Supporting Information

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SI Materials and Methods

Mice. C57BL/6 mice were purchased from CLEA Japan and μMT mice were obtained from The Jackson Laboratory. GFP transgenic mice were provide by M. Okabe, (Osaka University, Osaka). To generate *Pkn1*$^{-/-}$ mice, a targeting vector that disrupts the *Pkn1* gene was introduced into E14 (129) ES cells. The mutant ES cells were microinjected into C57BL/6 mice and heterozygous offspring were intercrossed to produce homozygous mutant animals. The *Pkn1*$^{-/-}$ mice were backcrossed to C57BL/6 mice for 15 generations. All mice were maintained in a specific pathogen-free animal facility in accordance with the Osaka University Guidelines for Animal Experimentation; this study was approved by the institutional animal care and use committee (permission number: H21-28-0).

Antibodies. The following antibodies were used for FACS analyses and immunohistochemistry: anti-CD19-APC-Cy7, anti-B220 (PE-Cy7 or PE conjugated), anti-Fas (CD95)-FITC, anti-GL7-FITC, anti-CD90.2-FITC, anti-CD4-PE-Cy7, anti-CD8-APC-Cy7, anti-Gr-1-FITC, anti-CD11b-FITC, anti-IgM-FITC, streptavidin (PE or APC-Cy7 conjugated) (all obtained from BD Biosciences), anti-PNA biotinylated (Vector Laboratories), anti-IgG1-APC, goat antimouse IgG, antigoat-FITC (all obtained from Jackson ImmunoResearch), anticomplement C3-FITC (MP Biomedicals), and anti-IgD-FITC (Nordic Immunology). Anti-NP-PE was prepared by conjugating the NP-coupled imide-ester with PE (1).

The following antibodies were used for Western blot or immunoprecipitation analyses: antiphospho-Akt, anti-Akt, anti-phospho-FoxO4, anti-FoxO4, anti-Bcl-xL, anti-FoxO1, anti–α-tubulin (all obtained from Cell Signaling Technology), as well as anti-FLAG and anti-HA (both obtained from Sigma), and anti-PKN1 (obtained from BD Biosciences).

Plasmids. To construct FLAG-PKN1FL, FLAG-PKN1CA, pMSCV-PKN1CA, and pMSCV-PKN1KD, the corresponding cDNA fragments were cloned into pCMVFLAG7.1 (Sigma) or pMSCVpuro (Clontech). To construct HA-Akt1, the corresponding cDNA fragment was cloned into pcDNA3HA (Invitrogen). MyrAkt1 was generated by fusing the c-Src myristoylation sequence to the N terminus of Akt1. To generate pMIGRI-MyrAkt1, MyrAkt1 was inserted into the pMIGRI retroviral vector, which was provided by W. Pear (University of Pennsylvania, Philadelphia).

Cell Culture and Reagents. Mature B cells were purified from splenocytes with Mouse B Lymphocyte Enrichment Set-DM (BD Bioscience) or a Magnetic cell sorting (MACS)-based B-cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocol. Ramos cells, a human Burkitt's lymphoma cell line, were cultured in RPMI1640 medium containing 10% (vol/vol) FBS, 100 ug/ml streptomycin/100 units/ml penicillin, and 50 μM 2-mercaptoethanol. 293T, 3Y1, and Plat E cells were maintained in Dulbecco's modified Eagle medium containing 10% (vol/vol) FBS at 37 °C in 5% CO_2.

Transfection and Retrovirus Production. 293T cells were transfected with each plasmid using FuGENE 6 transfection reagent (Roche). Ecotropic retroviruses were generated by cotransfecting pMIGRI, pMIGRI-MyrAkt1, pMSCVpuro, pMSCVpuro-PKN1CA, or pMSCVpuro-PKN1KD into PlatE cells using FuGENE 6 (Roche).

ELISA. Anti-dsDNA antibody production was measured by ELISA according to the manufacturer's protocol (Shibayagi). To detect IL-6 production, purified B cells (1×10^6) were cultured with 10 μg/mL of an anti-IgM antibody (Jackson ImmunoResearch) and 10 μg/mL of an anti-CD40 antibody (BD Biosciences). The cultured supernatants were harvested at the indicated times and IL-6 was detected using an IL-6–specific ELISA kit (R&D Systems). Serum Ig production was measured by ELISA with antibodies specific for each Ig isotype as previously described (2).

Proliferation Assay. Purified B cells (5×10^4) were cultured in triplicate in a flat-bottomed 96-well plate with 10 μg/mL anti-IgM F(ab′)$_2$ (Jackson ImmunoResearch), 10 μg/mL anti-CD40 antibody, 20 ng/mL BAFF (R&D Systems), 20 ng/mL IL-4, and 10 μg/mL LPS. The cultures were incubated for 72 h and pulsed for 12 h before the culture was terminated.

Cell Survival Assay. B cells purified from either control or *Pkn1*$^{-/-}$ mice were incubated for the indicated time periods in a 24-well plate coated with 100 μg/mL of immobilized anti-IgM F(ab′)$_2$ fragment (Southern Biotechnology). The cells were stained with Annexin V-FITC and propidium iodide according to the manufacturer's protocol (BioVision) and viable cells were determined by flow cytometry.

BrdU Incorporation Assay. To detect BrdU incorporation, purified B cells (5×10^5) were cultured in either the presence or absence of 10 μg/mL of anti-IgM F(ab′)$_2$ fragment with 10 μM BrdU. After a 48-h incubation, the BrdU incorporation assay was performed according to the manufacturer's protocol (BD Biosciences).

Flow Cytometric Analyses and Single Cell Sorting. Flow cytometric analyses were performed using a FACSCalibur and Cell Quest software (BD Biosciences) or a FACSCanto II and FACS Diva software (BD Biosciences). To sequence *V_H186.2-DFL16.1* DNA, cells were stained with fluorescently labeled antibodies against CD3, CD90.2, CD4, CD8, CD11b, and Gr-1, (lineage) together with labeled anti-IgM, IgD, IgG1, and NP-conjugated PE. Lineage$^-$IgM$^-$IgD$^-$NP-binding$^+$ IgG$^+$ B cells were obtained by single cell sorting using a FACSAria II (BD Biosciences).

Colony Transforming Assay. A colony transforming assay was performed as previously described (3). Briefly, to examine growth in soft agar, 35-mm dishes were prepared with a lower layer of 0.7% agar solution in DMEM with 10% (vol/vol) FBS containing 1 μg/mL of puromycin and then overlaid with a 0.35% agar solution also containing 10% (vol/vol) FBS and a supplement containing 1×10^5 retrovirally transduced cells. Twenty-one days after plating, colonies larger than 0.1 mm in diameter were counted.

Akt Kinase Assay. Akt activity was measured using an Akt enzyme activity kit (Millipore). Briefly, 293T cells were lysed and the supernatants were immunoprecipitated with a mouse anti-Akt antibody. Protein-G sepharose-bound immune complexes were resuspended in a buffer containing the GSK substrate peptide (RPRAATF) and γ-[^{32}P]ATP (GE Healthcare). Radioactivity was analyzed using a liquid scintillation counter.

Sequence Analysis of the *V_H186.2-DFL16.1* Gene. To extract total RNA from each cell, an isolated single cell was lysed in cell lysis buffer [first strand buffer (Invitrogen), RNase inhibitor (WAKO), 0.2 mM dNTPs, 0.25% (vol/vol) Nonidet P-40, 0.1 mg/mL BSA,

0.01 M DTT, and 3 μg/mL of random primer (Invitrogen)]. Reverse transcriptase (Invitrogen) was added to synthesize the first strand cDNA, which was then used as a template for two rounds of nested PCR. The first set of primers included GCTGTATCATGCTCTTCTTG for the $V_H186.2$ leader sequence and GGATGACTCATCCCAGGGTCACCATGGAGT for the constant region of the *IgG1* gene. The second PCR was performed using the GGTGTCCACTCCCAGGTCCA and CCAGGGGCCAGTGGATAGAC primers. The PCR products were cloned into pCR2.1-TOPO (Invitrogen). The obtained plasmid DNA was subjected to DNA sequencing, and the sequences were compared with the $V_H186.2$-*DFL16.1* germline sequence.

Statistical Analysis. Statistical analyses were performed using the unpaired, two-tailed Student *t* test. A P value <0.05 was considered statistically significant.

1. Yasui T, et al. (2002) Dissection of B cell differentiation during primary immune responses in mice with altered CD40 signals. *Int Immunol* 14(3):319–329.
2. Uchida J, et al. (1999) Mimicry of CD40 signals by Epstein-Barr virus LMP1 in B lymphocyte responses. *Science* 286(5438):300–303.
3. Bromberg JF, et al. (1999) Stat3 as an oncogene. *Cell* 98(3):295–303.

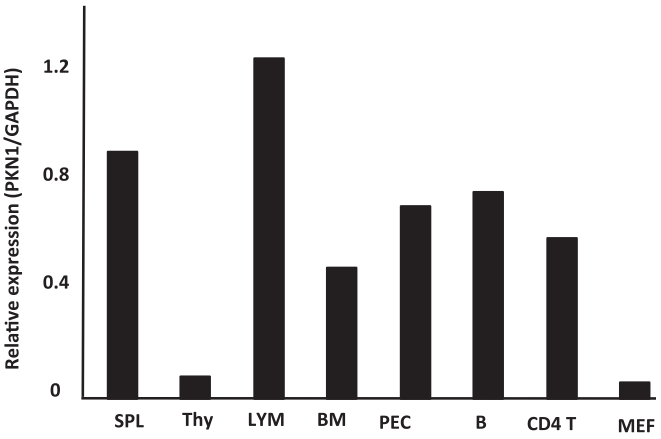

Fig. S1. PKN1 expression in mouse lymphoid tissues. PKN1 mRNA levels were determined by quantitative RT-PCR. Lymphoid cells in the spleen (SPL), thymus (THY), mesenteric lymph node (LYM), bone marrow (BM), and peritoneal cavity (PEC), as well as purified splenic B cells (B) and CD4$^+$ T cells (CD4T) were isolated from C57BL/6 mice. Mouse embryonic fibroblasts (MEFs) were also prepared as a nonlymphoid cell control. Total RNA was purified and cDNA was synthesized. Quantitative RT-PCR was performed using TaqMan probes specific for the *Pkn1* and *Gapdh* genes (Life Technologies). Expression data for *Pkn1* were normalized to *Gapdh* expression.

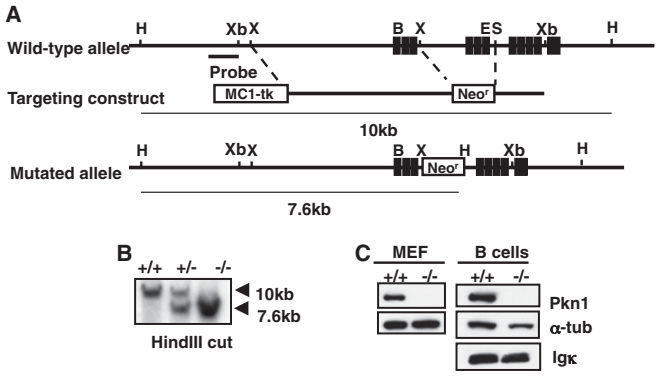

Fig. S2. Generation of $Pkn1^{-/-}$ mice. To generate PKN1-deficient mice, the targeting vector used to disrupt the *Pkn1* gene was introduced into ES cells using conventional methods. (*A*) Schematic of the WT *Pkn1*-targeting construct and mutated allele. Exons are depicted by filled boxes. Restriction sites: H, HindIII; Xb, XbaI; X, XhoI; B, BamHI; S, SalI; E, EcoRI. *Neo^r*, neomycin resistance gene cassette. *MC1-tk*, MC1 promoter-driven thymidine kinase gene. (*B*) Southern blot analysis of the *Pkn1* gene using the probe described in *A*. $^{+/+}$, wild-type; $^{+/-}$, *Pkn1* heterozygote; $^{-/-}$, *Pkn1* homozygote. (*C*) PKN1 protein expression in mouse embryonic fibroblasts (MEFs) and B cells from $Pkn1^{-/-}$ mice.

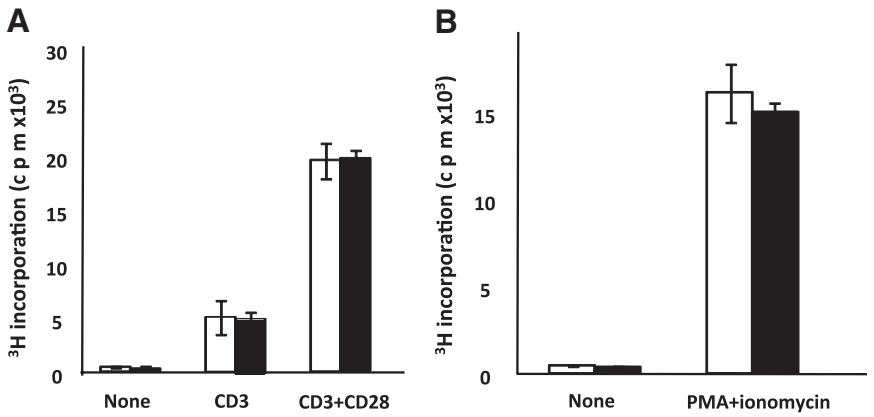

Fig. S3. Proliferative responses of splenic CD4$^+$ T cells from *Pkn1*$^{-/-}$ mice. CD4$^+$ T cells were isolated from splenocytes using a MACS CD4$^+$ T-cell isolation kit (Miltenyi Biotec). (*A*) Purified CD4$^+$ T cells (1×10^5) from WT and *Pkn1*$^{-/-}$ mice were cultured in triplicate in a flat-bottomed 96-well plate coated with 1 μg/mL of an immobilized anti-CD3 antibody (e-Bioscience) in the presence or absence of 1 μg/mL anti-CD28 antibody (e-Bioscience). (*B*) CD4$^+$ T cells were also stimulated with both 1 μg/mL phorbol 12-myristate acetate (PMA) and 1 μg/mL ionomycin (both obtained from Sigma). The cultures were incubated for 48 h and pulsed with 1 μCi ^{3}[H]thymidine 12 h before terminating the culture. Data indicate the mean ± SEM of three points per group. Representative data are shown from three independent experiments.

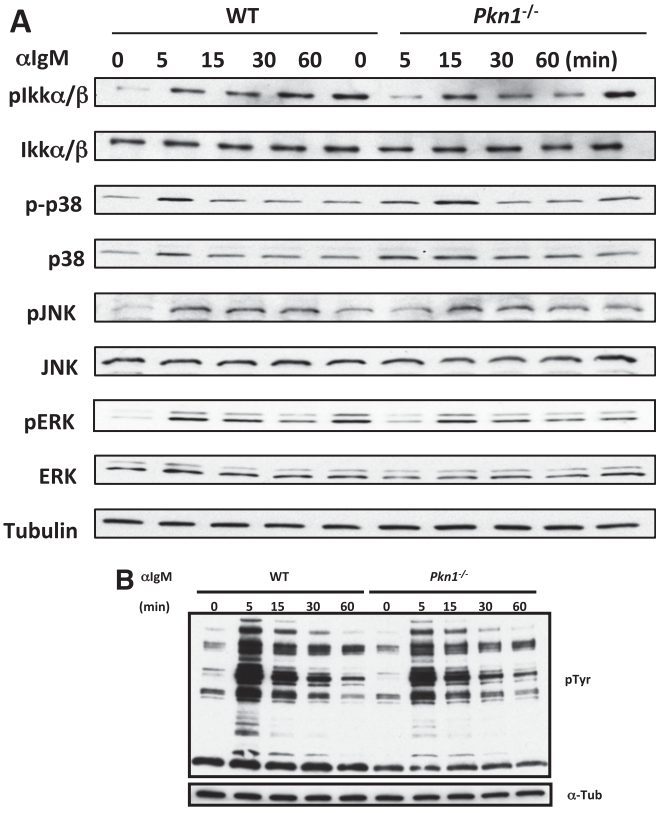

Fig. S4. Activation of signaling molecules downstream of the BCR. B cells (5×10^4) were stimulated with 10 μg/mL of an anti-IgM F(ab)$_2$ for the indicated time periods. (*A*) Cell lysates were prepared and subjected to Western blot analysis to detect phosphorylated IKKa/b, p38, JNK, and ERK. (*B*) Tyrosine phosphorylation induced by BCR ligation was detected using an antiphospho-tyrosine antibody (4G10; Millipore).

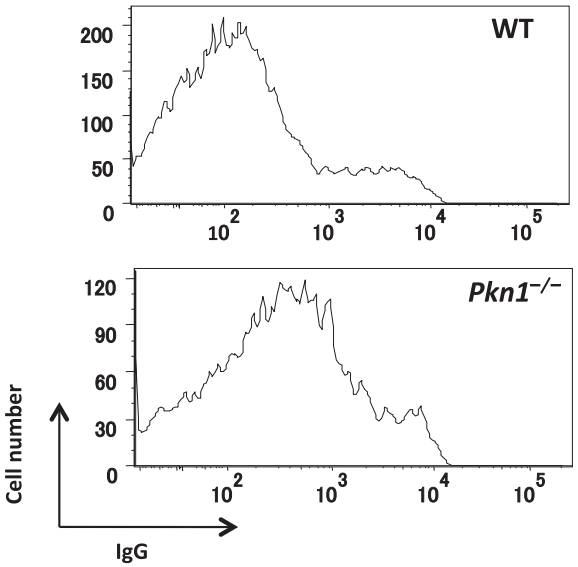

Fig. S5. Selective induction of surface IgG1$^+$ B-cell subsets in *Pkn1*$^{-/-}$ mice. WT or *Pkn1*$^{-/-}$ mice were intraperitoneally immunized with 100 μg nitrophenyl-acetyl (NP)-conjugated chicken γ-globulin (NP-CGG)/alum. Fourteen days after immunization, splenocytes were stained with the lineage surface markers NP-PE and anti-IgG1. Data are presented as histograms of IgG expression after gating on NP-binding$^+$ B cells.

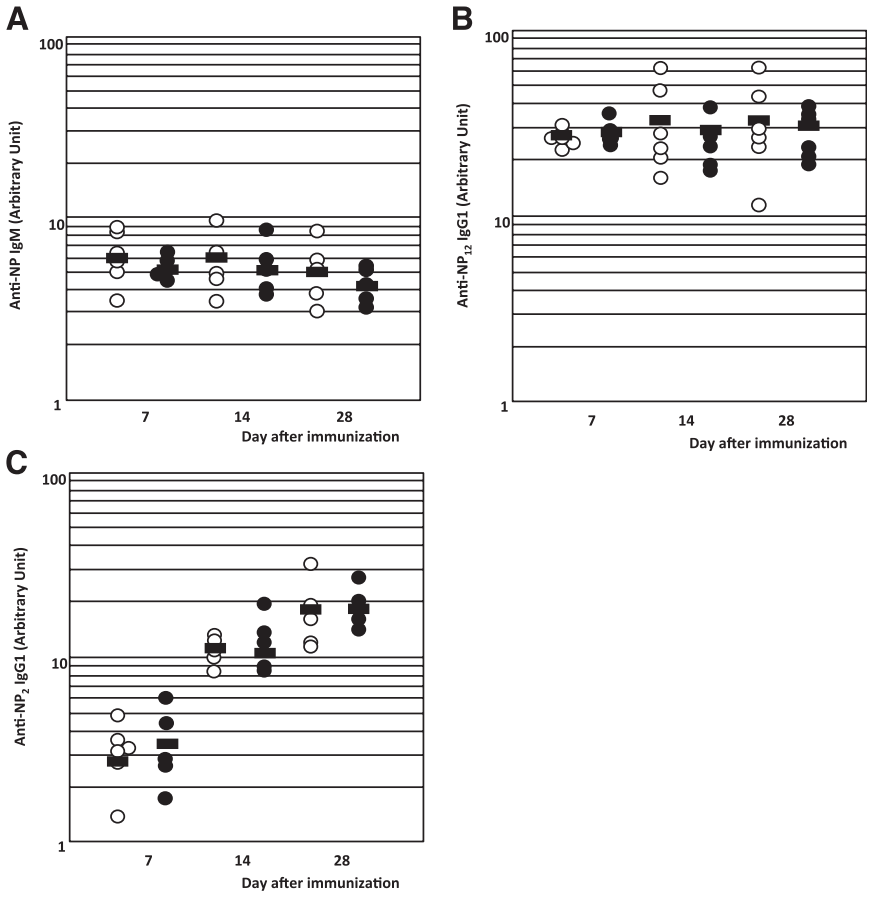

Fig. S6. T-cell–dependent antibody responses in NP-CGG–immunized WT and *Pkn1*$^{-/-}$ mice. Mice were immunized with NP-CGG intraperitoneally and bred at the indicated periods. The serum titers of NP-specific IgM and IgG1 were determined by ELISA using standard protocols. Total IgM (*A*) and IgG_1 (*B*) antibodies with NP affinity were captured using NP_{12}-BSA. (*C*) High-affinity antibodies against NP were examined using NP_2-BSA–bound plates.

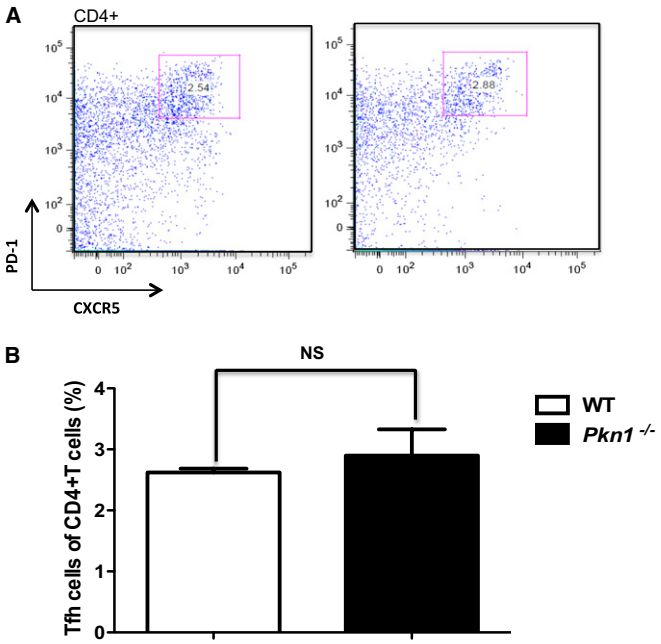

Fig. S7. Normal differentiation of follicular helper T (Tfh) cells in *Pkn1*$^{-/-}$ mice. WT and *Pkn1*$^{-/-}$ mice were intraperitoneally immunized with sheep red blood cells. After 7 d, splenocytes were stained with anti-CD4, anti–PD-1, and anti-CXCR5 antibodies and analyzed by flow cytometry. (*A*) Representative data are shown for Tfh cells, which were identified based on PD-1^{+}CXCR5^{+} expression after gating on total CD4^{+} T cells. (*B*) Statistical analysis of the population of Tfh cells for at least three mice per group. NS, not significant.

Table S1. Somatic genetics at the Ig locus of NP-binding GC B cells

R/S ratio	WT (n = 56)	*Pkn1*$^{-/-}$ (n = 59)
CDR1*	13.5	11.0
CDR2	5.8	2.5
CDR1+CDR2	8.0	4.3
Framework	2.2	2.0
DFL16.1, %[†]	93.0	80.0
YYGS, %[‡]	23.0	25.0
W to L 33, %[§]	60.7	37.3

*Value of R/S ratio in $V_H186.2$ gene.
[†]Percentage of rearranged DFL16.1 gene segment coupled to $V_H186.2$ gene.
[‡]Percentage of rearranged YYGS in CDR3 coupled to rearranged $V_H186.2$ gene.
[§]The percentage of rearranged *VH186.2* gene carrying W to L mutation at position 33.