

Down-regulation of B Cell Receptor Signaling by Hematopoietic Progenitor Kinase 1 (HPK1)-mediated Phosphorylation and Ubiquitination of Activated B Cell Linker Protein (BLNK)*[§]

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Background: HPK1 is a hematopoiesis-specific Ste20-like serine/threonine kinase that suppresses immune responses and autoimmunity.

Results: HPK1 knock-out B cells show loss of Thr-152 phosphorylation, 14-3-3 binding, and Lys-37/38/42-ubiquitination of BLNK.

Conclusion: HPK1 attenuates BCR signaling via inducing phosphorylation and ubiquitination of BLNK.

Significance: This is the first report of BLNK ubiquitination that is initiated by HPK1-induced phosphorylation.

Hematopoietic progenitor kinase 1 (HPK1) is a Ste20-like serine/threonine kinase that suppresses immune responses and autoimmunity. B cell receptor (BCR) signaling activates HPK1 by inducing BLNK/HPK1 interaction. Whether HPK1 can reciprocally regulate BLNK during BCR signaling is unknown. Here, we show that HPK1-deficient B cells display hyper-proliferation and hyper-activation of I κ B kinase and MAPKs (ERK, p38, and JNK) upon the ligation of BCR. HPK1 attenuates BCR-induced cell activation via inducing BLNK threonine 152 phosphorylation, which mediates BLNK/14-3-3 binding. Furthermore, threonine 152-phosphorylated BLNK is ubiquitinated at lysine residues 37, 38, and 42, leading to attenuation of MAPK and I κ B kinase activation in B cells during BCR signaling. These results reveal a novel negative feedback regulation of BCR signaling by HPK1-mediated phosphorylation, ubiquitination, and subsequent degradation of the activated BLNK.

B lymphocytes produce antibodies that play an important role in eliminating pathogenic challenge or, conversely, causing autoimmune diseases (1). B cells are activated by B cell receptor (BCR)³ signaling via a highly ordered signaling cascade (2). The

recognition of antigens by BCR rapidly induces the activation of cytoplasmic Src family protein-tyrosine kinases FYN and LYN. Followed by the activation of FYN and LYN, immunoreceptor tyrosine kinase-based activation motifs of Ig α and β chains are phosphorylated, which in turn recruit the tyrosine kinase SYK. Activated SYK then induces tyrosine phosphorylation of B cell linker protein (BLNK, also named SLP-65), which acts as a central adaptor to assemble multiple signaling molecules, including phospholipase C γ 2 (PLC γ 2), Bruton tyrosine kinase, GRB2, and hematopoietic progenitor kinase 1 (HPK1) (3). Bruton tyrosine kinase activates PLC γ 2, which in turn stimulates the PKC β -IKK-NF- κ B cascade (4). GRB2 facilitates the activation of RAS-RAF-MEK-ERK signaling (5), but the role of HPK1 in BCR downstream signaling remains unclear.

HPK1 (also named MAP4K1) is a Ste20-like serine/threonine kinase and is mainly expressed in hematopoietic cell compartments (6, 7). Because HPK1 specifically activates JNK through the signaling cascade MAP3K (e.g. MEKK1, TAK1, or MLK3) to MAP2K (e.g. MKK4 or MKK7) in overexpression systems (6–11), HPK1 belongs to the MAP4K subfamily that also includes the other five members GCK/MAP4K2, GLK/MAP4K3, HGK/MAP4K4, GCKR/MAP4K5, and MINK/MAP4K6 (12–14). In addition to regulating the JNK pathway, HPK1 negatively regulates AP-1 and ERK activation in T cell receptor signaling (7, 10). The role of HPK1 in NF- κ B activation is complex. HPK1 binds to and phosphorylates CARMA1, resulting in the activation of IKK β /NF- κ B (15, 16). In addition, HPK1 can be processed by caspases into the N-terminal kinase domain and C-terminal regulatory domain in apoptotic cells (17); the cleaved HPK1 C-terminal fragment has a suppressive activity on NF- κ B activation (18). The *in vivo* roles of HPK1 are demonstrated using HPK1-deficient mice that show enhanced immune responses upon antigen immunization and are more susceptible to experimentally induced autoimmune encephalo-

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³ The abbreviations used are: BCR, B cell receptor; BLNK, B cell linker protein; PLC γ 2, phospholipase C γ 2; IKK, I κ B kinase.

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myelitis (10). Furthermore, HPK1 attenuation may also promote human autoimmune diseases, as the HPK1 expression level is down-regulated in peripheral blood mononuclear cells of human patients with psoriatic arthritis or systemic lupus erythematosus (19–21).

We investigated the role of HPK1 in the regulation of BCR signaling. Our results demonstrate that HPK1 attenuates BCR-induced B cell activation by inducing BLNK Thr-152 phosphorylation and subsequent BLNK/14-3-3 binding; more importantly, we show that Thr-152 phosphorylation induces ubiquitination and proteasomal degradation of the activated BLNK. Furthermore, Lys-37, Lys-38, and Lys-42 are identified as BLNK ubiquitination sites, which attenuate ERK, JNK, and IKK activation during BCR signaling.

EXPERIMENTAL PROCEDURES

Mice—C57BL/6 (B6) WT and HPK1-deficient mice were bred in a specific pathogen-free environment within the Transgenic Mouse Facility at Baylor College of Medicine. All animal experiments were done according to institutional guidelines and regulations.

Antibodies—Rabbit polyclonal antibodies against phosphorylated Thr-152 (underlined) of a BLNK peptide (CRLASpTL-PAPN) were generated and purified by Eurogentec Inc. Other antibodies used in this study were from the following sources: anti-p-ERK (Thr-202/Tyr-204), anti-p-p38 (Thr-180/Tyr-182), anti-p-JNK (Thr-183/Tyr-185), anti-p-IKK α (Ser-180)/IKK β (Ser-181), anti-p-PLC γ 2 (Tyr-759), anti-p-BLNK (Tyr-96), anti-p-SYK (Tyr-323), anti-ERK, anti-p38, anti-IKK β , anti-BLNK, anti-HA (6E2), and anti-ubiquitin (P4D1) Abs were from Cell Signaling, Inc. Anti-HPK1 (N-19), anti-BLNK (H-80), anti-JNK1 (F-3), and anti-GST (B-14) Abs were all from Santa Cruz Biotechnology. Anti-FLAG (M2) mAb was from Sigma. Anti-14-3-3 Hu (θ/τ) mAb was from BIOSOURCE. Anti-ubiquitin (Lys-48) Ab was from Millipore, Inc. Anti-phosphotyrosine (4G10) was from Upstate. Pre-CP-Cy5.5-conjugated anti-B220 was from Pharmingen. FITC anti-B220 (RA3-6B2), phycoerythrin-anti-IgD (11–26c.2a), phycoerythrin/Cy7-anti-CD23 (B3B4), allophycocyanin-anti-IgM (RMM-1), and Pacific Blue anti-CD21/35 (7E9) were from BioLegend. Biotinylated and nonbiotinylated goat anti-mouse IgM F(ab')₂ antibodies were purchased from Jackson ImmunoResearch.

B Cell Purification, Stimulation, and in Vitro Proliferation—B cells were purified from splenocytes using MACS columns (Miltenyi Biotec) according to the manufacturer's instructions. The purity of B cells (CD19⁺ cells) was more than 95% as determined by flow cytometry. For *in vitro* B cell proliferation, purified B cells (1×10^5 or 2×10^5 cells/well) were stimulated with different doses of anti-IgM F(ab')₂ or LPS for 40–64 h in 96-well plates. In the final 16 h of cell culture, the cells were pulsed with [³H]thymidine (1 μ Ci/well). To study BCR signaling, purified B cells were either untreated or incubated with 10 μ g/ml biotin-labeled goat anti-mouse IgM F(ab')₂ for 15 min on ice and then stimulated by BCR cross-linking by using 20 μ g/ml streptavidin and incubating in a 37 °C water bath. The stimulation was immediately stopped by cold spin, and cells were lysed using the lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyro-

phosphate, 1 mM β -glycerophosphate, 0.5 mM PMSF, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, and 1 mM Na₃VO₄) for 30 min on ice.

Plasmid Construction and Mutagenesis—N-terminal FLAG-tagged full-length murine BLNK and BLNK(191–457) fragment were gifts from Dr. Hidetaka Yakura (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan). FLAG-tagged BLNK((1–190), (1–120), (121–340), and (121–190)) fragments were amplified by PCR and inserted into pEF-BOS vector using BamHI and XbaI site. FLAG-tagged BLNK mutants (S129A, S138A, S141A, S144A, S151A, or T152A) were generated by site-directed mutagenesis. BLNK WT or T152A mutant each containing two copies of FLAG tag at the N terminus was subcloned into pIRES2-AcGFP1 vector (Clontech) through the addition of FLAG sequence by PCR. FLAG-BLNK K37R/K38R/K40R/K42R mutant was made by site-directed mutagenesis of Lys-37, Lys-38, Lys-40, and Lys-42 to arginine. GST-tagged BLNK was constructed into pEBG vector (provided by Dr. J. Zhou, Nankai University, China). All WT and mutants were verified by DNA sequencing.

Cell Transfections—HEK293T cells were cultured in DMEM plus 10% FBS and transfected using calcium phosphate precipitation (Specialty Media Inc.) according to the manufacturer's instructions. HEK293T cells were harvested for experiments 40 h after transfection. BLNK-deficient chicken DT40 B cells (RIKEN BRC, Japan) were cultured in RPMI 1640 medium plus 10% FBS, 1% chicken serum, and 50 μ M 2-mercaptoethanol at 39.5 °C. BLNK-deficient DT40 B cells were transfected using NeonTM transfection system (Invitrogen) with the setting of 1435 V, 30 ms, and 1 pulse. WEHI-231 B cells were cultured in RPMI 1640 medium plus 10% FBS and transfected using NeonTM (1375 V, 40 ms, 1 pulse).

GST Pulldown, Co-immunoprecipitations, and Western Blotting—GST-14-3-3 or GST proteins were incubated with 400–800 μ g of cell lysates at 4 °C for 2 h and immobilized on glutathione-Sepharose 4B beads for 45 min at 4 °C. Beads were washed three times in cell lysis buffer and then boiled in 2 \times SDS loading buffer for 5 min. For co-immunoprecipitation, 2–5 μ g of antibody and 400–600 μ g cell lysate were mixed and rotated on a rocker at 4 °C for 2 h, followed by adding 30 μ l of protein-A/G plus conjugated agarose beads (Santa Cruz Inc.) and incubating for 1 h. Beads were washed four times with cell lysis buffer and boiled in 20 μ l of 2 \times SDS loading buffer. Samples were resolved by SDS-polyacrylamide gel, transferred to a PVDF filter, and subjected to Western blotting analyses for the detection of interested molecules.

Protein Denaturation and Re-immunoprecipitation—To determine BLNK ubiquitination, HEK293T cells overexpressing FLAG-BLNK and HA-ubiquitin were lysed and boiled in the presence of 1% SDS. Boiled cell lysates were diluted 10-fold to reduce SDS concentration, followed by immunoprecipitation with anti-FLAG antibody. The ubiquitination of BLNK was determined by Western blotting using anti-HA antibody.

Liquid Chromatography-Mass Spectrometry (LC-MS/MS)—Specific protein bands from silver-stained SDS-polyacrylamide gels were excised, destained, and digested with trypsin. The resulting peptide mixtures were loaded onto a 75- μ m \times 250-mm nanoACQUITY UPLC BEH130 column packed with

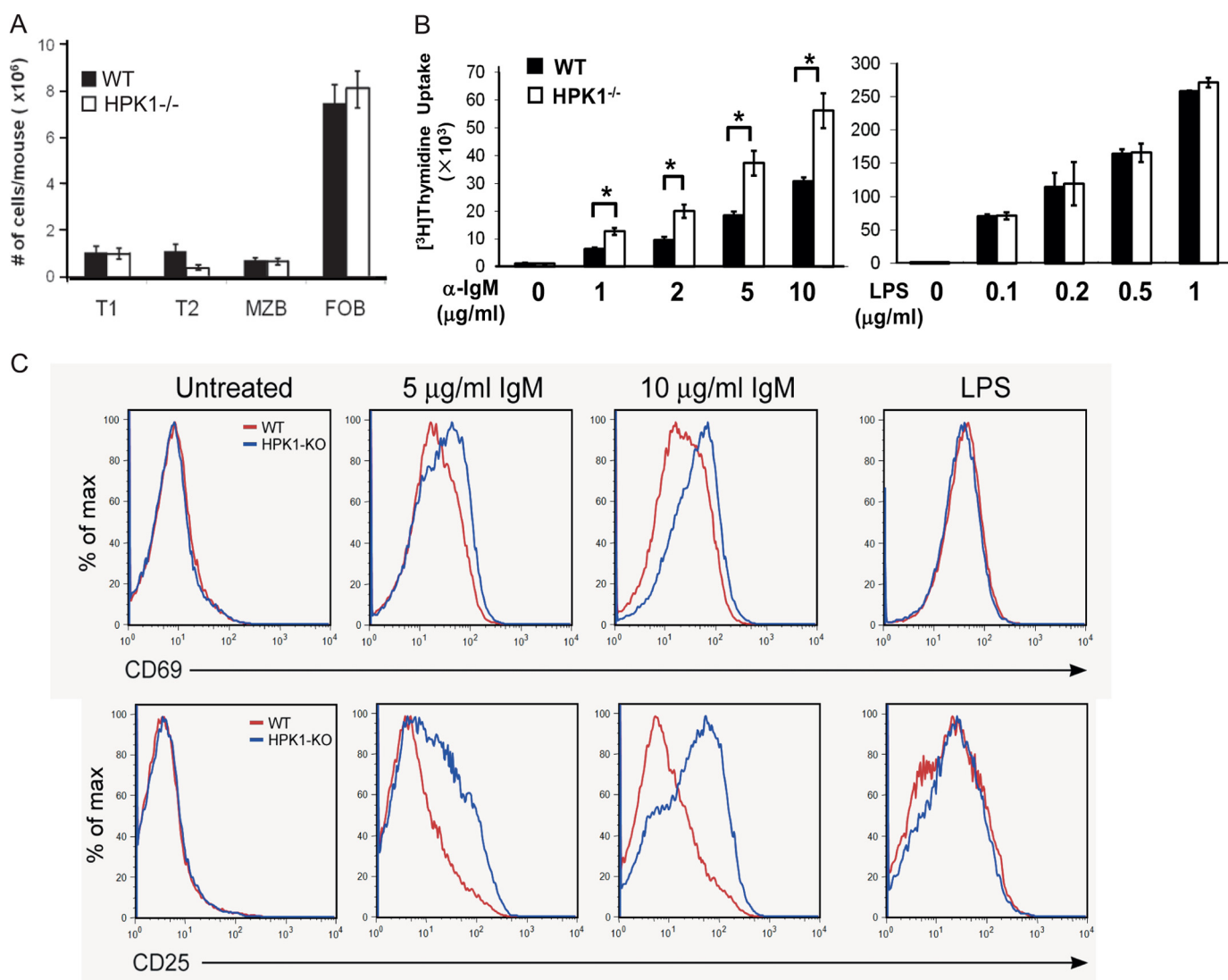


FIGURE 1. HPK1 deficiency enhances BCR-induced cell activation. *A*, B cell development is not affected in HPK1-deficient mice. Splenocytes from naive mice ($n = 3$) were stained for different developmental stages of B cells. Transitional 1 (T1, $CD23^{-}IgD^{lo}IgM^{hi}CD21^{-}$), transitional 2 (T2, $CD23^{+}IgD^{hi}IgM^{hi}CD21^{-}$), marginal zone (MZ, $CD23^{-}IgD^{lo}IgM^{hi}CD21^{+}$), and follicular (FO, $CD23^{+}IgD^{hi}IgM^{lo}CD21^{+}$) B cells were gated from $B220^{+}$ population from splenocytes. Numbers of B cell subsets were calculated based on total cell numbers of splenocytes and percentages of different B cell subsets. *B*, *in vitro* B cell proliferation of WT and HPK1-deficient B cells upon anti-IgM or LPS stimulation. Purified splenic B cells were incubated with anti-IgM or LPS for 40 h. The rate of cell proliferation was determined by the [³H]thymidine incorporation assay. *C*, HPK1-deficient B cells show increased activation markers upon the stimulation by anti-IgM but not LPS. Purified WT and HPK1-deficient splenic B cells were stimulated with anti-IgM (5–10 μ g/ml) or LPS (5 μ g/ml) for 6 h (CD69) or 24 h (CD25), respectively. The expression of CD69 (upper panel) and CD25 (lower panel) in these cells was analyzed by flow cytometry.

C18 resin (Waters, Milford, MA) and separated using a segmented gradient in 110 min from 5 to 85% solvent B (95% acetonitrile with 0.1% formic acid) at a flow rate of 300 nl/min. Solvent A was 0.1% formic acid in water. The samples were maintained at 4 °C in the autosampler. The LTQ-Orbitrap Velos hybrid mass spectrometer (Thermo Scientific) was operated in the positive ion mode with the following acquisition cycle: a full scan (m/z 350–1600) recorded in the orbitrap analyzer at resolution R 60,000 was followed by MS/MS of the 20 most intense peptide ions in the LTQ analyzer. All MS and MS/MS raw data were processed by Raw2MSM and searched against a target protein sequence data base using the Mascot Daemon 2.2 server. Search criteria used were as follows: trypsin digestion; variable modifications set as carbamidomethyl (Cys), oxidation (Met), phosphorylation (Ser, Thr, Tyr), and ubiquitination (Lys); up to two missed cleavages allowed; and mass

accuracy of 15 ppm for the parent ion and 0.60 Da for the fragment ions.

Statistical Analysis—Statistical analysis was performed using the unpaired Student's *t* test.

RESULTS

HPK1 Attenuates BCR-induced B Cell Activation—To study whether HPK1 regulates B cell development, different B cell populations in the spleen were analyzed. There was no significant difference between WT mice and HPK1-deficient mice in transitional 1 (T1), T2, marginal zone, and follicular B cells (Fig. 1A), suggesting that HPK1 is not essential for B cell development. To study the role of HPK1 in peripheral B cell activation, we isolated splenic B cells and studied *in vitro* B cell proliferation following anti-IgM and LPS treatment, which activates BCR and Toll-like receptor 4 signaling, respectively. B cell pro-

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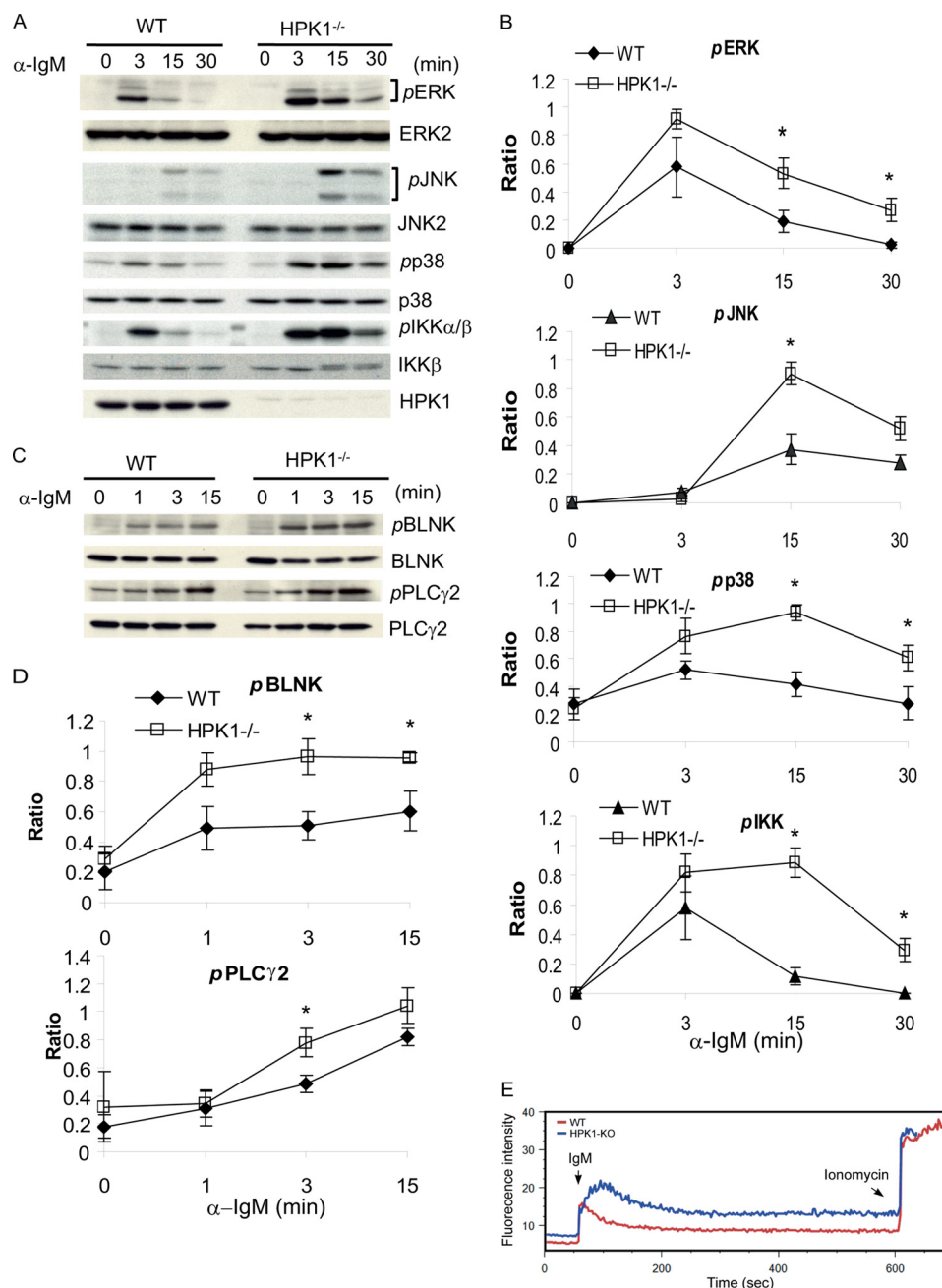


FIGURE 2. HPK1-deficient B cells show enhanced BCR signaling. Splenic B cells were isolated from WT and HPK1-deficient mice and stimulated with anti-IgM (10 μ g/ml) for indicated time periods. The activation of BCR signaling molecules was detected by Western blotting. Western blotting (A) and quantification (B) of phosphorylated and total ERK, p38, JNK, and IKK in WT and HPK1-deficient B cells are shown. Western blotting (C) and quantification (D) of phosphorylated and total BLNK and PLC γ 2 in WT and HPK1-deficient B cells are shown. The quantification of immunoblots was performed using Kodak Molecular Imaging Software Version 4.0. The ratios represent the relative phosphorylation levels. Statistical analysis was conducted on the data derived from four independent experiments. *, $p < 0.05$, Student's *t* test. E, HPK1-deficient B cells show increased calcium influx upon anti-IgM stimulation. The purified WT and HPK1-deficient splenic B cells were labeled with the calcium indicator Fluo-4. These labeled cells were stimulated with anti-IgM and then with ionomycin. The calcium influx was detected by flow cytometry.

liferation was significantly enhanced in HPK1-deficient B cells upon anti-IgM but not LPS stimulation (Fig. 1B). Similarly, B cell activation markers, including CD69 and CD25, were significantly increased in HPK1-deficient B cells by anti-IgM but not LPS stimulation (Fig. 1C). The enhanced B cell activation in HPK1-deficient B cells was not due to the increased expression of BCR in the cell surface (supplemental Fig. 1A). These data suggest that HPK1 regulates BCR intracellular signaling. Thus, we further determined the activation of intracellular BCR sig-

naling in wild-type (WT) and HPK1-deficient splenic B cells. The phosphorylation of MAPKs (ERK, p38, and JNK) was all significantly enhanced in HPK1-deficient B cells upon anti-IgM stimulation; IKK phosphorylation and I κ B degradation, which lead to NF- κ B activation, were also significantly enhanced in HPK1-deficient B cells (Fig. 2, A and B, and supplemental Fig. 1, B and C). In addition, we determined the phosphorylation of BCR proximal signaling molecules SYK, BLNK, and PLC γ 2. SYK phosphorylation was not affected in HPK1-deficient B

cells (supplemental Fig. 1, *D* and *E*); however, BLNK, which is downstream of SYK, displayed significantly enhanced tyrosine phosphorylation in HPK1-deficient B cells (Fig. 2, *C* and *D*). PLC γ 2, a downstream molecule of BLNK, also showed some enhancement of tyrosine phosphorylation in HPK1-deficient B cells (Fig. 2, *C* and *D*). An important outcome of PLC γ 2 activation is calcium flux, which leads to activation of MAPKs, NF-AT, and NF- κ B. Consistently, HPK1-deficient B cells showed increased calcium flux in response to anti-IgM stimulation (Fig. 2*E*). Taken together, these data suggest that HPK1 may target BLNK to attenuate BCR signaling and peripheral B cell activation.

HPK1 Induces BLNK/14-3-3 Interaction during BCR Signaling—As HPK1 interacts with BLNK in B cells and HPK1 induces SLP-76/14-3-3 interaction in T cells (10, 22), we hypothesized that HPK1 attenuated BCR signaling by inducing BLNK/14-3-3 interaction in B cells. In HEK293T cells, overexpression of HPK1 WT but not the kinase-dead mutant (M46) (6) induced BLNK/14-3-3 τ interaction as determined by GST-14-3-3 pull-down assays (Fig. 3*A*). Seven isoforms of 14-3-3 proteins (γ , ζ , τ , σ , η , ϵ , and β) have been described in mammals (23). We studied whether HPK1 also regulated the interaction of BLNK with other 14-3-3 isoforms in HEK293T cells. BLNK interacted with all seven isoforms of 14-3-3 in the presence of HPK1, with the relative affinity order $\gamma > \tau > \eta > \epsilon > \beta > \delta > \zeta$ (Fig. 3*B*). Next, we determined whether BLNK/14-3-3 interaction was induced in primary B cells during BCR signaling. BLNK/14-3-3 τ interaction was induced in WT splenic B cells upon anti-IgM stimulation, although this interaction was undetectable in HPK1-deficient splenic B cells (Fig. 3*C*). Furthermore, 14-3-3 τ -associated BLNK was tyrosine-phosphorylated in B cells after stimulation with anti-IgM (Fig. 3*D*), suggesting that the activated BLNK is targeted for 14-3-3-mediated regulation.

HPK1 Phosphorylates BLNK Thr-152, Which Mediates BLNK/14-3-3 Interaction and BCR Signaling Attenuation—14-3-3 mainly binds to phosphoproteins containing a conserved motif, which is a phosphorylated serine with consensus flanking sequences (RSXpSXP and RX(Y/F)XpSXP, where *X* represents any amino acid) (24). To identify the HPK1-induced phosphorylation site of BLNK that mediates BLNK/14-3-3 interaction, we searched the BLNK protein sequence for potential 14-3-3-binding motifs. However, unlike SLP-76, BLNK lacks a canonical 14-3-3 motif-like sequence. To map the HPK1-targeted BLNK phosphorylation site(s), we generated a series of FLAG-tagged BLNK fragments and tested these BLNK mutants for HPK1-dependent 14-3-3 binding ability in HEK293T cells using GST-pull-down assays (Fig. 4, *A* and *B*). BLNK(1–190) and BLNK(121–340) fragments were able to bind 14-3-3 (Fig. 3*B*), suggesting that the 14-3-3-binding site(s) is within the region from 121 to 190. We therefore mutated all the serine or threonine residues (Ser-129, Ser-138, Ser-141, and Thr-152) flanked with a conserved proline within the region of BLNK(121–190). Ser-144 and Ser-151, which are not flanked by any conserved sequence, were also mutated as a control. BLNK S129A, S138A, S141A, S144A, and S151A mutants still interacted with 14-3-3, whereas the BLNK T152A mutant completely lost the binding with 14-3-3 (Fig. 4*C*, data for S129A,

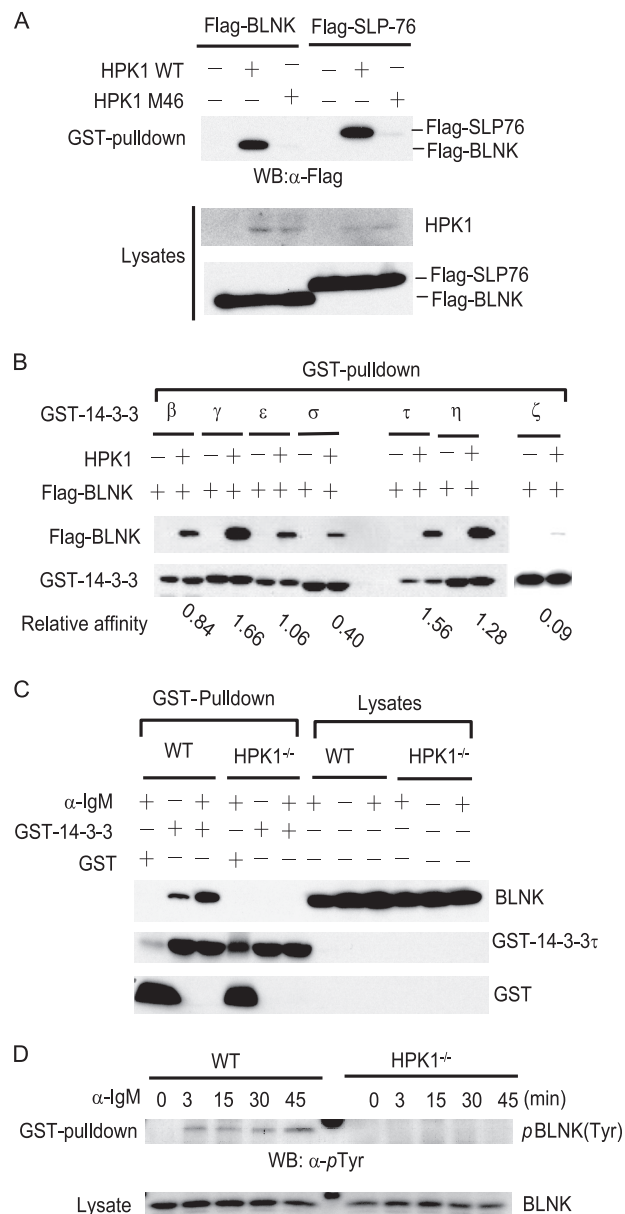


FIGURE 3. HPK1 induces BLNK/14-3-3 interaction in B cells. *A*, HPK1 induces BLNK/14-3-3 τ interaction in HEK293T cells. FLAG-tagged BLNK was transfected into HEK293T cells with HPK1 WT or kinase-dead mutant (M46). The interaction of FLAG-BLNK/GST-14-3-3 was detected by GST-14-3-3 pull-down assays. FLAG-SLP-76 was included as a positive control for GST-14-3-3 pull-down assays. *B*, interaction of BLNK with different 14-3-3 isoforms in the presence of HPK1. FLAG-tagged BLNK was transfected into HEK293T cells with HPK1. The interaction of BLNK/14-3-3 isoform was detected by GST pull-down assays. The relative binding affinity of BLNK with 14-3-3 isoforms was calculated by the amount of GST-14-3-3-bound BLNK divided by the amount of GST-14-3-3 input as determined by densitometry analyses. *C*, BCR stimulation induces BLNK/14-3-3 τ interaction in WT but not HPK1-deficient B cells. Splenic B cells were stimulated with anti-IgM for 3 min. Endogenous BLNK/14-3-3 τ interaction was pulled down either by GST-14-3-3 τ (2nd, 3rd, 5th, and 6th lanes) or by GST alone (1st and 3rd lanes) as controls. *D*, 14-3-3-associated BLNK is tyrosine-phosphorylated in B cells. WT and HPK1-deficient splenic B cells were stimulated with anti-IgM for indicated time periods and subjected to GST-pull-down assay using GST-14-3-3 τ . GST-14-3-3 τ -associated BLNK was detected by Western blotting (WB) using anti-phosphotyrosine antibody. Data are representative of three independent experiments.

S138A, and S141A mutants are not shown). These suggest that Thr-152 is an HPK1-targeted phosphorylation site that mediates BLNK/14-3-3 interaction. To demonstrate the phospho-

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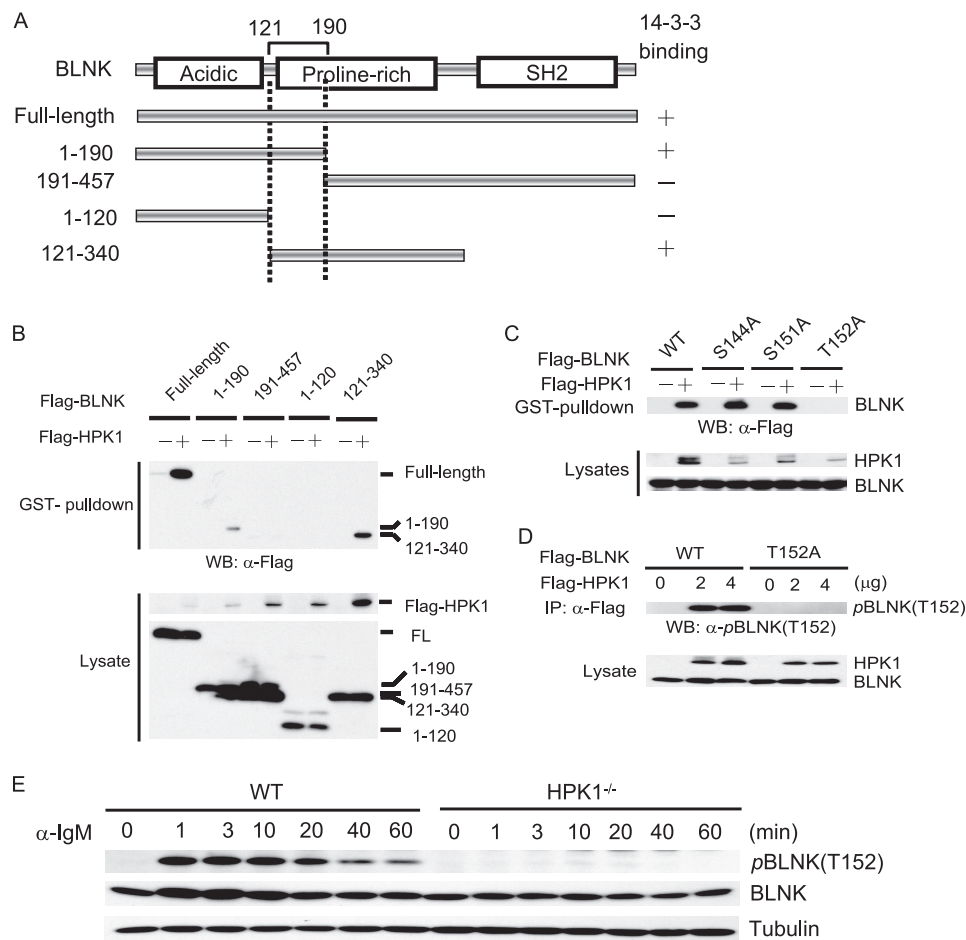


FIGURE 4. Thr-152 is phosphorylated by HPK1 and mediates BLNK/14-3-3 interaction. *A*, schematic diagram of identification of BLNK/14-3-3 binding region. *B*, interaction of BLNK fragments and 14-3-3 in HEK293T cells was determined by GST-pull-down assays. *C*, T152A mutation abolishes HPK1-dependent BLNK/14-3-3 interaction in HEK293T cells. Full-length FLAG-BLNK mutants containing S141A, S151A, or T152A mutation were transfected into HEK293T cells with HPK1. The interaction of FLAG-BLNK mutants with GST-14-3-3 was detected by GST-pull-down assays. *D*, HPK1 induces BLNK Thr-152 phosphorylation in HEK293T cells. FLAG-tagged BLNK WT or T152A mutants were transfected into HEK293T cells with HPK1. FLAG-BLNK was immunoprecipitated with anti-FLAG antibody and Western-blotted with anti-p-BLNK(Thr-152) antibody. *E*, BCR signaling induces BLNK Thr-152 phosphorylation in WT but not HPK1-deficient B cells. Purified splenic B cells were stimulated with anti-IgM. The phosphorylation of BLNK Thr-152 was detected by Western blotting (WB) with anti-p-BLNK(Thr-152) antibody. Data are representative of three independent experiments.

rylation of BLNK Thr-152 site, we generated an anti-phospho-BLNK(Thr-152) antibody, which specifically recognized BLNK WT but not T152A mutant when co-transfected with HPK1 in HEK293T cells (Fig. 4D). Using this phospho-antibody, we verified that BLNK Thr-152 phosphorylation was induced in WT splenic B cells as early as 1 min upon anti-IgM stimulation (Fig. 4E); in contrast, HPK1-deficient splenic B cells completely lost BLNK Thr-152 phosphorylation (Fig. 4E).

We further studied the functional significance of BLNK Thr-152 phosphorylation using DT40 B cells, which is a transformed chicken B cell line with a high frequency of homologous gene targeting (25). Anti-chicken IgM stimulation of DT40 B cells induces signaling cascades that are analogous to BCR signaling cascades in murine and human B cells (26). BLNK-deficient DT40 mutant B cells were further generated by gene targeting (27, 28); these BLNK-deficient DT40 mutant B cells display a defective activation of MAPKs (ERK, p38, and JNK) upon anti-chicken IgM stimulation (27, 28). We transfected murine FLAG-tagged BLNK WT or T152A mutant, both of which were subcloned into pIRES2-EGFP vector simultaneously expressing GFP, into BLNK-deficient DT40 mutant B cells. Transient

expression of murine BLNK WT partially restored BCR-induced activation of MAPKs and IKK in BLNK-deficient DT40 mutant B cells (Fig. 5, A and B). As expected, BLNK-deficient DT40 mutant B cells that were transfected with the BLNK T152A mutant showed significantly enhanced phosphorylation of all three MAPKs and IKK (Fig. 5, A and B). BLNK T152A mutant also displayed significantly enhanced tyrosine phosphorylation in the transfected BLNK-deficient DT40 mutant B cells (Fig. 5, C and D). Consistent with the role of BLNK Thr-152 phosphorylation in mediating 14-3-3 interaction in HEK293T cells, the BLNK T152A mutant showed drastically reduced binding with 14-3-3 τ in BLNK-deficient DT40 mutant B cells upon anti-IgM stimulation (Fig. 5, C and D).

Thr-152 Phosphorylation Induces BLNK Ubiquitination in B Cells—Next, we aimed to delineate the underlying mechanism of BCR signaling attenuation by HPK1-induced Thr-152 phosphorylation and subsequent 14-3-3 binding of BLNK. 14-3-3 proteins may regulate the functions of its binding partners through different mechanisms as follows: (i) change the conformation of its binding proteins, leading to the change of enzymatic properties or protein interactions of the binding part-

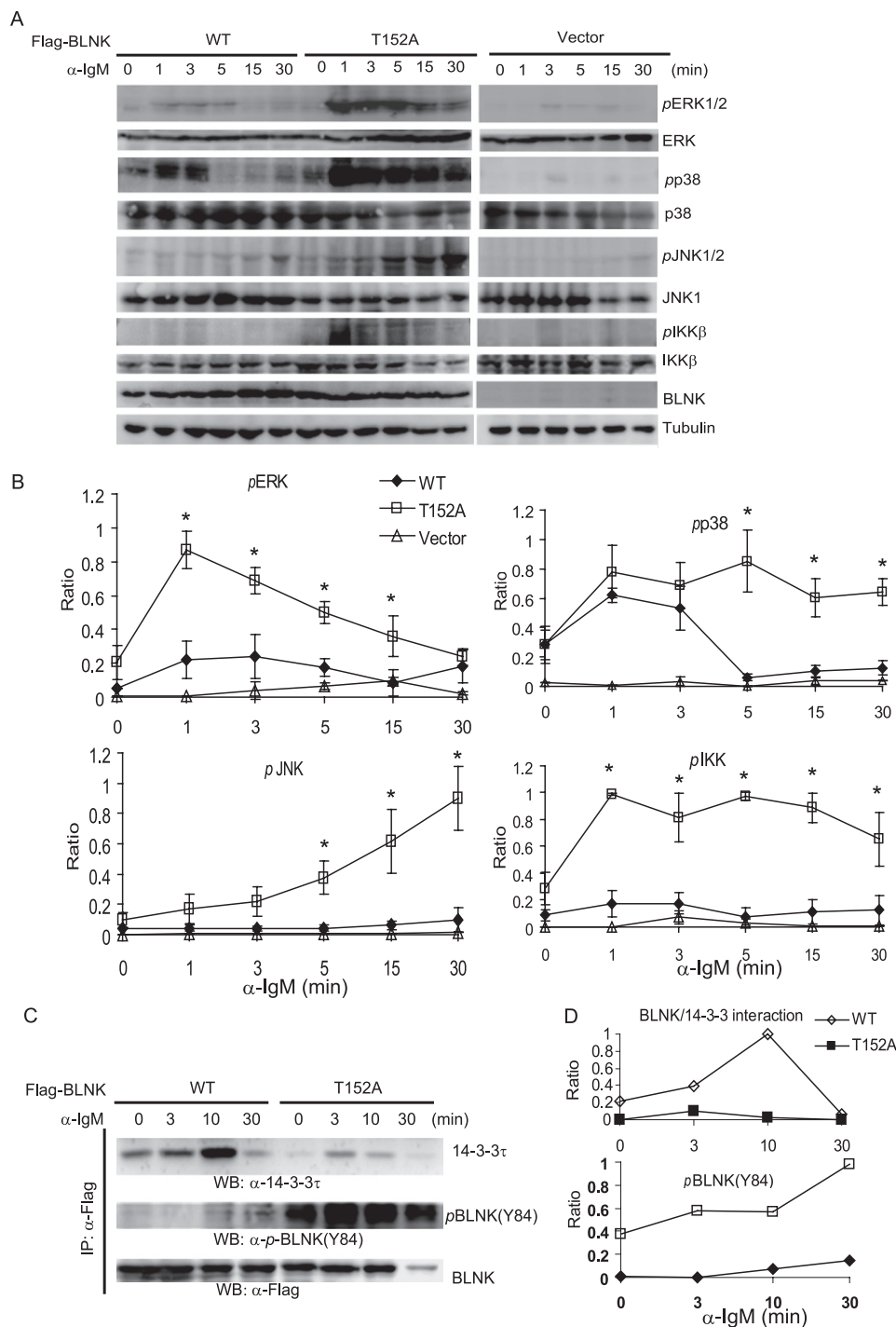


FIGURE 5. BLNK Thr-152 phosphorylation down-regulates BCR signaling in DT40 B cells. FLAG-tagged BLNK WT, FLAG-tagged BLNK(T152A), and an empty vector control were transfected into BLNK-deficient DT40 mutant chicken B cells. 36 h after transfection, cells were stimulated with anti-chicken IgM (5 μ g/ml). Western blotting (A) and quantification (B) of phosphorylated and total ERK, p38, JNK, and IKK in transfected BLNK-deficient DT40 mutant B cells are shown. Statistical analysis was conducted on the data derived from three independent experiments. *, $p < 0.05$, Student's t test. C, BLNK T152A mutant shows reduced 14-3-3 binding and increased tyrosine phosphorylation in BLNK-deficient DT40 B cells. BLNK-deficient DT40 B cells were transfected as described above. BLNK was immunoprecipitated by anti-FLAG antibody followed by Western blotting (WB) with anti-14-3-3 τ or anti-p-BLNK(Y84) antibody. D, quantification of BLNK/14-3-3 interaction and BLNK tyrosine phosphorylation for C.

ners; (ii) physically block the interaction of other proteins with 14-3-3 binding partners; and (iii) function as an obligatory dimer and may bridge an interaction between two proteins, each associated with a 14-3-3 monomer (29, 30). As BLNK interactions with PLC γ 2 and other signaling molecules are important for the activation of BCR downstream signaling (3),

we first studied whether HPK1 regulates BLNK and PLC γ 2 signaling complexes. The anti-IgM-induced BLNK- and PLC γ 2-interacting complexes were not changed in HPK1-deficient B cells upon anti-IgM stimulation (Fig. 6A). We also studied whether the recruitment of 14-3-3 to the activated BLNK led to BLNK degradation. The proteasomal inhibitor MG132

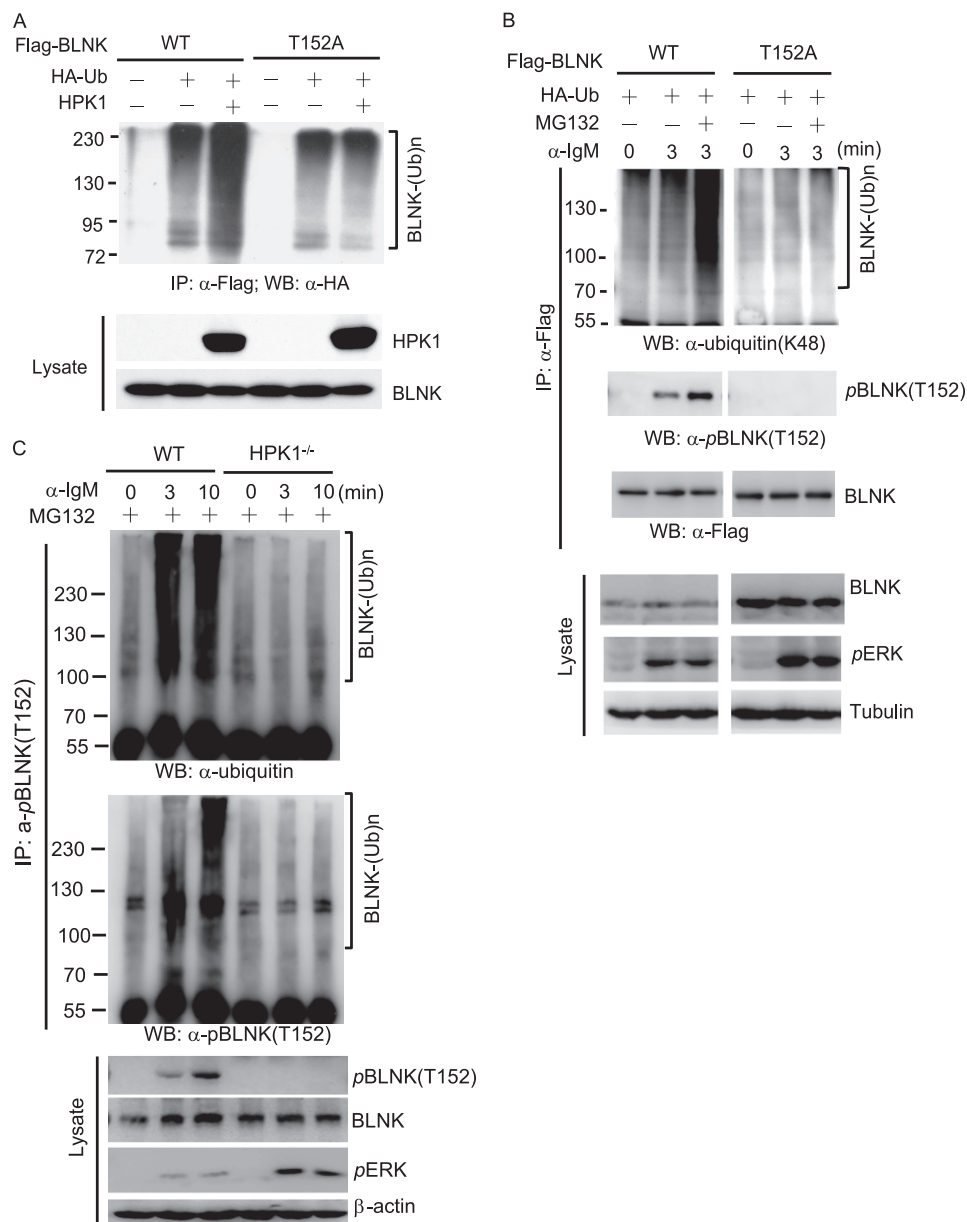


FIGURE 7. Thr-152 phosphorylation induces BLNK ubiquitination. *A*, T152A mutation abolishes HPK1-induced BLNK ubiquitination in HEK293T cells. FLAG-tagged BLNK WT or T152A mutant was transfected into HEK293T cells with HA-ubiquitin and HPK1. BLNK was immunoprecipitated (IP) using anti-FLAG antibody followed by Western blotting (WB) analyses with anti-HA antibody. *B*, BCR stimulation induces ubiquitination of BLNK WT but not T152A mutant in DT40 B cells. BLNK-deficient DT40 chicken B cells were transfected with FLAG-tagged BLNK WT or T152A mutant with HA-tagged ubiquitin. The transfected cells were either untreated or treated with MG132 for 4 h, followed by stimulation with anti-IgM antibody and immunoprecipitation using anti-FLAG antibody. The ubiquitination of BLNK was detected using anti-ubiquitin (Lys-48) antibody. *C*, Thr-152-phosphorylated BLNK is ubiquitinated in WT B cells. WT splenic B cells were stimulated with anti-IgM stimulation. BLNK was immunoprecipitated by anti-p-BLNK(Thr-152) followed by Western blotting analyses with anti-ubiquitin or anti-p-BLNK(Thr-152) antibody. HPK1-deficient B cells were included as a negative control. Data are representative of three independent experiments.

tion. We co-transfected FLAG-tagged BLNK WT or T152A mutant with HPK1 and HA-ubiquitin into HEK293T cells. HPK1 co-expression significantly enhanced ubiquitination of BLNK WT, whereas T152A mutation abrogated the effect of HPK1 on BLNK ubiquitination (Fig. 7A). To study whether Thr-152 phosphorylation induced BLNK ubiquitination in B cells, we co-transfected FLAG-BLNK WT or T152A mutant with HA-ubiquitin into BLNK-deficient DT40 mutant B cells. FLAG-BLNK WT but not T152A mutant was polyubiquitinated through Lys-48 linkage in the transfected BLNK-deficient DT40 mutant B cells treated with anti-IgM and MG132

(Fig. 7B). Consistently, immunoprecipitates of anti-phospho-BLNK(Thr-152) from anti-IgM-stimulated WT splenic B cells displayed significant ubiquitination, which was detected by both anti-ubiquitin antibody and anti-pBLNK(Thr-152) antibody (Fig. 7C). These data indicate that Thr-152 phosphorylation targeted BLNK for ubiquitination during BCR signaling.

Lys-37, Lys-38, and Lys-42 Are BLNK Ubiquitination Sites That Attenuate BCR Signaling in B Cells—To identify BLNK ubiquitination site(s), we isolated transfected BLNK from WEHI-231 mouse B cell line treated with anti-IgM and MG132, followed by mass spectrometry analyses. Three lysine residues

ubiquitination sites (Fig. 8B). Furthermore, anti-IgM-induced phosphorylation of ERK, JNK, and IKK was significantly enhanced in BLNK-deficient DT40 mutant B cells transfected with BLNK K37R/K38R/K40R/K42R mutant (Fig. 8C). These data indicate that loss of BLNK ubiquitination enhances activation of BCR downstream signaling.

DISCUSSION

BCR signaling is delicately controlled by positive and negative regulatory mechanisms. Imbalance of BCR signaling may result in autoimmune diseases or immune deficiency disorders. In this study, we have found that HPK1 plays a negative role in BCR signaling. HPK1-deficient B cells are hyperactive in response to BCR ligation, resulting in enhanced B cell proliferation and activation (Figs. 1, B and C, and 2). The enhanced BCR signaling in HPK1-deficient B cells may contribute to increased antibody productions and experimental autoimmune encephalomyelitis induction in HPK1-deficient mice as shown previously (10).

Previous studies show that HPK1 activates IKK (15, 16, 19) and JNK (6–9). In contrast, our data show an inhibitory role of HPK1 for IKK and JNK activation in B cells (Fig. 2, A and B). The difference may be due to using different experimental systems. The positive role of HPK1 in JNK and IKK activation was mainly demonstrated using overexpression systems. One caveat of these overexpression systems is that these cells have limited or no expression of the target proteins (such as BLNK) required for the HPK1-mediated feedback inhibition. Therefore, HPK1 may be skewed to activate JNK and IKK in these cells. HPK1-deficient primary B cells show enhanced JNK and IKK phosphorylation during BCR signaling, indicating that the inhibitory roles of HPK1 in JNK and IKK activation overcome the positive roles HPK1 in JNK and IKK activation in primary B cells.

Our study shows a negative feedback regulation of BLNK by HPK1-induced Thr-152 phosphorylation, which recruits 14-3-3 to BLNK and subsequently attenuates BCR downstream signaling. Previous studies show that HPK1 attenuates T cell receptor signaling via inducing SLP-76 Ser-376 phosphorylation and SLP-76/14-3-3 interaction in T cells (10, 31). Thus, HPK1 negatively regulates BCR and T cell receptor signaling via a similar mechanism. However, in contrast to partial loss of SLP-76/14-3-3 binding in HPK1-deficient T cells (10), BLNK/14-3-3 interaction was undetectable in HPK1-deficient B cells (Fig. 3C). This suggests that HPK1 plays an exclusive role in B cells. It is notable that the transfected BLNK WT is weakly tyrosine-phosphorylated in BLNK-deficient DT40 mutant B cells (Fig. 5C). Consistently, transient expression of BLNK WT in BLNK-deficient DT40 mutant B cells does not strongly induce downstream MAPK and IKK activation (Figs. 5A and 8C). One potential explanation is that murine BLNK has only 56% identity as chicken BLNK. The transfected murine BLNK may not fully reconstitute BCR signaling of BLNK-deficient DT40 chicken B cells. However, after transfection with murine BLNK WT or T152A, chicken DT40 cells displayed a remarkable difference in BLNK tyrosine phosphorylation and downstream MAPK and IKK phosphorylation (Fig. 5). Future generation and characterization of knock-in or transgenic mice carrying BLNK(T152A) would provide more insights on *in vivo*

functions of BLNK(Thr-152) phosphorylation in immune responses and autoimmune diseases.

A novel finding in this report is that the activated BLNK is ubiquitinated at Lys-37, Lys-38, and Lys-42 residues in B cells during BCR signaling and that the loss of BLNK ubiquitination results in enhanced BCR signaling. Ubiquitination has emerged as a key mechanism regulating the immune function (32). We showed that BLNK was regulated by polyubiquitination via Lys-48 linkage in BCR signaling (Figs. 6, D and E, and 7). The proteasomal inhibitor MG132 stabilized the phosphorylated BLNK proteins but not the total BLNK proteins (Fig. 6B), suggesting that only the phosphorylated BLNK is targeted for ubiquitination and degradation. As phosphorylation and ubiquitination events of a protein only account for a small portion of the total protein, this could explain why the stabilization of total BLNK proteins by MG132 was not discernible. More interestingly, BLNK ubiquitination is initiated by HPK1-induced Thr-152 phosphorylation as HPK1 deficiency or loss of BLNK Thr-152 phosphorylation abolished BLNK ubiquitination (Figs. 6E and 7, B and C). We further identify that Lys-37, Lys-38, and Lys-42 residues are BLNK ubiquitination sites in mouse B cells. These three lysine residues and Thr-152 are also conserved in human BLNK protein sequence (supplemental Fig. 3), suggesting that mutations or post-translational modification of these residues could regulate B cell activation in humans as well. As HPK1 phosphorylates SLP-76 in T cells, our findings in B cells suggest that HPK1-induced SLP-76 phosphorylation may also regulate SLP-76 ubiquitination. Our study has implied that an E3 ligase is recruited to BLNK via 14-3-3-mediated binding and induces BLNK ubiquitination. Although c-Cbl is shown to interact with 14-3-3 (33, 34), BLNK ubiquitination was not reduced in c-Cbl-deficient splenic B cells (data not shown), suggesting that an E3 ligase other than c-Cbl is responsible for BLNK ubiquitination. The identification of the elusive E3 ubiquitin ligases for BLNK is not the focus of this report and will be studied in the future.

In summary, our studies have uncovered a novel negative feedback regulation of the activated BLNK mediated by HPK1-induced Thr-152 phosphorylation, 14-3-3 binding, and subsequent ubiquitination of BLNK at Lys-37, Lys-38, and Lys-42 (Fig. 8D). The activated BLNK is tagged with Thr-152 phosphorylation and subsequently targeted for ubiquitination and degradation. BLNK ubiquitination results in the subsequent attenuation of ERK, JNK, and IKK activation, which in turn prevents B cell overactivation and hyperproliferation. This report in combination with our previous studies of HPK1-deficient mice (10) suggest that HPK1 down-regulation, BLNK mutations (at Thr-152, Lys-37, Lys-38, and Lys-42), and dysregulation of BLNK phosphorylation and ubiquitination in B cells may contribute to the pathogenesis of autoimmune diseases.

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REFERENCES

1. Yanaba, K., Bouaziz, J. D., Matsushita, T., Magro, C. M., St Clair, E. W., and Tedder, T. F. (2008) B-lymphocyte contributions to human autoimmune disease. *Immunol. Rev.* **223**, 284–299
2. Townsend, M. J., Monroe, J. G., and Chan, A. C. (2010) B-cell targeted therapies in human autoimmune diseases: an updated perspective. *Immunol. Rev.* **237**, 264–283
3. Koretzky, G. A., Abtahian, F., and Silverman, M. A. (2006) SLP76 and SLP65. Complex regulation of signaling in lymphocytes and beyond. *Nat. Rev. Immunol.* **6**, 67–78
4. Datta, S. R., Katsov, A., Hu, L., Petros, A., Fesik, S. W., Yaffe, M. B., and Greenberg, M. E. (2000) 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. *Mol. Cell* **6**, 41–51
5. Jang, I. K., Zhang, J., and Gu, H. (2009) Grb2, a simple adapter with complex roles in lymphocyte development, function, and signaling. *Immunol. Rev.* **232**, 150–159
6. Hu, M. C., Qiu, W. R., Wang, X., Meyer, C. F., and Tan, T. H. (1996) Human HPK1, a novel human hematopoietic progenitor kinase that activates the JNK/SAPK kinase cascade. *Genes Dev.* **10**, 2251–2264
7. Liou, J., Kiefer, F., Dang, A., Hashimoto, A., Cobb, M. H., Kurosaki, T., and Weiss, A. (2000) HPK1 is activated by lymphocyte antigen receptors and negatively regulates AP-1. *Immunity* **12**, 399–408
8. Ling, P., Yao, Z., Meyer, C. F., Wang, X. S., Oehrl, W., Feller, S. M., and Tan, T. H. (1999) Interaction of hematopoietic progenitor kinase 1 with adapter proteins Crk and CrkL leads to synergistic activation of c-Jun N-terminal kinase. *Mol. Cell. Biol.* **19**, 1359–1368
9. Ma, W., Xia, C., Ling, P., Qiu, M., Luo, Y., Tan, T. H., and Liu, M. (2001) Leukocyte-specific adaptor protein Grap2 interacts with hematopoietic progenitor kinase 1 (HPK1) to activate JNK signaling pathway in T lymphocytes. *Oncogene* **20**, 1703–1714
10. Shui, J. W., Boomer, J. S., Han, J., Xu, J., Dement, G. A., Zhou, G., and Tan, T. H. (2007) Hematopoietic progenitor kinase 1 negatively regulates T cell receptor signaling and T cell-mediated immune responses. *Nat. Immunol.* **8**, 84–91
11. Chuang, H. C., Lan, J. L., Chen, D. Y., Yang, C. Y., Chen, Y. M., Li, J. P., Huang, C. Y., Liu, P. E., Wang, X., and Tan, T. H. (2011) The kinase GLK controls autoimmunity and NF- κ B signaling by activating the kinase PKC- θ in T cells. *Nat. Immunol.* **12**, 1113–1118
12. Diener, K., Wang, X. S., Chen, C., Meyer, C. F., Keesler, G., Zukowski, M., Tan, T. H., and Yao, Z. (1997) Activation of the c-Jun N-terminal kinase pathway by a novel protein kinase related to human germinal center kinase. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 9687–9692
13. Chen, Y.-R., and Tan, T. H. (1999) Mammalian c-Jun N-terminal kinase pathway and STE20-related kinases. *Gene Ther. Mol. Biol.* **4**, 83–98
14. Yao, Z., Zhou, G., Wang, X. S., Brown, A., Diener, K., Gan, H., and Tan, T. H. (1999) A novel human STE20-related protein kinase, HGK, that specifically activates the c-Jun N-terminal kinase signaling pathway. *J. Biol. Chem.* **274**, 2118–2125
15. Hu, M. C., Wang, Y., Qiu, W. R., Mikhail, A., Meyer, C. F., and Tan, T. H. (1999) Hematopoietic progenitor kinase-1 (HPK1) stress response signaling pathway activates I κ B kinases (IKK- α/β) and IKK- β is a developmentally regulated protein kinase. *Oncogene* **18**, 5514–5524
16. Brenner, D., Brechmann, M., Röhling, S., Tapernoux, M., Mock, T., Winter, D., Lehmann, W. D., Kiefer, F., Thome, M., Krammer, P. H., and Arnold, R. (2009) Phosphorylation of CARMA1 by HPK1 is critical for NF- κ B activation in T cells. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 14508–14513
17. Chen, Y. R., Meyer, C. F., Ahmed, B., Yao, Z., and Tan, T. H. (1999) Caspase-mediated cleavage and functional changes of hematopoietic progenitor kinase 1 (HPK1). *Oncogene* **18**, 7370–7377
18. Arnold, R., Liou, J., Drexler, H. C., Weiss, A., and Kiefer, F. (2001) Caspase-mediated cleavage of hematopoietic progenitor kinase 1 (HPK1) converts an activator of NF κ B into an inhibitor of NF κ B. *J. Biol. Chem.* **276**, 14675–14684
19. Batliwalla, F. M., Li, W., Ritchlin, C. T., Xiao, X., Brenner, M., Laragione, T., Shao, T., Durham, R., Kemshtetti, S., Schwarz, E., Coe, R., Kern, M., Baechler, E. C., Behrens, T. W., Gregersen, P. K., and Gulko, P. S. (2005) Microarray analyses of peripheral blood cells identifies unique gene expression signature in psoriatic arthritis. *Mol. Med.* **11**, 21–29
20. Stoeckman, A. K., Baechler, E. C., Ortmann, W. A., Behrens, T. W., Michet, C. J., and Peterson, E. J. (2006) A distinct inflammatory gene expression profile in patients with psoriatic arthritis. *Genes Immun.* **7**, 583–591
21. Zhang, Q., Long, H., Liao, J., Zhao, M., Liang, G., Wu, X., Zhang, P., Ding, S., Luo, S., and Lu, Q. (2011) Inhibited expression of hematopoietic progenitor kinase 1 associated with loss of jumonji domain containing 3 promoter binding contributes to autoimmunity in systemic lupus erythematosus. *J. Autoimmun.* **37**, 180–189
22. Tsuji, S., Okamoto, M., Yamada, K., Okamoto, N., Goitsuka, R., Arnold, R., Kiefer, F., and Kitamura, D. (2001) B cell adaptor containing src homology 2 domain (BASH) links B cell receptor signaling to the activation of hematopoietic progenitor kinase 1. *J. Exp. Med.* **194**, 529–539
23. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J., and Cantley, L. C. (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell* **91**, 961–971
24. Bunnell, S. C., Diehn, M., Yaffe, M. B., Findell, P. R., Cantley, L. C., and Berg, L. J. (2000) Biochemical interactions integrating Itk with the T cell receptor-initiated signaling cascade. *J. Biol. Chem.* **275**, 2219–2230
25. Buerstedde, J. M., and Takeda, S. (1991) Increased ratio of targeted to random integration after transfection of chicken B cell lines. *Cell* **67**, 179–188
26. Kosugi, A., Hayashi, F., Liddicoat, D. R., Yasuda, K., Saitoh, S., and Hamaoka, T. (2001) A pivotal role of cysteine 3 of Lck tyrosine kinase for localization to glycolipid-enriched microdomains and T cell activation. *Immunol. Lett.* **76**, 133–138
27. Ishiai, M., Kurosaki, M., Pappu, R., Okawa, K., Ronko, I., Fu, C., Shibata, M., Iwamatsu, A., Chan, A. C., and Kurosaki, T. (1999) BLNK required for coupling Syk to PLC γ 2 and Rac1-JNK in B cells. *Immunity* **10**, 117–125
28. Chiu, C. W., Dalton, M., Ishiai, M., Kurosaki, T., and Chan, A. C. (2002) BLNK. Molecular scaffolding through “cis”-mediated organization of signaling proteins. *EMBO J.* **21**, 6461–6472
29. Hermeking, H. (2003) The 14-3-3 cancer connection. *Nat. Rev. Cancer* **3**, 931–943
30. Obsilova, V., Silhan, J., Boura, E., Teisinger, J., and Obsil, T. (2008) 14-3-3 proteins. A family of versatile molecular regulators. *Physiol. Res.* **57**, Suppl. 3, S11–S21
31. Di Bartolo, V., Montagne, B., Salek, M., Jungwirth, B., Carrette, F., Fourtane, J., Sol-Foulon, N., Michel, F., Schwartz, O., Lehmann, W. D., and Acuto, O. (2007) A novel pathway down-modulating T cell activation involves HPK-1-dependent recruitment of 14-3-3 proteins on SLP-76. *J. Exp. Med.* **204**, 681–691
32. Bhoj, V. G., and Chen, Z. J. (2009) Ubiquitylation in innate and adaptive immunity. *Nature* **458**, 430–437
33. Liu, Y. C., Elly, C., Yoshida, H., Bonnefoy-Berard, N., and Altman, A. (1996) Activation-modulated association of 14-3-3 proteins with Cbl in T cells. *J. Biol. Chem.* **271**, 14591–14595
34. Thien, C. B., and Langdon, W. Y. (2001) Cbl. Many adaptations to regulate protein tyrosine kinases. *Nat. Rev. Mol. Cell. Biol.* **2**, 294–307