ABSTRACT

Constitutive activation of NF-κB is a hallmark of the activated B cell-like (ABC) subtype of diffuse large B-cell lymphoma (DLBCL), owing to upstream signals from the B-cell receptor (BCR) and MYD88 pathways. The linear polyubiquitin chain assembly complex (LUBAC) attaches linear polyubiquitin chains to IkB kinase-γ, a necessary event in some pathways that engage NF-κB. Two germline polymorphisms affecting the LUBAC subunit RNF31 are rare among healthy individuals (~1%) but enriched in ABC DLBCL (7.8%). These polymorphisms alter RNF31 α-helices that mediate binding to the LUBAC subunit RBCK1, thereby increasing RNF31–RBCK1 association, LUBAC enzymatic activity, and NF-κB engagement. In the BCR pathway, LUBAC associates with the CARD11–MALT1–BCL10 adapter complex and is required for ABC DLBCL viability. A stapled RNF31 α-helical peptide based on the ABC DLBCL–associated Q622L polymorphism inhibited RNF31–RBCK1 binding, decreased NF-κB activation, and killed ABC DLBCL cells, credentialing this protein–protein interface as a therapeutic target.

SIGNIFICANCE: We provide genetic, biochemical, and functional evidence that the LUBAC ubiquitin ligase is a therapeutic target in ABC DLBCL, the DLBCL subtype that is most refractory to current therapy. More generally, our findings highlight the role of rare germline-encoded protein variants in cancer pathogenesis.

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See related commentary by Grumati and Dikic, p. 394.

Essential Role of the Linear Ubiquitin Chain Assembly Complex in Lymphoma Revealed by Rare Germline Polymorphisms

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INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) can be divided into two main molecular subtypes, termed activated B cell-like (ABC) and germinal center B cell-like (GCB) DLBCL, which differ in their gene expression profiles, oncogenic abnormalities, and clinical behavior (1, 2). In ABC DLBCL, regulatory pathways normally associated with B-cell activation are constitutively engaged (1). In particular, the NF-κB pathway plays an essential role in its pathogenesis by promoting malignant cell survival and inducing expression of the master regulatory transcription factor IRF4 (3, 4).

Recent genomic and functional studies have elucidated the molecular mechanisms underlying constitutive NF-κB activity in ABC DLBCL, highlighting the central role of the B-cell receptor (BCR) and MYD88 signaling pathways. The involvement of BCR signaling in ABC DLBCL was first revealed by the dependence of these lymphomas on BCR signaling, a phenomenon dubbed “chronic active” BCR signaling (7). In more than 20% of ABC DLBCL cases, mutations in the immunoreceptor tyrosine-based activation motifs (ITAM) of the BCR subunits CD79B and CD79A augment chronic active BCR signaling (7), providing genetic evidence that BCR signaling is central to the pathogenesis of this lymphoma subtype. A second pathway activating NF-κB in ABC DLBCL is mediated by MYD88, the central adapter in Toll-like receptor (TLR) signaling (8). MYD88 silencing is lethal to ABC DLBCL cells due to inhibition of NF-κB and autocrine interleukin (IL)-6/IL-10 signaling through Janus-activated kinase (JAK) and STAT3 (8, 9). In 39% of ABC DLBCL cases, this pathway is activated by somatic, gain-of-function MYD88 mutations (8). The most common MYD88 mutant, L265P, spontaneously coordinates a signaling complex in which IRAK4 phosphor-ylates IRAK1, leading to IKK and NF-κB activation (8).

Protein ubiquitination is involved in various steps of the NF-κB pathway (10). A recently identified type of polyubiquitin, the linear polyubiquitin chain, plays important roles in NF-κB activation (11–14). This polyubiquitin chain is generated by linkages between the C- and N-terminal amino acids of ubiquitin with wild-type (WT) CARD11, CARD11 is, nonetheless, essential for survival, revealing the dependence of these lymphomas on BCR signaling, a phenomenon dubbed “chronic active” BCR signaling (7). In more than 20% of ABC DLBCL cases, mutations in the immunoreceptor tyrosine-based activation motifs (ITAM) of the BCR subunits CD79B and CD79A augment chronic active BCR signaling (7), providing genetic evidence that BCR signaling is central to the pathogenesis of this lymphoma subtype. A second pathway activating NF-κB in ABC DLBCL is mediated by MYD88, the central adapter in Toll-like receptor (TLR) signaling (8). MYD88 silencing is lethal to ABC DLBCL cells due to inhibition of NF-κB and autocrine interleukin (IL)-6/IL-10 signaling through Janus-activated kinase (JAK) and STAT3 (8, 9). In 39% of ABC DLBCL cases, this pathway is activated by somatic, gain-of-function MYD88 mutations (8). The most common MYD88 mutant, L265P, spontaneously coordinates a signaling complex in which IRAK4 phosphor-ylates IRAK1, leading to IKK and NF-κB activation (8).
modules, resulting in a head-to-tail linear polyubiquitin polymer. The E3 ligase complex responsible for linear polyubiquitin chain formation is the linear ubiquitin chain assembly complex (LUBAC), composed of RNF31 (HOIP), RBCK1 (HOIL-1L), and SHARPIN. In the canonical NF-κB pathway, LUBAC specifically recognizes and conjugates linear polyubiquitin chains onto the IKKγ/NEMO subunit, which is considered to be an essential event that activates IKK and NF-κB (11, 15). Cells derived from both cpdm (Sharpin-mutant) and Rbck1−/− mice have reduced activation of the canonical NF-κB pathway in response to multiple stimuli as well as increased TNF-α-induced apoptosis, highlighting the critical role of LUBAC in NF-κB activation (11–14). Although the full physiologic function of LUBAC is still largely unknown, it seems to regulate B-cell function and innate immune responses (12, 13, 16–19). The fact that the BCR and MYD88 signaling pathways are recurrently targeted by genetic changes in ABC DLBCL suggests that the constitutive activation of NF-κB by genetic changes in ABC DLBCL could depend on LUBAC function.

Although NF-κB is an attractive therapeutic target in ABC DLBCL, no IKK inhibitors have been developed for clinical use due to concerns about the pleiotropic effects of IKK and potential on-target toxicities. Unlike mice with disruption of the genes encoding IKK-β or NEMO, which succumb to massive liver cell death, knockout animals for the LUBAC component RBCK1 are born healthy, suggesting that therapies targeting this pathway might have tolerable side effects. Because ABC DLBCL is the subtype of DLBCL that is most refractory to current therapy (20), new therapeutic strategies are needed. In the present report, we investigate the role of LUBAC in ABC DLBCL and its potential as a therapeutic target.

**RESULTS**

**Enrichment of Two Rare SNPs among ABC DLBCL Tumors**

Given the importance of LUBAC activity in NF-κB signaling, we searched for mutations affecting LUBAC components using RNA-seq data from ABC DLBCL biopsies and identified two recurrent missense RNF31 mutations that change glutamine 584 to histidine (Q584H; n = 2) and glutamine 622 to leucine (Q622L; n = 3). Both of these mutations have been identified previously as rare single-nucleotide polymorphisms (SNP); among the healthy individuals studied in the 1000 Genomes Project (n = 1,094; ref. 21) and the Grand Opportunity (GO) Exome Sequencing Project (n = 8,413; ref. 22), Q584H (SNP accession rs184184005) had a minor allele frequency (MAF) of 0.19% and 0.13%, respectively, whereas Q622L (SNP accession rs149481717) had a MAF of 0.24% and 0.49%, respectively. Both SNPs are located in a highly conserved region of RNF31 that encodes the ubiquitin-associated (UBA) domain, which interacts with the ubiquitin-like (UBL) domain of RBCK1, leading to LUBAC enzyme formation (Fig. 1A and B; refs. 11, 23).

![Figure 1](https://cancerdiscovery.aacrjournals.org/content/early/2014/02/03/cdis.113.0915/F1.large.jpg)

**Figure 1.** Enrichment of two rare SNPs among ABC DLBCL tumors. **A,** amino acid sequence (based on accession NP_060469) of a region of the UBA domain of RNF31 showing the residues altered by two SNPs, Q584H and Q622L, and the number and type of lymphoma biopsies in which they were identified. **B,** location of the residues altered by the RNF31 SNPs in two views of the three-dimensional structure of the RNF31 UBA domain. **C,** frequencies of RNF31 SNPs in biopsy samples from different lymphoma subtypes.
We resequenced RNF31 exon 10, which includes these SNPs, in 561 biopsy samples of various lymphoma subtypes. In 103 ABC DLBCL biopsies, we detected Q584H in 2 cases (1.94%) and Q622L in 6 cases (5.83%), with an overall frequency of 7.77% [95% confidence interval (CI), 4.05%–16.65%], which is 8.22-fold (95% CI, 4.05%–16.65%) higher than in healthy individuals studied in the GO Exome Sequencing Project (22) and 7.77% [95% confidence interval (CI), 3.41%–14.73%], which is similar to the frequency in the healthy cohorts (0.95%), but 11.9-fold lower than the frequency in ABC DLBCL (P = 1.02E−5; Fig. 1C; Supplementary Table S1; ref. 21). Among 458 samples of other lymphoid malignancies, 3 Q622L cases were identified, one each in the GCB subtype of DLBCL, follicular lymphoma, and Hodgkin lymphoma, and no cases had Q584H, yielding a frequency of both SNPs in non–ABC DLBCL cases of 0.66%, which is similar to the frequency in the healthy cohorts (0.95%), but 11.9-fold lower than the frequency in ABC DLBCL (P = 1.03E−4).

Of note, all of the ABC DLBCL cases carrying these SNPs had either a MYD88 mutation or genetic aberrations affecting A20, whereas only one had a mutation in the CD79B subunit of the BCR (Supplementary Table S2). In 5 ABC DLBCL cases with available germline DNA, both Q584H and Q622L were constitutively modified by polyubiquitin, and depletion of RNF31 or SHARPIN decreased this modification (Fig. 2B).

**LUBAC Is Essential for NF-κB Activity in ABC DLBCL**

RNA interference–mediated depletion of RNF31 and SHARPIN was toxic for most of ABC DLBCL lines, but had little effect on the GCB DLBCL lines tested (Fig. 2A). The attachment of linear ubiquitin chains to the IKK-β subunit is required for NF-κB activity in response to various stimuli (11, 15). In ABC DLBCL cells, NEMO was constitutively modified by polyubiquitin, and depletion of RNF31 or SHARPIN decreased this modification (Fig. 2B).
Accordingly, depletion of these LUBAC components decreased two indicators of IKK activity, phosphorylation of IKK-β and its substrate IκBα (Fig. 2B). The activity of IKK-β can be measured using a reporter construct in which IκBα is fused to luciferase (24). Knockdown of RNF31 in an ABC DLBCL line caused a rise in the IκBα luciferase reporter, indicating IKK-β inhibition (Fig. 2C). Likewise, RNF31 and SHARPIN depletion decreased nuclear NF-κB p65 DNA binding (Fig. 2D), and RNF31 depletion reduced NF-κB transcriptional activity in ABC DLBCL, as indicated by a luciferase reporter driven by an NF-κB response element (Fig. 2E). Hence, LUBAC is essential for maintaining NF-κB activity and viability of ABC DLBCL cells.

**Role of LUBAC in CBM Complex–Mediated NF-κB Activation**

We next investigated the role of LUBAC in the BCR and MYD88 pathways, which govern NF-κB activity in ABC DLBCL. By communoprecipitation, RNF31 associated with MALT1 and, to a lesser extent, IRAK1 in ABC DLBCL lines, suggesting that the LUBAC complex could play a role in both pathways (Fig. 3A). Using an antibody specific for linear ubiquitin (11, 13), this modification was detectable on IKK-γ/NEMO immunoprecipitated from ABC DLBCL cells, as expected, but also on IRAK1 (Supplementary Fig. S2). Neither protein was modified by linear ubiquitin in the control GCB DLBCL line. In contrast, linear ubiquitin was not detectable in immunoprecipitates of MALT1 or CARD11.

**Figure 3.** LUBAC is involved in CBM complex mediated NF-κB activation in ABC DLBCL. A, ABC DLBCL lines engineered to express Myc epitope-tagged RNF31 were immunoprecipitated using antibodies to IRAK1 or MALT1, or control immunoglobulin G (IgG; ctrl1) and were analyzed by immunoblotting for the indicated proteins. B, HBL1 cells were engineered to express the indicated shRNAs, treated with MALT1 inhibitor Z-VRPR-fmk (75 μmol/L) for 24 hours or left untreated. Whole-cell lysate was immunoblotted for indicated proteins. MALT1-dependent A20 cleavage products are indicated. C, HBL1 cells expressing the indicated shRNAs were activated by anti-IgM treatment (10 μg/mL) for indicated times and analyzed by immunoblotting for the indicated proteins. D, viability of ABC DLBCL lines expressing control or RNF31 shRNAs were treated with DMSO, ibrutinib (1 nmol/L), or lenalidomide (2 μmol/L) and analyzed by fluorescence-activated cell sorting (FACS) for viable GFP+/shRNA-expressing cells over a time course.

Chronic active BCR signaling in ABC DLBCL causes MALT1 to proteolytically cleave A20, a negative regulator of NF-κB/NF-kB and, to a lesser extent, IRAK1 in ABC DLBCL, suggesting that the LUBAC complex could play a role in NF-κB signaling during BCR cross-linking by anti-immunoglobulin M (IgM) antibodies in a GCB DLBCL line (BJAB) or in an ABC DLBCL line (HBL1) rapidly increased IKK-β phosphorylation, but knockdown of RNF31 compromised this induction, reinforcing the view that LUBAC plays a key role in NF-κB activation during BCR signaling (Fig. 3C and Supplementary Fig. S3A). In keeping with these results, ABC DLBCL lines depleted of RNF31 were sensitized to the Bruton agammaglobulinemia tyrosine kinase (BTK) kinase inhibitor ibrutinib, which blocks...
chronic active BCR signaling in ABC DLBCL (7), and to lenalidomide, which reduces CARD11 levels by inhibiting IRF4 (Fig. 3D; Supplementary Fig. S3B; ref. 4). Together, these results show that LUBAC is associated with the CBM complex and contributes to BCR signaling in ABC DLBCL cells.

**RNF31 SNPs Promote NF-κB Activity in ABC DLBCL**

A small region of the RNF31 UBA domain, from amino acids 579 to 623, binds to the UBL domain of RBC1 (23). The RNF31 Q584H and Q622L mutants reside in this region, suggesting that they might promote LUBAC complex formation and subsequent NF-κB activation. When these RNF31 mutants or WT RNF31 were expressed in ABC DLBCL cells at equivalent levels, Q622L and Q584H increased the activity of an NF-κB-driven luciferase reporter more effectively than WT RNF31 (Fig. 4A). Expression levels of two well-known NF-κB target genes, NFKBIA and IRF4, were elevated by the RNF31 mutants more than by WT RNF31 (Fig. 4B). The RNF31 mutants were also more active in stimulating IKK activity than WT RNF31, as judged by the IκBα luciferase reporter (Fig. 4C), and, accordingly, were also superior in stimulating phosphorylation of IKK-β and its substrate IκBα (Fig. 4D) and in inducing nuclear NF-κB p65 DNA-binding activity (Fig. 4E). When expressed in the GCB DLBCL BJAB, the RNF31 mutants induced expression of the NF-κB target CD83, especially in response to anti-IgM-induced BCR activation (Fig. 4F), supporting the notion that LUBAC contributes to BCR-induced engagement of NF-κB (Fig. 3C and Supplementary Fig. S3A).

In keeping with this hypothesis, RNF31 mutants were more effective than WT RNF31 in stimulating MALT1-dependent cleavage of A20 in ABC DLBCL cells (Fig. 4G). Although both mutant and WT RNF31 isoforms interacted with MALT1 equivalently, A20 was more effectively recruited to the CBM complex in ABC DLBCL BJAB cells selected for expression of either the indicated Myc epitope-tagged RNF31 isoforms.
complex in ABC DLBCL cells expressing mutant RNF31 than in cells expressing WT RNF31 (Fig. 4H). Recent reports demonstrated that zinc finger 7 of A20 binds linear polyubiquitin chains preferentially, thereby facilitating its recruitment to receptor signaling complexes containing LUBAC and IKK (27, 28). In light of this, our results suggested that the RNF31 mutants may promote A20 cleavage by stimulating LUBAC ubiquitination activity and increasing A20 recruitment to the CBM complex.

**RNF31 SNPs Enhance LUBAC Formation and E3 Ligase Activity**

To investigate the possibility that the RNF31 mutants have elevated E3 ligase activity, we studied LUBAC-mediated ubiquitination of IKK-γ/NEMO, a modification that is necessary for IKK activation (11). RNF31 depletion decreased the constitutive NEMO linear ubiquitination in the HBL1 ABC DLBCL line, and the RNF31 mutants were more effective in restoring NEMO linear ubiquitination than WT RNF31 (Fig. 5A). We next transduced HBL1 ABC DLBCL cells with Myc epitope-tagged WT or mutant RNF31 isoforms and evaluated E3 ligase activity in anti-Myc immunoprecipitates using an ELISA-based assay as well as in an *in vitro* ubiquitination assay, the products of which were analyzed by immunoblotting for the indicated proteins (top). The relative NEMO linear ubiquitination signal intensity was determined by densitometric analysis (bottom). Also shown are immunoblots for the indicated proteins in whole-cell lysates subjected to immunoprecipitation with an anti-NEMO antibody, followed by immunoblotting for indicated proteins (top). The relative NEMO linear ubiquitination signal intensity was determined by densitometric analysis (bottom). Also shown are immunoblots for the indicated proteins in whole-cell lysates subjected to immunoprecipitation with an anti-NEMO antibody, followed by immunoblotting for indicated proteins (top). The relative NEMO linear ubiquitination signal intensity was determined by densitometric analysis (bottom). Also shown are immunoblots for the indicated proteins in whole-cell lysates subjected to immunoprecipitation with an anti-NEMO antibody, followed by immunoblotting for indicated proteins (top). The relative NEMO linear ubiquitination signal intensity was determined by densitometric analysis (bottom). Also shown are immunoblots for the indicated proteins in whole-cell lysates subjected to immunoprecipitation with an anti-NEMO antibody, followed by immunoblotting for indicated proteins (top). The relative NEMO linear ubiquitination signal intensity was determined by densitometric analysis (bottom). Also shown are immunoblots for the indicated proteins in whole-cell lysates subjected to immunoprecipitation with an anti-NEMO antibody, followed by immunoblotting for indicated proteins (top). The relative NEMO linear ubiquitination signal intensity was determined by densitometric analysis (bottom). Also shown are immunoblots for the indicated proteins in whole-cell lysates subjected to immunoprecipitation with an anti-NEMO antibody, followed by immunoblotting for indicated proteins (top).

**Targeting the RBCK1–RNF31 Interface with Stapled α-Helical RNF31 Peptides**

We next considered the possibility that the RBCK1–RNF31 interaction surface that is altered by the RNF31 polymorphisms could be a therapeutic target. To address this, we synthesized a series of peptides modeled on RNF31 α-helices 8 and 9 (Fig. 1A and B) that reside at the RBCK1–RNF31 interface using “hydrocarbon stapling” (31) to stabilize their α-helical structure (Fig. 6A). Stapling of the amino-terminal α-helix 8 in the RNF31 WT and Q622L peptides increased their α-helical character, as expected (Fig. 6B). In cultures of HBL1 cells exposed to fluorescein isothiocyanate (FITC)–conjugated derivatives of these peptides, all cells internalized these peptides to roughly equivalent levels, as judged by flow cytometry and confocal microscopy (Supplementary Fig. S4A and S4B). The ability of these FITC-conjugated peptides to bind to recombinant RBCK1 in *vitro* was examined quantitatively using fluorescence polarization spectroscopy. Although both stapled peptides interacted more strongly with RBCK1 than did the unstapled peptide, the stapled FITC–RNF31 N-Q622L peptide was clearly superior to the stapled WT RNF31 peptide (Fig. 6C). We next set up a competition assay in which binding of FITC–RNF31 N-Q622L to RBCK1 was inhibited by increasing concentrations of unlabeled peptides. On the basis of the difference in IC₅₀ values, the N-Q622L RNF31 peptide had an approximately 6-fold higher relative affinity for RBCK1 than the WT RNF31 peptide (Fig. 6D), in keeping with the previous coimmunoprecipitation results (Fig. 5D).
RNF31 Germline Polymorphisms Activate NF-κB in ABC DLBCL

Biologic Effects of RNF31 Stapled Peptides in ABC DLBCL

The N-Q622L peptide prevented endogenous LUBAC formation in ABC DLBCL cells to a greater degree than WT RNF31, as judged by RNF31 coimmunoprecipitation with RBCK1, and the unstaed peptide had little, if any, effect (Supplementary Fig. S5A). NF-κB pathway activity in ABC DLBCL cells, as measured by the IkBα luciferase assay for IKK-β activity and the NF-κB–driven luciferase reporter, was inhibited by the stapled peptides, with the N-Q622L being more active than N-WT, whereas the unstaed peptide and an unrelated stapled peptide were inactive (Fig. 7A and B). RNF31 N-Q622L killed two ABC DLBCL lines in a dose-dependent fashion but had no toxicity for two GCB DLBCL lines (Fig. 7C). Ectopic expression of a constitutively active IKK-β mutant mitigated the effects of RNF31 N-Q622L on ABC DLBCL viability, consistent with IKK being a major target of LUBAC activity in this lymphoma subtype (Supplementary Fig. S5B). Moreover, this stapled peptide sensitized ABC DLBCL lines to the lethal effects of the topoisomerase inhibitor etoposide in response to the topoisomerase inhibitor etoposide, as measured by phosphorylation of IKK-β and IkBα, whereas ectopic provision of either of the RNF31 mutants restored the NF-κB response to a greater extent than WT RNF31 (Fig. 7D). The RNF31 stapled peptides cooperated with etoposide in killing ABC DLBCL cells, with the N-Q622L stapled peptide having more activity than the WT version (Fig. 7E).

DISCUSSION

We report two rare germline polymorphisms affecting the LUBAC subunit RNF31 that were enriched among patients with ABC DLBCL relative to patients with other lymphoma subtypes and to healthy individuals. This genetic observation uncovered an essential role for LUBAC enzyme function in maintaining constitutive NF-κB activity in ABC DLBCL cells, which is the central feature of its pathogenesis. The ABC DLBCL–associated SNPs promote LUBAC complex formation, ubiquitin ligase activity, and stimulation of the NF-κB pathway. We credentialed the RNF31–RBCK1 interface as a therapeutic target using stapled α-helical peptides based on the RNF31 SNPs. This work highlights the potential for rare SNPs in the human population to play a pathogenic role in human disease. The ABC DLBCL–associated SNPs promote LUBAC formation and the ubiquitin E3 ligase activity. Although the exact mechanism by which these SNPs affect LUBAC activity will require structural studies, their position in the available RNF31–RBCK1 crystal structure offers some insight. The RNF31 Q622L mutant is located in an unusual, bent α-helical region that makes direct contact with RBCK1 (23). On the basis of the analysis of other proteins (35), the proline residue at position 619 would be predicted to create a 26° bend between the two adjacent α-helices, but the observed angle
in RNF31 is approximately 53°. This acute bend seems to be reinforced by electrostatic interactions between glutamic acid 618 (E618) and both Q622 and arginine 621. The non-polar leucine residue introduced by the Q622L polymorphism should not interact with E618 in this way, allowing the carboxy-terminal α-helix to adopt an alternative orientation with respect to RBCK1 and potentially promoting the observed stronger interaction between RNF31 and RBCK1. The Q584H polymorphism, which was functionally weaker than Q622L, is nonetheless located in the α6-helix that makes contact with the bent α8/α9-helix containing Q622. Q584 sits at a sharp kink between the α6- and α7-helices, making contact with E591. Conceivably, the introduction of a histidine at position 584 could alter this arrangement and indirectly affect the interaction of the α8/α9-helix with RBCK1.

The present study describes a new role for LUBAC in the CBM complex, whereby LUBAC promotes MALT1 cleavage of A20 during BCR signaling, presumably contributing to greater IKK activity. LUBAC coimmunoprecipitated with MALT1 in ABC DLBCL cells and was required for full IKK activity following BCR cross-linking in a GCB DLBCL line. The association of MALT1 with its substrate A20 was enhanced by the expression of RNF31, and mutant RNF31 isoforms that promote greater LUBAC activity increased MALT1/A20 association. One model to explain these observations would be that LUBAC-mediated ubiquitination of protein(s) in the CBM complex attracts A20, owing to the ability of A20 to bind to linear ubiquitin (28), thereby increasing the access of MALT1 to its substrate. Although neither MALT1 nor CARD11 was detectably modified by linear ubiquitin, IKK itself could attract A20 to the CBM complex, as it was heavily modified by linear ubiquitin, thereby increasing the intrinsic proteolytic activity of MALT1, by an unknown mechanism.

Previous studies have implicated LUBAC in various signaling pathways that engage NF-κB, including those triggered by TNF, IL-1β, and CD40 ligand (CD40L; refs. 11, 12, 14, 15). In the TNFR1-mediated pathway, LUBAC is recruited to the receptor complex in a TRAF2/c-IAP1/c-IAP2–dependent fashion...
by binding to c-IAP1/2-generated ubiquitin linkages (15). Thus, it is likely that LUBAC is recruited to the CBM complex via polyubiquitin chains attached to one or more subunits in this complex. Indeed, the CBM subunits MALT1 and BCL10 are modified by ubiquitin following T-cell receptor stimulation (36–38). Moreover, MALT1 is monoubiquitinated in ABC DLBCL cells, and this modification promotes its survival (38). Chronic active BCR signaling in ABC DLBCL presumably stimulates MALT ubiquitination in ABC DLBCL, but the mechanism by which this occurs remains to be elucidated.

Targeting the RNF31–RBCK1 interface using stapled α-helical RNF31 peptides specifically kills ABC DLBCL cells, supporting the development of LUBAC inhibitors for the therapy of ABC DLBCL. Despite the oncogenic role of many E3 ligases in cancer, small-molecule inhibitors of the active site of these enzymes have not yet emerged as therapies. Our work suggests that focusing small-molecule screens on the RNF31–RBCK1 interface might be a useful strategy. LUBAC inhibitors would be expected to have direct cytotoxic effects on the malignant ABC DLBCL cells but also, by inhibiting NF-κB, sensitize these cells to the apoptotic effects of conventional chemotherapeutic agents (32). Indeed, the RNF31 stapled peptides sensitized ABC DLBCL cells to etoposide. Our studies also suggest that LUBAC inhibitors should sensitize ABC DLBCLs to targeted agents that affect the NF-κB pathway, including ibritinib and lenalidomide. When contemplating LUBAC as a therapeutic target, it is important to emphasize that LUBAC seems to participate in some, but not all, signaling events that activate IKK and NF-κB. In particular, mice with mutations that disrupt LUBAC components do not phenocopy mice with loss of IKK-β or -γ, which are characterized by massive liver apoptosis during development (11–14). Likewise, a rare inherited human immunodeficiency disease caused by loss-of-function RNF31 mutations was characterized by loss of NF-κB activity in some cell types but gain of NF-κB activity in others (39). Thus, drugs targeting LUBAC would have a different spectrum of activities than IKK-β inhibitors. Given the presumed importance of BCR and TLR signaling in autoimmune/inflammatory diseases (40), LUBAC inhibitors might prove useful beyond ABC DLBCL.

Finally, our study demonstrates the value of interrogating rare germline polymorphisms for their role in cancer. Recent examples of rare germline polymorphisms contributing to tumorigenesis have been described in neuroblastoma and melanoma (41–44). These alleles are difficult to discover by standard genome-wide association methods, but can confer a high relative risk, as is the case for the RNF31 polymorphisms in ABC DLBCL. Our study highlights the importance of functional analysis in evaluating the contribution of rare SNPs to disease pathogenesis.

METHODS

Patient Samples, PCR Amplification, and Sanger Sequencing

Tumor biopsy specimens before treatment were obtained from 302 patients with de novo DLBCL, which had previously been classified by gene expression profiling, and 116 patients with follicular lymphoma (FL), 75 patients with chronic lymphocytic leukemia, and 68 patients with Hodgkin lymphoma. All samples were studied according to a protocol approved by the National Cancer Institute Institutional Review Board. Genomic DNA from patient samples was extracted with the DNeasy Tissue Kit (Qiagen) according to the manufacturer’s instructions. The primers used to amplify RNF31 exon 10 are RNF31_E10_F_5′-CTGGGCTTGGTGCCTTTTCTGCAGG-3′ and RNF31_E10_R_5′-GAGTAATTCTTGAGCAGGTATCG-3′. The PCR products were purified using the MinElute UF PCR Purification Kit (Qiagen) and subsequently sequenced using the BigDye sequencing system (Applied Biosystems) from both strands.

Cell Culture

ABC- and GCB-derived DLBCL cell lines BJAB, HT, HBL1, DLBCL2, TMD8, OYB, SUDHL2, and TK were grown in RPMI-1640 medium (Invitrogen) + 10% FBS (Hyclone, Defined) + penicillin/streptomycin (Invitrogen); OCI-Ly3, OCI-Ly10, OCI-Ly8, and OCI-Ly19 cell lines were grown in Iscove’s Modified Dulbecco’s Medium (IMDM; Invitrogen) + 20% human serum + pen/strep (Invitrogen). All cell lines were grown to log phase at 37°C, 5% CO₂ when experiments started. All cell lines had previously been modified to express an ecotropic retroviral receptor and a fusion protein of the Tet repressor and the bacterial resistance gene, as described previously (5).

Cell Lines

ABC and GCB DLBCL cell lines were obtained from the following sources: Martin Dyer (University of Leicester, Leicester, United Kingdom; HBL1; ref. 45), Hans Messner (University of Toronto, Toronto, Canada; OCI-Ly3, OCI-Ly10, and OCI-Ly19; ref. 46), Shuji Tohda (Tokyo Medical and Dental University, Tokyo, Japan; TMD8; ref. 47), Momoko Nishikori (Kyoto University, Kyoto, Japan; OYB, DLBCL2; ref. 48), the Japanese Collection of Research Bioreresources (JCRB) cell bank (TK; http://cellbank.nibio.go.jp), the American Type Culture Collection (HT, SUDHL2; http://www.atcc.org), and DMSZ (BJAB; http://www.dsmz.de). Cell lines have been characterized extensively by gene expression profiling (1) and cancer gene resequencing (6, 7, 49).

Retroviral Vectors and Transduction for shRNA Expression

The retroviral vectors for short hairpin RNA (shRNA) expression were described previously (8). In brief, the shRNA oligos were constructed into a pMSCV-based retroviral vector (pRSMX_Puro) with constitutive expression of a puromycin resistance marker fused with GFP. The inductive expression of shRNA was released after binding of the bacterial tetracycline repressor by doxycycline (50 ng/mL). For retroviral production, shRNA constructs were mixed with a mutant ecotropic envelope-expressing plasmid pHIT/E6 × 3 and gag-pol-expressing plasmid pHIT60 and were transfected into 293T cells using the Lipofectamine 2000 reagent (Invitrogen) as described previously (5). The produced retroviral was used to infect doxycycline-inducible lymphoma cells in the presence of 8-μg/mL polybrene in a single spin infection, and puromycin was used to select for stable integrants over 6 days.

shRNA Sequences

Sequences for control- and target-specific shRNAs are as follows: control shRNA (CTCTCAACCCCTTTAAATGTCGA), MYC shRNA (GGAGCCCCCTAAGACAGGCT), RNF31 shRNA #3 (GCGAGAGCT GTACCTTGGAA), RNF31 shRNA #10 (GAAGACACCGTTGAA GATGAT), SHARPIN shRNA #4 (GGAACCCTTGGATCCTTAC), and SHARPIN shRNA #6 (GAGGCAGGCTTGGCCTCTTAC).

shRNA Toxicity and Complementation Assays

The toxicity assay of shRNA was described previously (5). In brief, 2 days after infection with a retrovirus-expressing shRNA and GFP, the fraction of GFP-positive live cells was measured by flow cytometry. Doxycycline was then added to induce shRNA expression, and the cells were incubated for another 2 days after infection. The toxicity assay of shRNA was repeated. The fraction of GFP-positive live cells was measured by flow cytometry. Doxycycline was then added to induce shRNA expression.
the fraction of GFP-positive live cells was measured at various time points during subsequent culture. The GFP-positive fraction from the test shRNA cultures was normalized to the GFP-positive fraction on day 0.

Retroviral construct used for ectopic expression of WT and mutant RNF31 was described previously (8). In brief, the retroviral vector for inducible DNA expression was pMSCV based, with the DNA fragment inserted at the transcription start site (derived from pCDNA4/TO; Invitrogen). The Myc-tagged human RNF31 cDNA was described previously (11) and was cloned into retroviral vector for expression. RNF31 shRNA rescue retroviral constructs, additional mutations were introduced to RNF31 shRNA-targeted sequence with the primers:

Forward, p-GCCTGGAGAGCAAATAGTGGATGATGCTGCCTTGTAATCTTTCAACAGC
Reverse, p-GCAGATATCATCTTCTCCTTGTGTATCCACAGC

Antibody and Reagents

The antibody against linear ubiquitin chains and RBCK1 was described previously (13). Other antibodies were purchased as follows: anti-IKKβ, anti–phospho-IKKβ, anti-IκBα, anti–phospho-IκBα, and anti-CARD11 from Cell Signaling Technology; anti-ubiquitin polyclonal anti–NEMO/IKK-γ antibody. Secondary horseradish peroxidase–conjugated antibodies were obtained from the same company as each experimental antibody. Secondary horseradish peroxidase–conjugated antibodies were obtained from GE Healthcare.

The Myc-tag elution peptide and epitope were obtained from Sigma. The IMCD compound lenalidomide was obtained from Celgene Corporation. The IKKβ inhibitor MLN120B was obtained from Millennium Pharmaceuticals. The BTK inhibitor ibutinib was obtained from Pharmacycins, Inc. The MALT1 inhibitor Z-VPRP-fmk was obtained from Enzo Life Sciences. Tissue culture–grade dimethyl sulfoxide (DMSO) vehicle control was obtained from Sigma-Aldrich.

Western Blotting

Cell pellets were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris–HCl pH 7.5, 150 mmol/L NaCl, 1% NP40, 0.25% deoxycholic acid, and 1 mmol/L EDTA) supplemented with protease inhibitor tablet and phosphatase inhibitor tablet (Roche), 1 mmol/L dichothreitol (DTT), 1 mmol/L Na3V04, and 1 mmol/L phenethylsulfonylfluoride (PMSF). Protein concentration was measured by the BCA Protein Assay Kit (Thermo Scientific). Total proteins were separated on 4% to 12% SDS-PAGE gels and transferred to nitrocellulose membranes.

NEMO Immunoprecipitation and Ubiquitination

For NEMO ubiquitination, cells were boiled for 15 minutes in 1% SDS before immunoprecipitation. Boiled lysates were diluted to 0.1% SDS with a modified RIPA buffer [50 mmol/L Tris–HCl pH 7.5, 150 mmol/L NaCl, 1% NP40, 0.25% deoxycholic acid, and 1 mmol/L EDTA, supplemented with protease inhibitors, and 5 mmol/L N-ethylmaleimide (Sigma)]. Cleared lysates were incubated overnight with polyclonal anti–NEMO/IKK-γ antibody (Santa Cruz Biotechnology FL-419). Immunoprecipitates were washed five times with lysis buffer, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with an anti-ubiquitin antibody. The method for the detection of NEMO linear polyubiquitination was described previously (13). In brief, cells were boiled for 15 minutes in 1% SDS and then diluted to 0.1% SDS with a modified RIPA buffer. Cleared lysates were subjected to immunoprecipitation with an anti-NEMO monoclonal antibody (BD Pharmingen). Immunoprecipitates were separated on 4% to 12% SDS-PAGE gels and transferred to nitrocellulose membranes, autoclaved with distilled water at 121°C for 30 minutes, and then autoclaved again for 15 minutes without water. Membranes were analyzed by immunoblotting with an anti-linear ubiquitin antibody.

Communoprecipitation

Cells were lysed in an endogenous lysis buffer (20 mmol/L Tris–HCl pH 7.6, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 30 mmol/L NaF, and 2 mmol/L sodium pyrophosphate) supplemented with complete protease inhibitor cocktail (Roche), phosphatase inhibitor tablet (Roche), 1 mmol/L DTT, 1 mmol/L Na3V04, and 1 mmol/L PMSF. Cleared lysates were incubated overnight with polyclonal anti-MALT, anti-IRAK1, and control antibodies. Immunoprecipitates were washed five times with 0.5 mol/L NaCl lysis buffer, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting.

E3 Ubiquitin Ligase Assay and In Vitro Ubiquitination Assay

Engineered HBL1 lines induced to express various isoforms of Myc-tagged RNF31 were lysed in an endogenous lysis buffer (20 mmol/L Tris–HCl pH 7.6, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 30 mmol/L NaF, and 2 mmol/L sodium pyrophosphate) supplemented with complete protease inhibitor cocktail (Roche), phosphatase inhibitor tablet (Roche), 1 mmol/L DTT, 1 mmol/L Na3V04, and 1 mmol/L PMSF. Cleared lysates were incubated overnight with an anti-Myc antibody. Immunoprecipitates were washed five times with lysis buffer, eluted with Myc-specific peptides, and subjected to E3 ubiquitin ligase assay using an E3LITE customizable ubiquitin ligase kit obtained from Life Sensors, following the manufacturer’s instructions. For the in vitro ubiquitination assay, the immunoprecipitated LUBAC complex was washed five times with lysis buffer, eluted with Myc-specific peptides, and resuspended in 40 μL of 20 mmol/L Tris–HCl pH 7.5, 2 mmol/L DTT, 0.1 mmol/L UBE1, 0.4 μmol/L UBE2, 10 μmol/L ubiquitin, 5 mmol/L MgCl2, and 2 mmol/L ATP. Reaction mixtures were incubated for 1 hour at 30°C and stopped by boiling for 10 minutes with SDS. The formation of linear-ubiquitin chains was analyzed by immunoblotting with an anti-ubiquitin antibody.

NF-κB p65 DNA-Binding ELISA

NF-κB p65 DNA-binding activity was measured using a TransAM NF-κB p65 ELISA kit obtained from Active Motif, following the manufacturer’s instructions.

IkB Kinase Activity Reporter Assay

The assay for IκB kinase activity using the IκBα-Photinus luciferase reporter has been described previously (6). In brief, stable clones of TMDB were constructed with vectors to express a fusion protein between IκBα and Photinus luciferase (from pGL3; Promega) as the reporter, and Renilla luciferase (from pRL-TK; Promega) for normalization. The ratio of IκBα-Photinus to Renilla luminescence was measured by the Dual-Glo Luciferase Assay System (Promega), and was normalized to that in untreated or uninduced controls.

NF-κB Reporter Assays

NF-κB transcriptional reporter ABC DLBCL lines were generated by transduction with lentiviral particles containing an inducible reporter, and...
NF-κB responsive luciferase reporter construct (SA Biosciences) and selected with puromycin. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) on a Microtiter Plate Luminometer (Dyn-Ex Technologies).

Flow Cytometry
Flow cytometry for NF-κB activation was performed 3 days after transgene infection. CD83 expression in BJAB cells was determined by staining with an anti-human CD83 antibody (BioLegend).

Peptide Synthesis
Peptide synthesis, olefin metathesis, FITC derivatization, reverse-phase high-performance liquid chromatography (HPLC) purification, and amino acid analysis were performed as described previously (50).

Circular Dichroism Spectroscopy
Peptides (dry, powder form) were dissolved in H2O to prepare 50 μmol/L solutions. The spectra were obtained on a Jasco J-715 spectropolarimeter at 20°C. The spectra were collected using a 0.1-cm pathlength quartz cuvette with the following measurement parameters: wavelength, 185–255 nm; step resolution, 0.2 nm; speed, 20 nm/min; accumulations, 3; and bandwidth, 1 nm.

Fluorescence Polarization Assay and Competition Assay
For binding assays, FITC-peptide (Lc = 14.1 mmol/L) was incubated with a broad range of GST-RBCK1 concentrations in 50 mmol/L Tris, 150 mmol/L NaCl, pH 8.0 at 4°C. Binding activity was measured by fluorescence polarization on a SpectraMax M5 Microplate Reader (Molecular Devices) at 20°C. The spectra were collected using a 0.1-cm pathlength quartz cuvette with the following measurement parameters: wavelength, 185–255 nm; step resolution, 0.2 nm; speed, 20 nm/min; accumulations, 3; and bandwidth, 1 nm.

RBCK1 Recombinant Protein
The codon-optimized cDNA for Escherichia coli expression of full-length human RBCK1 was a kind gift of Dr. Tita K. Sixma (Division of Biochemistry, The Netherlands Cancer Institute, Amsterdam, the Netherlands). For expression, the transformed E. coli BL21 (DE3) pLysS cells were induced with 0.8 mmol/L isopropyl-β-D-thio-galactopyranoside (IPTG) and 0.2 mmol/L ZnSO4 for 8 hours at 22°C. Cells were lysed by using the B-PER bacterial protein extraction reagent (Thermo Scientific), and full-length human RBCK1 was purified with a Pierce GST spin purification kit (Thermo Scientific).

Confocal Microscopy
Images were acquired using a Zeiss LSM510 Meta laser scanning confocal microscope equipped with a 40× C-Apochromat (N.A., 1.2) objective lens, transmitted light detector, and differential interference contrast optical components. Confocal fluorescence images were collected with consistent detector settings for all samples, including 0.11-μm X-Y pixel size, 1.5-μm optical slice thickness, and 4× frame averaging. The final images were exported as TIFF files and arranged into figures using Adobe Photoshop (v.9.0). The brightness and contrast was adjusted equally for all images, using histogram stretching and adjustment of gamma to a value of 1.10.

Cell Viability (MTS) Assay
Cells were plated in triplicate at a density of 10,000 cells per well in 96-well plates. Cell viability after indicated treatments was assessed by adding MTS reagents (Promega), incubating for 1 hour, and measuring the amount of 490-nm absorbance using a 96-well plate reader. The background was subtracted using a media-only control.

Statistical Analysis
All experiments presented have been repeated and results reproduced. Where possible, error bars or P values are shown to indicate statistical significance. For the SNP enrichment analysis, two-sided P values for differences in prevalence were calculated using a Fisher exact test. Confidence levels for the enrichment values were calculated using a normal approximation to the log enrichment. CIs for prevalence estimates were calculated using the Clopper–Pearson method.

Disclosure of Potential Conflicts of Interest
W.C. Chan is a consultant/advisory board member of the Lymphoma Research Foundation. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Essential Role of the Linear Ubiquitin Chain Assembly Complex in Lymphoma Revealed by Rare Germline Polymorphisms

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