

The formation of linear ubiquitin chains on NEMO by LUBAC requires that the catalytic activity of HOIP is targeted both towards lysines on NEMO and towards the amino-terminus of the target ubiquitin. Currently, it is unknown how this dual target specificity of the complex is regulated. To gain insight into the dual target recognition by LUBAC, we investigated the requirements of the complex for the ubiquitination of NEMO and the N-terminus of ubiquitin.

Results and discussion

HOIP is not sufficient for NEMO ubiquitination

LUBAC mediates the linear ubiquitination on NEMO K285 and K309[4]. To gain more insight into the regulation of the targeted ubiquitination of LUBAC towards the lysines of NEMO, we tested whether the N-terminal deletion construct HOIP$^{RBR\text{-}LDD}$ is sufficient for *in-vitro* ubiquitination of NEMO. Our *in-vitro* ubiquitination assays were set up with a short Strep-NEMO$^{242\text{-}419}$ construct, which includes the known minimal domain of NEMO (amino acids 241-344) that is required for its ubiquitination[18]. We used purified Strep-NEMO$^{242\text{-}419}$, HOIP$^{RBR\text{-}LDD}$, full-length HOIP and full-length HOIL-1L to compare the activity of full-length HOIP/HOIL-1L to HOIP$^{RBR\text{-}LDD}$ (Figure 1A). The full-length HOIP/HOIL-1L complex and HOIP$^{RBR\text{-}LDD}$ formed free ubiquitin chains in solution, but only the full-length complex ubiquitinated the substrate NEMO. In addition, full-length HOIP alone formed some free ubiquitin chains, but the presence of HOIL-1L was needed for the ubiquitination of NEMO (Figure 1B). This result indicates that HOIP requires the presence of HOIL-1L for the priming ubiquitination reaction on NEMO.

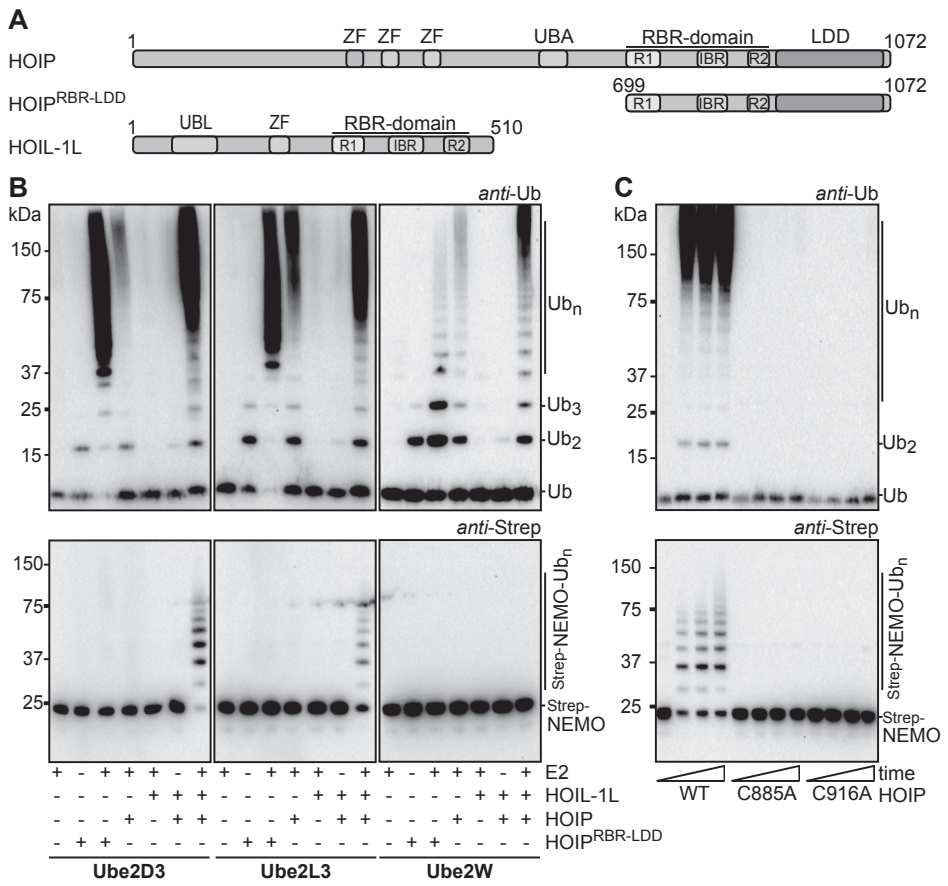

Figure 1. NEMO ubiquitination requires the presence of HOIP and HOIL-1L (**A**) E3-ligase constructs used in this study. HOIP Ubiquitin-Like domain (UBL), Npl4 Zinc Finger (ZF), Ubiquitin-Associated domain (UBA), Linear chain Determining Domain (LDD) and a RING-in-Between-RING domain (RBR) consisting of two RING domains (R1 and R2) and an in-between RING domain (IBR). The domain borders of the UBL, ZF, UBA and RBR domains are drawn to scale according to Uniprot definitions (www.uniprot.org). (**B**) Free ubiquitin chain formation and Strep-NEMO$^{242\text{-}419}$ ubiquitination by LUBAC after 2 hours in the presence of different E2s. (**C**) HOIP RING2 and LDD are essential for NEMO$^{242\text{-}419}$ ubiquitination. The blots show the ubiquitin chain formation and NEMO$^{242\text{-}419}$ modification with Ube2L3 after 0, 30, 60, 120 min.

E2 conjugases play a role in NEMO ubiquitination

The LUBAC specificity for linear ubiquitin chain formation is embedded in the RBR-LDD domains of HOIP[21]. This linear chain specificity is retained with a broad spectrum of different E2s, and even overrules the ubiquitin chain specificity of several E2s[8]. To test if the E2 or the E3 determines the specificity of the ubiquitination reaction towards NEMO, we compared HOIP/HOIL-1L mediated chain formation and NEMO modification in the presence of the

E2s Ube2D3 (UbcH5c), Ube2L3 (UbcH7) and Ube2W. The same pattern of linear ubiquitin chains was formed with all three E2s (Figure 1B, Supplementary figure S1A), confirming that the E3-ligase drives this reaction. In contrast, on the substrate NEMO, the HOIP/HOIL-1L complex was only active with Ube2D3 and Ube2L3, whereas Ube2W did not mediate the ubiquitination of this target. Apparently, the specificity for the priming ubiquitination reaction on NEMO is more dependent on the E2 enzyme than the linear ubiquitin chain formation step.

NEMO ubiquitination requires the HOIP RBR-LDD domain

The E2-dependent modification of NEMO suggests that the initial modification of NEMO is mediated via a RING-type mechanism and not via the RING/HECT-type mechanism, which is used in linear ubiquitin chain elongation[21, 22, 25]. We tested if the HECT-type thioester intermediate that is essential for linear ubiquitin chain formation[22], is dispensable for the initial modification of NEMO by introducing a point mutation of the active site Cys885 (Figure 1C, Supplementary figure S1B). The HOIP^{C885A}/HOIL-1L complex formed neither free ubiquitin chains, nor mono-ubiquitinated NEMO, revealing that HOIP C885 is also essential for the initial modification on NEMO. Interestingly, the introduction of the C916A point mutation in the HOIP LDD domain also abolished the ubiquitination of NEMO (Figure 1C, Supplementary figure S1B). Since this mutation does not disrupt the HOIP/HOIL-1L complex formation[21], the results indicate that the C-terminal domain outside the RBR is important for both linear ubiquitin chain formation and the priming ubiquitination of NEMO. These results illustrate that the initial modification of NEMO is mediated via the RING/HECT-type mechanism of HOIP and requires the proper organization of the HOIP C-terminus. In sum, the full LUBAC complex is needed to direct the catalytic activity of the HOIP RBR-LDD domain towards NEMO.

Our data show that the HOIP mediated initial ubiquitination of NEMO and linear ubiquitin chain elongation both rely on the RING/HECT-type mechanism. Nevertheless in NEMO a lysine is targeted, and in ubiquitin the amino terminus. Therefore we wondered how this amino terminus is selected for modification by HOIP.

The side-chain of ubiquitin residue Met1 is not involved in linear chain formation

The N-terminal modification of the target ubiquitin is very precisely targeted, since the potential ubiquitination site K63 that is located very close to the amino-terminus, is not modified by HOIP. This suggests that the N-terminus of the target ubiquitin has unique features for the reaction. To test different aspects of this site, we designed and chemically synthesized ubiquitins with various N-termini, changing either the side chain of the N-terminal residue or the positioning the N-terminal amino-group (Figure 2A, Supplementary figure S2A, B, C).

To test the importance of the ubiquitin Met1 side chain, we changed it into various natural and non-natural amino acids. The *in-vitro* ubiquitin chain formation activity of HOIP$^{RBR\text{-}LDD}$ with the ubiquitin M1 point-mutants was compared to ubiquitin chain formation with chemically

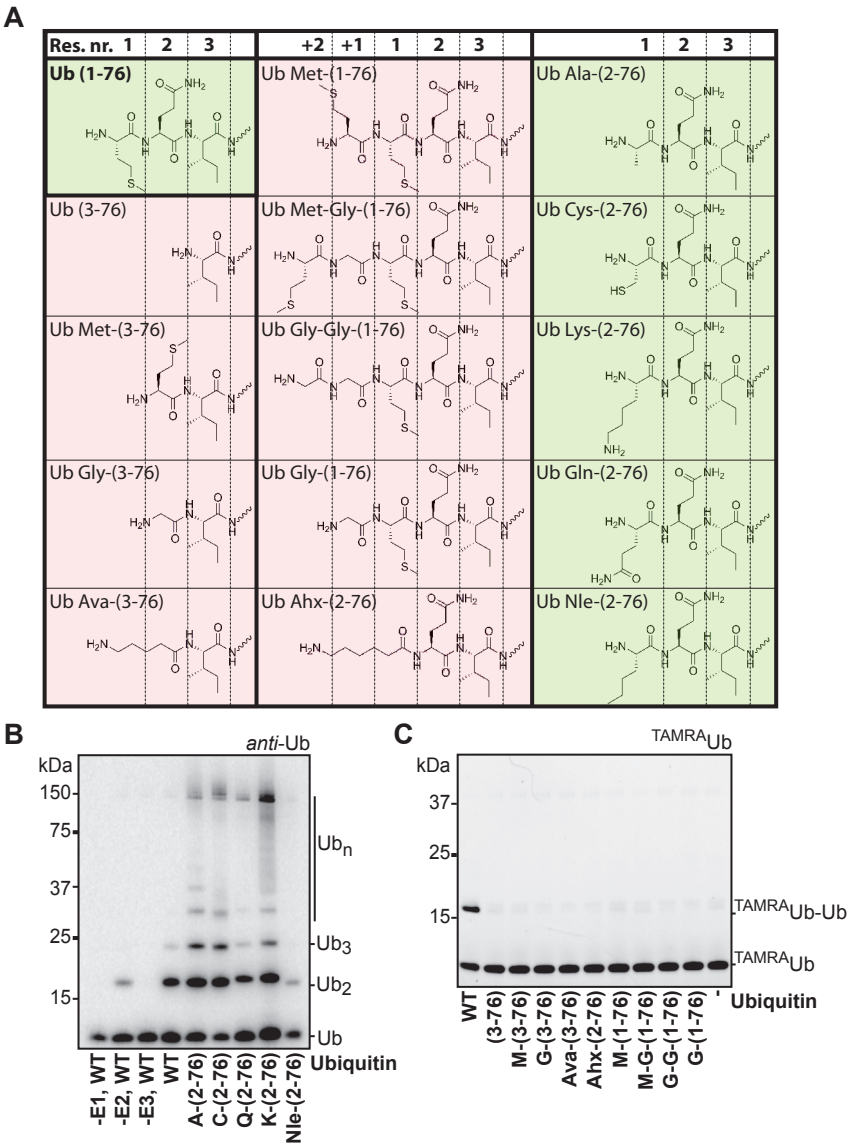

Figure 2. HOIPRBR-LDD mediated ubiquitin chain formation with N-terminal modified synthetic ubiquitins (**A**) Schematic representation of the N-terminal residues of the synthetic N-terminally modified ubiquitins. Construct names represent the wild type amino acids between brackets and additional amino acids by their 3 letter code. Norleucine (Nle) was used as a steric equivalent of Met1. 5-Aminovaleric acid (Ava), 6-aminohexanoic acid (Ahx). Ubiquitin mutants that were successfully used by HOIP are shown in green and ubiquitins that were not used by HOIP are represented in red. (**B**) HOIP$^{RBR\text{-}LDD}$ mediates free ubiquitin chain formation with ubiquitin Met1 point-mutants after 30 min. (**C**) HOIP$^{RBR\text{-}LDD}$ does not mediate di-ubiquitin formation with ^{TAMRA}ubiquitin and the N-terminally shortened or elongated ubiquitins.

synthesized wild type ubiquitin. The M1A, -C, -Q and -K point mutants formed side reactions with the E1 (Figure 2B). Nevertheless, the ubiquitin~E2 thioester-intermediate was still formed (Supplementary figure S2D), and HOIP$^{RBR\text{-}LDD}$ mediated the final ubiquitin chain formation with all M1 point-mutants (Figure 2B). The differences in the efficiency by which the ubiquitin M1 mutants were used are reflecting the variation in the efficiency of the loading of the E2 (Supplementary figure S2D). Together these results show that the side-chain of ubiquitin Met1 is not essential for the HOIP-mediated N-terminal modification.

The position of the N-terminus on ubiquitin is critical for its modification

We next tested the importance of the position of the N-terminal amine-group within the target ubiquitin structure. For this purpose, we designed N-terminally extended and shortened synthetic ubiquitins, and modified a shortened ubiquitin with a chemical group (5-Aminovaleric acid (Ava) to potentially allow an amine-group to extend to the same position as the wild type amino-terminus (Figure 2A, Supplementary figure S2A, B, C). These novel ubiquitin variants were tested for their capacity to function as target ubiquitin in linear ubiquitin chain formation assays.

First we tested the N-terminally extended ubiquitins as targets for ^{TAMRA}ubiquitin. None of the extended ubiquitins was ubiquitinated by HOIP$^{RBR\text{-}LDD}$ with ^{TAMRA}ubiquitin (Figure 2C), indicating that the N-terminus of these ubiquitins was not a suitable target for linear ubiquitination. Nonetheless, the E1/E2 dependent activation of the N-terminally extended ubiquitins was the same as for wild type ubiquitin (Supplementary figure S2E, F), and the K63-linked ubiquitin chain formation with Ube2N/Ube2V2 (Ubc13/Mms2) was normal (Supplementary figure S2E). This shows that the modified ubiquitins behaved the same in the initial steps of ubiquitin chain formation. However, in agreement with the impaired ^{TAMRA}ubiquitin modification, the N-terminally extended ubiquitins were not used in Ube2L3/HOIP$^{RBR\text{-}LDD}$ mediated linear ubiquitin chain formation (Supplementary figure S2G). Consequently, the changed position of the amine-group in the N-terminally extended ubiquitins impaired the HOIP mediated N-terminal ubiquitination of these ubiquitins.

Next we tested the effect of shortening the ubiquitin N-terminus. Similar to the N-terminally extended ubiquitins, the deletion of the ubiquitin N-terminus disabled the capacity of the ubiquitins to function as targets for HOIP$^{RBR\text{-}LDD}$ mediated ubiquitination with ^{TAMRA}ubiquitin (Figure 2C). In addition, even though some non-natural E1-dependent di-ubiquitin was formed (Supplementary figure S2H), HOIP$^{RBR\text{-}LDD}$ did not cooperate with the loaded Ube2L3~ubiquitin to mediated free linear ubiquitin chain formation (Supplementary figure S2F, G). These data show that the deletion of the ubiquitin N-terminal residues, as well as the modification with Ava, disabled the HOIP mediated N-terminal ubiquitination. In sum, all N-terminally modified ubiquitins were impaired as targets for linear ubiquitination by HOIP, revealing that the position of the N-terminal amine-group on the target ubiquitin is crucial for linear ubiquitin chain formation.

E16 and E18 on the target ubiquitin are essential for linear ubiquitin chain formation

The restricted positioning of the N-terminus within the target ubiquitin illustrates that the modification of the ubiquitin N-terminus is highly specific. Since the linear ubiquitin chain formation is solely mediated by the interplay between the LDD domain of HOIP and the target ubiquitin[21], we mutated the outer surface of the ubiquitin to identify the interaction sites on the target ubiquitin. Previously we already showed that the ubiquitin hydrophobic patch (L8, I44, V70) is neither essential for linear ubiquitin chain formation, nor for the interaction between the HOIP LDD-domain and the target ubiquitin[21]. Furthermore, none of the ubiquitin lysine residues is important for LUBAC mediated linear ubiquitin chain formation[8]. Now, we designed and purified 16 additional single point-mutants of the ubiquitin surface and tested them in an *in-vitro* ubiquitin chain formation assay with $HOIP^{RBR\text{-}LDD}$.

Most of the ubiquitin surface mutants did not impair the linear ubiquitin chain formation (Figure 3A). However, ubiquitins mutated with E16K or E18K were not used in chain forming reactions by $HOIP^{RBR\text{-}LDD}$ (Figure 3A), even though these ubiquitin mutants were used in K63 chain forming reactions with Ube2N/Ube2V2 (Supplementary figure S3A). These data show that the ubiquitin E16K and E18K mutants are well folded and loaded on the E1 and E2, but cannot be used in $HOIP^{RBR\text{-}LDD}$ mediated linear ubiquitin chain formation.

Ubiquitin E16 and E18 are positioned next to the ubiquitin N-terminus on the 'top' of ubiquitin, opposite from K63 (Figure 3D) and in most crystal structures either one or both of these residues form a salt-bridge with the amino-terminus (Supplementary figure S3B). The charge swap point mutants of E16K and E18K do not resolve whether these sites affect the catalysis of the reaction, or the positioning of the amino terminus. Therefore, we introduced different amino acids at position 16 and 18 to further analyze the role of the negative charges at these positions.

Ubiquitin E16A, -Q, -D and –K point-mutants could all be loaded on the E2 (Supplementary figure S3C), showing that the initial activation by E1-E2 was not effected. Despite this, $HOIP^{RBR\text{-}LDD}$ mediated ubiquitin chain formation was strongly impaired with all E16 mutants (Figure 3B). In addition, no detectable di-ubiquitin was formed when the E16 mutants were tested as target ubiquitin for ^{TAMRA}ubiquitin (Figure 3C). These results show that $HOIP^{RBR\text{-}LDD}$ is incompetent to use any E16 point mutant as a target ubiquitin for linear ubiquitin chain formation. Nevertheless, the full-length HOIP/HOIL-1L complex did form some free ubiquitin chains and also ubiquitinated NEMO with the ubiquitin E16A, E16Q and E16D mutants (Figure 3E, supplementary figure S3D). Only the ubiquitin E16K charge swap point-mutant could not be used by HOIP/HOIL-1L for NEMO ubiquitination and ubiquitin chain elongation, revealing that this mutant could neither be used as a target, nor as a donor ubiquitin. These results reveal that the reversal of the charge in E16K completely abolishes the target and donor capacity of the ubiquitin. The less dramatic E16A, Q and D mutants weaken the efficiency by which the ubiquitins can be used as target ubiquitin by HOIP, but do not completely impair the N-terminal modification. Consequently, the negative charge

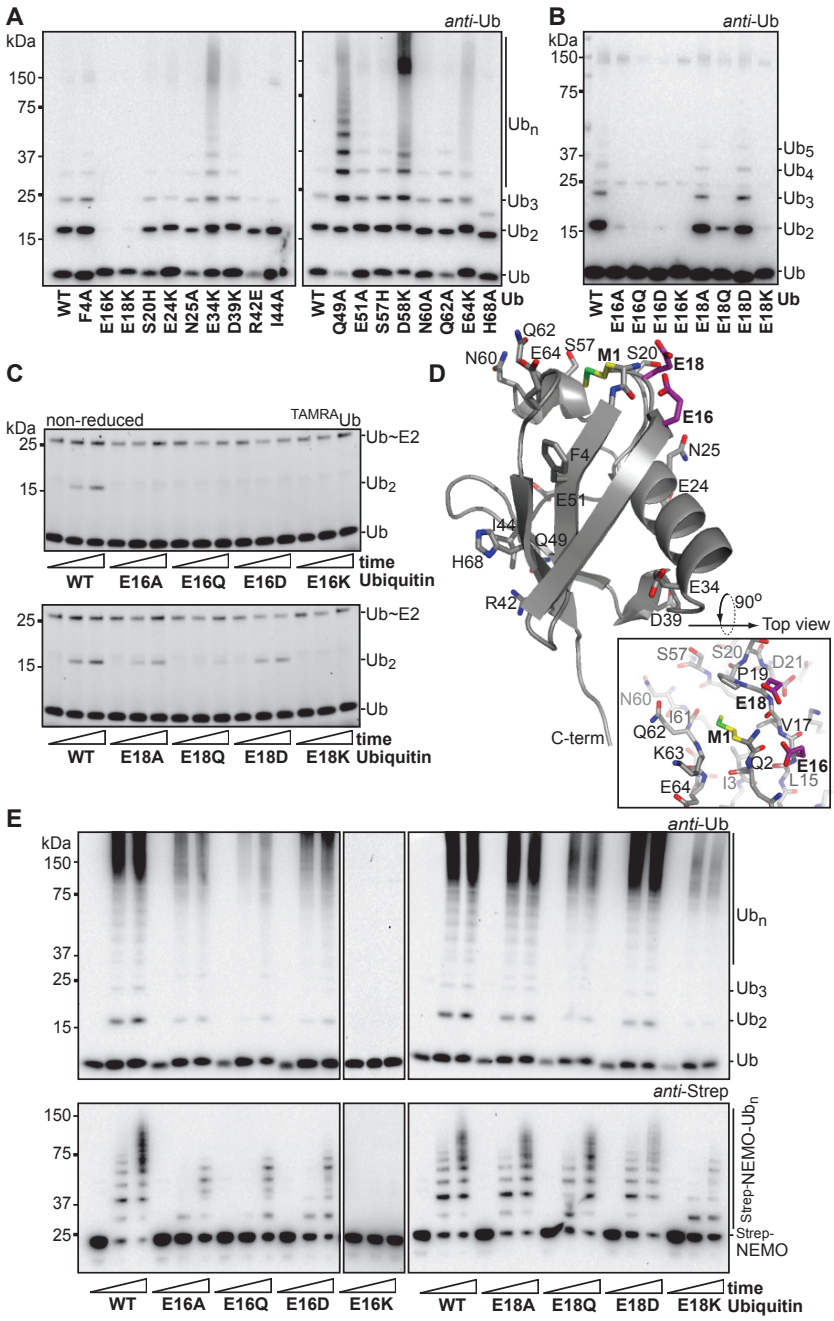

Figure 3. Target ubiquitin residues E16 and E18 are critical for ubiquitin chain formation (**A**) Ubiquitin chain formation by HOIP$^{RBR\text{-}LDD}$ with different ubiquitin point-mutants in 1 hour reactions. (**B**) HOIP$^{RBR\text{-}LDD}$ mediated chain formation with E16 and E18 ubiquitin point-mutants. Reactions were stopped after 1 hour. (**C**) Di-ubiquitin formation between ^{TAMRA}ubiquitin and different ubiquitin E16 and E18 point-mutants by HOIP$^{RBR\text{-}LDD}$. (**D**) Crystal structure of ubiquitin (PDB: 3PRM, ►

of E16 is not directly involved in the peptide bond catalysis, but plays an indirect role in the chain formation catalysis.

The ubiquitin E18 point-mutants showed the same pattern of results as the E16 point-mutants, however the efficiency of HOIP with the E18 mutants was less strongly impaired (Figure 3B, C, E, Supplementary figure S3D). Apparently, the negative charge of E16 and E18 is not essential for the chemistry of the bond formation on the N-terminal amine-group. Nevertheless, the removal of either of these residues does affect the efficiency of the chain formation reaction, suggesting that they facilitate the N-terminal ubiquitination of the target ubiquitin indirectly by facilitating the correct positioning of the ubiquitin N-terminus on HOIP LDD for its modification

Conclusion

LUBAC mediated linear ubiquitin chain formation on NEMO requires a dual target specificity of the complex. First the ubiquitination activity of LUBAC is directed towards NEMO, after which the initial ubiquitin on NEMO becomes the target for linear ubiquitin chain extension. We showed that the initial ubiquitination of NEMO$^{242\text{-}419}$ requires contributions from HOIL-1L as well as from HOIP (Figure 4A), while the linear ubiquitin chain elongation is solely controlled by the interplay of HOIP LDD and the 'top' of the target ubiquitin that specifically positions the target ubiquitin N-terminus for its modification (Figure 4B). Consequently, LUBAC is a complex that activates the catalytic center of HOIP and specifically directs the released HOIP-activity towards target proteins that have to be modified with linear ubiquitin chains. Therefore, the possible different combinations of HOIP with HOIL-1L, Sharpin and E2s, elucidates a tightly regulated mechanism for the selection of targets towards which the HOIP linear ubiquitin chain formation activity will be addressed.

Materials and methods

Construction of plasmids

E.coli expression constructs of HOIP, HOIP$^{RBR\text{-}LDD}$ and HOIL-1L have been described previously[21]. Full-length HOIP C885A and C916A were subcloned from the previously described pcDNA3.1-Myc-HOIP into pGEX-6P-1 vectors (GE Healthcare) with an N-terminal GST tag for E. coli expression[21]. The pASK-IBA3plus Strep-NEMO$^{242\text{-}419}$ expression construct was kindly provided by Prof. Dr. D. Krappmann (Helmholtz Zentrum München, Germany)[27]. The Ube2W expression construct was kindly provided by Prof. Dr. R. E. Klevit (University of Washington, Seattle, USA). Ubiquitin single point mutations were introduced in a pET3a-ubiquitin construct by using the Quick-Change Mutagenesis Kit from Stratagene (La Jolla, CA, USA).

► chain d), illustrating the position of the tested ubiquitin surface point mutations in sticks (M1 in yellow, E16 and E18 in purple). The top view of the ubiquitin structure (PDB: 3PRM, chain d) illustrates the local environment of Met1 (**E**) Full-length HOIP/HOIL-1L mediated free ubiquitin chain formation and NEMO$^{242\text{-}419}$ ubiquitination with different ubiquitin E16 and E18 point-mutants. The reactions were stopped after 0, 1, 2 hours.

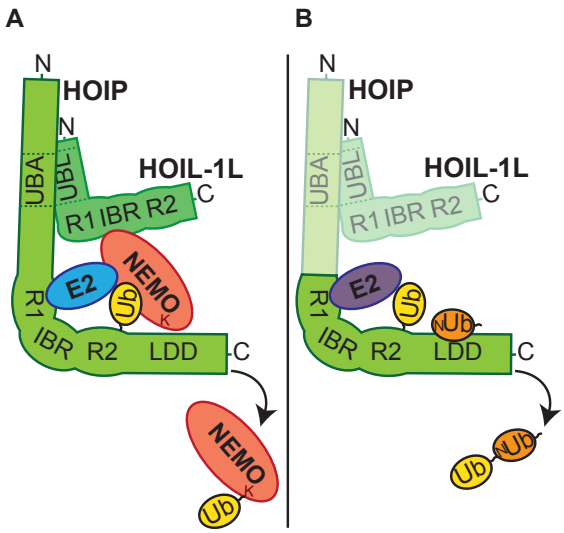

Figure 4. Model for LUBAC-mediated ubiquitination of NEMO and the ubiquitin N-terminus (**A**) The LUBAC complex recruits NEMO in the presence of HOIL-1L, HOIP and specific E2s, after which the donor ubiquitin that forms a thioester-bond with C885 on RING2, can be transferred onto a lysine residue on NEMO. (**B**) Linear ubiquitin chain formation is mediated by the interplay of the RBR-LDD of HOIP and E16/E18 of the target ubiquitin. The N-terminal modification of a target ubiquitin is mediated in the presence of a broader spectrum of E2 enzymes than the ubiquitination of NEMO.

Protein expression and purification

Ubiquitin, hUba1, Ube2D3, Ube2L3, Ube2W, Ube2N/Ube2V2, HOIP$^{RBR\text{-}LDD}$ and HOIL-1L were expressed and purified as described previously[21, 28-32]. Purification of full-length HOIP was as described previously, modified by using a Bead Beater (Mixer Mill MM400, Retsch, Germany) for cell lysis[21]. Strep-NEMO$^{242\text{-}419}$ was expressed in *E. coli* Bl21 (DE3) pLysS cells by induction with 0.8 mM isopropyl-1-thio-β-D-galacto-pyranoside (IPTG) overnight at 18°C. Cells were resuspended in 20 mM Tris/HCl pH 8.0, 100 mM NaCl, 5 mM β-mercapto-ethanol (βME) and Complete EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). Cells were lysed by a high-pressure EmulsiFlex-C5 device (Avestin, Mannheim, Germany). Initial purification was achieved by binding the protein to StrepTactin high performance resin (GE Healthcare) and elution in buffer containing 2.5 mM desthiobiotin. The protein was further purified over a Resource Q column, followed by gel filtration (Superdex 75) in 20 mM Hepes/HCl pH 8, 150 mM NaCl and 5 mM βME.

Ubiquitin synthesis

Synthetic ubiquitin, synthetic ubiquitin N-terminal variants and ^{TAMRA}ubiquitin were synthesized as described by El Oualid et al.[29], and subsequently purified over a Resource S and gel filtration (Superdex 75) according to the same protocol as for wild type ubiquitin.

In-vitro ubiquitin chain formation

In-vitro ubiquitin chain formation reactions were performed in standard conditions, containing 100 nM hUba1, 600 nM Ube2L3 (unless indicated otherwise), 1 μM E3, 10mM ubiquitin and 10 mM ATP in buffer containing 20 mM Hepes/HCl pH 8, 150 mM NaCl, 10 mM MgCl2, 5 mM βME, unless specified otherwise. Samples were separated on 4–12% Nu-PAGE gels (Invitrogen) in MES buffer and analyzed by western blot using ubiquitin antibody (P4D1, Santa Cruz biotechnology) and HRP conjugated anti-Mouse antibody (Bio-Rad), Hercules, CA, USA), or anti-Strep antibody (StrepMAB-Classic-HRP, IBA).

Di-ubiquitin formation with TAMRAUbiquitin

Di-ubiquitin formation assays with ^{TAMRA}ubiquitin were performed in the same buffer conditions as described for the ubiquitin chain formation. ^{TAMRA}ubiquitin (500 nM) was loaded onto Ube2L3 (600 nM) in the presence of ATP (1 mM) and hUba1 (100 nM) for 15 min at 37°C. Subsequently, HOIP$^{RBR\text{-}LDD}$ (1μM) and ubiquitin (500 nM) were added to the reactions and incubated for 20 minutes. The reactions were stopped by the addition of protein loading buffer. Samples were analyzed on 4–12% NU-PAGE gels (Invitrogen) in MES buffer and the TAMRA signal was visualized on a ChemiDoc XRS (Bio-Rad).

Acknowledgements

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Author contribution

JJS designed and performed the *in-vitro* experiments and wrote the manuscript. WJvD contributed to protein expression, purification and western blots. DEA synthesized ubiquitin variants. RM synthesized ^{TAMRA}ubiquitin and analyzed the purified end-products of the ubiquitin synthesis. HO designed and supervised the synthesis of the ubiquitins. TKS designed and supervised experiments and wrote the manuscript. All authors critically read the manuscript.

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Supplementary information

Materials and methods

LC-MS analysis of synthetic ubiquitins

LC-MS measurements were performed on a system equipped with a Waters 2795 Seperation Module (Alliance HT), Waters 2996 Photodiode Array Detector (190-750nm), Phenomenex Kinetex C18 (2.1x50, 2.6 μm) column and LCTTM Orthogonal Acceleration Time of Flight Mass Spectrometer. Samples were run using 2 mobile phases: A = 1% CH3CN, 0.1% formic acid in water and B = 1% water and 0.1% formic acid in CH3CN. Flow rate= 0.8 mL/min, runtime= 6 min, column T= 40°C. Gradient: 0 – 0.5 min: 5% B; 0.5 – 4 min: à 95% B; 4 –5.5 min: 95% B. All synthetic peptides eluted as a single peak, data processing was performed using Waters MassLynx Mass Spectrometry Software 4.1 (deconvulation with Maxent1 function).

In-Silico superposition of ubiquitin structures

The superposition of 24 ubiquitin structures on PDB: 1UBI, was made by secondary structure matching (SSM), using the PDBeFold server[1]. PyMOL(TM) Molecular Graphics System (Version 1.5.0.3, Schrodinger, LLC) was used for generating the graphical images of ubiquitin. Ubiquitin structures used (PDB-chain nr.): 1UBI-a, 1WR6-h, 2C7M-b, 2C7N-f, 2C7N-h, 2C7N-l, 2FIF-c, 2FIF-e, 2J7Q-b, 2J7Q-d, 30J3-e, 30J3-f, 30NS-a, 3A1Q-a, 3A1Q-d, 3BY4-b, 3C0R-d, 3EFU-a, 3IFW-b, 3KW5-b, 3LDZ-e, 3LDZ-f, 3M3J-e, 3PRM-d, 3PTF-c.

Supplementary reference

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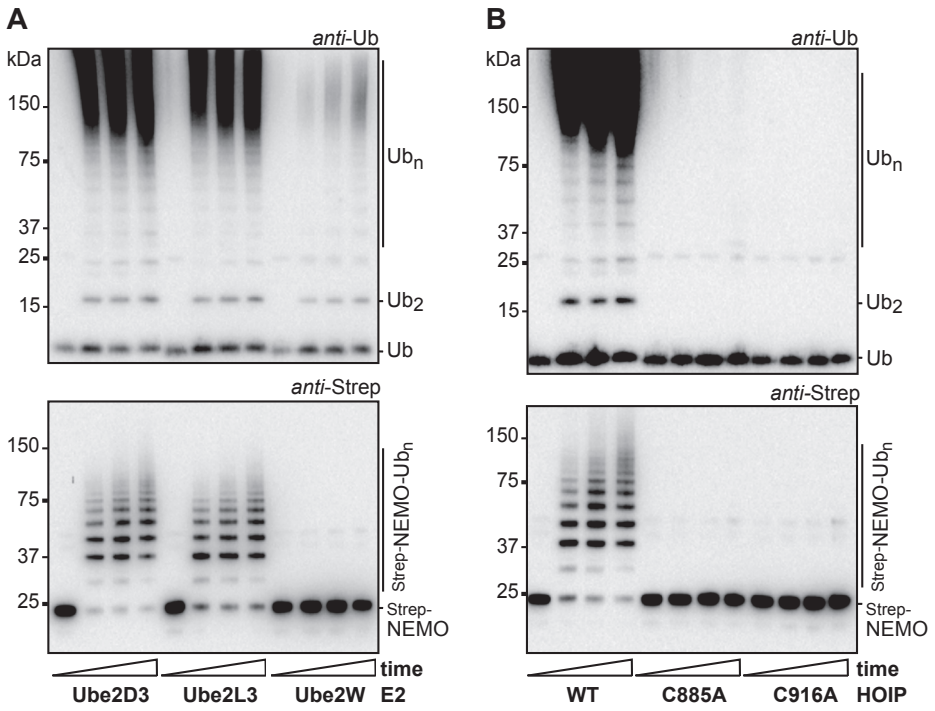
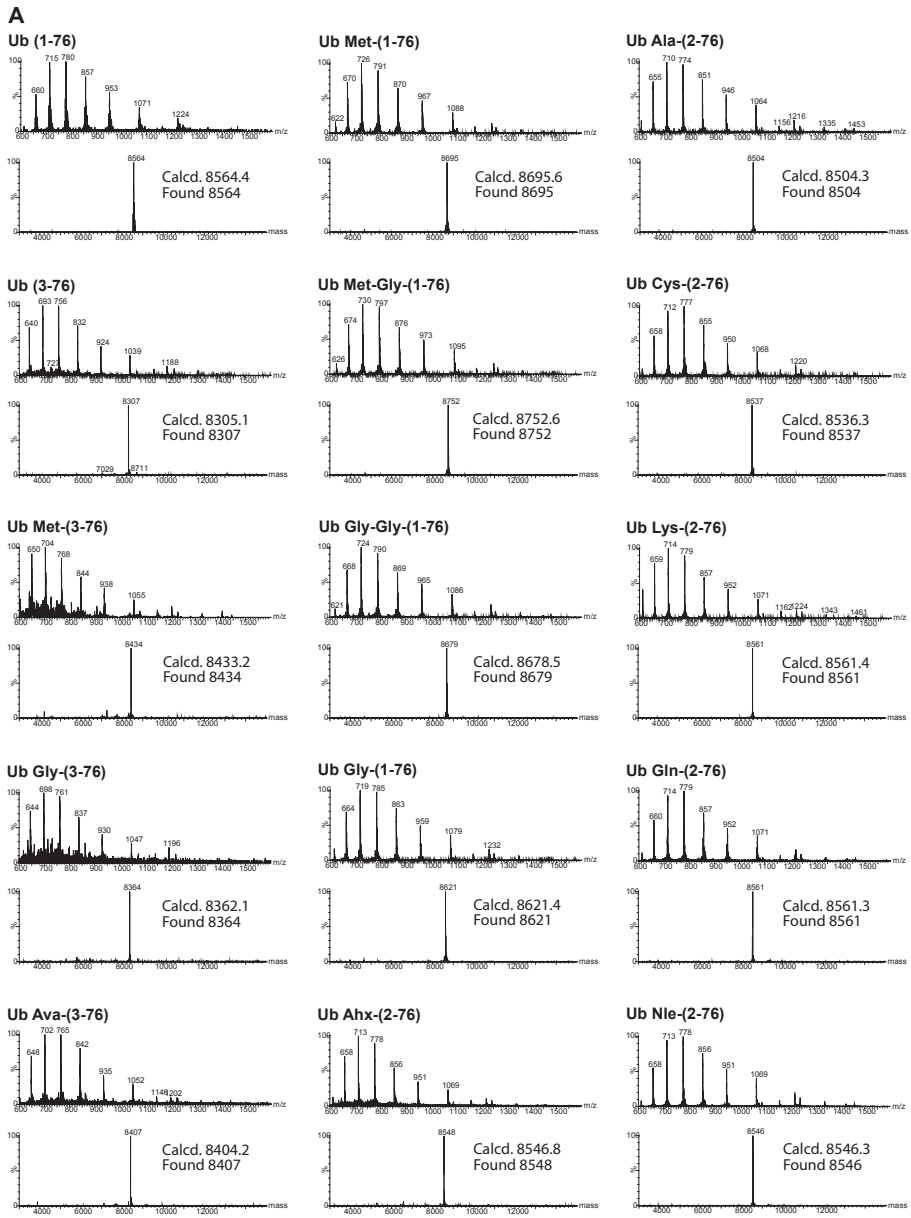
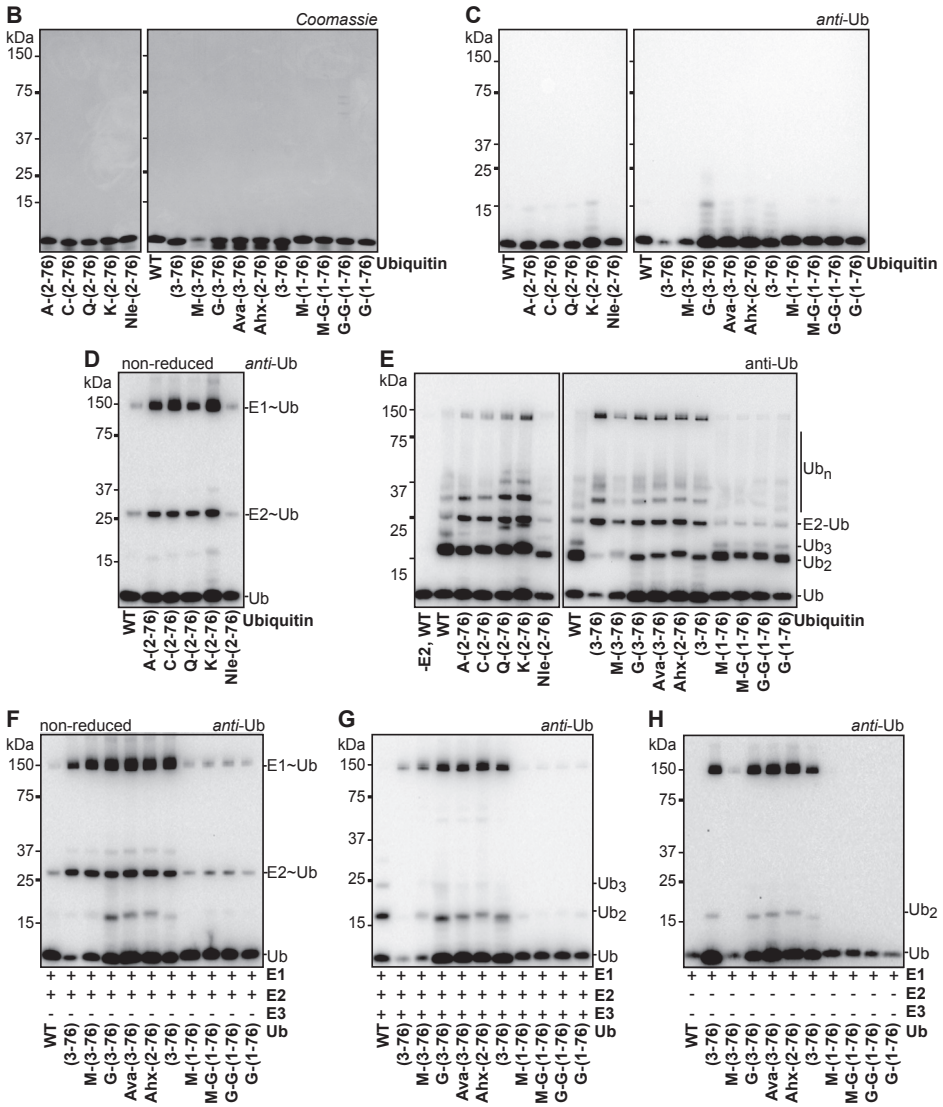


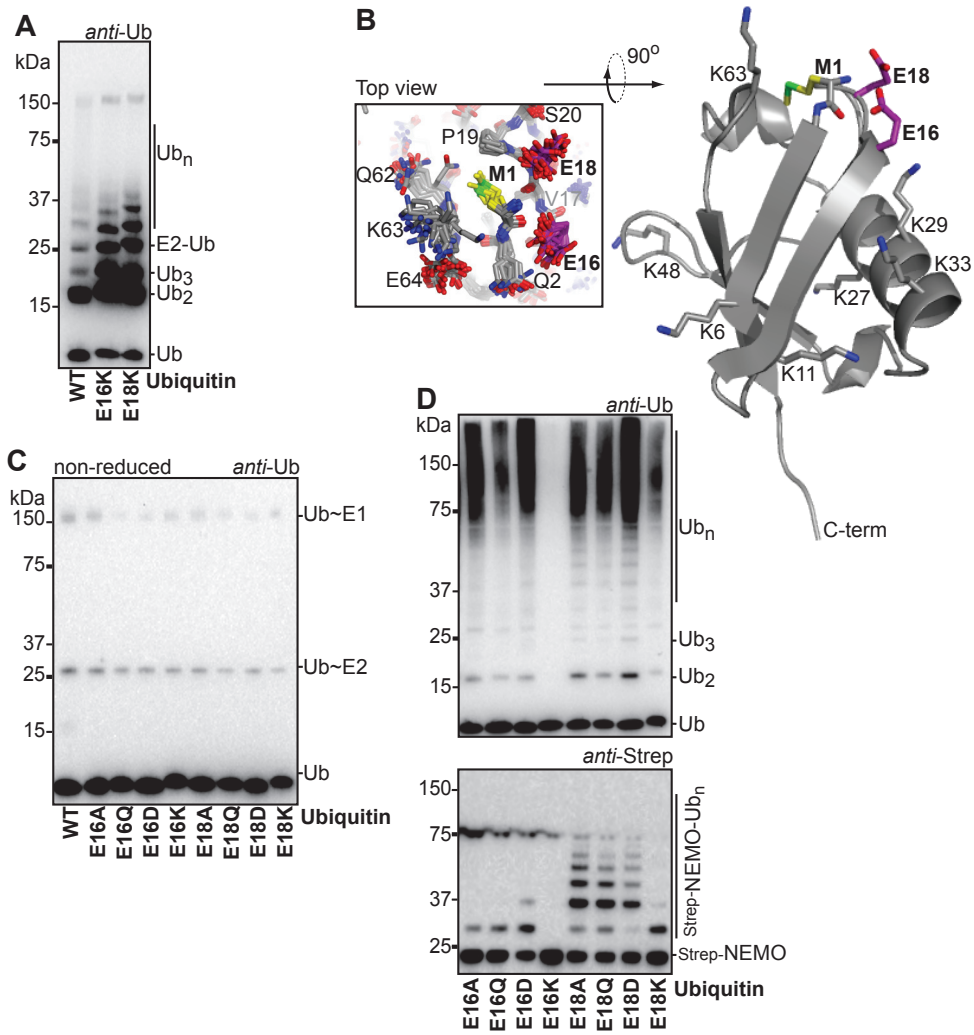
Supplementary figure S1. NEMO ubiquitination by LUBAC (**A**) Free ubiquitin chain formation and Strep-NEMO$^{242\text{-}419}$ ubiquitination by full length HOIP/HOIL-1L in the presence of different E2s. Reactions were stopped after 0, 30, 60, 120 minutes. (**B**) Ubiquitination activity of full length HOIP and full length HOIP point-mutants in the presence of HOIL-1L and Ube2D3 after 0, 30, 60, 120 minutes.



Supplementary figure S2. HOIP$^{RBR\text{-}LDD}$ mediated ubiquitin chain formation with N-terminal modified synthetic ubiquitins (**A**) ESI-MS analysis of the purified synthetic ubiquitins. Spectrum of the ubiquitin peak using LC-MS (top spectrum) and deconvoluted mass of the peak (calculated and found) in the top spectra (bottom spectrum). (**B**) Coomassie gel of the purified synthetic ubiquitins. (**C**) Anti-ubiquitin western blot of the purified synthetic ubiquitins. (**D**) Ubiquitin thioester intermediates on hUba1 (E1~ub) and Ube2L3 (E2~ub) with synthetic ubiquitin Met1 point-mutants. ►



► Samples were taken from reactions without E3 (T= 2 hours), showing no background ubiquitin chain formation. (**E**) Ubiquitin K63-chain formation in the presence of hUba1 (E1) and Ube2N/Ube2V2 (E2) with the different synthetic ubiquitin N-terminal mutants. Reactions were stopped after 1 hour. (**F**) Non-reduced blot of ubiquitin thioester intermediates on hUba1 (E1~ub) and Ube2L3 (E2~ub) with synthetic ubiquitin mutants. Samples were taken from reactions without E3 (T= 2 hours). (**G**) Ubiquitin chain formation capacity of HOIP$^{RBR\text{-}LDD}$ with synthetic ubiquitin mutants after 30 minutes (hUba1 (E1), Ube2L3 (E2)). (**H**) Control reaction for the ubiquitin N-terminal mutants in the absence of E2 and E3. Reactions with hUba1 (E1) and ATP were stopped after 30 minutes.



Supplementary figure S3. Acceptor ubiquitin residues E16 and E18 are critical for ubiquitin chain formation (**A**) Ubiquitin chain formation with ubiquitin E16A and E18A by hUba1 and Ube2N/Ube2V2. Samples were taken after T = 30 minutes. (**B**) Illustration of the position of E16 and E18 (purple sticks) on ubiquitin (PDB: 3PRM, chain d). The 7 lysines and M1 (yellow) are represented as sticks. The orientation of the residues around Met1 in 25 different ubiquitin structures is illustrated by the top view of the superposition of 24 ubiquitins on ubiquitin PDB: 1UBI. (**C**) Ube2L3~ubiquitin thioester formation with ubiquitin E16 and E18 point-mutants. Reactions were stopped after 30 minutes, showing no background activity in the absence of E3. (**D**) Ubiquitin chain formation and Strep-NEMO$^{242\text{-}419}$ modification with ubiquitin E16 and E18 point-mutants by full length HOIP/HOIL-1L.

General discussion

6

Ubiqutination is an important posttranslational modification that may target proteins for degradation, but also has many other signaling functions in cells[1-3]. The ubiquitination of proteins is mediated by the concerted action of E1, E2, and E3 enzymes that specifically attach the small ubiquitin protein to their targets[4]. The work in this thesis focuses on the molecular characterization of the functioning of Triad1 and HOIP, which are members of the RING-in-between-RING (RBR) class of ubiquitin E3-ligases.

The RBR-class of E3-ligases was first described in 1999[5, 6] and the 15 different human RBR proteins have been shown to play important roles in many different pathways that are essential for cellular functioning (Chapter 1)[7]. Therefore, they form interesting potential targets for the development of molecular medicine, which requires a thorough understanding of the mechanisms by which they mediate ubiquitination. In the following part we will discuss the work that is described in this thesis in the context of the growing field of research on the RBR E3-ligases, and provide future directions for research.

RBRs mediate ubiquitination via a RING/HECT-type mechanism

RBR proteins contain a highly conserved unit of two RING domains that are separated by an in-between RING zinc finger (IBR) (see Chapter 1). The RING1 domain forms a classical RING-fold (PDB: 1wim), but the sequence of the RING2 domain is shorter than classical RINGs and is only found within the context of RBRs. The high conservation of the RINGs and IBR together suggests that they function together. In 2011 it was illustrated for Parkin and HHARI that the RING-domains of these RBRs indeed cooperate to mediate the transfer of the ubiquitin from the E2 onto its target via a RING/HECT-type mechanism[8]. The RING1 domain interacts with the E2 to mediate the transfer of the ubiquitin onto a cysteine residue in RING2 before it is transferred onto its target. Our work on HOIP in Chapter 4 and the work of Rittinger and coworkers that was published around the same time, confirms that this RBR protein also mediates the transfer of ubiquitin via the RING/HECT-type mechanism[9, 10].

The fact that Parkin, HHARI and HOIP form a thioester intermediate with ubiquitin makes them suitable to cooperate with the E2 Ube2L3 (UbcH7), which discharges its ubiquitin only onto cysteine residues[8]. We confirm in chapter 2 and 3 that Triad1 is also functionally active with Ube2L3[11, 12]. Therefore, Triad1 is likely to function via the RING/HECT-type mechanism. Also other RBR proteins have been shown to be functionally active with Ube2L3 (Chapter 1, table 2)[8, 13-15], therefore we expect that the RING/HECT-type mechanism is generally used by the RBR E3-ligases. Nevertheless, more *in-vitro* studies that aim to trap the thioester intermediate on the RING2 domain of these RBRs are needed to confirm that all RBRs work via this mechanism.

Even though the general features of the RING/HECT-type mechanism have been revealed, the molecular details on the separate RING-domains and how they form functional units is limited. At first sight, RING1 of the RBR resembles classical RING-domains (PDB: 1wim), and E2-E3 interactions with the separate RING domains are dependent on the same residues of the RING[16]. However, there are a couple of differences between the interactions of E2s with RBR RING1 and classical RINGs that we do not understand. First, classical RINGs interact with

E2~ubiquitin to position and activate the E2~ubiquitin thioester-intermediate to enhance its transfer onto a target lysine[17, 18]. However, the interaction of an E2~ubiquitin with RING1 of HHARI[8], Parkin[8] and HOIP (Chapter 4)[9] does not enhance the reactivity of the E2~ubiquitin thioester. Second, the interaction between an RBR RING1 and Ube2L3 is suggested to be tighter than classical RING-E2 interactions, because it can be identified by pull-down experiments and immunoprecipitations[16]. Interestingly, we could not detect the interaction between purified Triad1 and Ube2L3, even though the interaction was detected by yeast-two-hybrid (Chapter 2)[11] and the purified proteins are functionally active in *in-vitro* assays that are directed towards p53 (Chapter 3). Consequently, the interaction between Ube2L3 and Triad1 RING1 is likely to require the presence of the ubiquitin on the E2, or needs the presence of a substrate to be bound to Triad1. Thus, even though the RBR RING1 structurally resembles classical RING domains, the E2-E3 interactions with these RINGs are not completely the same.

Besides RING1, RING2 of the RBR has been shown to be essential for the RING/HECT-type mechanism by forming a RING2~ubiquitin thioester intermediate on a cysteine residue[8-10]. Most likely, the cysteine in RING2 is available because the domain only coordinates one zinc ion and leaves the three cysteines that coordinate a zinc ion in a classical RING free, as was shown in the solution structure of HHARI RING2[19]. One specific cysteine (Cys357) in RING2 of HHARI[8] and the corresponding cysteine in HOIP[10] have been shown to form a thioester intermediate with ubiquitin during the transfer towards its target. Interestingly, RING2 also interacts with E2s in Triad1 (Chapter 2)[11] and Parkin[20, 21], which suggests that it more closely resembles RING-domains than was initially suggested by the solution structure of HHARI[19]. Thus, the functioning of RING2 needs further research to explain how it can interact with E2s and form thioester intermediates with ubiquitin.

Finally, the highly conserved RBR-unit contains an IBR zinc finger of which the function remains to be revealed. We show that mutations in the IBR of HOIP are disruptive for its E3-ligase activity (Chapter 4)[9], but the inhibitory effect of Triad1 on clonogenic growth is not affected when the IBR is removed from the protein (Chapter 2)[11]. Nevertheless, the high conservation of this domain within the RBR suggests that it is critical for E3-ligase functioning. As shown above, many questions regarding the functioning of the RBR-domain remain unanswered, which highlights the need for crystal structures of RBRs to gain further insight in their functioning.

Auto-regulation of RBR E3-ligase activity

The activity of the RBR domains of HOIP (Chapter 4) and Parkin is auto-inhibited by domains outside the RBR[9, 10, 22]. The N-terminal UBL domain in Parkin binds to its RBR to block the ubiquitin transfer and needs to interact with effectors to release the inhibition[22]. Moreover, HOIP needs to form a complex with HOIL-1L or Sharpin to release the N-terminal inhibition of the RBR (Chapter 4)[9, 10]. However, the N-terminal auto-inhibition of HOIP is not mediated by the direct binding of the N-terminus to the RBR, since the constitutively active isolated HOIP RBR-domain cannot be inhibited in trans by the addition of the N-terminal domain (Chapter 4)[9, 10]. Thus, the internal regulation of the activity of HOIP and Parkin is mediated by different mechanisms.

The RBR domain of Triad1 does not need the addition of cofactors to be active and is only slightly inhibited by its C-terminus (Chapter 5). Instead, the activity of Triad1 is regulated by the tight control of its concentration in cells (see Chapter 1). We observed that the *in-vitro* activity of Triad1 for ubiquitin chain formation is optimal at specific concentrations of Triad1 with respect to the other proteins in the reaction (Chapter 4 and 5)[11]. This concentration-dependent auto-inhibition of Triad1 was seen in multiple experiments, but it could not be systematically reproduced to study the underlying mechanism. Since the concentration-dependent auto-inhibition of Triad1 is sensitive to minor variations in the *in-vitro* reactions, the observed inhibition may reflect an *in-vitro* artifact. However, we do not completely exclude that it reflects a subtle *in-vivo* effect of the tightly regulated cellular Triad1-levels. In summary, the studies on Parkin, HOIP and Triad1 show that the tightly controlled activity of the separate RBR proteins is controlled by different mechanisms.

Ubiquitination specificity by RBR E3-ligases

The ubiquitination of proteins with ubiquitin chains requires a dual target specificity of the RBR E3-ligases. The target-protein has to be recognized to selectively modify it with the first ubiquitin, and specific sites on ubiquitin need to be selected to mediate the formation of well-defined ubiquitin chains. The work in this thesis shows that the E3-ligases Triad1 and HOIP regulate the formation of ubiquitin signals on targets by different mechanisms.

The E3-ligase activity of Triad1 is important for the regulation of the proliferation of myeloid progenitor cells and is dependent on its RING domains, but not on the IBR and coiled-coil regions outside its RBR[11]. The RING domains are important for the interactions with E2s that are used to select the target and ubiquitin chain formation specificity (Chapter 2[11] and 3). Interestingly, the K48-linked ubiquitin chain forming E2 Ube2L3 is used for the *in-vitro* mono-ubiquitination of p53 and is not used by Triad1 to mediate the formation of ubiquitin chains, while Triad1 mediates the formation of ubiquitin chains with Ube2n/Ube2V2 (Ubc13/Mms2), but does not link them to p53. Therefore, Triad1 might limit the activity of Ube2L3 to mono-ubiquitination and work together with other E2s to specifically mediate the elongation of the ubiquitin signal on the first ubiquitin.

In contrast, the RBR E3-ligase HOIP solely mediates the formation of linear ubiquitin chains in cooperation with a variety of E2s. The ubiquitin chain formation specificity of the E3 is embedded within the RING2-LDD domain, in which the LDD positions the ubiquitin N-terminus (Chapter 4)[9]. The position of the ubiquitin N-terminus within the ubiquitin is critical, since the positioning of the N-terminal amine-group with respect to the HOIP~ubiquitin thioester intermediate needs to be accurate (Chapter 5). The ubiquitin chain determination by the LDD overrules the non-covalent interaction between ubiquitin and E2s that is normally used for chain formation. Possibly, HOIP blocks these sites on the E2s, or specific residues on the LDD might be needed to facilitate the transfer of the very labile thioester-intermediate onto the ubiquitin N-terminus.

HOIP is the only E3-ligase that is known to target the N-terminal amine-group of ubiquitin for ubiquitination. Therefore, the highly specific reaction is expected to be tightly regulated. Interestingly, HOIP mediates the transfer of ubiquitin onto the N-terminal amine-group of different amino-acids when they are used to replace the N-terminal methionine (Chapter 5). Nevertheless, it does neither mediate the modification of other available amine-groups on ubiquitin, nor targets the N-termini of random proteins. Thus, the N-terminal modification of ubiquitin by HOIP is highly dependent on the position of the targeted amine-group and is likely to be facilitated by a combination of specific contributions of residues on HOIP and the target-ubiquitin.

Even though the ubiquitin chain formation specificity of HOIP is embedded within the protein, the ubiquitination of Nemo with linear ubiquitin chains requires more than HOIP alone. Other components of the LUBAC complex direct the chain formation of HOIP towards its target and the reaction may even be dependent on the E2 that is used (Chapter 5).

Our studies on Triad1 and HOIP show that individual RBRs select their targets via different mechanisms. Therefore, the RBR proteins need to be characterized individually to learn about the target selection of each individual RBR. Also the mechanism by which the ubiquitin chain-types are determined by RBR proteins differs between the individual RBRs. The activity of HOIP is highly dependent on its unique LDD domain, and there are no other RBRs known that overrule the ubiquitin chain formation specificity of the E2. Nevertheless, there may be other ubiquitin positioning-domains in the uncharacterized RBRs that have a similar function. The mechanism by which Triad1 functions may be more general to the RBR class of E3-ligases, since multiple RBRs have been suggested to follow the ubiquitin chain formation specificity of the E2s (see Chapter 1)[23-25]. But, future research is needed to explain how the RING/HECT-type mechanism uses different E2s to determine the ubiquitin chain formation specificity.

Concluding remarks

The novel insights in the function of RBR proteins that have been published in the past couple of years have provided us with a first glimpse on the molecular functioning by which these proteins mark their targets with ubiquitin signals. However, more research is needed to identify how the formation of specific ubiquitin signals is regulated by the RBRs.

To be able to fully understand the functionality of the different RBRs, a complete overview of all E2s that interact with the RING domains of the RBRs is needed. Unfortunately, the published yeast-two-hybrid studies that map E2/RING E3-ligase interactions did not systematically screen the separate RING domains of the RBRs[26, 27], leaving the proper analysis of the interactions to be done. Furthermore, the functionality of the E2-RBR complexes should be systematically analyzed. To serve this purpose, many techniques can be used to detect the formation of ubiquitin chains. However, novel techniques need to be developed to identify functional E2-E3 complexes that solely mediate the formation of mono-ubiquitination of unknown targets, since these E2/E3 pairs will appear to be nonfunctional in ubiquitin chain formation

assays. Possibly, the formation of the ubiquitin-RBR thioester intermediate can be stabilized or trapped to detect the full set of E2s that is functionally active with a certain RBR. Finally, a major challenge in the field is the identification of targets for the separate RBR E3-ligases and the molecular mechanisms by which they are recognized. Several RBRs have been shown to interact with potential target-proteins via different regions outside the RBR or by cofactors, indicating that there is no general mechanism for target recognition by these E3s. Therefore, *in-vivo* cross-linking may be an interesting technique to identify targets for the RBRs[28].

The functional analysis of the RBRs will provide novel insights in the functioning of these proteins and may reveal novel pathways and cellular processes in which they are involved. However, to fundamentally understand how the RBR domains form functional units, the structural characterization of the RBR domain, E2~ubiquitin/RBR complexes, and RBR~ubiquitin complexes is critical.

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Addendum

Summary

Ubiquitination is an important posttranslational modification that plays a role in virtually all cellular pathways. Ubiquitin signals are made out of one or multiple ubiquitin proteins that are targeted towards substrates by a cascade of E1, E2 and E3 enzymes, where the E3-ligase catalyzes the transfer of the ubiquitin from the E2 onto the target. The RING-in-between-RING (RING1-IBR-RING2 / RBR) subfamily of RING E3-ligases is a novel identified class of E3-ligases that are characterized by the presence of a RBR domain. The RBRs are involved in major pathways such as the nuclear factor of kappa-B (NF-kB) pathway and play a role in the development of diseases like Parkinson disease and cancer. Therefore, a detailed understanding of the molecular mechanisms underlying their functioning is critical to understand the implication of mutations and to facilitate the development of molecular medicine. In this thesis we analyze the molecular mechanisms by which the RBR E3-ligases Triad1 and HOIP mediate the transfer of ubiquitins from the E2s onto their targets, to expand our knowledge on the functioning of this class of E3-ligases.

In **chapter 2** we show that Triad1 is induced during monocytic differentiation and that both RING domains of the RBR are essential for the inhibitory effect of Triad1 on the clonogenic growth of myeloid cells. We show that Triad1 interacts via its RING1 with the E2 Ube2L3, and via RING2 with Ube2N. Interestingly, Triad1 catalyzes the formation of K48 and K63-linked ubiquitin chains, which are the respective ubiquitin chain-types of which the formation is mediated by Ube2L3 and Ube2N/Ube2V2. In summary, we show that the E3-ligase activity of Triad1 by its RING1 and RING2 domains is important for myelopoiesis.

In **chapter 3** we further characterize the functioning of Triad1 *in vitro* and analyze its activity in cooperation with various E2s. The ubiquitin chain-formation with Triad1 is only mediated by a subset of the E2s and depends on the presence of an E2 SPA-motif. Furthermore, we show that the E2s determine the types of ubiquitin chains that are formed by Triad1 and play an important role in the recognition of p53 as an *in-vitro* target for ubiquitination. Thus, the activity of Triad1 is highly regulated by the E2 that is involved in the reaction and can mediate the formation of different ubiquitin signals in cooperation with separate E2s.

In **chapter 4** we characterize the linear ubiquitin chain formation that is mediated by the Linear Ubiquitin Chain Assembly Complex (LUBAC) E3 consisting of HOIP, HOIL-1L and Sharpin. We show that both LUBAC catalytic activity and LUBAC specificity for linear ubiquitin chain formation are embedded within the C-terminal RBR domain of the E3-ligase HOIP that is autoinhibited by its N-terminus. The catalytic RING-IBR-RING domain of HOIP mediates linear ubiquitin chain formation via a two-step mechanism, involving both RING and HECT-type activities. First, an E2~ubiquitin interacts with RING1-IBR (RING-type function) to facilitate the transfer of the ubiquitin onto RING2 to form a HECT-like covalent thioester intermediate. Second, the ubiquitin is transferred from HOIP onto the N-terminus of a target ubiquitin. This transfer is facilitated by a unique region in the C-terminus of HOIP that we termed 'Linear

ubiquitin chain Determining Domain' (LDD). The precise positioning of the acceptor ubiquitin on the LDD is essential for the highly specific ubiquitin N-terminal modification.

In **chapter 5**, we show that the position of the N-terminal amine-group within the target ubiquitin is critical for its modification by HOIP. Furthermore, we identify two negatively charged residues on the 'top' of ubiquitin that are critical for the HOIP-mediated linear ubiquitin chain formation.

The activity of HOIP within the LUBAC complex is critical for the activation of the NF-kB pathway that plays a central role in inflammatory and immune responses. In **chapter 5** we illustrate that multiple LUBAC-components are required to target the HOIP-mediated ubiquitination towards NEMO. Thus, even though the linear ubiquitin chain formation of LUBAC is completely embedded within HOIP, the full LUBAC complex is required to release its autoinhibition and direct its activity towards NEMO.

In **chapter 6** we discuss our findings on Triad1 and HOIP in the context of the RBR-class of RING E3-ligases and provide future directions for research. Finally, we conclude that the RING/HECT-type mechanism is likely to be general for all RBRs, while the target selection and ubiquitin chain formation determination are regulated in different ways for the separate RBRs.

Samenvatting

Ubiquitinatie is een belangrijke posttranslationele modificatie die een rol speelt in de regulatie van de meeste cellulaire processen. Ubiquitinesignalen bestaan uit één of meerdere ubiquitine-eiwitten die aan andere eiwitten zijn vastgemaakt om hun functie of cellulaire locatie te veranderen. Het ubiquitinesignaal wordt gemaakt door een cascade van E1, E2 en E3 enzymen, waarbij de E3 in de laatste stap de overgang van de ubiquitine vanaf de E2 naar het doelwit faciliteert. Recentelijk is een nieuwe subfamilie van de RING E3-ligases ontdekt: de RING-in-between-RING E3-ligases (RING1-IBR-RING2/ RBR). Hoewel alle E3s in deze subfamilie worden gekenmerkt door hun RBR-domein, zijn ze betrokken bij de regulering van verschillende cellulaire processen, zoals de activatie van nuclear factor of kappa-B (NF-kB) en het reguleren van de proliferatie van cellen. Daarnaast spelen RBRs een rol in de ontwikkeling van ziektes zoals de ziekte van Parkinson en kanker. Omdat de RBRs zo belangrijk zijn voor het functioneren van cellen, is het van belang dat we de moleculaire processen waarmee ze hun functies uitoefenen beter leren begrijpen. Op die manier kan de invloed van mutaties in deze eiwitten worden verklaard en kan een begin worden gemaakt aan de ontwikkeling van medicijnen die de functie van de RBRs kunnen beïnvloeden. In dit proefschrift beschrijven we de analyse van de moleculaire mechanismen die ten grondslag liggen aan het functioneren van de RBRs Triad1 en HOIP om het begrip van het functioneren van deze groep E3s te vergroten.

In **hoofdstuk 2** laten we zien dat Triad1 wordt geïnduceerd tijdens de monocytaire differentiatie en dat beide RING-domeinen van belang zijn voor de remmende rol die Triad1 heeft op de clonogene groei van myeloïde progenitor cellen. De RING-domeinen zijn van belang voor de interactie met verschillende E2s. Het RING1-domein interageert met de E2 Ube2L3 (UbcH7) en het RING2-domein met de E2 Ube2N (Ubc13). Deze interacties correleren met het feit dat Triad1 de formatie van K48- en K63-gelinkte ubiquitineketens medieert die specifiek door deze E2s worden gevormd. Kortom, de E3-ligase activiteit van Triad1 via zijn RING1- en RING2-domeinen is essentieel voor de ontwikkeling van bloedcellen.

In **hoofdstuk 3** wordt de *in-vitro* activiteit van Triad1 verder gekarakteriseerd in de aanwezigheid van verschillende E2s. De samenwerking van Triad1 met E2s is afhankelijk van het SPA-motief van E2s en is mede daardoor alleen met een subset van de E2s mogelijk. Verder ontdekken we dat de E2s een cruciale rol spelen in de ubiquitineringsreacties met Triad1, omdat ze bepalen welk type ubiquitineketen gemaakt wordt en daarnaast een rol spelen in de selectie van p53 als target voor de ubiquitinatie.

In **hoofdstuk 4** karakteriseren we de lineaire ketenformatie door het Linear Ubiquitin Chain Assembly Complex (LUBAC) dat bestaat uit HOIP, HOIL-1L en Sharpin. We laten zien dat alle lineaire ketenformatie-activiteit en -specificiteit van het complex ligt opgeslagen in het C-terminale RBR-domein van HOIP dat wordt ge-autoinhibeerd door de N-terminus. HOIP medieert de transfer van ubiquitine vanaf de E2 naar zijn doelwit via een mechanisme dat de eigenschappen van RING- en HECT-E3s combineert. Het RING1-IBR-domein rekruteert een E2~ubiquitine (RING-functie) en faciliteert daarmee de formatie van een ubiquitine~HOIP

thioester op het RING2-domain (HECT-functie). Ten slotte wordt de ubiquitine vastgemaakt aan de N-terminus van een target-ubiquitine. Deze laatste stap wordt gefaciliteerd door een unieke extensie van het RBR-domein in HOIP dat de target-ubiquitine specifiek positioneert voor zijn modificatie. Om die reden hebben wij dit domein 'Linear ubiquitin chain Determining Domain' (LDD) genoemd.

In **hoofdstuk 5** laten we zien dat de target-ubiquitine specifieke eigenschappen heeft voor zijn N-terminale modificatie door HOIP. Ten eerste is de positie van de ubiquitine-N-terminus binnen het eiwit belangrijk voor zijn modificatie. Daarnaast zijn er twee negatief geladen residuen op de 'bovenkant' van de target-ubiquitine die essentieel zijn om de ubiquitine-N-terminus correct te positioneren op het LDD-domein van HOIP.

De activiteit van HOIP in het LUBAC complex is cruciaal voor de activatie van de NF-kB cascade voor het reguleren van ontstekingsreacties en immuunreacties. In **hoofdstuk 5** laten we zien dat meerder componenten van het LUBAC-complex nodig zijn om de activiteit van HOIP op NEMO te richten, terwijl de lineaire ketenformatie alleen afhankelijk is van HOIP en de target-ubiquitine.

De belangrijkste resultaten uit de voorgaande hoofdstukken worden besproken in de context van de RBR subfamilie van RING E3-ligases in **hoofdstuk 6**. Het RING/HECT-achtige mechanisme wordt waarschijnlijk door alle RBRs gebruikt, maar de herkenning van targets en de ubiquitine ketenspecificiteit worden per RBR verschillend gereguleerd.

Curriculum vitae

Judith Janny Smit werd geboren op 23 maart 1984 te Heerenveen. Ze behaalde haar VWO diploma in 2002 aan het Bornego College te Heerenveen en startte aansluitend met de bacheloropleiding Biomedische wetenschappen aan de Vrije Universiteit te Amsterdam (VU). Als afstudeerstage heeft zij onderzoek verricht op de afdeling moleculaire en cellulaire neurobiologie van Prof. dr. Guus A.B. Smit. In 2005 startte ze met de masteropleiding Neuroscience aan de VU. Haar eerst afstudeerstage deed zij in de groep van Prof. dr. Titia K. Sixma op de afdeling moleculaire carcinogenese aan het Nederlands Kanker Instituut te Amsterdam waarin ze onder begeleiding van dr. Chris Ulens onderzoek heeft gedaan naar de structurele kenmerken van homologen van de nicotine acetylcholine receptor. Haar tweede afstudeerstage deed zij onder begeleiding van dr. M. Wijtmans en dr. I.J.P. de Esch in de groep van Prof. dr. R. Leurs op de afdeling Medische Chemie aan de VU. Tijdens deze stage heeft zij bijgedragen aan het ontwerpen, synthetiseren en *in silico* analyseren van liganden in de histamine bindingspocket van de histamine H_4 receptor.

Sinds september 2007 is ze werkzaam als onderzoeker in opleiding op de afdelingen moleculaire carcinogenese en biochemie aan het Nederlands Kanker Instituut. Hier heeft zij onder leiding van Prof. dr. Titia K. Sixma de experimenten verricht die in dit proefschrift staan beschreven.

List of publications

Requirements for LUBAC-mediated NEMO priming and linear ubiquitin chain elongation.
Submitted for publication
J.J. Smit, W.J. van Dijk, D. El Atmioui, R. Merkx, H. Ovaa, T.K. Sixma

The E3 ligase HOIP specifies linear ubiquitin chain assembly through its RING-IBR-RING domain and the unique LDD extension.
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