The paracaspase MALT1 cleaves the LUBAC subunit HOIL1 during antigen receptor signaling

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SHORT REPORT

The paracaspase MALT1 cleaves the LUBAC subunit HOIL1 during antigen receptor signaling

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ABSTRACT

Antigen-receptor-mediated activation of lymphocytes relies on a signalosome comprising CARMA1 (also known as CARD11), BCL10 and MALT1 (the CBM complex). The CBM activates nuclear factor κB (NF-κB) transcription factors by recruiting the 'linear ubiquitin assembly complex' (LUBAC), and unleashes MALT1 paracaspase activity. Although MALT1 enzyme shapes NF-κB signaling, lymphocyte activation and contributes to lymphoma growth, the identity of its substrates continues to be elucidated. Here, we report that the LUBAC subunit HOIL1 (also known as RBCK1) is cleaved by MALT1 following antigen receptor engagement. HOIL1 is also constitutively processed in the 'activated B-cell-like' (ABC) subtype of diffuse large B-cell lymphoma (DLBCL), which exhibits aberrant MALT1 activity. We further show that the overexpression of MALT1-insensitive HOIL1 mitigates T-cell-receptor-mediated NF-κB activation and subsequent cytokine production in lymphocytes. Thus, our results unveil HOIL1 as a negative regulator of lymphocyte activation cleaved by MALT1. This cleavage could therefore constitute an appealing therapeutic target for modulating immune responses.

KEY WORDS: Lymphocyte, MALT1, LUBAC, Signaling, Lymphoma, NF-κB

INTRODUCTION

The engagement of antigen receptors in B and T lymphocytes assembles a large signaling complex of CARMA1 (also called CARD11), BCL10, and MALT1 (the CBM complex), which plays a pivotal role in lymphocyte activation and in cellular homeostasis in the immune system (Thome et al., 2010). The CBM serves as a docking platform to recruit and activate the IκB kinase (IKK) complex, which phosphorylates IκBs [nuclear factor κB (NF-κB) inhibitors], marking them for proteasomal degradation (Thome et al., 2010). This allows NF-κB to initiate transcription of its target genes in the nucleus. In addition to its scaffold function during NF-κB activation, MALT1 catalytic activity shapes the immune response (Bornancin et al., 2015; Gewies et al., 2014; Jaworski et al., 2014). MALT1 protease dictates T-cell receptor (TCR)-mediated proliferation, optimal IL-2 production, and Th17 differentiation, and MALT1 enzyme inactivation in mice establishes a lethal multi-organ inflammatory syndrome (Bornancin et al., 2015; Gewies et al., 2014; Jaworski et al., 2014). Known substrates include regulators of NF-κB [A20 (also known as TNFAIP3), RelB and MALT1], adhesion (BCL10), JNK and AP-1 (CYLD), and mTORC1, as well as mRNA stability factors (Regnase-1 and Roquin-1/2) (Demeyer et al., 2016). In the 'activated B-cell-like' (ABC) subset of diffuse large B-cell lymphoma (DLBCL), a combination of genetic lesions drives the constitutive assembly of the CBM (Shaffer et al., 2012). The resulting aberrant activation of NF-κB and of MALT1 counteracts cell death and promotes unlimited growth (Shaffer et al., 2012). In return, ABC DLBCL cells develope a profound addiction to the CBM–NF-κB nexus and to MALT1 catalytic activity (Ferch et al., 2009; Fontan et al., 2012; Hailfinger et al., 2009; Nagel et al., 2012; Ngo et al., 2006).

The fundamental functions of MALT1 protease in lymphocytes and lymphoma urge us to define the landscape of MALT1 substrates (Hailfinger et al., 2014). Here, we have discovered that a new substrate of MALT1 is the E3 ligase HOIL1 (also called RBCK1). HOIL1 is a subunit of the linear ubiquitin assembly complex (LUBAC) together with the E3 ligase HOIP (also known as RNF31), the SHANK-containing protein SHARPIN, and the deubiquitinylase OTULIN (Iwai et al., 2014). This complex catalyzes linear ubiquitylation and participates in multiple signaling pathways converging on NF-κB (Iwai et al., 2014). Although the LUBAC is a central part of the CBM needed for IKK activation in lymphocytes and in ABC DLBCL cells, the exact role of HOIL1 remains elusive (Dubois et al., 2014; Yang et al., 2014). We now report that HOIL1 is a substrate of MALT1 that is cleaved in TCR-stimulated cells and in ABC DLBCL cells, and that this processing contributes to the optimal activation of NF-κB in lymphocytes.

RESULTS AND DISCUSSION

MALT1 is a cysteine protease, which specifically cleaves after an arginine residue when embedded in a consensus S/PR↓G motif (Coornaert et al., 2008; Rebeaud et al., 2008) (Fig. 1A). To uncover additional MALT1 substrates, we performed an *in silico* analysis of known partners of the CBM complex in the literature. Examination of HOIL1 sequence revealed a putative MALT1 cleavage site at LQPR^{165}G (Fig. 1B). The transfection of HEK293T cells with a FLAG-tagged HOIL1 plasmid together with BCL10 and MALT1 resulted in the generation of a COOH-terminal HOIL1 cleavage fragment (HOIL1^{Cter}) of >35 kDa (Fig. 1C). However, replacement of R^{165} with an alanine or with a glycine residue abolished this cleavage (Fig. 1C). Hence, MALT1 drives HOIL1 processing at R^{165} when overexpressed.

Because MALT1 catalytic activity is unleashed in the vicinity of the CBM complex (Pelzer et al., 2013; Rebeaud et al., 2008), where the LUBAC is dynamically recruited (Dubois et al., 2014), we next investigated the status of HOIL1 in Jurkat T lymphocytes. Stimulation with either antibodies to CD3 and CD28 or with phorbol 12-myristate 13-acetate (PMA) plus ionomycin, which both

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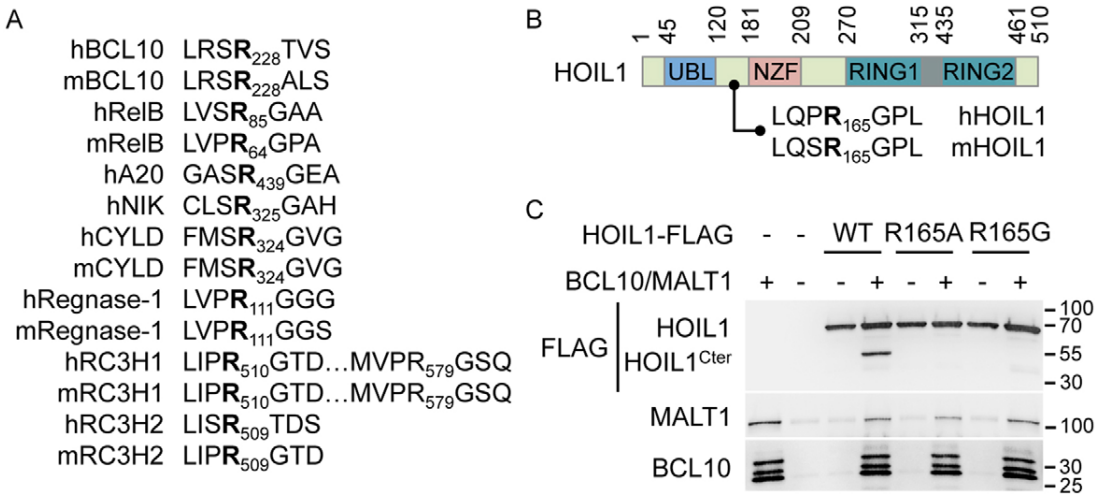

Fig. 1. HOIL1 cleavage by MALT1 at R165. (A) Alignment of the known MALT1 cleavage site in human (h) and mouse (m) proteins. MALT1 cleaves these targets after the arginine residue in bold. (B) Schematic of HOIL1. The putative MALT1 cleavage site after the R165 residue in human and mouse sequences is shown. UBL, ubiquitin-like motif; NZF, novel zinc finger; RING, really interesting new gene. (C) Immunoblots of lysates from HEK293T cells transfected with WT-, R165A-, R165G-HOIL1–FLAG together with BCL10 and MALT1. The positions of molecular mass markers (kDa) are shown. Data are representative of at least three independent experiments.

mimic TCR ligation, led to a robust cleavage of HOIL1 (Fig. 2A). Of note, the known MALT1 substrate CYLD (Staal et al., 2011) is processed with similar kinetics. HOIL1 and CYLD, however, remained intact in response to tumor necrosis factor-α (TNFα, also known as TNF), which operates independently of MALT1 (Fig. 2A). Importantly, similar results were obtained with mouse primary T lymphocytes (Fig. 2B). We further observed that FLAG-tagged wild-type (WT) HOIL1 (HOIL1^{WT}–FLAG) and not HOIL1^{R165A}–FLAG was efficiently processed upon TCR stimulation, further confirming R^{165} as MALT1 cleavage site (Fig. 2C). The silencing of MALT1 and of CARMA1 with small interfering RNA (siRNA) abolished TCR-mediated cleavage of HOIL1 and CYLD (Fig. 2D). This was also true when MALT1 enzyme activity was blocked with the tetrapeptide protease inhibitor zVRPR.fmk or with Mepazine (Nagel et al., 2012; Rebeaud et al., 2008), suggesting that HOIL1 cleavage results from MALT1 protease activity (Fig. 2E,F; Fig. S1). We next examined HOIL1 status in ABC DLBCL cells, which display aberrant MALT1 activity (Ferch et al., 2009; Hailfinger et al., 2009). As a control, MALT1-independent germinal center B-cell-like (GCB) DLBCL cells were used (Shaffer et al., 2012). We found that HOIL1 was only processed in ABC DLBCL lines, and that zVRPR.fmk treatment abrogated this cleavage to restore full-length HOIL1 (Fig. 2G). However, MALT1 inhibition did not affect HOIP, SHARPIN and OTULIN levels, reinforcing the idea that HOIL1 proteolysis does not destabilize the LUBAC. In line with this, HOIP similarly bound to SHARPIN in ABC DLBCL and GCB DLBCL cells although less HOIL1 was detected in ABC DLBCL lysates (Fig. S2). Taken together, these data suggest that HOIL1 is a bona fide MALT1 substrate cleaved after the R^{165} residue.

MALT1 exerts dual complementary roles in TCR signaling (Hailfinger et al., 2014). Its scaffold function marshals NF-κB activation, whereas proteolytic activity governs optimal proliferation and cytokine production (Bornancin et al., 2015; Gewies et al., 2014; Jaworski et al., 2014). MALT1 enzyme also regulates NF-κB signaling independently of the IKK complex by cleaving substrates including A20, RelB and MALT1 itself (Baens et al., 2014; Coornaert et al., 2008; Hailfinger et al., 2011). To explore the effect of HOIL1 cleavage on NF-κB activation, we first expressed HOIL1 or MALT1-resistant HOIL1 in Jurkat cells. This led to a significant reduction in TCR-mediated NF-κB activation, with a more pronounced effect when HOIL1^{R165G} was overexpressed (Fig. 3A). The stable overexpression of HOIL1^{R165G} also resulted in a significant reduction in NF-κB transcriptional activation combined with a decrease in IL-2 secretion following stimulation with PMA plus ionomycin (Fig. 3B,C). However, IκBα was phosphorylated normally, and A20 and RelB were cleaved normally by MALT1 (Fig. 3D,E). HOIL1 fragments resulting from MALT1 cleavage (HOIL1^{Nter} and HOIL1^{Cter}) were shown to exert opposing functions on NF-κB when overexpressed together with the LUBAC subunits in HEK293T cells (Elton et al., 2015). Whereas HOIL1^{Nter} promotes LUBAC-mediated NF-κB signaling, HOIL1^{Cter} thwarts it (Elton et al., 2015). We therefore assessed their effect on NF-κB activation following TCR engagement. In contrast to full-length HOIL1, its cleaved products had no overt impact on NF-κB signaling (Fig. 3F). Supporting previous reports (Klein et al., 2015; Tokunaga et al., 2009), HOIL1^{Cter} was not part of the LUBAC, as evidenced by co-immunoprecipitation experiments of endogenous SHARPIN in lysates from lymphocytes or from ABC DLBCL cells (Fig. 3G; Fig. S2). In stimulated Jurkat, HOIL1, but not HOIL1^{Cter}, bound to CK1α (also known as CSNK1A1), a kinase that dynamically interacts with the CBM and the LUBAC (Dubois et al., 2014), reinforcing the idea that HOIL1^{Cter} is not part of these complexes (Fig. 3H). Taken together, our results suggest that HOIL1 negatively regulates NF-κB independently of the LUBAC and IKK complex, and is inactivated once cleaved by MALT1 upon TCR engagement.

MALT1 paracaspase activity exerts a central function in optimally orchestrating an immune response (Bornancin et al., 2015; Gewies et al., 2014; Jaworski et al., 2014). Yet, the full spectrum of its substrates continues to be elucidated (Demeyer et al., 2016). We now report that the LUBAC subunit HOIL1 mitigates TCR-mediated NF-κB signaling and is cleaved by MALT1 after the residue R^{165}, as well as in MALT1-dependent ABC DLBCL cells. Our data suggest that HOIL1 impedes NF-κB independently of IKK, although the exact mechanism remains unclear. In addition to

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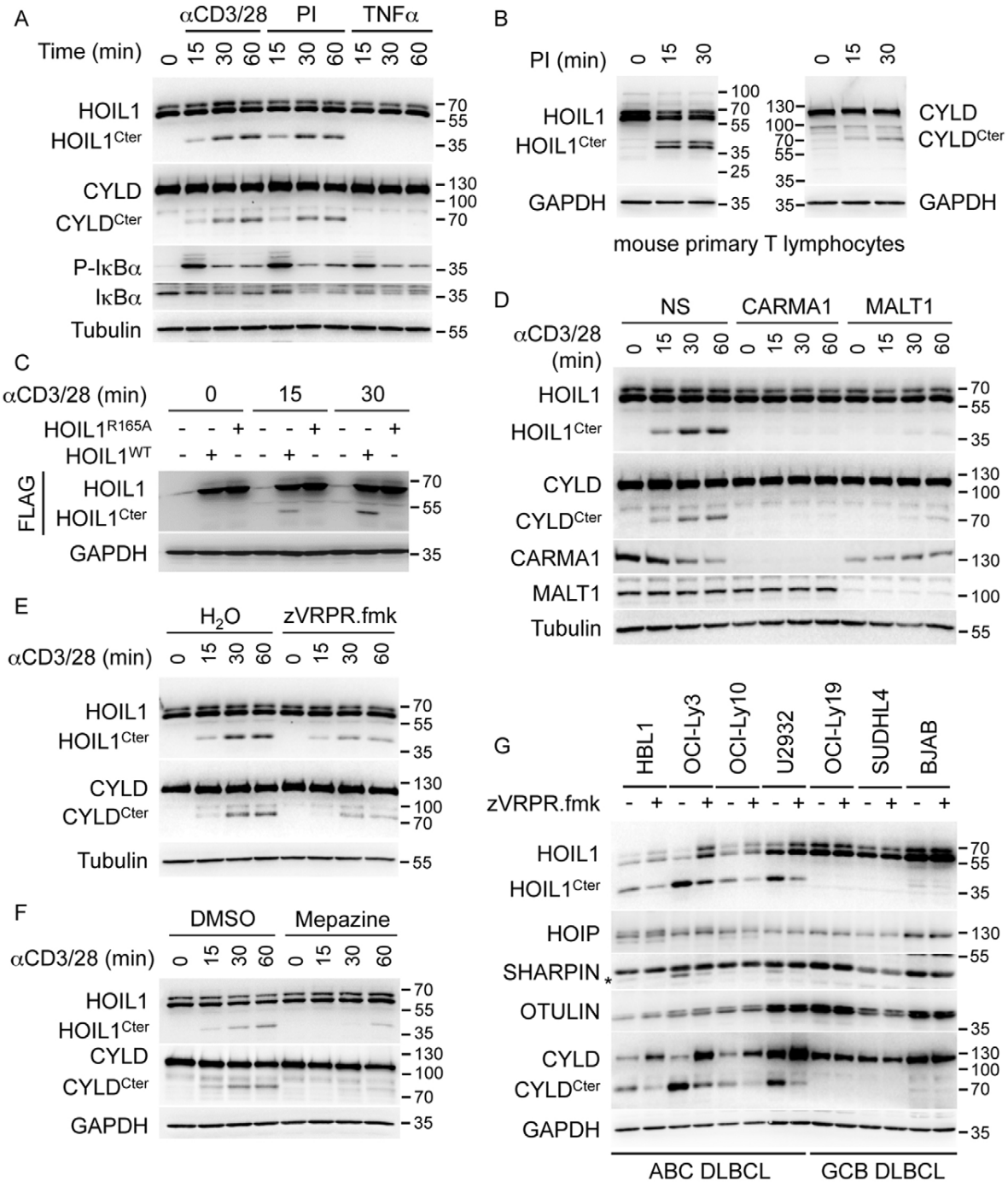

Fig. 2. HOIL1 is cleaved by MALT1 following T-cell receptor engagement and in ABC DLBCL lines. (A) Immunoblot analysis of Jurkat cells stimulated with 1 µg ml^{-1} anti-CD3 and anti-CD28 (αCD3/28), or with 20 ng ml^{-1} PMA plus 300 ng ml^{-1} ionomycin (PI), or with 10 ng ml^{-1} TNFα. (B) Immunoblot of primary mouse T lymphocytes stimulated as in A. (C) Immunoblot of Jurkat cells overexpressing WT- or R165A-HOIL1–FLAG and stimulated as in A. (D) Immunoblot of cells transfected with siRNA for CARMA1, MALT1 or scramble nonspecific (NS) siRNA, and stimulated as in A. (E,F) Immunoblot of Jurkat cells pretreated for 30 min with 75 µM of zVRPR.fmk (E) or 90 min with 20 µM of Mepazine (F), and stimulated as in A. (G) ABC DLBCL lines (HBL1, OCI-Ly3, OCI-Ly10, U2932) and GCB DLBCL lines (OCI-Ly19, SUDHL4, BJAB) were treated with 75 µM zVRPR.fmk for 16 h. Cell lysates were prepared and analyzed by immunoblotting as indicated. The asterisk indicates residual HOIL1 staining. The positions of molecular mass markers (kDa) are shown. Data are representative of at least three independent experiments.

inactivating the NF-κB negative regulators A20 and RelB (Coornaert et al., 2008; Hailfinger et al., 2011), MALT1 initiates an IKK-independent NF-κB pathway through its own auto-proteolysis (Baens et al., 2014) and participates in c-Rel activation (Ferch et al., 2009, 2007). Nevertheless, our data suggests that HOIL1 belongs, together with A20 and RelB (Coornaert et al., 2008; Hailfinger et al., 2011), to a group of proteins that curtail NF-κB when not cleaved by MALT1. In that

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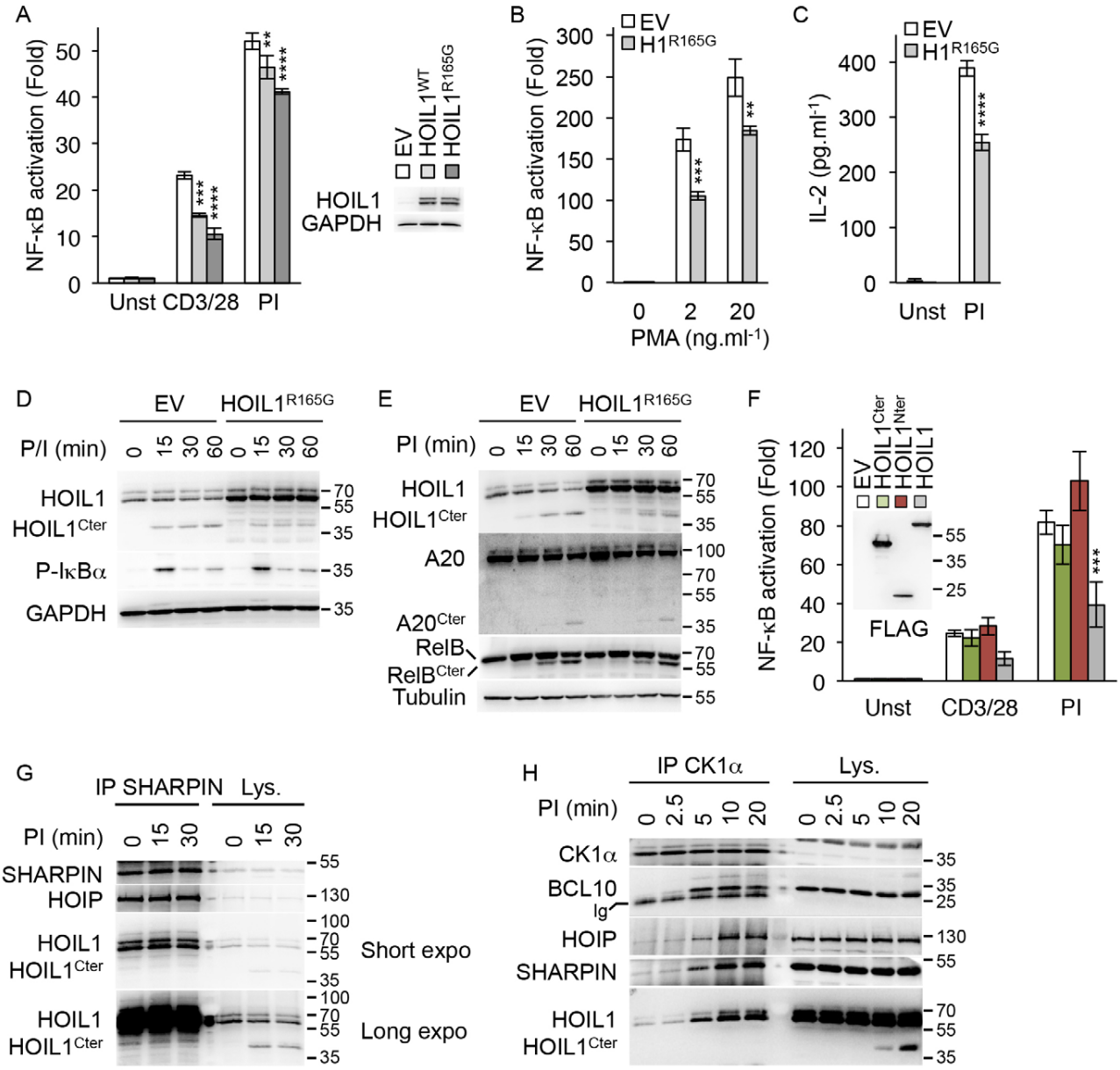

Fig. 3. HOIL1 cleavage participates in the optimal activation of NF-κB. (A) NF-κB reporter luciferase assay (mean±s.e.m.; *n*=3) of cells transfected with 10 µg of plasmids encoding for HOIL1^{WT}, HOIL1^{R165G} or with an empty vector (EV). Cells were stimulated with 0.5 µg ml^{-1} anti-CD3 plus anti-CD28 (CD3/28), or with 20 ng ml^{-1} PMA plus 300 ng ml^{-1} ionomycin (PI or P/I). The inset panel shows the expression of the plasmids when overexpressed in HEK293T cells. Unst, unstimulated. ***P*<0.01; ****P*<0.001; *****P*<0.0001 (ANOVA). (B) NF-κB luciferase assay (mean±s.e.m. of triplicate experiments) of Jurkat cells stably expressing HOIL1^{R165G} or an empty vector (EV), and stimulated with 0-20 ng ml^{-1} PMA plus 300 ng ml^{-1} ionomycin (PI). ***P*<0.01; ****P*<0.001 (ANOVA). (C) Cells as in B were stimulated for 16 h. IL-2 secretion in the culture supernantants was determined by ELISA. Shown is the mean±s.e.m. of triplicate experiments. *****P*<0.0001 (ANOVA). (D,E) Immunoblots of cells as in B. In E, cells were pretreated with 25 µM of the proteasome inhibitor MG132 for 30 min prior to stimulation. (F) NF-κB luciferase assay (mean±s.e.m.; *n*=5; ****P*<0.001 by ANOVA) of Jurkat cells transfected with the indicated FLAG-tagged plasmids. Cells were stimulated as in A. The inset panel shows the expression of the constructs when overexpressed in HEK293T cells. (G,H) Jurkat lymphocytes were stimulated as in A. Cell lysates were immunoprecipitated (IP) with antibodies to SHARPIN (G) or to CK1α (H), and immunoblotting was performed as indicated. Lys., lysates; Ig, light chain immunoblogulin. The positions of molecular mass markers (kDa) are shown. Data are representative of three independent experiments.

sense, defining whether HOIL1 is harmful when left intact in lymphocytes and contributes to the striking phenotype of MALT1 protease-dead mice would be of interest (Bornancin et al., 2015; Gewies et al., 2014; Jaworski et al., 2014). We also provide evidence that exacerbated MALT1 activity in ABC DLBCL cells results in the constitutive cleavage of HOIL1. In addition to interfering with the LUBAC stability (Yang et al., 2014), targeting HOIL1 cleavage might therefore offer a new angle for therapeutic targeting in ABC DLBCL.

How exactly HOIL1 exerts its negative function remains unclear. Two fragments emanate from HOIL1 cleavage (Elton et al., 2015; Klein et al., 2015, and this work), and further investigations will need to clarify their exact role. HOIL1^{Nter} encompasses an ubiquitin-like (UBL) domain sufficient to preserve the LUBAC

architecture and allow NF-κB signaling (Elton et al., 2015; Klein et al., 2015; Tokunaga et al., 2009). This N-terminal fragment likely maintains the LUBAC activity and mediates aberrant NF-κB in ABC DLBCL (Dubois et al., 2014; Yang et al., 2014). HOIL1^{Cter} essentially bears the E3 ligase catalytic activity of HOIL1. Although HOIL1^{Cter} restrains the ability of the LUBAC to activate NF-κB when overexpressed in HEK293T (Elton et al., 2015), it had little impact on TCR-mediated NF-κB activation. In keeping with this, we observed that endogenous HOIL1^{Cter} is not retained in the LUBAC or the CBM. It also massively accumulates in ABC DLBCL cells, which exhibit aberrant NF-κB activation. Because HOIL1 has been shown to catalyze degradative K^{48}-linked ubiquitylation (Elton et al., 2015), it is tempting to speculate that intact HOIL1 promotes the proteasomal degradation of substrates involved in NF-κB signaling, and that MALT1 cleavage counteracts HOIL1 enzyme activity. Our future work will therefore be aimed at defining the nature of HOIL1 substrates when uncleaved.

MATERIALS AND METHODS

Cell culture and reagents

Jurkat E6.1, BJAB and HEK293T were purchased from ATCC. U2932, RIVA, OCI-Ly3 and SUDHL4 were from DSMZ. OCI-Ly10 and OCI-Ly19, and HBL1 cell lines were kindly given by Karin Tarte (INSERM U917, France) and Martin Dyer (University of Leicester, UK), respectively. Mouse primary T lymphocytes were purified with a pan T cell isolation kit (Miltenyi Biotec) from spleens of C57bl/6 (Janvier). Cells were stimulated with a mixture of 20 ng ml^{-1} PMA (Sigma) and 300 ng ml^{-1} ionomycin (Calbiochem), or with 1 µg ml^{-1} anti-CD3 plus 1 µg ml^{-1} anti-CD28 antibodies (both from BD Biosciences), or with 10 ng ml^{-1} of TNF-α (R&D systems). MALT1 protease activity was blocked with 75 µM zVRPR.fmk (Enzo Life Sciences), or with 20 µM Mepazine (Chembridge). siRNA against CARMA1 (HSS130975), and MALT1 (HSS116800) were from Life Technologies.

Expression plasmids, transfections and antibodies

pCMV3flag8HOIL1 was a gift from Martin Dorf (Department of Microbiology and Immunobiology, Harvard Medical School, USA) (Addgene plasmid no. 50016; Fu et al., 2014). HOIL1 was further cloned into a pCDH1-MSCV-EF1α-GreenPuro vector (SBI). MALT1-resistant expression mutants (R165A and R165G) were generated by site-directed mutagenesis, and were verified by sequencing (Genomics and Bioinformatics Core Facility of Nantes, Nantes, France). FLAG-tagged constructs for HOIL1^{Cter}, HOIL1^{Nter} and HOIL1 were previously described (Elton et al., 2015). HEK293T cells were transfected according to standard calcium phosphate protocol, and Jurkat cells were transfected by electroporation (BTX ECM 830, Harvard Apparatus) as previously described (Bidère et al., 2009). Luciferase gene reporter assays (Promega), ELISA for IL-2 secretion (R&D Systems), and cell transduction were performed as previously described (Bidère et al., 2009; Dubois et al., 2014). Antibodies against A20 (59A426, 1:1000), BCL10 (A-6, 1:1000), CK1α (C-19, 1:1000), CYLD (H-6, 1:1000), GAPDH (6C5, 1:20,000), HOIL1 (H-1, 1:1000), MALT1 (B-12, 1:1000), and to tubulin (TU-02, 1:1000) were from Santa Cruz Biotechnology. Antibodies against CARMA1 (1D12, 1:1000), IκBα (cat. no. 9242, 1:1000), phosphorylated IκBα (5A5, 1:2000) and RelB (cat. no.4922, 1:1000) were from Cell Signaling and Technologies. Antibodies to HOIP (A303-560A, 1:1000), SHARPIN (A303-559A, 1:2000), USP34 (A300-824A) and to phosphorylated EZH2 (IHC-00388) were from Bethyl Laboratories. Antibodies to FLAG (M2, Sigma, 1:5000) were also used. Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Southern Biotechnology.

Immunoblotting and immunoprecipitation

Stimuli were washed away with ice-cold PBS prior to cell lysis with TNT buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% Igepal, 2 mM EDTA, protease inhibitors (Thermo Fisher Scientific)] for 30 min on ice. Samples were cleared by centrifugation at 9000 ***g*** and proteins concentration was determined by a BCA assay (Thermo Fisher Scientific). 5–10 µg proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare). Immunoprecipitation experiments were performed as previously described (Bidère et al., 2009; Dubois et al., 2014). Briefly, samples lysed with TNT buffer were precleared with Protein-G–agarose (Sigma) for 30 min and then incubated with 5 µg antibodies and Protein-G–agarose for 1–2 h at 4°C. After four washes, proteins were denaturated and resolved by SDS-PAGE.

Statistical analysis

Statistical significance was assessed with two-way ANOVA tests with post hoc Tukey's analysis (Prism GraphPad Software), and *P* values are indicated in the figure legends.

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Competing interests

The authors declare no competing financial interests.

Author contributions

T.D. designed the research, conducted experiments, analyzed the data; J.G. analyzed the data; and N.B. conceived the project, designed and performed experiments, analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

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Supplementary information

Supplementary information available online at
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