Defective immune responses in mice lacking LUBAC-mediated linear ubiquitination in B cells

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The linear ubiquitin chain assembly complex (LUBAC) plays a crucial role in activating the canonical NF- κ B pathway, which is important for B-cell development and function. Here, we describe a mouse model (B-HOIP^{Alinear}) in which the linear polyubiquitination activity of LUBAC is specifically ablated in B cells. Canonical NF- κ B and ERK activation, mediated by the tumour necrosis factor (TNF) receptor superfamily receptors CD40 and TACI, was impaired in B cells from B-HOIP^{Alinear} mice due to defective activation of the IKK complex; however, B-cell receptor (BCR)-mediated activation of the NF- κ B and ERK pathways was unaffected. B-HOIP^{Alinear} mice show impaired B1-cell development and defective antibody responses to thymus-dependent and thymus-independent II antigens. Taken together, these data suggest that LUBAC-mediated linear polyubiquitination is essential for B-cell development and activation, possibly via canonical NF- κ B and ERK activation induced by the TNF receptor superfamily, but not by the BCR.

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Introduction

The NF- κ B family of transcription factors plays crucial roles in many systems, including innate and adaptive immune

responses. After stimulation with various agents, NF- κ B participates in the expression of genes involved in a wide variety of biological phenomena, including immune responses, inflammation, and protection against apoptosis (Vallabhapurapu and Karin, 2009). NF- κ B is activated via the canonical or alternative pathways (Hayden and Ghosh, 2004). In the canonical NF- κ B pathway, the IKK complex, comprising IKK1(α), IKK2(β), and NEMO (also called IKK γ), is activated by several factors including tumour necrosis factor receptors (TNFRs), lymphocyte antigen receptors, and CD40. The IKK complex then phosphorylates I κ B proteins, leading to their degradation by the 26S proteasome. Stimulation of some TNFR family receptors, including BAFF-R and CD40, activates the alternative NF- κ B pathway, in which p100/NF- κ B2 is processed to p52/NF- κ B2, resulting in its translocation to the nucleus.

The ubiquitin system is intimately involved in NF- κ B signalling (Bhoj and Chen, 2009). In most cases, the ubiquitin system modifies and regulates the function of proteins via the attachment of polyubiquitin chains (Hershko and Ciechanover, 1998; Glickman and Ciechanover, 2002). Although it is believed that polyubiquitin chains are generated via one of the seven Lys residues within ubiquitin (Peng *et al*, 2003), we previously identified a novel ubiquitin ligase complex that synthesizes unique N-terminally-linked linear polyubiquitin chains; this complex is called linear ubiquitin chain assembly complex (LUBAC) (Kirisako *et al*, 2006; Tokunaga *et al*, 2009; Iwai, 2012). LUBAC comprises HOIP, HOIL-1L, and SHARPIN. The RING-IBR-RING domain of HOIP is the catalytic centre for linear polyubiquitination (Tokunaga *et al*, 2009, 2011; Gerlach *et al*, 2011; Ikeda *et al*, 2011; Iwai, 2012). Activation of the canonical NF- κ B pathway, which is induced by various stimuli, including TNF- α , is attenuated in HOIL-1L-knockout (KO) and cpdm (chronic proliferative dermatitis in mice) mice (Tokunaga *et al*, 2009, 2011; Gerlach *et al*, 2011; Ikeda *et al*, 2011). The latter are spontaneous mutant mice that lack SHARPIN (Gallardo Torres *et al*, 1995). Moreover, cpdm mice show pleomorphic phenotypes, including chronic dermatitis and immune disorders (Gallardo Torres *et al*, 1995; HogenEsch *et al*, 1999; Seymour *et al*, 2006, 2007), indicating a crucial role for LUBAC in canonical NF- κ B activation (Tokunaga *et al*, 2009, 2011; Gerlach *et al*, 2011; Ikeda *et al*, 2011). Moreover, deficiency of HOIL-1L provokes immunodeficiency, autoinflammatory responses, and amylopectinosis in humans (Boisson *et al*, 2012). However, canonical NF- κ B activation is not completely abrogated in individuals lacking either HOIL-1L or SHARPIN; this is possibly because LUBAC composed of HOIP and SHARPIN is present in individuals lacking HOIL-1L, or LUBAC composed of HOIP and HOIL-1L is present in cpdm mice, albeit in very tiny amounts (Tokunaga *et al*, 2009, 2011; Gerlach *et al*, 2011; Ikeda *et al*, 2011). Thus, the precise role played by LUBAC-mediated linear polyubiquitination has not fully been addressed.

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To examine the physiological function of linear polyubiquitination, we generated a mouse (HOIP^{FL}) strain in which the catalytic domain of HOIP can be ablated in a tissue-specific manner. We focused on B cells because both the canonical and alternative NF- κ B activation pathways play crucial roles in their development and function (Siebenlist *et al*, 2005; Gerondakis *et al*, 2006; Sen, 2006). The development of B1 cells is severely impaired in mice harbouring B cells lacking HOIP activity (B-HOIP^{Δlinear} mice), although the development of conventional B cells is not affected. Canonical NF- κ B activation upon stimulation by CD40 or transmembrane activator, and calcium modulator and cyclophilin ligand interactor (TACI), is heavily attenuated in B cells lacking LUBAC-mediated linear polyubiquitination. In addition, ERK activation by CD40 and TACI is also suppressed in these B cells. The reduced activation of both the canonical NF- κ B and ERK pathways is caused by insufficient activation of the IKK complex. However, to our surprise, B-cell receptor (BCR)-mediated activation of NF- κ B, which is thought to be indispensable for B1-cell development, was not overtly affected. As a result of defective CD40 and TACI signalling, both thymus-dependent (TD) and -independent type II (TI-II) humoral immune responses were severely impaired in B-HOIP^{Δlinear} mice. Taken together, these results show that LUBAC-mediated linear polyubiquitination plays an important role in the development and function of B lymphocytes, possibly via activation of the canonical NF- κ B and ERK by TNFR superfamily ligands, but not via the BCR.

Results

Generation of mice conditionally deficient in LUBAC-mediated linear polyubiquitination

To examine the physiological role(s) of LUBAC-mediated linear polyubiquitination, we generated a mouse strain (HOIP^{FL}) in which the RING-IBR-RING region of HOIP (the catalytic centre) can be ablated in a conditional manner (Supplementary Figure S1). The truncated form of the HOIP protein (HOIP Δ linear) is expressed from the targeted locus after the Cre-mediated removal of a loxP-flanked sequence (Figure 1A). As intercrossing HOIP^{Δlinear/+} mice did not generate any viable HOIP^{Δlinear/Δlinear} offspring, the lack of HOIP-mediated linear polyubiquitination activity appears to result in embryonic lethality. The analysis of HOIP^{Δlinear/Δlinear} mice will be described elsewhere. Therefore, considering that NF- κ B plays an important role in the development and function of B cells (Siebenlist *et al*, 2005; Gerondakis *et al*, 2006; Sen, 2006), we dissected the role(s) of LUBAC-mediated linear polyubiquitination in B cells by crossing HOIP^{FL} mice with mice carrying a Cre transgene driven by the B lymphocyte-specific *mb1* promoter (*Mb1-cre*) (Hobeika *et al*, 2006). Hereafter, we refer to this B cell-specific HOIP^{Δlinear} mouse as the B-HOIP^{Δlinear} mouse. B cells isolated from B-HOIP^{Δlinear} mice expressed mainly the HOIP Δ linear protein, although a tiny amount of wild-type (WT) HOIP protein was also expressed. The expression of HOIL-1L and SHARPIN was also reduced (Figure 1B). T cells from B-HOIP^{Δlinear} mice expressed mainly WT HOIP protein; however, small amounts of the HOIP Δ linear protein could be detected (Figure 1B), which may be due to the leaky expression of Cre protein in T cells of *Mb1-cre* mice (Hobeika *et al*, 2006). SHARPIN and

HOIL-1L co-precipitated with HOIP Δ linear because the HOIP Δ linear protein contains a crucial domain that interacts with HOIL-1L and SHARPIN (Figure 1C). Also, gel-filtration analysis showed that SHARPIN and HOIL-1L co-fractionated with HOIP Δ linear in the high molecular weight fraction, confirming the formation of HOIP Δ linear/HOIL-1L/SHARPIN complexes (LUBAC^{Δlinear}) (Figure 1D). LUBAC^{Δlinear}, successfully expressed in and immunoprecipitated from HEK293T cells, did not show linear polyubiquitination activity as expected (Figure 1E). Consistent with this, luciferase reporter assays showed that LUBAC^{Δlinear} could not activate the NF- κ B pathway (Figure 1F). Collectively, these results suggested that HOIP^{Δlinear} mice were a suitable model in which to examine the physiological functions of LUBAC-mediated linear polyubiquitination activity.

Development of B1 cells, but not that of conventional B cells, is impaired in mice lacking LUBAC-induced linear polyubiquitination

We first examined how the lack of LUBAC-mediated linear polyubiquitination activity affected B-cell development using flow cytometry (FACS). There was no gross developmental arrest of B cells in the bone marrow of B-HOIP^{Δlinear} mice (Figure 2A; Table I; Supplementary Figure S2A). The ratio of B cells to T cells, the expression of surface immunoglobulin M (IgM) and IgD on splenic B cells, and the number of transitional (T1, T2, and T3), mature follicular (Fo), and marginal zone (MZ) B cells were also comparable between control and B-HOIP^{Δlinear} mice (Figure 2B; Table I; Supplementary Figure S3). By contrast, the development of B1 cells in the peritoneal cavity was severely impaired in B-HOIP^{Δlinear} mice (Figure 2C; Table I; Supplementary Figure S2B). B1 cells are subdivided into B1a and B1b cells (Baumgarth, 2011). Both subtypes were reduced in the peritoneal cavity in B-HOIP^{Δlinear} mice, although the percentage of B1a cells was much lower than that of B1b cells (Figure 2C; Table I; Supplementary Figure S2B). There was no apparent difference in the T-cell populations between control and B-HOIP^{Δlinear} mice, although a small amount of HOIP Δ linear protein was detected in T cells from B-HOIP^{Δlinear} mice (Supplementary Figure S4). However, we did observe differences in the levels of different immunoglobulin isotypes in the serum of control and B-HOIP^{Δlinear} mice. Unimmunized B-HOIP^{Δlinear} mice showed much lower levels of all immunoglobulin isotypes examined than control mice (Figure 2D). Therefore, we next examined the induction of activation-induced deaminase (AID) and germline transcript- γ 1 (GL γ 1) mRNA, which are critical for class switch recombination, by CD40 (Pavri and Nussenzweig, 2011). We found that CD40-induced mRNA production was heavily suppressed in HOIP^{Δlinear} B cells compared with that in control B cells (Supplementary Figure S5).

CD40-mediated activation of the canonical NF- κ B and ERK pathways is severely impaired in B cells lacking LUBAC ligase activity

CD40 is a crucial receptor that mediates antibody responses and activates NF- κ B (Bishop and Hostager, 2003). To probe the mechanisms underlying the low levels of antibodies in the sera of unimmunized B-HOIP^{Δlinear} mice and the reduced induction of AID and GL γ 1 mRNA by CD40, we examined CD40-induced responses in B-HOIP^{Δlinear} mice. Phosphorylation and degradation of I κ B α , both of which are hallmarks of

Figure 1 Deletion of the RING-IBR-RING domain from HOIP does not prevent the formation of the LUBAC in B cells. (A) Schematic representation of wild-type HOIP and the HOIP mutant lacking the RING-IBR-RING domain (HOIP Δ linear). UBA, ubiquitin associated. (B) B cell-specific ablation of the RING-IBR-RING domain of HOIP was confirmed by immunoblotting. β -Actin was used as a loading control. (C) HOIP Δ linear co-immunoprecipitates with HOIL-1L and SHARPIN from HOIP Δ linear B cells. (D) HOIP Δ linear co-elutes with HOIL-1L and SHARPIN in the high molecular weight fraction. (E) HOIP Δ linear lacks linear polyubiquitination activity. (F) HOIP Δ linear lacks NF- κ B activation. 293T cells were cotransfected with plasmids containing a luciferase gene under NF- κ B binding site and an expression plasmid for WT HOIP or HOIP Δ linear with or without expression plasmids for HOIL-1L and SHARPIN. Luciferase activity was measured 24 h later. Data are representative of three independent experiments. Source data for this figure is available on the online supplementary information page.

canonical NF- κ B activation, were strongly attenuated in HOIP Δ linear B cells, although the level of CD40 expression on the surface of HOIP Δ linear B cells was almost identical to that on control B cells (Figure 3A; Supplementary Figure S6). Because LUBAC is thought to be involved in activating the IKK complex (Tokunaga *et al*, 2009, 2011; Gerlach *et al*, 2011; Ikeda *et al*, 2011), we examined the phosphorylation of IKK2 by immunoblotting with an anti-phospho-IKK2 antibody and by phosphate affinity SDS-PAGE (Kinoshita *et al*,

2006). CD40-mediated phosphorylation of IKK2 was severely impaired in HOIP Δ linear B cells (Figure 3B and C), indicating that the ligase activity of HOIP is essential for IKK activation. In addition, the nuclear translocation of RelA, c-Rel, and p50/NF- κ B1, and the CD40-mediated induction of mRNA for the NF- κ B target genes, I κ B α and Bcl-xL, were severely reduced in HOIP Δ linear B cells, confirming impaired CD40-induced canonical NF- κ B activation (Figure 3D and E; Supplementary Figure S7).

Figure 2 B-cell development in the absence of ligase activity for linear polyubiquitination. (A) Comparison of bone marrow-derived B cells from control and B-HOIP^{Δlinear} mice. FACS analysis of cells stained for IgM and B220 to identify pro-B and pre-B cells (B220^{low}IgM⁻), immature B cells (B220^{low}IgM⁺), and recirculating mature B cells (B220^{high}IgM⁺) (left panels). CD43 expression (gated on B220^{low}IgM⁻ cells) identifies pre-B cells (CD43⁻) and pro-B cells (CD43⁺) (right panels). (B) FACS analysis of B-cell subpopulations in the spleen. Analysis of splenocytes for surface expression of CD3 and B220 (left upper panels) and for B220 and AA4.1 (right upper panels). Transitional B cells are AA4.1⁺ and mature B cells are AA4.1⁻. Analysis of B220⁺AA4.1⁺ transitional B cells for surface expression of IgM and CD23; T2, IgM⁺CD23⁺; T3, IgM^{low}CD23⁺ (left lower panels). Analysis of B220⁺AA4.1⁻ mature B cells for surface expression of IgM and CD23; Fo B cells, IgM^{low}CD23⁺; MZ B cells, IgM^{high}CD23^{low} (right lower panels). (C) FACS analysis of B-cell subpopulations from the peritoneal cavity for surface expression of CD19 and B220 (B1 cells, B220^{low}CD19^{high}; B2 cells, B220⁺CD19^{low}; left upper panels), for expression of CD5 and IgM (B1a cells, CD5⁺IgM^{high}; right upper panels), and for B220 and CD11b expression (B1 cells, B220^{low}CD11b⁺; left lower panels). Histogram of CD5 expression (gated on B220^{low}CD11b⁺ B1 cells) showing B1a cells (CD5⁺) and B1b cells (CD5⁻) (right lower panels). The numbers refer to the percentage of lymphocytes within the individual gates, or to the percentage of cells in the gate indicated on the left-hand side of the plots. All results are representative of five independent experiments. (D) Titers of the different Ig isotypes in the sera of unimmunized mice. The bars indicate the geometric mean. **P* < 0.05 (Student's *t*-test).

CD40 activates several signalling pathways in addition to the canonical NF- κ B pathway (Bishop and Hostager, 2003). ERK activation was markedly impaired in CD40L-stimulated

HOIP^{Δlinear} B cells, whereas JNK activation was only marginally impaired (Figure 3F); however, we did not observe any substantial defects in CD40-induced p38MAP

Table 1 Number of B cells in the bone marrow, spleen, and peritoneal cavity

Genotype	Control ($\times 10^6$)	B-HOIP ^{Alinear} ($\times 10^6$)
<i>Bone marrow</i>		
Pro	0.36 $\pm$ 0.15	0.34 $\pm$ 0.04
Pre	2.0 $\pm$ 0.87	1.8 $\pm$ 0.34
IM	0.93 $\pm$ 0.42	0.9 $\pm$ 0.31
Recirculating	1.5 $\pm$ 0.74	1.8 $\pm$ 0.7
<i>Spleen</i>		
T1	2.6 $\pm$ 0.63	2.6 $\pm$ 1.1
T2	2.9 $\pm$ 0.86	3.1 $\pm$ 1.2
T3	3.0 $\pm$ 0.87	3.1 $\pm$ 1.4
Fo	35.7 $\pm$ 7.5	38.6 $\pm$ 4.4
MZ	5.4 $\pm$ 1.0	5.3 $\pm$ 1.9
T cells	25.5 $\pm$ 3.4	24.2 $\pm$ 2.6
<i>Peritoneal cavity</i>		
B1	0.76 $\pm$ 0.35	0.08 $\pm$ 0.05**
B1a	0.40 $\pm$ 0.18	0.006 $\pm$ 0.003**
B1b	0.36 $\pm$ 0.19	0.08 $\pm$ 0.05**
B2	0.59 $\pm$ 0.31	0.43 $\pm$ 0.19

Abbreviations: T1, T2, and T3, transitional type 1, 2, and 3 B cells; Fo, follicular B cells; MZ, marginal zone B cells. Bone marrow cells were obtained from two tibias. Cell numbers are based on the total cell counts and flow cytometry data. Data represent the mean $\pm$ standard deviation ($n = 7$ –10 mice). ** $P < 0.01$ (Student's *t*-test).

kinase or Akt activation (Figure 3F). The alternative NF- κ B pathway, which is triggered in B cells by both CD40- and BAFF-R-mediated stimulation, plays a role in cell survival (Sun, 2011). In contrast to the canonical NF- κ B pathway, there was no reduction in anti-CD40- or BAFF-induced processing of p100/NF- κ B2 to p52/NF- κ B2, or in the nuclear translocation of p52/NF- κ B2 and RelB, in HOIP^{Alinear} B cells (Figure 3G, Supplementary Figure S8A). The amount of p100/NF- κ B2 was reduced in HOIP^{Alinear} B cells compared with that in WT B cells. However, this reduction may have been due to the reduced induction of p100/NF- κ B mRNA because the induction of p100/NF- κ B2 mRNA upon CD40 stimulation was weaker in HOIP^{Alinear} B cells than in control B cells (Supplementary Figure S8B). Thus, the alternative pathway of NF- κ B activation appears not to be impaired in HOIP^{Alinear} B cells. We next examined the ability of an anti-CD40 antibody to induce activation markers and co-stimulatory molecules. FACS analyses showed that the CD40-mediated induction of CD80, CD69, and MHC class II was impaired in HOIP^{Alinear} B cells (Figure 3H). Labelling B cells isolated from control and B-HOIP^{Alinear} mice with carboxyfluorescein succinimidyl ester (CFSE) revealed that HOIP^{Alinear} B cells failed to divide when stimulated with the anti-CD40 antibody (Figure 3I). Stimulation with anti-CD40 supported the survival of HOIP^{Alinear} B cells to a level comparable to that of control B cells (Figure 3J); however, the survival of HOIP^{Alinear} B cells was superior to that of WT B cells in the absence of anti-CD40 stimulation (Figure 3J). Collectively, these results strongly indicated that LUBAC ligase activity is essential for CD40-induced activation of the canonical NF- κ B and ERK pathways.

The ligase activity of LUBAC is important for CD40-induced canonical NF- κ B activation

The expression of LUBAC^{Alinear} in HOIP^{Alinear} B cells was lower than that of WT LUBAC in control B cells. Because low levels of LUBAC comprising only HOIP and HOIL-1L result in

attenuated NF- κ B activation in cpdm cells (Gerlach *et al*, 2011; Ikeda *et al*, 2011; Tokunaga *et al*, 2011), we hypothesized that a decrease in the amount of LUBAC may be responsible for the attenuated CD40-mediated activation of the canonical NF- κ B pathway observed in HOIP^{Alinear} B cells. Therefore, we compared the LUBAC expression in HOIP^{Alinear} B cells and cpdm B cells. The results showed that HOIP^{Alinear} B cells contained much more LUBAC than cpdm B cells (Figure 4A). However, CD40-mediated canonical NF- κ B activation was more profoundly affected in HOIP^{Alinear} B cells than in cpdm B cells (Figure 4B). We examined CD40-induced linear polyubiquitination of NEMO using a newly established monoclonal antibody (LUB9), which specifically recognizes linear ubiquitin chains with high sensitivity, but does not recognize Lys-linked ubiquitin chains or monoubiquitin (Supplementary Figure S9). We found that CD40-mediated linear polyubiquitination of NEMO was completely suppressed in HOIP^{Alinear} B cells; however, it was observed at very low levels in cpdm B cells (Figure 4C). These results indicate that it is the loss of LUBAC linear polyubiquitination activity rather than the decrease in the amount of the LUBAC that is responsible for impaired activation of the CD40-induced canonical NF- κ B pathway in HOIP^{Alinear} B cells.

LUBAC activates the ERK pathway by controlling IKK-mediated Tpl-2 activation

We next examined the mechanism underlying attenuated ERK activation in HOIP^{Alinear} B cells. The induction of mRNA for ERK target genes (*Egr-1*, *Egr-2*, and *c-fos*) was markedly impaired in CD40-stimulated HOIP^{Alinear} B cells (Figure 5A), confirming that CD40-mediated ERK activation is also impaired in these cells. Phosphorylation of MEK1/2 (the MAPKK for ERK) upon CD40 stimulation was also attenuated in HOIP^{Alinear} B cells (Figure 5B), indicating that activation of the MAP3K for MEK1/2 is impaired. Tpl-2 (also known as Cot) acts as an MAP3K for ERK during CD40-mediated signalling (Eliopoulos *et al*, 2003). IKK-mediated phosphorylation and subsequent degradation of p105/NF- κ B1, which form a complex with Tpl-2 and ABIN-2, are essential for Tpl-2 activation (Gantke *et al*, 2012). Phosphorylation of p105/NF- κ B1, along with that of Tpl-2, was severely impaired in HOIP^{Alinear} B cells (Figures 5B and C). An *in vitro* kinase assay revealed that the kinase activity of Tpl-2 was markedly reduced in HOIP^{Alinear} B cells, confirming that CD40-induced activation of Tpl-2 is impaired (Figure 5D). Treatment with PS-1145, an inhibitor of IKK2, markedly suppressed not only the phosphorylation and degradation of I κ B α , but also the phosphorylation of p105/NF- κ B1 and ERK (Figure 5E). Thus, impaired activation of the IKK complex in HOIP^{Alinear} B cells, due to the lack of LUBAC-mediated linear polyubiquitination activity, plays a crucial role in attenuating the activation of both the ERK and the canonical NF- κ B pathways upon CD40 stimulation. Collectively, these results strongly suggest that CD40-induced activation of the canonical NF- κ B and ERK pathways, in which LUBAC plays a major role, is central to many CD40-mediated events, but not to B-cell survival.

LUBAC-mediated linear polyubiquitination is dispensable for BCR-induced NF- κ B activation

Next, we examined BCR-induced signalling. Surprisingly, BCR-mediated phosphorylation and degradation of I κ B α ,

Figure 3 CD40-mediated activation of the canonical NF- κ B and ERK pathways is impaired in B cells from B-HOIP^{Δlinear} mice. (**A**, **B**) Splenic B cells from control and B-HOIP^{Δlinear} mice were stimulated with an anti-CD40 antibody (3.0 μ g/ml) and then analysed as indicated. β -Actin was used as a loading control. Data are representative of three independent experiments. (**C**) Splenic B cells from control and B-HOIP^{Δlinear} mice were stimulated with an anti-CD40 antibody (3.0 μ g/ml). Cell lysates were prepared and separated by phosphate affinity SDS-PAGE and then immunoblotted with anti-IKK2 or anti- β -actin antibodies. Data are representative of two independent experiments. (**D**) Nuclear translocation of NF- κ B subunits upon CD40 stimulation. Splenic B cells isolated from control and B-HOIP^{Δlinear} mice were stimulated with an anti-CD40 antibody (3.0 μ g/ml). The nuclear fraction was then prepared and analysed as indicated. Lamin B1 was used as the loading control. (**E**) Induction of I κ B α and Bcl-xL mRNA by an anti-CD40 antibody (3.0 μ g/ml) was examined in splenic B cells by quantitative real-time PCR. Expression was normalized to that of β -actin mRNA. Results are expressed as the mean $\pm$ s.d. ($n = 3$). * $P < 0.05$ and ** $P < 0.01$ (Student's t -test). (**F**) Activation of MAP kinases and Akt in splenic B cells stimulated with an anti-CD40 antibody (3.0 μ g/ml). Data are representative of three independent experiments. (**G**) Lysates of splenic B cells stimulated with an anti-CD40 antibody (3.0 μ g/ml) or with mouse BAFF (200 ng/ml) for 24 h were immunoblotted with an anti-p100/p52 antibody. Data are representative of three independent experiments. (**H**) FACS analysis of CD80, CD69, and MHC class II expression on splenic B cells stimulated with an anti-CD40 antibody (3.0 μ g/ml) for 24 h. The solid and dotted lines represent stimulated and unstimulated cells, respectively. Data are representative of three independent experiments. (**I**) CFSE (5 μ M)-labelled splenic B cells were cultured with or without an anti-CD40 antibody (3 μ g/ml) for 72 h and cell proliferation was examined. Data are representative of two independent experiments. (**J**) Splenic B cells were stimulated with an anti-CD40 antibody (3 μ g/ml) and the proportion of live cells was calculated as a percentage of TO-PRO-3-negative cells after 24, 48, and 72 h of culture. The dashed and solid lines represent unstimulated and stimulated cells, respectively. Results are expressed as the mean $\pm$ s.d. ($n = 4$). Source data for this figure is available on the online supplementary information page.

both hallmarks of canonical NF- κ B activation, were unaffected in HOIP^{Δlinear} B cells (Figure 6A). The same was true regarding the activation of all three MAP kinase pathways and Akt (Figure 6A). BCR-mediated induction of CD80, CD69, and MHC class II was not suppressed in HOIP^{Δlinear} B cells; neither was cell proliferation and survival (Figure 6B–D). To probe the involvement of linear polyubiquitination in BCR signalling, we examined the linear polyubiquitination of NEMO. As expected, stimulation of the B-cell line, BAL17.2, and splenic B cells isolated from WT mice with an anti-CD40 antibody induced the linear polyubiquitination of NEMO; however, linear polyubiquitination of NEMO was not detected upon BCR stimulation (Figure 6E). These results indicate that LUBAC-induced linear polyubiquitination activity is not necessary for BCR-mediated activation of the

canonical NF- κ B pathway, and may not always be required for the signalling pathways leading to canonical NF- κ B activation.

B-HOIP^{Δlinear} mice show impaired antibody responses to both TD and TI-II antigens

Next, we probed the *in vivo* effects of defective CD40-mediated canonical NF- κ B and ERK activation. We examined primary TD antibody responses, in which CD40 is involved (Bishop and Hostager, 2003), by immunizing mice with the TD antigen, 4-hydroxy-3-nitrophenylacetyl (NP)-coupled chicken γ globulin (NP-CGG). The production of NP-specific IgM and IgG₁ antibodies was severely impaired in B-HOIP^{Δlinear} mice (Figure 7A). The percentage of NP-specific IgG₁-positive splenic B cells in B-HOIP^{Δlinear} mice on day 14

Figure 4 Cpdm mice show residual LUBAC-mediated linear polyubiquitination activity. (A) Lysates of splenic B cells from control, B-HOIP^{Alinear}, or cpdm mice were immunoblotted with the indicated antibodies. Data are representative of three independent experiments. (B) Splenic B cells were stimulated with 5 ng/ml of CD40L for the indicated times and then immunoblotted with the indicated antibodies. Data are representative of three independent experiments. (C) Splenic B cells were stimulated with an anti-CD40 antibody (3 μg/ml) for the indicated times. Whole-cell lysates (WCL) were prepared and immunoprecipitated with an anti-NEMO antibody and then immunoblotted with an anti-linear ubiquitin antibody (LUB9) (upper panel). WCL were also immunoblotted with an anti-NEMO antibody (lower panel) to confirm that the lysates contained equal amounts of NEMO protein. Data are representative of two independent experiments. Source data for this figure is available on the online supplementary information page.

post immunization was lower than that in control mice, although the percentage of NP-specific B cells in unimmunized B-HOIP^{Alinear} mice was comparable to that in unimmunized control mice (Figure 7B and C; Supplementary Table S2). Thus, the defective TD responses observed in B-HOIP^{Alinear} mice were due to a failure of antigen-specific B-cell expansion. FACS analysis revealed that the proportion of germinal centre (GC) B cells, which express PNA and Fas (Sasaki *et al*, 2004), was profoundly reduced in B-HOIP^{Alinear} mice (Figure 7D). These results indicate that the lack of LUBAC-mediated linear polyubiquitination activity in B cells impairs immune response to TD antigens, possibly due to attenuated CD40-induced activation of the canonical NF-κB and ERK pathways.

We also examined the antibody response to TI-II by immunizing mice with the TI-II antigen, NP-Ficoll. The production of both NP-specific IgM and IgG₃ antibodies was severely impaired in B-HOIP^{Alinear} mice (Figure 7E). Also, control mice showed expansion of NP-specific IgM-positive B cells on day 7 post immunization, whereas B-HOIP^{Alinear} mice did not (Figure 7F; Supplementary Table S2). These results suggest that TI-II antibody responses are impaired in B-HOIP^{Alinear} mice.

B-HOIP^{Alinear} mice show impaired TACI-mediated NF-κB activation

It is notable that BCR signalling, which plays a crucial role in TI-II responses, was not overtly affected in B-HOIP^{Alinear} mice. Thus, we sought to identify the mechanism(s) underlying the lack of TI-II responses in these mice. TACI, a member of the TNFR superfamily, plays an essential role in TI-II responses (Yan *et al*, 2000, 2001; von Bulow *et al*, 2001).

BCR stimulation for 24 h upregulated TACI expression in B cells isolated from both B-HOIP^{Alinear} and control mice to a similar extent, whereas BCMA, a member of the TNFR superfamily (and the other receptor for APRIL besides TACI), was induced only weakly (Figure 8A). APRIL transmitted signals mainly through TACI, but not through BCMA, in BCR-stimulated cells (Figure 8B), but failed to effectively activate the canonical NF-κB or ERK pathways in BCR-primed HOIP^{Alinear} B cells. However, BCR-primed HOIP^{Alinear} B cells showed normal levels of JNK and p38MAPK activation (Figure 8C). APRIL did not activate the canonical NF-κB pathway in unstimulated splenic B cells isolated from control mice or from B-HOIP^{Alinear} mice, which did not express TACI (Figure 8C). Thus, the lack of LUBAC-induced linear polyubiquitination in HOIP^{Alinear} B cells causes impaired TACI-induced activation of the canonical NF-κB and ERK pathways, resulting in defective TI-II responses.

Toll-like receptor-mediated canonical NF-κB and ERK activation is impaired in HOIP^{Alinear} B cells

Stimulation of Toll-like receptor (TLR) family members induces B-cell activation; therefore, we next examined TLR-mediated responses in HOIP^{Alinear} B cells. Stimulation of HOIP^{Alinear} B cells with LPS, which binds to TLR4, showed that phosphorylation of IKK2 was heavily suppressed, and that the activation of both the canonical NF-κB and ERK pathways was impaired (although the latter appeared to be more profoundly affected; Figure 9A). However, activation of the JNK and p38MAP kinase pathways was not overtly affected. Also, stimulation with CpG DNA, which binds to TLR9, caused only a slight impairment of IKK2 phosphorylation. Consistent with this, activation of the canonical NF-κB was not overtly affected, although ERK activation was markedly impaired (Figure 9B). These results indicate that although linear polyubiquitin plays an important role in TLR signalling, particularly through ERK activation, its role in canonical NF-κB activation might differ depending on whether the signals are delivered via TLR4 or TLR9.

Discussion

The LUBAC E3 ligase complex, which comprises HOIL-1L, SHARPIN, and HOIP, specifically forms a linear polyubiquitin chain. We and other groups used mice lacking either HOIL-1L or SHARPIN to show that LUBAC plays a critical role in activating the canonical NF-κB pathway (Tokunaga *et al*, 2009, 2011; Gerlach *et al*, 2011; Ikeda *et al*, 2011). However, we found that NF-κB activation was severely impaired, although not completely abolished, in these mice. This may be due to the formation of trace amounts of LUBAC comprising HOIP and SHARPIN in HOIL-1L KO mice, or HOIP and HOIL-1L in cpdm mice. To address the role of LUBAC-mediated linear polyubiquitination in activating the canonical NF-κB pathway, we generated mice harbouring B lymphocytes lacking LUBAC-mediated linear polyubiquitination activity (B-HOIP^{Alinear} mice). We found that, in addition to CD40-, TACI-, and TLR-mediated canonical NF-κB activation, ERK activation was also severely impaired in B cells isolated from B-HOIP^{Alinear} mice; however, BCR-mediated activation of the NF-κB and ERK pathways was normal. As shown in Figure 4, defective activation of the canonical NF-κB and ERK pathways in HOIP^{Alinear} B cells

Figure 5 LUBAC activates ERK via IKK-mediated activation of Tpl-2. (A) Splenic B cells from control and B-HOIP^{Δlinear} mice were stimulated with an anti-CD40 antibody (3.0 μg/ml) for the indicated times and then examined for the induction of Egr-1, Egr-2, and c-fos mRNA (target genes of the ERK pathway) by quantitative real-time PCR. Data were normalized to the expression of β-actin mRNA. Results are expressed as the mean ± s.d. (*n* = 3). ***P* < 0.01 (Student's *t*-test). (B) Splenic B cells were stimulated with an anti-CD40 antibody (3.0 μg/ml) for the indicated times. Whole-cell lysates (WCL) were prepared and then immunoblotted with the indicated antibodies. β-Actin was used as a loading control. Data are representative of three independent experiments. (C) Splenic B cells were stimulated with an anti-CD40 antibody (3.0 μg/ml) for the indicated times. WCL were prepared and the proteins separated by phosphate affinity SDS-PAGE and immunoblotted with an anti-Tpl-2 antibody. Data are representative of two independent experiments. (D) Splenic B cells were stimulated for 8 min with an anti-CD40 antibody (3.0 μg/ml). Tpl-2 was immunoprecipitated with an anti-Tpl-2 antibody and its catalytic activity was measured in a kinase assay (KA) using His-tagged MEK1 as a substrate (left panels). His-tagged MEK1 levels are also shown to confirm that the same amount of substrate was present in each reaction. Immunoprecipitation of Tpl-2 was confirmed by immunoblotting with an anti-Tpl-2 antibody. Cell lysates were subjected to immunoblotting to confirm the phosphorylation of MEK1/2 (right panels). (E) Splenic B cells from C57Bl/6 mice were preincubated with 20 μM PS-1145 or DMSO for 2 h and then stimulated with an anti-CD40 antibody (3.0 μg/ml) for the indicated times. WCL were prepared and immunoblotted with the indicated antibodies. Data are representative of three independent experiments. Source data for this figure is available on the online supplementary information page.

upon CD40-, TACI-, or TLR-mediated stimulation appeared to be due to the loss of the linear polyubiquitination activity of LUBAC, rather than a result of the reduced expression of the LUBAC. However, it is of note that the HOIP^{Δlinear}

protein might have some positive function in addition to the linear polyubiquitination activity of LUBAC or might exhibit a dominant-negative function by competing other proteins. Although CD40- and TACI-induced canonical NF-κB

Figure 6 BCR-mediated activation of NF- κ B and MAP kinases is not impaired in B cells from B-HOIP $^{\Delta linear}$ mice. **(A)** Splenic B cells from control and B-HOIP $^{\Delta linear}$ mice were stimulated with an anti-IgM antibody (5.0 μ g/ml) for the indicated times, lysed, and then probed as indicated. Data are representative of three independent experiments. **(B)** FACS analysis of CD80, CD69, and MHC class II expression on splenic B cells stimulated with an anti-IgM antibody (5.0 μ g/ml) for 24 h. The solid and dotted lines represent stimulated and unstimulated cells, respectively. Data are representative of two independent experiments. **(C)** CFSE (5 μ M)-labelled splenic B cells were stimulated with an anti-IgM antibody (5.0 μ g/ml) for 72 h and cell proliferation was measured. Data are representative of two independent experiments. **(D)** Splenic B cells were stimulated with 5.0 μ g/ml of an anti-IgM antibody. After 24, 48, and 72 h of culture, the proportion of live cells was calculated as a percentage of TO-PRO-3-negative cells. The dashed and solid lines represent unstimulated and stimulated cells, respectively. Data represent the mean $\pm$ s.d. ($n = 4$). **(E)** NEMO-containing immunoprecipitates from primary B cells (left panel) or the B-cell line, Bal 17.2 (right panel), were stimulated with an anti-IgM (5.0 μ g/ml) or an anti-CD40 antibody (3 μ g/ml) for the indicated times and then immunoblotted with an anti-linear ubiquitin antibody (LUB9) (upper panel). Whole-cell lysates (WCL) immunoblotted with an anti-NEMO (lower panel) antibody are also shown. Data are representative of two independent experiments. Source data for this figure is available on the online supplementary information page.

activation was markedly reduced in B cells lacking LUBAC-mediated linear polyubiquitination, it was not completely abolished. It might be attributed by a positive function of the HOIP $\Delta linear$ protein. Generation of a mouse strain, which lacks HOIP protein completely, will clarify this possibility.

The ubiquitin-binding activity of NEMO plays a crucial role in NF- κ B activation, and the affinity of NEMO for linear di-ubiquitin is much higher than that for K63 di-ubiquitin (Lo *et al*, 2009; Rahighi *et al*, 2009). Because NEMO artificially fused to linear di-ubiquitin effectively induces the phosphorylation of IKK and activates NF- κ B (Kensche *et al*, 2012), linear polyubiquitination is thought to play a major role in activating the IKK complex. We found that TLR4-mediated canonical NF- κ B activation was markedly impaired, whereas TLR9-induced canonical NF- κ B activation was not overtly affected. Both TRAF6 E3 ligase, which generates K63 chains (Deng *et al*, 2000), and MyD88 are involved in TLR-mediated canonical NF- κ B activation, although Trif also plays a role in TLR4-mediated stimulation. Both K63- and K11-linked chains, which bind NEMO with considerable affinity, are implicated in signal-induced NF- κ B activation (Laplantine *et al*, 2009; Dynek *et al*, 2010). Therefore, K63 or K11 chains might play a redundant role in IKK2 phosphorylation and NF- κ B activation. Thus, CD40- and TACI-induced canonical NF- κ B activation might not be completely abolished in B cells lacking LUBAC-mediated linear polyubiquitination. However, involvement of K63 or K11 chains might be restricted in B cells, as canonical NF- κ B activation by IL-1 β , in which TRAF6- and MyD88-mediated

signalling are also involved, is strongly impaired in MEFs lacking LUBAC expression almost completely (cpdm MEFs in which HOIL-1L expression was suppressed by siRNA) (Tokunaga *et al*, 2011). However, linear chains most likely play a dominant role in IKK activation through signalling via TNFR superfamily receptors. LUBAC might also play an important role in Trif-mediated canonical NF- κ B activation because TLR4-mediated canonical NF- κ B activation was more markedly affected than TLR9-mediated activation. CD40-, TACI-, and TLR-mediated ERK activation was severely attenuated in HOIP $^{\Delta linear}$ B cells. IKK-mediated phosphorylation of p105/NF- κ B1, TPL-2, and ABIN-2 appears to be involved in CD40-mediated ERK activation; thus, our observations strongly indicate that LUBAC regulates the CD40-mediated activation of the canonical NF- κ B and ERK pathways by activating IKK via its linear polyubiquitin activity (Figure 9C). However, LUBAC may play an additional role in selecting the substrate for IKK because phosphorylation of p105/NF- κ B1, but not that of I κ B α , upon stimulation by CpG DNA was strongly attenuated in HOIP $^{\Delta linear}$ B cells. The alternative NF- κ B and Akt pathways were not overtly affected in HOIP $^{\Delta linear}$ B cells. CD40 supported the survival of HOIP $^{\Delta linear}$ B cells at a level comparable to that of WT B cells, a process that seems to be mediated by the alternative NF- κ B and Akt pathways, as both pathways play important roles in B-cell survival (Srinivasan *et al*, 2009; Sun, 2011; Limon and Fruman, 2012).

Another surprising finding of the present study was that the linear polyubiquitination activity of LUBAC appeared to be dispensable for BCR-mediated canonical NF- κ B activation. The CARMA1-BCL10-MALT1 (CBM) complex plays a central role in

Figure 7 B-HOIP^{Δlinear} mice show impaired TD and TI-II responses. (A) Sera from control or B-HOIP^{Δlinear} mice were collected before, and every week after, immunization with 100 μ g NP-CGG. Total anti-NP IgM and IgG₁ titers are shown ($n=5$ representative mice per genotype). (B) The percentage of NIP-binding/B220⁺ cells within the splenocyte population isolated from non-immunized control and B-HOIP^{Δlinear} mice. Data are representative of three independent experiments. (C) The percentage of NIP-binding/IgG₁⁺ cells within the B-cell population from mice on day 14 post immunization with NP-CGG. Representative data from three mice are shown. (D) FACS analysis of germinal centre (GC) B cells from the spleen 2 weeks post immunization with NP-CGG. The numbers refer to the percentage of GC B cells (FAS^{high}, PNA^{high}) within the CD19⁺ cell population. Representative data from three mice are shown. (E) Sera from control or B-HOIP^{Δlinear} mice were collected before, and every week after, immunization with 50 μ g NP-Ficoll. Total anti-NP IgM and IgG₃ titers are shown ($n=5$ representative mice per genotype). (F) The percentage of NIP-binding/IgM⁺ cells within the spleens of mice 7 days post immunization with NP-Ficoll. Representative data from three mice are shown.

lymphocyte antigen receptor-mediated NF- κ B activation (Thome, 2004). However, abrogating the ubiquitin-binding activity of NEMO abolished canonical NF- κ B activation upon stimulation by PMA/ionomycin, which mimics antigen receptor stimulation; this suggests that the ubiquitin system is involved in lymphocyte antigen receptor-mediated canonical NF- κ B activation (Laplantine *et al*, 2009). K63 chains, which are generated by the interaction between TRAF6 and the CBM complex, are thought to be involved in TCR-mediated

NF- κ B activation (Sun *et al*, 2004). However, BCR-induced proliferation still occurs normally in B cell-specific *Traf6* KO mice (Kobayashi *et al*, 2009). The ubiquitin system appears not to be involved in BCR-mediated ERK activation because ERK is activated via the RasGRP-Ras pathway (Coughlin *et al*, 2005). Thus, it is important to identify which ubiquitin chain(s) is involved in BCR signalling.

B-cell responses to both TD and TI-II antigens were severely impaired in B-HOIP^{Δlinear} mice. The development

Figure 8 The linear polyubiquitination system is required for TACI-induced canonical NF-κB activation. **(A)** FACS analysis of TACI and BCMA expression on the surface of B cells from control and B-HOIP^{Alinear} mice 24 h after BCR stimulation. Solid and dotted lines represent stimulated cells and unstimulated cells, respectively. The results are representative of at least three independent experiments. **(B)** Splenic B cells from control and B-HOIP^{Alinear} mice were stimulated with 5.0 μg/ml of an anti-IgM antibody for 24 h. These BCR-primed B cells were then preincubated with 10 μg/ml of either anti-BCMA (R&D MAB593) or anti-TACI (R&D MAB1041) antibodies for 1 h to block BCMA or TACI, and then stimulated with 200 ng/ml APRIL for the indicated times. Cell lysates were prepared and immunoblotted with the indicated antibodies. β-Actin was used as a loading control. Data are representative of two independent experiments. **(C)** Unstimulated splenic B cells or BCR-primed splenic B cells from control and B-HOIP^{Alinear} mice were stimulated with 250 ng/ml of APRIL for the indicated times. Cell lysates prepared and separated by SDS-PAGE followed by immunoblotting with the indicated antibodies. Data are representative of three independent experiments. Source data for this figure is available on the online supplementary information page.

of conventional B cells, including Fo B cells and MZ B cells, in the spleen was almost normal; however, the number of B1 cells in the peritoneal cavity was markedly reduced. As CD40 plays a critical role in TD responses, including GC formation (Bishop and Hostager, 2003), impaired CD40-induced activation of the canonical NF-κB and ERK pathways appears to be the main reason for the defective TD responses in B-HOIP^{Alinear} mice. Responses to TI-II antigens are mainly mounted by MZ B and B1 cells (Martin *et al*, 2001). Thus, the defective TI-II response observed in

B-HOIP^{Alinear} mice cannot be solely attributed to a reduction in the number of B1 cells. BCR stimulation plays a crucial role in the TI-II response (Vos *et al*, 2000); however, since neither BCR-induced proliferation nor canonical NF-κB and ERK pathway activation was affected in HOIP^{Alinear} B cells, signalling via receptor(s) other than BCR, which are involved in TI-II response, also appear to be impaired. Signalling via TACI mediates the differentiation of TI-II antigen-activated B cells into plasma cells (Mantchev *et al*, 2007). We found that TACI-mediated signals were impaired in BCR-primed B cells

Figure 9 TLR-mediated activation of the canonical NF-κB and ERK pathways is impaired in B cells from B-HOIP^{Δlinear} mice. (A) Splenic B cells from control and B-HOIP^{Δlinear} mice were stimulated with 20 μg/ml LPS and then analysed as indicated. Data are representative of two independent experiments. (B) Splenic B cells from control and B-HOIP^{Δlinear} mice were stimulated with 100 nM CpG DNA and then analysed as indicated. Data are representative of three independent experiments. (C) Schematic showing the LUBAC-mediated activation of the canonical NF-κB and ERK pathways. Upon CD40 stimulation, LUBAC conjugates linear polyubiquitin chains to NEMO. Linear polyubiquitination of NEMO activates the IKK complex. The activated IKK complex phosphorylates IκBs and p105/NF-κB1 to activate the canonical NF-κB and ERK pathways, respectively. Source data for this figure is available on the online supplementary information page.

lacking LUBAC-mediated linear polyubiquitination activity. These results indicate that the impaired TD and TI-II responses observed in B-HOIP^{Δlinear} mice are likely caused by defective CD40- and TACI-mediated activation of the canonical NF-κB and ERK pathways, respectively.

The number of B1 cells in the peritoneal cavity of B-HOIP^{Δlinear} mice was markedly reduced. This might be due to a defect in B1-cell growth and survival because the majority of B1 cells are of fetal origin and their numbers are maintained by a process of self-replenishment (Baumgarth, 2011). The strength of BCR signalling is important for B1-cell development, and a deficiency in the positive regulators of BCR signalling leads to a lack of B1 cells (Casola *et al*, 2004; Hardy, 2006). However, BCR-induced proliferation and activation of the canonical NF-κB and ERK pathways were not affected in B-HOIP^{Δlinear} mice. The mechanisms by which B1 cells are maintained are not well understood. However, signalling via TLRs or the IL-1 receptor family, which requires MyD88 and Trif (the latter is involved in canonical NF-κB activation-induced by some TLRs) (Yamamoto *et al*, 2003), appears not to be involved in the process because B1-cell numbers (as well as TD and TI-II responses) are not overtly affected in MyD88/Trif double-KO mice (Gavin *et al*, 2006). Therefore, any as yet unidentified receptor(s) that appear to be indispensable for B1-cell development may belong to the TNFR family. The number of B1 cells in B cell-specific *Traf6* KO mice is low, although BCR-induced proliferation remains normal (Kobayashi *et al*, 2009). TRAF6 is involved in NF-κB activation mediated by some TNFR family members. Since LUBAC is also involved in canonical NF-κB and ERK activation via these receptors, both LUBAC and TRAF6 might function in these signalling pathways via receptor(s) that are necessary for B1-cell development.

Here, we showed that the linear polyubiquitin ligase activity of HOIP in B cells is essential for both B1-cell development and

immune responses to TD and TI-II antigens. The phenotypes of B-HOIP^{Δlinear} mice may be caused by defective signalling via TNFR superfamily receptors because BCR signalling is not overtly affected in these mice; however, CD40- and TACI-mediated activation of the canonical NF-κB and ERK pathways is impaired.

Materials and methods

Mice

HOIP^{FL} mice were generated as described in Supplementary experimental procedures. HOIP^{FL} mice were crossed with *mb1-cre* mice to ablate the ligase activity of HOIP in B cells. The *mb1-cre* mice were kindly provided by Dr M Reth (Max Planck Institute for Immunobiology, Freiburg, Germany). Since B-cell development and CD40-mediated responses in HOIP^{FL/+}/*mb1-cre* mice were comparable to those in HOIP^{FL/FL} mice, HOIP^{FL/FL} mice were used as the control for all experiments. All mice were bred and maintained under specific pathogen-free conditions. All animal protocols were approved by Kyoto and Osaka Universities.

Flow cytometry analysis

Single-cell suspensions, prepared from various lymphoid organs, were stained with fluorochrome-conjugated antibodies. NP-binding B cells were detected using (4-hydroxy-5-endo-3-nitrophenyl)-acetyl-(NIP)-BSA-PE. All samples were acquired on a FACSCanto II (BD Biosciences) and the results were analysed with the Flow-Jo software (Tree star).

Immunization and serum analyses

Mice were injected intraperitoneally (i.p.) with 100 μg of a TD Ag, NP-CGG, suspended in Imject Alum (Pierce). TI-II immune responses were elicited by the i.p. injection of 50 μg of NP-Ficoll (Bioressearch Technologies). Mice were bled via the tail vein both before and after immunization. Serum immunoglobulin concentrations were determined by ELISA, as previously described (Sasaki *et al*, 2004).

Immunoblot analysis

Whole-cell lysates were prepared as previously described, separated by SDS-PAGE, and transferred onto PVDF membranes (Millipore). For phosphate affinity SDS-PAGE, Phos-tagTM AAL-107 (NARD

Institute) and Mn^{2+} were added to the SDS-PAGE gel at final concentrations of 100 and 200 μ M, respectively. To detect *in vivo* ubiquitination of NEMO, NEMO was immunoprecipitated and immunoblotted with an anti-linear polyubiquitin monoclonal antibody (LUB9).

In vitro kinase assay

To assay Tpl-2 MEK kinase activity, CD40-stimulated B cells were lysed in $1 \times$ kinase lysis buffer (0.5% NP-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM β -glycerophosphate, 20 mM NaF, 1 mM Na_3VO_4 , 10 mM okadaic acid, 10% glycerol, and 0.1% 2-ME). Lysates were then subjected to immunoprecipitation with an anti-Tpl-2 antibody (M-20; Santa Cruz) coupled to protein A-Sepharose beads. Immunoprecipitates were washed three times with $1 \times$ kinase lysis buffer and then twice with $1 \times$ kinase reaction buffer (20 mM Hepes (pH 7.6), 20 mM $MgCl_2$, 20 mM β -glycerophosphate, 1 mM EGTA, and 2 mM DTT). The beads were then resuspended in 25 μ l of $1 \times$ kinase reaction buffer containing 1 mM ATP and 200 ng of His-tagged MEK1 (Abcam) and incubated at 37°C for 30 min. The reaction was terminated by adding 25 μ l of 2 $\times$ SDS-sample buffer. The samples were separated by SDS-PAGE and phosphorylation of His-tagged MEK1 was examined by immunoblotting with an anti-phospho-MEK1/2 antibody (Cell Signaling Technology).

In vitro ubiquitination assay

Reaction mixtures containing 5 μ g/ml of E1 and 10 μ g/ml of UbcH5c were immunoprecipitated with WT LUBAC or LUBAC^{Δlinear} and then incubated with 250 μ g/ml of ubiquitin at 37°C for 1 h in 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 2 mM DTT with or without ATP, followed by immunoblotting with an anti-ubiquitin antibody.

Quantitative RT-PCR (QPCR) analysis

Total RNA was isolated using RNeasy Micro or Mini Kits (Qiagen). DNase-treated RNA (20 ng) was reverse transcribed to cDNA using the RevaTra Ace qPCR RT kit (Toyobo). Real-time PCR was performed with Power SYBR Green PCR master mix (Applied Biosystems) in an ABI 7900 Real-time PCR system (Applied

Biosystems) using the primers listed in Supplementary Table S1. All gene expression data were normalized to the expression of β -actin.

Statistical analysis

Statistical analyses were performed using a two-tailed unpaired Student's t-test.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: YS, SS, and MN performed the experiments and prepared the figures; SM generated the mice; SS, YW, and KK contributed the reagents; YS and KI wrote the paper; YA, KT, TK, and KI offered scientific advice. YS, TK, and KI designed the experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

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