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EDITOR'S CHOICE

MALT1 cleaves the E3 ubiquitin ligase HOIL-1 in activated T cells, generating a dominant negative inhibitor of LUBAC-induced NF-κB signaling

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Human paracaspase 1 (PCASP1), better known as mucosa associated lymphoid tissue lymphoma translocation 1 (MALT1), plays a key role in immunity and inflammation by regulating gene expression in lymphocytes and other immune cell types. Deregulated MALT1 activity has been implicated in autoimmunity, immunodeficiency and certain types of lymphoma. As a scaffold MALT1 assembles downstream signaling proteins for nuclear factor-κB (NF-κB) activation, while its proteolytic activity further enhances NF-κB activation by cleaving NF-κB inhibitory proteins. MALT1 also processes and inactivates a number of mRNA destabilizing proteins, which further fine-tunes gene expression. MALT1 protease inhibitors are currently developed for therapeutic targeting. Here we show that T cell activation, as well as overexpression of the oncogenic fusion protein API2–MALT1, induces the MALT1-mediated cleavage of haem-oxidized IRP2 ubiquitin ligase 1 (HOIL-1). In addition, to acting as a K48-polyubiquitin specific E3 ubiquitin ligase for different substrates, HOIL-1 co-operates in a catalytic-independent manner with the E3 ubiquitin ligase HOIL-1L interacting protein (HOIP) as part of the linear ubiquitin chain assembly complex (LUBAC). Intriguingly, cleavage of HOIL-1 does not directly abolish its ability to support HOIP-induced NF-κB signaling, which is still mediated by the N-terminal cleavage fragment, but generates a C-terminal fragment with LUBAC inhibitory properties. We propose that MALT1-mediated HOIL-1 cleavage provides a gain-of-function mechanism that is involved in the negative feedback regulation of NF-κB signaling.

Introduction

Mucosa associated lymphoid tissue lymphoma translocation gene 1 (MALT1) or paracaspase-1 (PCASP1) [1], plays a central role in nuclear factor-κB (NF-κB) signaling initiated by several immunoreceptor tyrosine-

Abbreviations
BCL10, B-cell lymphoma 10; CARD11, caspase recruitment domain family member 11; CARMA1, CARD-containing MAGUK protein 1; CBM complex, CARMA1–BCL10–MALT1 complex; CYLD, cylindromatosis; DLBCL, diffuse large B cell lymphoma; HOIL-1L, haem-oxidized IRP2 ubiquitin ligase 1L; HOIP, HOIL-1L interacting protein; IKK, IκB kinase; LUBAC, linear ubiquitin chain assembly complex; MALT1, mucosa associated lymphoid tissue lymphoma translocation 1; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB; NZF, Npl4-like zinc finger; PCASP1, paracaspase-1; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RBR, RING in-between–RING RING; RING, really interesting new gene; SHARPIN, SHANK-associated RH domain-interacting protein; TAB2/3, TAK binding protein 2/3; TAK1, transforming growth factor-β-activated kinase 1; TCR, T cell receptor; Th17, T helper 17; TNF, tumor necrosis factor; TRAF6, tumor necrosis factor receptor associated factor 6; UBL, ubiquitin-like.

based activation motif (ITAM) containing immunoreceptors, of which its role in T cell receptor (TCR) signaling is best described [2]. MALT1-dependent TCR signaling is strictly required for the development of regulatory T cells and effector T cells of the Th17 type. MALT1-deficient mice show impaired T-cell responses to immunization and are resistant to experimental autoimmune encephalomyelitis [3,4], while human patients with defective MALT1 expression or function were recently shown to suffer from combined immunodeficiency, characterized by severe recurrent infections and impaired cellular and humoral immune responses [5–7].

MALT1-mediated TCR signaling involves the TCR-induced activation of protein kinase C (PKC)θ, which phosphorylates the adaptor protein CARD-containing MAGUK protein 1 [CARMA1; also known as caspase recruitment domain family member 11 (CARD11)]. This facilitates the recruitment of the CARD-containing adaptor protein B-cell lymphoma 10 (BCL10) through homotypic CARD–CARD interactions, which is itself associated with MALT1, thus forming a CARMA1–BCL10–MALT1 (CBM) complex [8–11]. As part of the BCM complex, MALT1 recruits the E3 ubiquitin ligase TRAF6 (tumor necrosis factor receptor associated factor 6), which mediates the K63-linked polyubiquitination of MALT1, its binding partner BCL10 and TRAF6 itself. This provides docking sites for the recruitment of the kinase transforming growth factor-β-activated kinase 1 (TAK1) via TAK binding protein (TAB)2/3 adaptor proteins [12]. The CBM complex also associates with the linear ubiquitination chain assembly complex (LUBAC) [13], which consists of the E3 ubiquitin ligases HOIL-1L (haem-oxidized IRP2 ubiquitin ligase 1L; also known as RBCK1 or RNF54) and HOIP (HOIL-1L interacting protein; also known as RNF31), together with the adaptor protein SHANK-associated RH domain-interacting protein (SHARPIN). In the case of tumor necrosis factor (TNF) receptor signaling, catalytic activity of HOIP mediates the M1-linked (also known as linear) polyubiquitination of signaling proteins, which is thought to form docking sites for the physical recruitment of the IκB kinase (IKK) complex. The current model is that this stabilizes the TNF receptor signaling complex and brings IKKβ into close proximity to TAK1, allowing TAK1 to phosphorylate and thereby activate IKKβ, leading to NF-κB activation [14]. However, the precise role of LUBAC in TCR signaling is less clear. Knockdown of HOIP or SHARPIN decreased TCR-mediated NF-κB activation, but expression of catalytically inactive HOIP did not influence NF-κB activation, implying that the role of LUBAC in TCR-mediated NF-κB activation is independent of the E3 ligase activity of HOIP [13,15]. In contrast to HOIP, HOIL-1 does not directly mediate linear ubiquitination, but binding of HOIL-1 to the N-terminal region of HOIP is believed to induce a conformational change that unlocks the C-terminal catalytic domain of HOIP [16]. In addition, binding of HOIL-1 is known to stabilize HOIP. HOIL-1 also acts as a K48 polyubiquitin-specific E3 ligase (reviewed in [17]), but a role for HOIL-1-mediated ubiquitination in TCR signaling has not yet been described.

A conceptual breakthrough in the TCR signaling field was the identification that MALT1 not only promotes NF-κB activation by acting as a scaffold, but also has proteolytic activity that is essential for NF-κB signal amplification and persistence (reviewed in [18]). This is thought to be mainly achieved by the cleavage of the NF-κB subunit RelB and the deubiquitinating enzyme A20 [19,20], two negative regulators of the NF-κB pathway. Besides these proteins, only a limited number of other MALT1 substrates have been identified so far, including BCL10 [21], the deubiquitinating enzyme CYLD [22], the mRNA-destabilizing proteins Regnase-1 (also known as MCPIP1 or Zc3h12a), Roquin-1 and -2 [23,24], and MALT1 itself [25]. The discovery of MALT1 proteolytic activity has stimulated the development of MALT1 inhibitors for therapeutic targeting, which have already shown promising results in preclinical models [26–29]. On the other hand, the recent finding that mice expressing catalytically inactive MALT1 spontaneously develop signs of autoimmunity [30–33], indicates that MALT1 targeting may not be without risk. It is therefore important to further explore its molecular function and to identify the full spectrum of its substrates. Here, we show that TCR stimulation triggers the rapid cleavage of the E3 ubiquitin ligase HOIL-1 and identify MALT1 as the protease responsible. In addition, we show that the C-terminal HOIL-1 cleavage fragment negatively regulates LUBAC-mediated NF-κB signaling. These data demonstrate a complex role for MALT1 proteolytic activity in the regulation of TCR-induced NF-κB signaling.

Results

HOIL-1 is cleaved upon TCR stimulation

During our studies on the role of LUBAC in TCR signaling, we noticed the appearance of an additional faster running band of approximately 36 kDa in a HOIL-1 immunoblot of anti-CD3 plus anti-CD28 stimulated Jurkat T cells (Fig. 1A). Similar results were obtained when Jurkat cells were stimulated with phorbol 12-myristate 13-acetate (PMA) plus ionomycin, which

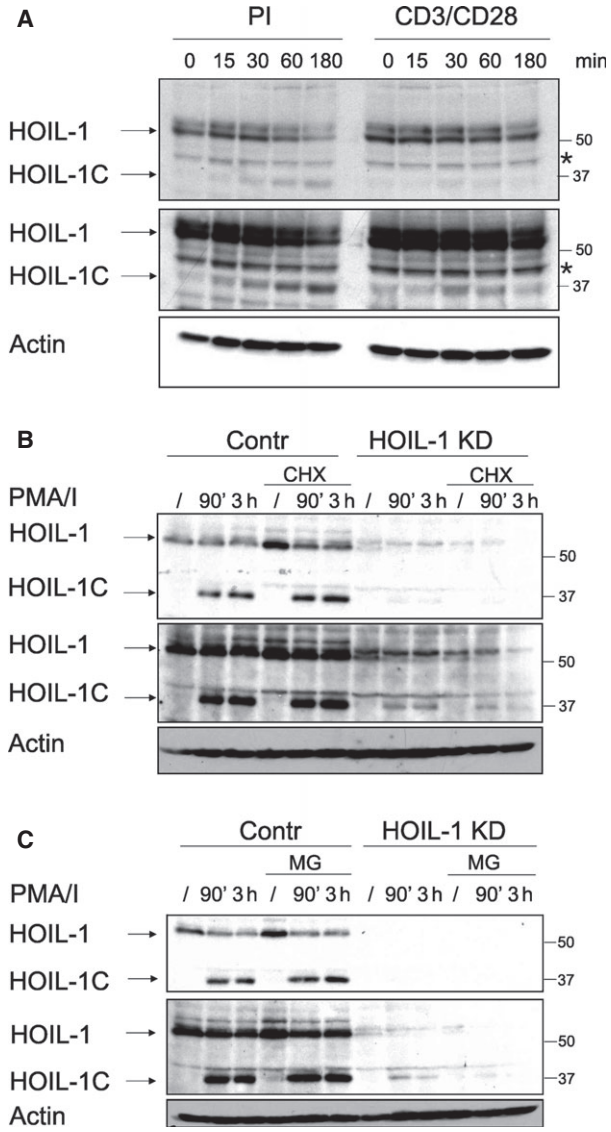

Fig. 1. HOIL-1 is cleaved in activated T cells. (A) Jurkat T cells were stimulated for the indicated times with anti-CD3 plus anti-CD28 (CD3/CD28) or PMA plus ionomycin (PI). Cell extracts were analyzed by immunoblotting for expression of HOIL-1 or actin as a loading control. HOIL-1C represents the C-terminal cleavage fragment of HOIL-1. * indicates a non-specific band. (B, C) Jurkat cells (contr) were treated with PMA plus ionomycin for the times indicated after pretreatment for 3 h with cycloheximide (CHX) or MG132 (MG) where indicated. Similar treatments were applied to cells in which HOIL-1 was stably silenced (HOIL-1 KD). Results are representative of at least three independent experiments.

mimics TCR signaling downstream of PKCθ (Fig. 1A). Because we detected HOIL-1 with an antibody raised against the C-terminal part of HOIL-1, the faster migrating band must represent the C-terminal part of HOIL-1 (from now on referred to as HOIL-1C). The TCR-induced generation of HOIL-1C was already detectable within 15 min after stimulation and could not be prevented by treatment of cells with the protein translation inhibitor cycloheximide (Fig. 1B), suggesting a role for post-translational processing rather than *de novo* synthesis. HOIL-1 and HOIL-1C were no longer detectable upon siRNA-mediated silencing of HOIL-1, demonstrating specificity (Fig. 1B). Treatment of Jurkat T cells with the proteasome inhibitor MG132 did not affect the levels of HOIL-1C, suggesting it is rather stable (Fig. 1C). Together, these results indicate that TCR stimulation leads to the proteolytic processing of HOIL-1, generating a stable 36 kDa C-terminal MALT1 cleavage product.

TCR-induced HOIL-1 cleavage is dependent on MALT1 catalytic activity

Because TCR stimulation is known to activate MALT1 proteolytic activity, leading to the processing of a limited number of substrates, we hypothesized a potential role for MALT1 in the TCR-induced generation of HOIL-1C. To explore this further we first tested the effect of siRNA-mediated MALT1 silencing on TCR-induced HOIL-1 cleavage in Jurkat T cells. In the absence of MALT1, anti-CD3 plus anti-CD28-induced HOIL-1 cleavage was completely abolished (Fig. 2A). Similar results were obtained upon stimulation with PMA plus ionomycin (Fig. 2A). We next tested if MALT1-dependent HOIL-1 cleavage could also be detected in primary cells. Indeed, PMA plus ionomycin treatment also induced the rapid HOIL-1 cleavage in primary mouse $CD4^+$ T cells, which was absent in $CD4^+$ T cells isolated from MALT1 knockout mice (Fig. 2B).

To demonstrate that MALT1-dependent HOIL-1 cleavage also required MALT1 catalytic activity, we analyzed the effect of different MALT1 protease inhibitors on TCR-induced HOIL-1 cleavage in Jurkat T cells. Pretreatment of cells with the tetrapeptide MALT1 inhibitor z-VRPR-fmk [21] decreased PMA plus ionomycin- as well as anti-CD3 plus anti-CD28-induced HOIL-1 cleavage to a similar extent as it decreased cleavage of the known MALT1 substrate CYLD [22] (Fig. 2C). Almost complete inhibition of HOIL-1 processing was obtained with the more potent MALT1 inhibitor mepazine [27] (Fig. 2D). Together, these data demonstrate that TCR-induced HOIL-1 cleavage is dependent on MALT1 catalytic activity.

HOIL-1 is directly cleaved by MALT1 between its UBL and NZF domain

The ability of MALT1 to cleave HOIL-1 was further investigated in HEK293T cells by co-expressing

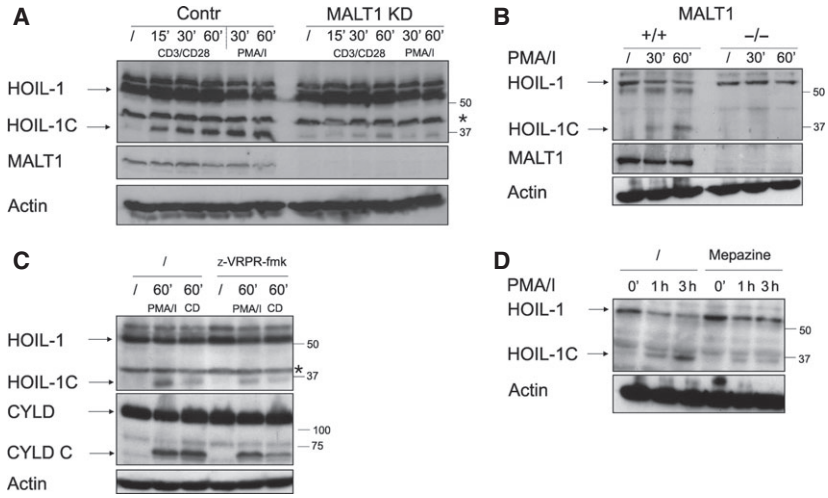

Fig. 2. HOIL-1 cleavage requires MALT1 catalytic activity. (A) Jurkat T cells treated with control siRNA (contr) or MALT1-specific siRNA (MALT1 KD) were stimulated with anti-CD3 plus anti-CD28 (CD3/CD28) or PMA plus ionomycin (PMA/I) for the indicated times. HOIL-1C represents the C-terminal cleavage fragment of HOIL-1. (B) Primary spleen $CD4^+$ T cells isolated from either wild-type (+/+) or MALT1 deficient (−/−) mice were stimulated with PMA plus ionomycin for 30 or 60 min and analyzed as in (A). (C, D) Jurkat cells were treated with PMA plus ionomycin (PMA/I) or anti-CD3 plus anti-CD28 (CD) for different times after pretreatment with z-VRPR-fmk or mepazine as indicated. Cell extracts were analyzed by immunoblotting for expression of HOIL-1, CYLD, or actin as a loading control. Results are representative of at least three independent experiments.

Flag-tagged HOIL-1 with either wild-type MALT1 or a catalytic inactive MALT1(C464A) mutant. MALT1 was activated by either co-expressing BCL10, or by expressing MALT1 as a fusion protein with a helix–loop–helix (HLH) motif that induces MALT1 oligomerization [34]. Immunoblotting of cell lysates with anti-HOIL-1 showed that overexpression of BCL10 plus MALT1 or HLH-MALT1 induced the generation of a C-terminal HOIL-1 cleavage fragment with the same size as the fragment generated in PMA plus ionomycin-treated Jurkat cells (Fig. 3A). Since cells were transfected with N-terminal Flag-tagged HOIL-1, anti-Flag immunoblotting also allowed detection of the 22 kDa N-terminal cleavage fragment of HOIL-1. Mutation of the active site cysteine in the catalytic domain of MALT1 each time completely abolished HOIL-1 cleavage. Similar results were obtained upon expression of an API2–MALT1 fusion protein, which results from a chromosomal translocation that juxtaposes the *API2* gene at 11q21 to the *MALT1* gene at 18q21 and results in the in-frame fusion of the N-terminal region of *API2* with the C-terminal region of *MALT1*. This fusion protein is found in 50% of MALT lymphomas with cytogenetic abnormalities and is thought to have an important role in lymphomagenesis through its impact on survival and proliferation via the NF-κB-mediated pathway [35,36].

HOIL-1 consists of an N-terminal UBL (ubiquitin-like) domain, followed by a ubiquitin-binding NZF (Npl4-type zinc finger) domain and a C-terminal catalytic RING in-between-RING RING (RBR) motif (Fig. 3B). The generation of an N-terminal 22 kDa fragment and a C-terminal 36 kDa fragment upon co-expression of HOIL-1 with activated MALT1 points to a MALT1 cleavage site between the UBL and NZF domains of HOIL-1. MALT1 cleaves its substrates directly after an arginine in the P1 position, with a fairly strict requirement for a leucine residue in P4 [37]. This information together with the alignment of cleavage sites in known substrates led us to postulate LQPR^{165}G as a potential cleavage site in human HOIL-1 (Fig. 3B), which is conserved in mouse HOIL-1 (LQSRG). To verify this, we compared the cleavage of human wild-type HOIL-1 and mutant HOIL-1(R165A), in which arginine at position 165 was replaced by alanine, upon co-expression with MALT1 plus BCL10 or the constitutively active fusion proteins HLH–MALT1 and API2–MALT1 in HEK293T cells. In contrast to wild-type HOIL-1, HOIL-1(R165A) was no longer cleaved by MALT1 in the above experiments (Fig. 3C). We also tested the ability of recombinant MALT1 to cleave *in vitro* translated [^{35}S]methionine-labeled HOIL-1 and HOIL-1(R165A) *in vitro*. Again, MALT1 induced the formation of two 36 and 22 kDa products, corresponding to the C-terminal and N-terminal HOIL-1 fragments

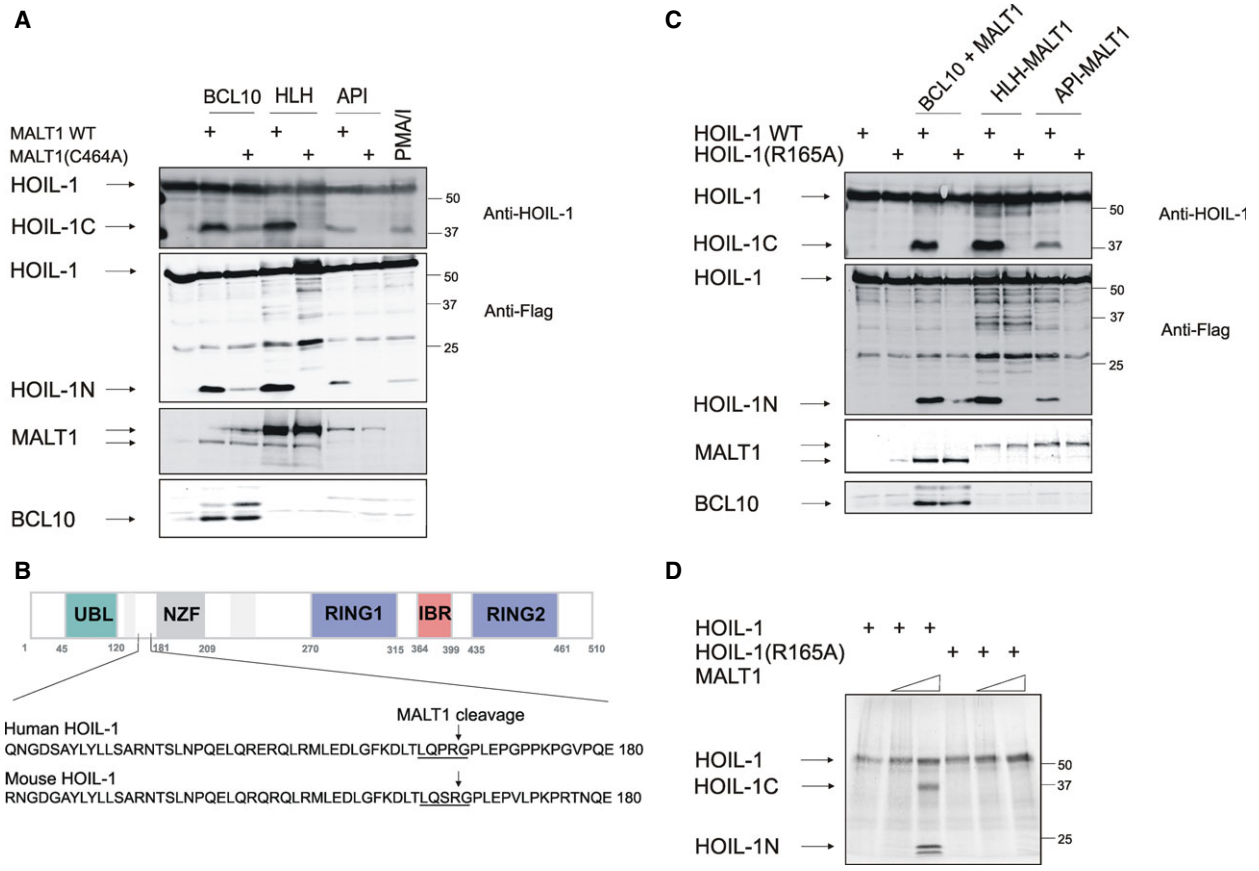

Fig. 3. HOIL-1 is directly cleaved by MALT1 at R165. (A) HEK293T cells were transiently transfected with Flag-tagged HOIL-1 together with either wild-type (WT) MALT1 or mutant MALT1(C464A) plus BCL10, wild-type HLH-MALT1 or mutant HLH-MALT1(C464A), wild-type API2-MALT1 or mutant API2-MALT1(C464A) as indicated. Cell extracts were analyzed by immunoblotting for expression of HOIL-1 using a HOIL-1 antibody specifically detecting the C-terminal fragment of HOIL-1 (HOIL-1C), or an anti-flag antibody detecting HOIL-1 and the N-terminal fragment of HOIL-1 (HOIL-1N). MALT1, HLH–MALT, API2–MALT1 and BCL10 were detected using anti-MALT1 and anti-BCL10 antibodies. Results are representative of at least three independent experiments. Cell extracts from Jurkat cells stimulated for 6 h with PMA plus ionomycin were used as a control for HOIL-1 cleavage (last lane). Results are representative of three independent experiments. (B) Domain structure of HOIL-1 illustrating the UBL domain, the NZF domain and the RBR-domain. Part of the amino acid sequence between the UBL and NZF domain of human and mouse HOIL-1 is shown, indicating the MALT1 cleavage site. (C) HEK293T cells were transfected with wild-type (WT) flag-tagged HOIL-1 or mutant flag-tagged HOIL-1(R165A), together with different MALT1 expression constructs as described in (A). Cell extracts were analyzed by immunoblotting for the proteins indicated. Results are representative of three independent experiments. (D) *In vitro* cleavage of ^{35}S-labeled HOIL-1 or HOIL-1(R165A) by increasing concentrations of recombinant MALT1. Processing was analyzed by SDS/PAGE and autoradiography. Results are representative of two independent experiments.

observed in cells, which was completely abolished in the case of HOIL-1(R165A) (Fig. 3D). These results demonstrate that MALT1 directly cleaves human HOIL-1 at LQPR^{165}G, between the UBL and ZNF domains.

The C-terminal HOIL-1 cleavage fragment negatively regulates LUBAC-induced NF-κB signaling

HOIL-1 is best known as a component of the LUBAC complex, where its N-terminal UBL domain mediates binding to the catalytic component HOIP. Binding of HOIP to HOIL-1, or SHARPIN, is believed to release HOIP's catalytic RBR domain from its auto-inhibitory interaction with the N-terminal UBA domain and to promote the ability of HOIP to activate NF-κB signaling [16,38,39]. To test the effect of MALT1-mediated HOIL-1 cleavage on HOIP-induced NF-κB activation, we compared the ability of wild-type HOIL-1 and its N- or C-terminal fragments to promote HOIP-induced NF-κB activation upon co-expression in HEK293T cells. Co-expression of HOIP with HOIL-1 or HOIL-1N enabled HOIP-induced NF-κB activation (Fig. 4,

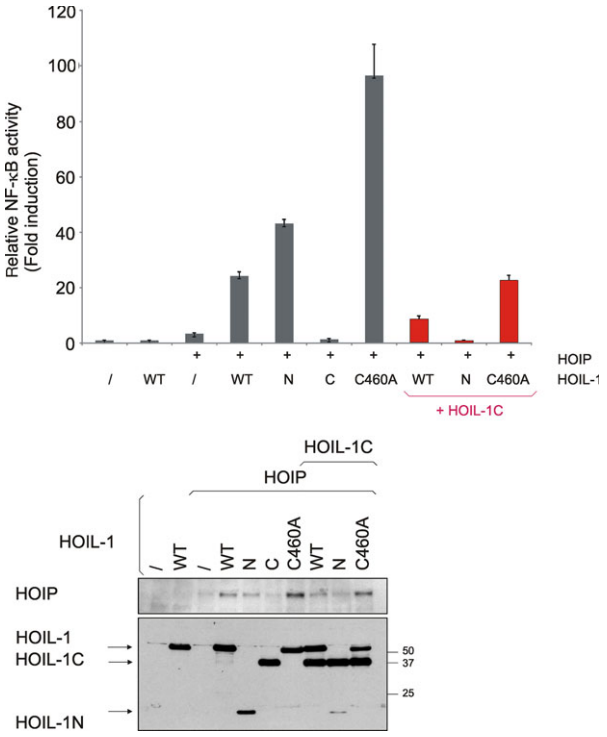

Fig. 4. HOIL-1C inhibits LUBAC-mediated NF-kB activation. HEK293T cells were transiently transfected with an NF-κB-dependent luciferase reporter plasmid, an expression plasmid for β-galactosidase, and expression plasmids for His-tagged HOIP, flag-tagged wild-type HOIL-1 (WT) or the indicated mutants HOIL-1N (N), HOIL-1C (C) or HOIL-1(C460A) as indicated. After 24 h cell lysates were analyzed for luciferase and β-galactosidase activity, corrected for possible differences in transfection efficiency by calculating the luciferase/β-galactosidase ratio, and plotted as fold NF-κB activation. Values are the mean of triplicates ± SD. Expression of HOIL-1 and HOIP was verified by immunoblotting with anti-Flag or anti-His, respectively. Results are representative of three independent experiments.

upper panel), consistent with their known ability to bind HOIP via the N-terminal UBL domain, leading to HOIP stabilization [38]. Co-expression of a HOIL-1 catalytically inactive mutant [HOIL-1(C460A)] promoted HOIP-induced NF-κB activity more efficiently compared with wild-type HOIL-1, suggesting a negative regulatory role for the E3 ubiquitin ligase activity of HOIL-1. No NF-κB activation could be detected upon co-expression of HOIP and HOIL-1C, which is consistent with the inability of HOIL-1C to bind HOIP due to the absence of a UBL domain. However, HOIL-1C significantly reduced or completely abolished NF-κB activation in response to HOIP/HOIL-1 or HOIP/HOIL-1N expression, respectively, indicating a dominant-negative effect of the newly generated C-terminal HOIL-1 fragment. Of note, expression levels of HOIP and especially HOIL-1N were lower in the presence of HOIL-1C, indicating that HOIL-1C leads to their destabilization, which may also explain the inhibitory effect of HOIL-1C on HOIP/HOIL-1N-induced NF-κB signaling. Together, these data indicate that MALT1-mediated cleavage of HOIL-1 generates a C-terminal fragment that acts as a dominant-negative inhibitor of LUBAC-mediated NF-κB signaling, most likely by decreasing the stability of the LUBAC complex.

Discussion

The identification of HOIL-1 as a novel substrate of MALT1 and the oncoprotein API2–MALT1 generates a number of interesting perspectives for future studies. We show that HOIL-1 cleavage at R165 generates an N-terminal fragment that, similar to full length HOIL-1, is still able to support HOIP-induced NF-κB activation. This is also consistent with the presence of the HOIP-binding UBL domain in the N-terminal HOIL-1 fragment. However, the C-terminal fragment that is generated behaves as a dominant-negative inhibitor of HOIP/HOIL-1- or HOIP/HOIL-1N-induced NF-κB signaling. We did not investigate the effect of HOIL-1 cleavage on LUBAC-mediated linear ubiquitination because previous findings indicated a catalytic-independent role of LUBAC in antigen receptor-induced NF-κB activation and failed to see linear ubiquitination in PMA plus ionomycin-treated Jurkat T cells [13]. How exactly HOIL-1C inhibits LUBAC signaling remains to be determined, but our finding that HOIP and HOIL-1N expression levels are reduced in the presence of HOIL-1C suggests that HOIL-1C decreases the stability of the LUBAC complex. Since HOIL-1 has been shown to act as an E3 ubiquitin ligase that is responsible for the K48-polyubiquitination of multiple signaling proteins [17], targeting them for proteasomal degradation, it is tempting to speculate that HOIL-1C affects the levels of HOIP and HOIL-1N by similar mechanisms. Of note, HOIL-1 shows strong structural similarities with the E3 ubiquitin ligase Parkin, whose C-terminal catalytic RBR domain has been reported to be autoinhibited by its own N-terminal UBL domain [40]. It is therefore tempting to speculate that MALT1-mediated cleavage and removal of the UBL domain also releases HOIL-1 from autoinhibition, thus activating HOIL-1 catalytic activity. If this is the case, MALT-mediated HOIL-1 cleavage may affect the ubiquitination and stability of several other HOIL-1 interacting proteins, including PKC, that have been shown to be polyubiquitinated by HOIL-1 [17]. Since many other proteins bind HOIL-1 at the UBL domain

(e.g. PKC) or near its cleavage site (e.g. NF-κB essential modulator (NEMO), the viral RNA receptor retinoic acid inducible gene 1 (RIG-I) and TAB2/3) [41–44], HOIL-1 cleavage may also directly affect the HOIL-1 protein–protein interaction network and associated activities.

Interestingly, HOIL-1 has also been characterized as a transcription factor that is regulated by nucleocytoplasmic shuttling. Mutational analysis of HOIL-1 indicated an N-terminal nuclear export signal and a C-terminal nuclear localization signal [45]. MALT1-mediated cleavage of HOIL-1 physically separates these two signals and may redirect the N- and C-terminal protein parts to the cytoplasm and nucleus, respectively. Interestingly, RBCK2, a splice variant of HOIL-1 that only contains the N-terminal part (amino acids 1–240), of which the last 20 amino acids are different from HOIL-1, can repress HOIL-1's transcriptional activity by tethering HOIL-1 in the cytoplasm [46]. It will therefore be interesting to also investigate the effect of MALT1 on the transcriptional activity of HOIL-1.

In conclusion, our results reveal a complex role for MALT1 proteolytic activity in the regulation of NF-κB-dependent gene expression. While cleavage of A20 and RelB has been shown to promote NF-κB signaling, HOIL-1 cleavage seems to have the opposite effect and could provide a novel negative feedback mechanism to regulate the dynamics of NF-κB signaling. Moreover, HOIL-1 cleavage may affect other signaling pathways in which HOIL-1 has been implicated. Studies using cells or mice expressing non-cleavable HOIL-1 mutant proteins will undoubtedly be very helpful in elucidating the physiological role of HOIL-1 cleavage in the context of T cell-mediated immunity and beyond. As HOIL-1 is also cleaved by the oncoprotein API2–MALT1, our findings might also have implications in the context of lymphomagenesis.

Materials and methods

Cells and transfection

Spleen $CD4^+$ T cells were isolated from wild-type or MALT1 knockout mice using a MACS $CD4^+$ T cell isolation kit II (Miltenyi Biotec, Leiden, The Netherlands), according to the manufacturer's instructions. HEK293T cells and Jurkat T cells were cultured as described before [19]. HEK293T cells were transiently transfected via the calcium phosphate DNA coprecipitation method. Transient silencing MALT1 in Jurkat T cells was obtained by electroporating the cells twice (300 V and 1050 mF; Genepulser, Bio-Rad, Hercules, CA, USA), once 2 days and once 3 days before the start of stimulation, with 400 nM specific siRNA (M-005936-02; GE Healthcare Dharmacon, Diegem, Belgium) in 500 μL serum-free medium per 5 million cells. Stable silencing of HOIL-1 was obtained by electroporating the cells on two consecutive days with an shRNA specifically targeting HOIL-1 (Sigma Mission 1.5 collection, TRCN0000007599 Sigma Aldrich, St Gallen, Switzerland) in 500 μL serum-free medium per 5 million cells. Single clones were isolated by limiting dilution in a 96-well format using conditional RPMI medium and 1 $\mu g \cdot mL^{-1}$ puromycin. HOIL-1 silenced clones were analyzed for HOIL-1 expression by immunoblotting and detection with anti-HOIL-1.

Plasmids, anitbodies and reagents

Constructs in the pcDNA3 vector encoding Flag and Strep tagged HOIL-1 (F-HOIL-1-S) (LMBP 7317), F-HOIL-1(R165A)-S (LMBP 7363), F-HOIL-1Nt-S (LMBP 7390), F-HOIL-1Ct-S (LMBP 7681), F-HOIL-1(C460A)-S (LMBP 8220), as well as pCD-MK MALT1 (LMBP 5536), pCD-MK MALT1p20mutC464A (LMBP 5541), pCD-F-HLH-MALT1-MycHis (LMBP 6102), pCD-F-HLH-MALT1C464A-MycHis (LMBP 6101), and pCD-F-BCL10 (LMBP 5535) were obtained from the LMBP/BCCM plasmid collection, Ghent University, Belgium. Jurkat T cells were stimulated with 5 $\mu g \cdot mL^{-1}$ anti-CD3 (UCHT-1; Pharmingen) plus 5 $\mu g \cdot mL^{-1}$ anti-CD28 (28.2; Pharmingen, San Diego, CA, USA) or 200 $ng \cdot mL^{-1}$ PMA (Sigma-Aldrich) plus 1 mM ionomycin (Calbiochem, San Diego, CA, USA). Where indicated, Jurkat T cells were pretreated for 3 h with 2.5 μM MG132 (Calbiochem, San Diego, CA, USA) or 2 $\mu g \cdot mL^{-1}$ cycloheximide (Sigma-Aldrich) or for 30 min with 100 μM of the MALT1 inhibitors z-VRPR-fmk (Alexis Biochemicals, Lausen, Switzerland) or mepazine (Vitas-M Laboratory, Apeldoorn, The Netherlands). The primary antibodies that were used for detection on western blot were anti-HOIL-1 recognizing the C-terminal part (aa 203–237) (sc-365523; Santa Cruz Biotechnology, Dallas, TX, USA), anti-HOIP (ab85294; Abcam), anti-MALT1 (H-300; Santa Cruz Biotechnology), anti-actin (MP 6472J; MP Biomedicals, Santa Ana, CA, USA), anti-CYLD (sc-74435; Santa Cruz Biotechnology), anti-His6 (Roche-11922416001; Sigma Aldrich) and anti-flag (F-3165; Sigma Aldrich).

Immunoblot analysis

Cells were lysed in 50 mM Hepes, pH 7.6, 250 mM NaCl, 5 mM EDTA and 0.5% (vol/vol) NP-40, plus phosphatase (2.1 μM leupeptin, 0.15 μM aprotinin and 1 mM pefabloc) and protease inhibitors (200 μM sodium orthovanadate, 10 mM sodium fluoride and 5 $\mu g \cdot mL^{-1}$ β-glycerophosphate). Proteins were separated by SDS/PAGE and

analyzed by semi-dry immunoblotting and detection via enhanced chemiluminescence (Perkin-Elmer Life Sciences, Waltham, MA, USA) or by Odyssey infrared imaging (LI-COR, Westburg, Leusden, The Netherlands).

NF-κB-dependent reporter gene assay

NF-κB-dependent luciferase reporter assays were performed as previously described [47]. To normalize for potential differences in transfection efficiency between set-ups, NF-κB dependent expression of luciferase is expressed relative to the constitutive expression of β-galactosidase.

In vitro cleavage of HOIL-1

pCDNA3 expression vectors of HOIL-1 and HOIL-1 (R165A) under the control of the T7 promoter were used for *in vitro* coupled transcription–translation of [^{35}S]methionine-labeled HOIL-1 using reticulocyte extracts according to the manufacturer's instructions (Promega Biosystems, Madison, WI, USA). *In vitro* cleavage was performed with 1.45 μg or 4.35 μg recombinant MALT1 (gift from M. Baens, Human Genome Laboratory, KULeuven, Belgium) in a buffer containing 1 M sodium citrate, 150 mM NaCl, 10% glycerol, 50 mM Mes and 10 mM DTT (pH6.8) for 1.5 h at 37 °C. The resulting cleavage products were analyzed using SDS/PAGE and autoradiography.

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Author contributions

L.E., I.C. and R.B. planned the experiments and analyzed the data. L.E., I.C., Y.D. and M.H. performed the experiments. Y.D. made the HOIL-1 deficient Jurkat T cell clones. J.S. made several expression plasmids. L.E., I.C. and R.B. wrote the manuscript.

References

1 Hulpiau P, Driege Y, Staal J & Beyaert R(2015) MALT1 is not alone after all: identification of novel paracaspases. *Cell Mol Life Sci*, in press, doi: 10.1007/s00018-015-2041-9.
2 Blonska M & Lin X (2011) NF-κB signaling pathways regulated by CARMA family of scaffold proteins. *Cell Res* **21**, 55–70.
3 Brüstle A, Brenner D, Knobbe CB, Lang PA, Virtanen C, Hershenfield BM, Reardon C, Lacher SM, Ruland J, Ohashi PS *et al.* (2012) The NF-κB regulator MALT1 determines the encephalitogenic potential of Th17 cells. *J Clin Invest* **122**, 4698–4709.
4 Mc Guire C, Wieghofer P, Elton L, Muylaert D, Prinz M, Beyaert R & van Loo G (2013) Paracaspase MALT1 deficiency protects mice from autoimmune-mediated demyelination. *J Immunol* **190**, 2896–2903.
5 Jabara HH, Ohsumi T, Chou J, Massaad MJ, Benson H, Megarbane A, Chouery E, Mikhael R, Gorka O, Gewies A *et al.* (2013) A homozygous mucosa-associated lymphoid tissue 1 (MALT1) mutation in a family with combined immunodeficiency. *J Allergy Clin Immunol* **132**, 151–158.
6 McKinnon ML, Rozmus J, Fung S-Y, Hirschfeld AF, Del Bel KL, Thomas L, Marr N, Martin SD, Marwaha AK, Priatel JJ *et al.* (2014) Combined immunodeficiency associated with homozygous MALT1 mutations. *J Allergy Clin Immunol* **133**, 1458–1462.
7 Punwani D, Wang H, Chan AY, Cowan MJ, Mallott J, Sunderam U, Mollenauer M, Srinivasan R, Brenner SE, Mulder A *et al.* (2015) Combined immunodeficiency due to MALT1 mutations, treated by hematopoietic cell transplantation. *J Clin Immunol* **35**, 135–146.
8 Matsumoto R, Wang D, Blonska M, Li H, Kobayashi M, Pappu B, Chen Y, Wang D & Lin X (2005) Phosphorylation of CARMA1 plays a critical role in T Cell receptor-mediated NF-kappaB activation. *Immunity* **23**, 575–585.
9 Ruland J, Duncan GS, Elia A, del Barco Barrantes I, Nguyen L, Plyte S, Millar DG, Bouchard D, Wakeham A, Ohashi PS *et al.* (2001) Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. *Cell* **104**, 33–42.
10 Ruland J, Duncan GS, Wakeham A & Mak TW (2003) Differential requirement for Malt1 in T and B cell antigen receptor signaling. *Immunity* **19**, 749–758.
11 Ruefli-Brasse AA, French DM & Dixit VM (2003) Regulation of NF-kappaB-dependent lymphocyte activation and development by paracaspase. *Science* **302**, 1581–1584.
12 Sun L, Deng L, Ea C-K, Xia Z-P & Chen ZJ (2004) The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. *Mol Cell* **14**, 289–301.

13 Dubois SM, Alexia C, Wu Y, Leclair HM, Leveau C, Schol E, Fest T, Tarte K, Chen ZJ, Gavard J *et al.* (2014) A catalytic-independent role for the LUBAC in NF-κB activation upon antigen receptor engagement and in lymphoma cells. *Blood* **123**, 2199–2203.
14 Verhelst K, Verstrepen L, Carpentier I & Beyaert R (2011) Linear ubiquitination in NF-κB signaling and inflammation: What we do understand and what we do not. *Biochem Pharmacol* **82**, 1057–1065.
15 Beyaert R (2014) An E3 ubiquitin ligase-independent role of LUBAC. *Blood* **123**, 2131–2133.
16 Stieglitz B, Morris-Davies AC, Koliopoulos MG, Christodoulou E & Rittinger K (2012) LUBAC synthesizes linear ubiquitin chains via a thioester intermediate. *EMBO Rep* **13**, 840–846.
17 Elton L, Carpentier I, Verhelst K, Staal J & Beyaert R (2015) The multifaceted role of the E3 ubiquitin ligase HOIL-1: beyond linear ubiquitination. *Immunol Rev* **266**, 208–221.
18 Afonina IS, Elton L, Carpentier I & Beyaert R (2015) MALT1 - a universal soldier: multiple strategies to ensure NF-κB activation and target gene expression. *FEBS J* **282**, 3286–3297.
19 Hailfinger S, Nogai H, Pelzer C, Jaworski M, Cabalzar K, Charton J-E, Guzzardi M, Décaillet C, Grau M, Dörken B *et al.* (2011) Malt1-dependent RelB cleavage promotes canonical NF-kappaB activation in lymphocytes and lymphoma cell lines. *Proc Natl Acad Sci USA* **108**, 14596–14601.
20 Coornaert B, Baens M, Heyninck K, Bekaert T, Haegman M, Staal J, Sun L, Chen ZJ, Marynen P & Beyaert R (2008) T cell antigen receptor stimulation induces MALT1 paracaspase-mediated cleavage of the NF-kappaB inhibitor A20. *Nat Immunol* **9**, 263–271.
21 Rebeaud F, Hailfinger S, Posevitz-Fejfar A, Tapernoux M, Moser R, Rueda D, Gaide O, Guzzardi M, Iancu EM, Rufer N *et al.* (2008) The proteolytic activity of the paracaspase MALT1 is key in T cell activation. *Nat Immunol* **9**, 272–281.
22 Staal J, Driege Y, Bekaert T, Demeyer A, Muyllaert D, Van Damme P, Gevaert K & Beyaert R (2011) T-cell receptor-induced JNK activation requires proteolytic inactivation of CYLD by MALT1. *EMBO J* **30**, 1742–1752.
23 Uehata T, Iwasaki H, Vandenbon A, Matsushita K, Hernandez-Cuellar E, Kuniyoshi K, Satoh T, Mino T, Suzuki Y, Standley DM *et al.* (2013) Malt1-induced cleavage of regnase-1 in CD4(+) helper T cells regulates immune activation. *Cell* **153**, 1036–1049.
24 Jeltsch KM, Hu D, Brenner S, Zöller J, Heinz GA, Nagel D, Vogel KU, Rehage N, Warth SC, Edelmann SL *et al.* (2014) Cleavage of roquin and regnase-1 by the paracaspase MALT1 releases their cooperatively repressed targets to promote T(H)17 differentiation. *Nat Immunol* **15**, 1079–1089.
25 Baens M, Bonsignore L, Somers R, Vanderheydt C, Weeks SD, Gunnarsson J, Nilsson E, Roth RG, Thome M & Marynen P (2014) MALT1 auto-proteolysis is essential for NF-κB-dependent gene transcription in activated lymphocytes. *PLoS One* **9**, e103774.
26 Mc Guire C, Elton L, Wieghofer P, Staal J, Voet S, Demeyer A, Nagel D, Krappmann D, Prinz M, Beyaert R *et al.* (2014) Pharmacological inhibition of MALT1 protease activity protects mice in a mouse model of multiple sclerosis. *J Neuroinflammation* **11**, 124.
27 Nagel D, Spranger S, Vincendeau M, Grau M, Raffegerst S, Kloo B, Hlahla D, Neuenschwander M, Peter von Kries J, Hadian K *et al.* (2012) Pharmacologic inhibition of MALT1 protease by phenothiazines as a therapeutic approach for the treatment of aggressive ABC-DLBCL. *Cancer Cell* **22**, 825–837.
28 Ferch U, Kloo B, Gewies A, Pfänder V, Düwel M, Peschel C, Krappmann D & Ruland J (2009) Inhibition of MALT1 protease activity is selectively toxic for activated B cell-like diffuse large B cell lymphoma cells. *J Exp Med* **206**, 2313–2320.
29 Fontan L, Yang C, Kabaleeswaran V, Volpon L, Osborne MJ, Beltran E, Garcia M, Cerchietti L, Shaknovich R, Yang SN *et al.* (2012) MALT1 small molecule inhibitors specifically suppress ABC-DLBCL in vitro and in vivo. *Cancer Cell* **22**, 812–824.
30 Gewies A, Gorka O, Bergmann H, Pechloff K, Petermann F, Jeltsch KM, Rudelius M, Kriegsmann M, Weichert W, Horsch M *et al.* (2014) Uncoupling Malt1 threshold function from paracaspase activity results in destructive autoimmune inflammation. *Cell Rep* **9**, 1292–1305.
31 Jaworski M, Marsland BJ, Gehrig J, Held W, Favre S, Luther SA, Perroud M, Golshayan D, Gaide O & Thome M (2014) Malt1 protease inactivation efficiently dampens immune responses but causes spontaneous autoimmunity. *EMBO J* **33**, 2765–2781.
32 Bornancin F, Renner F, Touil R, Sic H, Kolb Y, Touil-Allaoui I, Rush JS, Smith PA, Bigaud M, Junker-Walker U *et al.* (2015) Deficiency of MALT1 paracaspase activity results in unbalanced regulatory and effector T and B cell responses leading to multiorgan inflammation. *J Immunol* **194**, 3723–3734.
33 Yu JW, Hoffman S, Beal AM, Dykon A, Ringenberg MA, Hughes AC, Dare L, Anderson AD, Finger J, Kasparcova V *et al.* (2015) MALT1 protease activity is required for innate and adaptive immune responses. *PLoS One* **10**, e0127083.
34 Malinverni C, Unterreiner A, Staal J, Demeyer A, Galaup M, Luyten M, Beyaert R & Bornancin F (2010) Cleavage by MALT1 induces cytosolic release of A20. *Biochem Biophys Res Commun* **400**, 543–547.
35 Akagi T, Motegi M, Tamura A, Suzuki R, Hosokawa Y, Suzuki H, Ota H, Nakamura S, Morishima Y,

Taniwaki M *et al.* (1999) A novel gene, MALT1 at 18q21, is involved in t(11;18) (q21;q21) found in low-grade B-cell lymphoma of mucosa-associated lymphoid tissue. *Oncogene* **18**, 5785–5794.
36 Dierlamm J, Baens M, Wlodarska I, Stefanova-Ouzounova M, Hernandez JM, Hossfeld DK, De Wolf-Peeters C, Hagemeijer A, Van den Berghe H & Marynen P (1999) The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18) (q21;q21) associated with mucosa-associated lymphoid tissue lymphomas. *Blood* **93**, 3601–3609.
37 Hachmann J, Snipas SJ, van Raam BJ, Cancino EM, Houlihan EJ, Poreba M, Kasperkiewicz P, Drag M & Salvesen GS (2012) Mechanism and specificity of the human paracaspase MALT1. *Biochem J* **443**, 287–295.
38 Kirisako T, Kamei K, Murata S, Kato M, Fukumoto H, Kanie M, Sano S, Tokunaga F, Tanaka K & Iwai K (2006) A ubiquitin ligase complex assembles linear polyubiquitin chains. *EMBO J* **25**, 4877–4887.
39 Smit JJ, van Dijk WJ, El Atmioui D, Merkx R, Ovaa H & Sixma TK (2013) Target specificity of the E3 ligase LUBAC for ubiquitin and NEMO relies on different minimal requirements. *J Biol Chem* **288**, 31728–31737.
40 Chaugule VK, Burchell L, Barber KR, Sidhu A, Leslie SJ, Shaw GS & Walden H (2011) Autoregulation of Parkin activity through its ubiquitin-like domain. *EMBO J* **30**, 2853–2867.
41 Nakamura M, Tokunaga F, Sakata S & Iwai K (2006) Mutual regulation of conventional protein kinase C and a ubiquitin ligase complex. *Biochem Biophys Res Commun* **351**, 340–347.
42 Tokunaga F, Sakata S, Saeki Y, Satomi Y, Kirisako T, Kamei K, Nakagawa T, Kato M, Murata S, Yamaoka S *et al.* (2009) Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. *Nat Cell Biol* **11**, 123–132.
43 Inn K-S, Gack MU, Tokunaga F, Shi M, Wong L-Y, Iwai K & Jung JU (2011) Linear ubiquitin assembly complex negatively regulates RIG-I- and TRIM25-mediated type I interferon induction. *Mol Cell* **41**, 354–365.
44 Tian Y, Zhang Y, Zhong B, Wang Y-Y, Diao F-C, Wang R-P, Zhang M, Chen D-Y, Zhai Z-H & Shu H-B (2007) RBCK1 negatively regulates tumor necrosis factor- and interleukin-1-triggered NF-kappaB activation by targeting TAB2/3 for degradation. *J Biol Chem* **282**, 16776–16782.
45 Tatematsu K, Yoshimoto N, Koyanagi T, Tokunaga C, Tachibana T, Yoneda Y, Yoshida M, Okajima T, Tanizawa K & Kuroda S (2005) Nuclear-cytoplasmic shuttling of a RING-IBR protein RBCK1 and its functional interaction with nuclear body proteins. *J Biol Chem* **280**, 22937–22944.
46 Yoshimoto N, Tatematsu K, Koyanagi T, Okajima T, Tanizawa K & Kuroda S (2005) Cytoplasmic tethering of a RING protein RBCK1 by its splice variant lacking the RING domain. *Biochem Biophys Res Commun* **335**, 550–557.
47 Heyninck K, De Valck D, Vanden Berghe W, Van Criekinge W, Contreras R, Fiers W, Haegeman G & Beyaert R (1999) The zinc finger protein A20 inhibits TNF-induced NF-kappaB-dependent gene expression by interfering with an RIP- or TRAF2-mediated transactivation signal and directly binds to a novel NF-kappaB-inhibiting protein ABIN. *J Cell Biol* **145**, 1471–1482.