Supporting Information

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SI Materials and Methods

Generation of 14-3-3σ–Deficient Mice. The targeting vector (Fig. S1*B*) was constructed by inserting a PGK-neomycin resistance cassette into the single exon of the genomic 14-3-3σ locus using standard DNA cloning methods. The culture, transfection, and selection of murine embryonic stem (ES) cells were as described previously (1). Briefly, E14K ES cells derived from 129-Ola were electroporated with linearized targeting vector and selected in G418 (300 μg/mL) for approximately 10 d. Colonies were screened for homologous recombination of the mutated 14-3-3σ allele by Southern blotting. BglII-digested genomic DNA samples from ES cells were hybridized to an external probe to yield bands of 3 kb for the WT locus and 5 kb for the targeted locus. Correctly targeted ES cell clones were injected into 3.5-d C57BL/6 blastocytes, and the blastocytes were transferred to the uteri of pseudopregnant (C57BL/6-BALB/c) F1 foster mothers. Male chimeric mice were mated with C57BL/6 females to generate mice bearing the 14-3-3σ KO allele. Germline transmission was scored by coat color. All animal experiments were approved by University Health Network (Toronto, ON, Canada) Animal Care Committee.

Adoptive Transfer. One day before adoptive transfer, $RAG1^{-/-}$ mice or μMT mice were γ-irradiated with 6 Gy. Single-cell suspensions of 5×10^6 cells from BM of WT or KO mice were prepared and i.v. injected into the irradiated mice. Mice were fed antibiotic-containing water for 1 wk. At 8 wk after transfer, the reconstitution of B cells in the recipient mice was analyzed by flow cytometry.

BrdU Incorporation Assay. Mice were fed drinking water containing 1 mg/mL BrdU for 3 d. Single-cell suspensions were prepared from spleen and immunostained with anti-B220 Ab to detect B cells. Fixation and staining for BrdU detection were performed using a BrdU-Flow kit according to the manufacturer's instructions (BD Biosciences).

Cell Culture and B-cell Purification. Single-cell suspensions were prepared from mouse spleens by sieving and gentle pipetting through 70-μm nylon mesh filters (Falcon; BD Biosciences). Splenocytes were suspended in RPMI medium 1640 (JRH Biosciences) supplemented with 2 mM L-glutamine (Invitrogen), 100 U penicillin/streptomycin (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 100 mM Hepes (Sigma-Aldrich), 0.055 mM 2-mercaptoethanol (Gibco), and 10% FCS (HyClone). To purify B cells, splenocytes were suspended in 2% BSA/PBS solution, incubated with anti-mouse CD16/CD32 monoclonal Ab (BD Biosciences) for 15 min on ice, and then incubated for a further 15 min on ice with biotin-conjugated Abs, including anti-mouse CD3, CD4, CD8, Thy1.2, NK1.1, Ter119, CD11b, CD11c, and Gr-1. After washing in ice-cold 2% BSA/PBS solution, cell pellets were resuspended with streptavidin-conjugated magnetic beads (BD Biosciences) and incubated on ice for 15 min. Non-B cells were depleted using the BD IMag Cell Separation Magnet system, resulting in a fivefold enrichment of B cells. The average B-cell purity was estimated to be approximately 94% to 98% as determined by anti-B220 immunostaining.

CFSE Decay Assay. Splenic B cells (5×10^6) were purified, washed twice with PBS solution, and incubated with 5 μM CFSE for 7 min at room temperature (RT) (2). Cells were washed twice with PBS solution/2% FCS immediately after CFSE incubation, and subsequently injected into 6-Gy–irradiated $RAG1^{-/-}$ mice. At 4 and 7 d after injection, $CD19^+$-gated splenocytes were analyzed by FACS.

ELISA. For analysis of total Ig and specific isotype titers, 96-well ELISA plates (Thermo Fisher Scientific) were precoated overnight with 5 μg/mL of the appropriate goat anti-mouse Ab (Southern Biotech) in 50 μL ice-cold PBS solution. Plates were washed three times in 0.05% Tween/PBS solution and blocked with 100 μL 1% BSA/PBS solution at RT for 1 h. Serial dilutions of serum samples in 100 μL were applied to precoated plates and incubated overnight. On the third day, the coated plates were washed three times with 200 μL PBS solution per well, followed by incubation for 1 h with the appropriate goat anti-mouse Ig conjugated to HRP. After thorough washing, the plate was developed using HRP substrate 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma-Aldrich), and the reaction was stopped by the addition of 1% SDS. Colors developing in wells were read at 405 nm using a Thermomax Microplate Reader (MDS Analytical Technologies). Titers of Ig in serum samples were calculated as the log serum concentration required to achieve 50% maximum OD.

Flow Cytometry. Single-cell suspensions of 1×10^6 cells were prepared as described earlier, except that cells were maintained in the dark at 4 °C throughout the experiment. Cells were washed twice in ice-cold FACS buffer (2% FCS, 0.1% NaN3 in PBS solution), incubated with desired Ab for 15 min, and washed thoroughly with FACS buffer. Flow cytometers (FACSCalibur and LSR II; BD Biosciences) and software (CellQuest or FACSDiva, respectively; BD Biosciences) were used to acquire flow cytometric data, and FlowJo software (Tree Star) was used for analysis. Antibodies and streptavidin conjugates for flow cytometry were from eBioscience and included B220-FITC, IgM-PE-Cy5, IgM-PE-Cy7, CD5-PE, CD19-APC, CD21-PE, CD23-PE-Cy5, CD25-PE-Cy7, CD69-PerCP, CD80-APC, CD86-PE, IgG_1-APC, IgG_3-FITC, and streptavidin–PerCP-Cy5.5. For apoptosis assays, single-cell suspensions of 1×10^6 splenic cells were stained with anti–B220-APC plus FITC-Annexin V and examined using the Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturer's instructions.

Immunohistochemistry. Spleens were fixed in excess 10% neutral-buffered formalin and incubated overnight at 4 °C with shaking. Fixed spleens were washed three times in ice-cold PBS solution, embedded in paraffin, and sectioned with a microtome set to 5 μm. Tissue sections were mounted on slides coated with amino-propyl-triethoxy-silane and dried overnight. Dried slides were deparaffinized by three washes in xylene (5 min each), and rehydrated by immersion three times in 100% ethanol (3 min each) followed by immersion in 95% ethanol for 2 min. Slides were treated with 3% hydrogen peroxidase in methanol for 30 min and rehydrated by immersion in 90% ethanol for 2 min followed by washing in PBS solution for 5 min. Antigen was retrieved by incubating slides in 0.125% trypsin at RT for 15 min, followed by washing in PBS solution for 5 min. Slides were blocked by incubation in 10% normal serum in TBS for 2 h at RT. For primary Ab binding, blocked slides were incubated overnight at 4 °C with rat anti-mouse B220 (BD Biosciences), rabbit anti-human/mouse CD3 (Sigma), rat anti-mouse MOMA-1 (AbD Serotec), peroxidase-conjugated anti-mouse IgM (Sigma), rabbit monoclonal anti-Ki67 (Thermo Scientific), or PNA-biotin (Vector Laboratories). Slides were washed three times and incubated at RT for

60 min with secondary Ab: biotin-conjugated donkey anti-rat IgG (Research Diagnostic) or alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Zymed). Biotin-conjugated signals were detected with Vectastain Elite ABC reagent (Vector Laboratories) and DAB substrate (Vector Laboratories) according to the manufacturer's instructions. After washing, slides were incubated with 0.5 M levamisole for 30 min. Alkaline phosphatase-conjugated signals were visualized by incubating slides at RT for 30 min with NBT/BCIP solution (Roche). Sections were dehydrated, mounted using Immu-Mount (Thermo Fisher Scientific), and viewed under a microscope (IX71; Olympus).

TI and TD Immune Responses. WT and KO mice were immunized i.p. with 25 μg of the TI antigen TNP-Ficoll in PBS solution or 100 μg of the alum-precipitated TD antigen $NP_{(15)}$-CG. Assays were performed by using 96-well ELISA plates coated with TNP-BSA (20 μg/mL) or $NP_{(15)}$-BSA (10 μg/mL), respectively. Serum samples collected on day 0 (before immunization) and on days 7 and 21 after immunization were serially diluted and applied to the ELISA plates. Bound Abs were revealed by the addition of goat anti-mouse IgM, IgG_1, and IgG_3 and developed as described earlier.

[^{3}H]Thymidine Incorporation. Purified splenic B cells from WT and KO mice were seeded (10^5 cells/200 μL) in triplicate in 96-well U-bottom plates. Cells were left untreated or stimulated with 5 μg/mL anti-IgM (Jackson ImmunoResearch Laboratories), 5 μg/mL anti-IgM plus 5 μg/mL anti-CD40 (BD Pharmingen), or 5 μg/mL anti-IgM plus 5 ng/mL BAFF (PeproTech). At 15, 39, or 63 h after seeding with stimuli, [^{3}H]thymidine (1 μCi) was added to each well and cells were cultured for another 9 h to determine proliferation at 24, 48, and 72 h. [^{3}H]thymidine uptake was assessed using a liquid scintillation β-counter (Canberra Packard).

qRT-PCR. Splenic B cells (10^7) were left untreated or stimulated in triplicate with 10 μg/mL anti-IgM (Southern Biotech) for 2 or 6 h, with or without 1 μg/mL recombinant murine soluble CD40 Ligand (PeproTech). RNA was extracted using an RNeasy kit (Qiagen) according to the manufacturer's instructions, and cDNA was produced from 500 ng RNA using SuperScript III (Invitrogen) according to the manufacturer's instructions. Primers used were as follows: FOXO1 (forward, 5′-ACGAGTGGATGGTGAAGAGC-3′; reverse, 5′-TGCTGTGAAGGGACAGATTG-3′); Bim (forward, 5′-CGGATCGGAGACGAGTTCA-3′; reverse, 5′-TTCAGCCTCGCGGTAATCA-3′); p21 (forward, 5′-CGCGGTGTCAGAGTCTAGG-3′; reverse, 5′-GGACATCACCAGGATTGGAC-3′); Mxi1 (forward, 5′-TACCTGGAGCAGATCGAGAAA-3′; reverse, 5′-GGGGCTTCGAGTGCTGTAG-3′); p27 (forward, 5′-CCTGACTCGTCAGACAATCC-3′; reverse, 5′-TCTGTTCTGTTGGCCCTTTT-3′); 14-3-3σ (forward, 5′-TGTGTGCGACACTGTGCTC-3′; reverse, 5′-TCGGCTAGGTAGCGGTAGTAG-3′); 14-3-3β (forward, 5′-TGGATAAGAGTGAGCTGGTACA-3′; reverse, 5′-CGTGTCCCTGCTCTGTTACG-3′); 14-3-3ε (forward, 5′-AGTGACATTGCGATGACAGAAC-3′; reverse, 5′-ACGGTCGGGGGAATTAAGAAT-3′); 14-3-3η (forward, 5′-ACGAAGATCGAAATCTCCTCTCT-3′; reverse, 5′-CCGGTAGGCTTTAACTTTCTCCA-3′); 14-3-3γ (forward, 5′-GTGACCGAGCTGAACGGAAC-3′; reverse, 5′-GATGCTGCTGATGACCCTCC-3′); 14-3-3θ (forward, 5′-CCAAGAGGCGTTTGATATAAGCA-3′; reverse, 5′-AAAAGCCGTTTTAGCCAGTGT-3′); 14-3-3ζ (forward, 5′-GAAAAGTTCTTGATCCCCAATGC-3′; reverse, 5′-TGTGACTGGTCCACAATTCCTT-3′); and 18S RNA control (forward, 5′-AGTTCCAGCACATTTTGCGAG-3′; reverse, 5′-TCATCCTCCGTGAGTTCTCCA-3′). cDNAs were amplified in the presence of SYBR Green using a real-time PCR system (model 7900; Applied Biosystems). The gene-specific fold change, normalized to 18S, was calculated using the $2^{-\Delta\Delta ct}$ method by comparison with the untreated WT sample (3).

Transfection and Plasmids. One day before transfection, 293T cells were subcultured 1:3 into 15-cm Petri dishes and incubated to achieve confluence on the next day. pCDNA3.1-His-14-3-3σ (human sequence, 20 μg; Addgene) alone or mixed with 20 μg pCDNA3.1-flag-FOXO1 (human sequence; Addgene), was transfected into 293T cells using FuGENE 6 Transfection Reagent (Roche) following the manufacturer's instructions. At 2 d after transfection, 293T cells were lysed in 1% Nonidet P-40 lysis buffer and the lysates were subjected to pull-down by using Ni Sepharose 6 Fast Flow (GE Healthcare) or anti-FLAG M2 Affinity Gel (Sigma) according to the manufacturers' instructions.

Western Blotting and Nuclear/Cytosolic Fractionation. For total lysate preparation, purified B cells (1×10^7) were lysed in 100 μL 1% Nonidet P-40 lysis buffer (10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, protease inhibitor freshly added). Lysates were then fractionated by SDS/PAGE and blotted using standard procedures. Proteins were detected using anti-FOXO1, anti–phospho-S6-ribosomal protein (S240/244), anti–S6-ribosomal protein, anti–phospho-Erk1/2, anti–Erk1/2, anti–phospho-JNK1/2, anti–phospho-Akt/PKB (S473), or anti-Akt/PKB Ab (all from Cell Signaling), or anti-p27 (Santa Cruz), anti-Bim (Enzo Life Sciences), or anti–14-3-3σ (IBL) Ab. Blots were developed using ECL Plus Western blotting detection reagents (Amersham). For nuclear/cytosolic fractionation, B-cell pellets (1×10^7 cells) were washed once with ice-cold PBS solution, resuspended in buffer A (10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, protease inhibitor freshly added), and incubated on ice for 15 min. Nonidet P-40 was added to a final concentration of 0.65% with frequent vortexing. After incubation on ice for 15 min, nuclei were pelleted by centrifugation at 15,000 rpm (Eppendorf, centrifuge 5417R, Rotor F-45-30-11) for 2 min at 4 °C. The cytosolic fraction was collected from the supernatant and stored at −80 °C for Western blot analysis as described earlier. To prepare nuclear fractions, buffer C (20 mM Hepes, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, protease inhibitor freshly added) was added to the pellet followed by incubation on ice for 30 min with frequent vortexing. After centrifugation at 15,000 rpm for 10 min at 4 °C, the supernatant was recovered as the nuclear extract and stored at −80 °C for Western blot analysis as described earlier.

Inhibition of Proteasomal Degradation, Cell Stimulation, and Immunoprecipitation. Purified B cells (1×10^7) were left untreated or treated with proteasome inhibitor MG132 (20 μM) for 1 h at 37 °C, followed by nuclear/cytosolic fractionation as described earlier. For cell stimulation, purified B cells (1×10^7) were left untreated or stimulated with 10 μg/mL anti-IgM (Southern Biotech). Cell lysates were prepared as described earlier. For immunoprecipitation, 500 μg lysate was incubated with 1 μg anti–14-3-3σ (IBL) plus 20 μL Protein G Sepharose beads (GE Healthcare) with agitation at 4 °C overnight. After three washes with 1 mL ice-cold PBS solution, protein loading buffer was added to the beads and the immunoprecipitated proteins were subjected to Western blot analysis as described earlier.

VSV Infection. Mice were i.v. injected with 2×10^6 pfu VSV-Indiana (VSV-IND; Mudd–Summers isolate) as previously described (4). VSV was originally obtained from D. Kolakofsky (University of Geneva, Switzerland) and propagated on BHK-21 cells at a multiplicity of infection of 0.01 and plaqued on Vero cells. During survival experiments, health status of mice was checked twice daily. Upon appearance of clinical signs of VSV replication in the CNS, such as paralysis or scrubby pelt, mice were taken out of the experiment and counted as dead. Serum samples were collected on days 0, 2, 4, 6, and 8 for the detection of neutralizing IgM and IgG. VSV-specific neutralization assay has been carried out as previously described (5). Briefly, sera from different time points were diluted 40-fold in MEM and heat-inactivated for 30 min.

Different serum concentrations were coincubated with 500 pfu VSV-Indiana for 90 min. Next, the solution was transferred on a single cell layer of Vero cells, followed by an incubation of 1 h. After addition of the overlay (1:1 MEM:2% methylcellulose) cells were incubated over night and then fixed and stained for 1 h with 0.5% Crystal Violet (Sigma). To determine IgG titers, serum samples were treated with 0.1M β-ME in PBS solution for 1 h at RT before dilution. All mouse sera were heated at 56 °C for 30 min to inactivate complement. Immunoglobulins were measured in serially diluted serum samples using standard ELISA.

Statistical Analyses. The Student *t* test was used for statistical analyses. Values are expressed as the mean ± SD. A *P* value lower than 0.05 was considered statistically significant.

1. Torres R, Kuehn R (1997) *Laboratory Protocols for Conditional Gene Targeting* (Oxford University Press, Oxford, United Kingdom).
2. Lyons AB, Parish CR (1994) Determination of lymphocyte division by flow cytometry. *J Immunol Methods* 171:131–137.
3. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using realtime quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25:402–408.
4. Charan S, Zinkernagel RM (1986) Antibody mediated suppression of secondary IgM response in nude mice against vesicular stomatitis virus. *J Immunol* 136:3057–3061.
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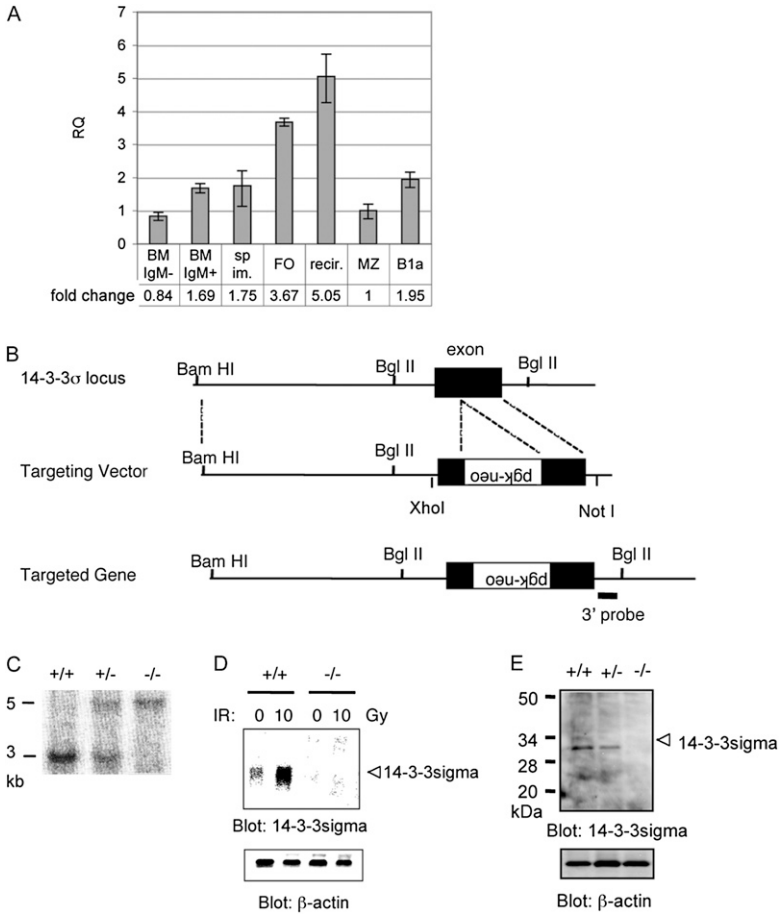

Fig. S1. 14-3-3σ expression in WT B cells and generation of 14-3-3σ–deficient mice. (*A*) Differential 14-3-3σ expression in B-cell subsets. qRT-PCR was used to analyze 14-3-3σ mRNA levels in BM B220^{low}IgM^{-} B-cell progenitors (BM IgM^{-}), BM B220^{low}IgM^{+} immature B cells (BM IgM^{+}), splenic B220^{+}CD21^{-}CD23^{-} immature B cells (sp im.), B220^{+}CD21^{low}CD23^{high} FO B cells (FO), BM B220^{high}IgM^{+} recirculating B cells (recir.), B220^{+}CD21^{high}CD23^{low} MZ B cells (MZ), and IgM^{+}CD5^{+} B1a B cells (B1a) from WT mice. Results are expressed as relative quantification (RQ) and are the specific mRNA level relative to the level of 18S RNA transcripts. Data shown are the mean ± SD of triplicates. ΔΔCt values were normalized to MZ B-cell values (fold change, 1) to determine fold change. (*B*) Gene targeting strategy. *Top*: Structure of mouse genomic 14-3-3σ locus, in which only one exon encodes the entire 14-3-3σ protein. *Middle*: Targeting vector designed to insert the PGK-neo cassette into the 14-3-3σ exon. *Bottom*: Structure of the targeted 14-3-3σ locus after homologous recombination in ES cells. The probe used for Southern blotting is indicated. (*C*) Confirmation of 14-3-3σ locus disruption. Genomic DNA extracted from WT ($^{+/+}$), heterozygote ($^{+/-}$), and KO ($^{-/-}$) ES cell clones was subjected to Southern blotting by using the probe in *B*. (*D*) Confirmation of lack of 14-3-3σ mRNA expression. WT ($^{+/+}$) and KO ($^{-/-}$) ES cells were left untreated (0) or subjected to 10 Gy irradiation for 10 h, followed by RNA extraction and Northern blotting to detect 14-3-3σ mRNA. (*E*) Confirmation of lack of 14-3-3σ protein. Intestinal epithelial cells from WT ($^{+/+}$), heterozygote ($^{+/-}$), and KO mice ($^{-/-}$) were subjected to Western blotting to detect 14-3-3σ protein.

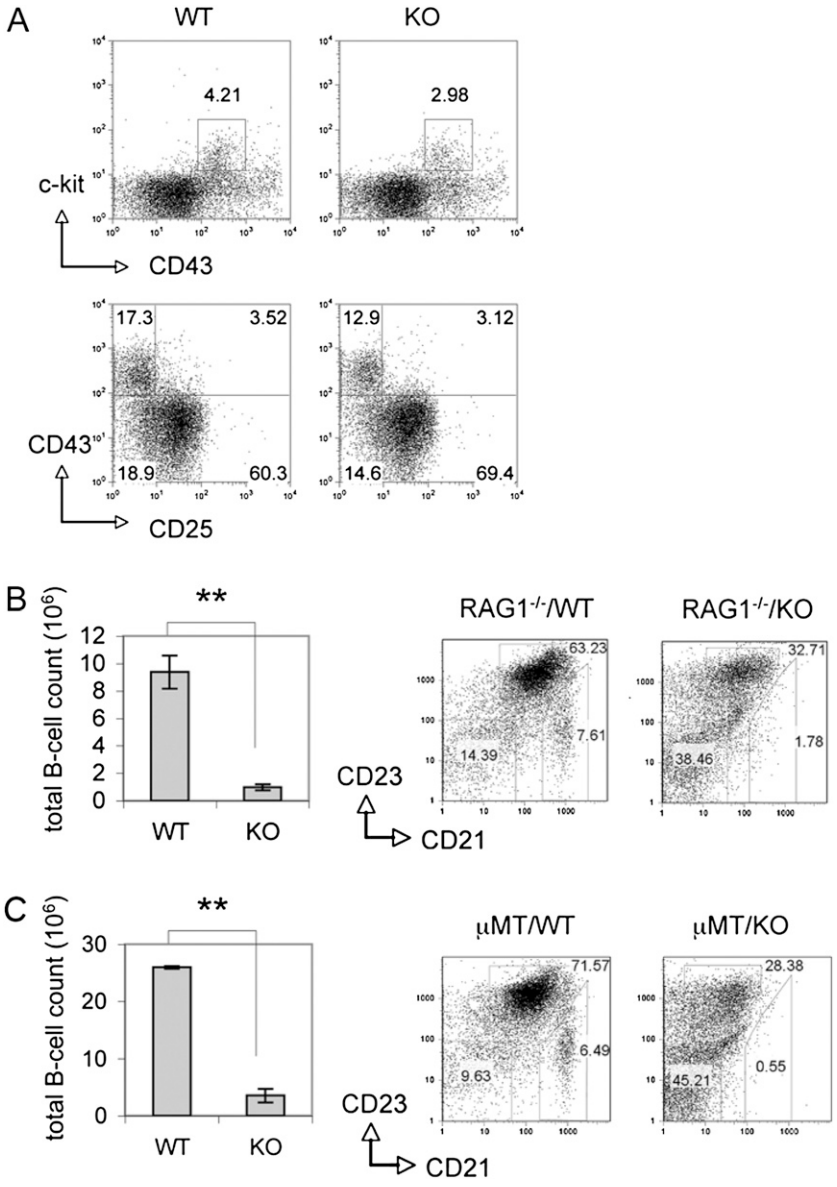

Fig. S2. Impaired B-cell homeostasis in 14-3-3σ–deficient mice. (*A*) Normal early B-cell development. FACS profile of c-kit versus CD43 (*Top*) and CD43 versus CD25 (*Bottom*). Data shown reflect the gating of 10,000 $B220^+IgM^-$ lymphocytes per group. Numbers shown in the top sections are the percentage of $B220^+IgM^-c\text{-}kit^+CD43^+$ proB cells relative to $B220^+IgM^-$ lymphocytes. Numbers in the upper left corners of the bottom sections are the percentage of $B220^+IgM^-CD43^+$ $CD25^-$ pro/preB cells relative to $B220^+IgM^-$ lymphocytes. Numbers in the lower right corners of the bottom sections are the percentage of $B220^+IgM^-CD43^-CD25^+$ preB cells relative to $B220^+IgM^-$ lymphocytes. (*B* and *C*) Adoptive transfer experiments in $RAG1^{-/-}$ (*B*) and μMT (*C*) mice. BM cells (5×10^6) from WT or KO mice were injected into 6-Gy–irradiated $RAG1^{-/-}$ or μMT mice ($n = 2$ per group). At 8 wk after injection, $CD19^+$-gated splenocytes from recipient spleens were counted (histograms; *Left*), and analyzed by FACS to determine CD23 versus CD21 expression (*Right*). Sections show the percentages of immature, FO, and MZ B cells (**$P < 0.005$).

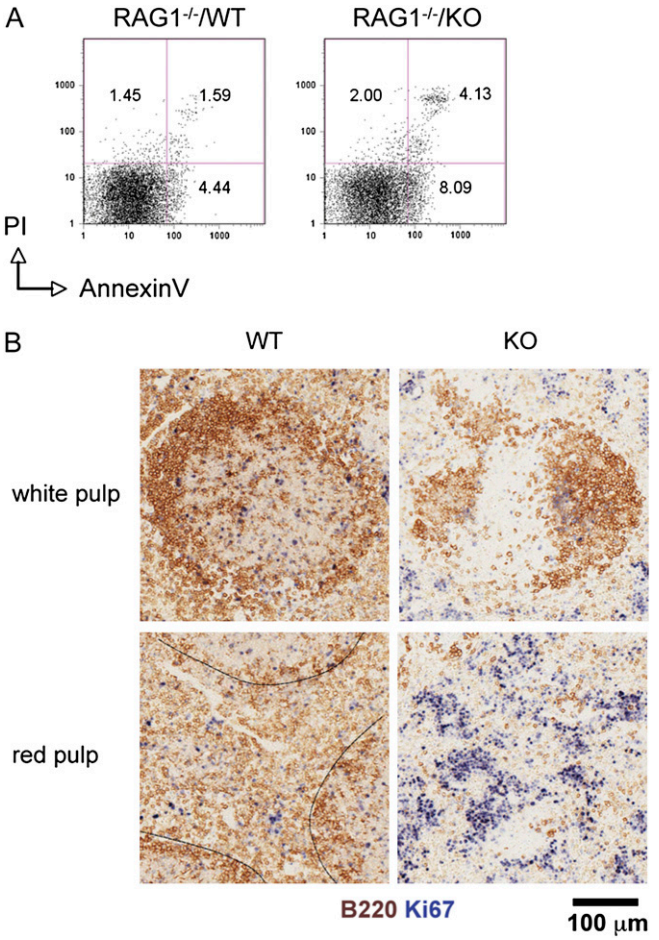

Fig. S3. Altered apoptosis and proliferation in 14-3-3σ–deficient B cells. (*A*) FACS profile of PI versus Annexin V staining of B220$^+$-gated splenocytes. BM cells (5×10^6) from WT and KO mice were injected into 6-Gy–irradiated RAG1$^{-/-}$ mice (n = 3 per group). At 8 wk after injection, B cells from recipient spleens were analyzed by FACS. Data shown reflect the gating of 10,000 B220$^+$ splenocytes. (*B*) Immunohistochemical staining of WT and KO spleen sections with anti-B220 plus anti-Ki67 Abs. Black lines (*Lower Left*) indicate the border between the red pulp and white pulp areas.

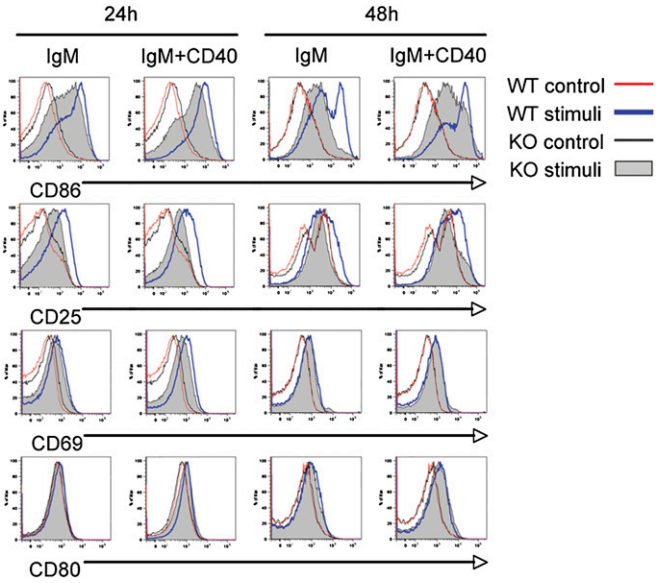

Fig. S4. Impaired up-regulation of CD86 and CD25 in 14-3-3σ–deficient mice. Splenic B cells (1×10^6) from WT and KO mice were left untreated (control) or stimulated for 24 or 48 h with 10 μg/mL anti-IgM or 10 μg/mL anti-IgM plus 1 μg/mL CD40L (IgM+CD40). B220$^+$-gated cells were analyzed by FACS to detect surface expression of CD86, CD25, CD69, and CD80.

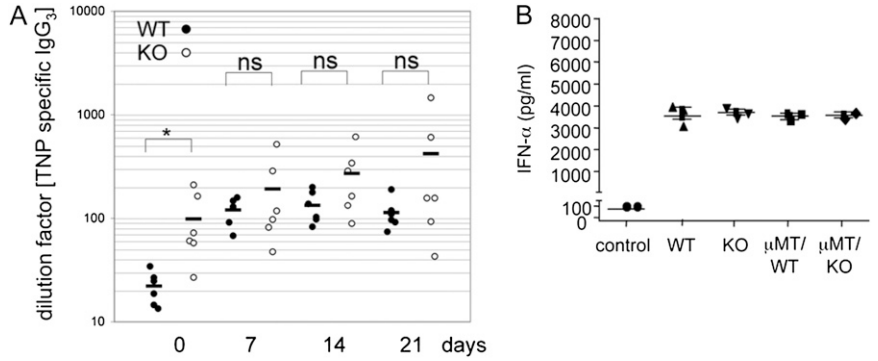

Fig. S5. TI immune responses in 14-3-3σ–deficient mice. (*A*) ELISA analysis of serum TNP-specific IgG_3 after TNP-Ficoll immunization. WT and KO mice ($n = 6$ per group) were i.p. injected with 25 μg TNP-Ficoll and serum samples were analyzed by ELISA on day 0 (before immunization) and on days 7, 14, and 21 d after immunization. (*B*) ELISA analysis of serum IFNα induced by VSV infection. WT, KO, μMT/WT, and μMT/KO chimeric mice (8 wk after adoptive transfer; $n = 3$ per group) were infected with 10^5 pfu VSV and serum samples were analyzed by ELISA on day 1 after infection to detect IFNα. Control, serum samples from WT mice taken before VSV infection (*$P < 0.05$; ns, not significant).

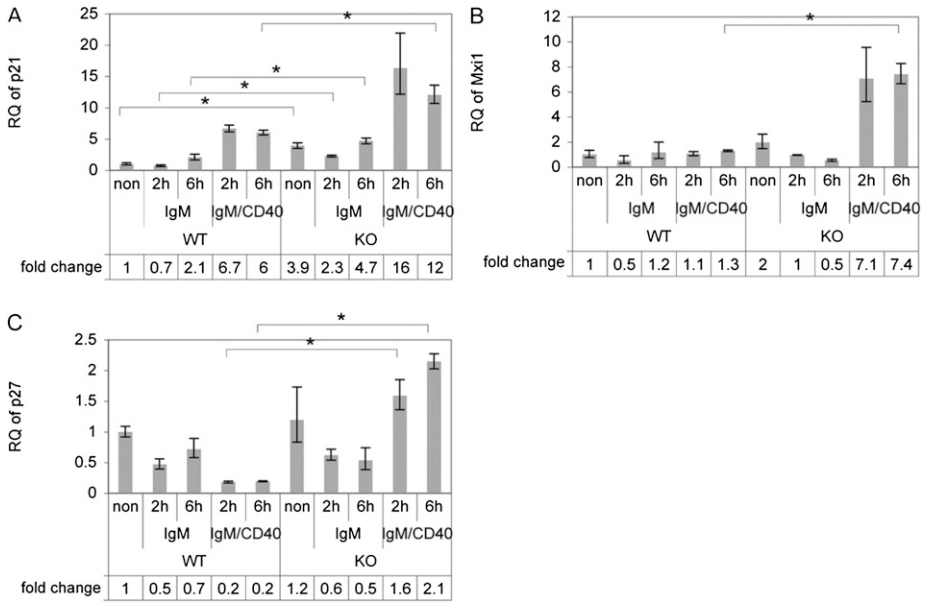

Fig. S6. Increased FOXO transcriptional activity in 14-3-3σ–deficient B cells. (*A*–*C*) Increased FOXO target gene mRNA levels. Splenic purified B cells (1×10^7) from WT and KO mice were left untreated (non) or stimulated with 10 μg/mL anti-IgM (IgM) or 10 μg/mL anti-IgM plus 1 μg/mL anti-CD40 (IgM/CD40) for 2 or 6 h. P21 (*A*), Mxi1 (*B*), and p27 (*C*) mRNA levels were determined using qRT-PCR. Data are expressed as for Fig. S1*A* (*$P < 0.05$).

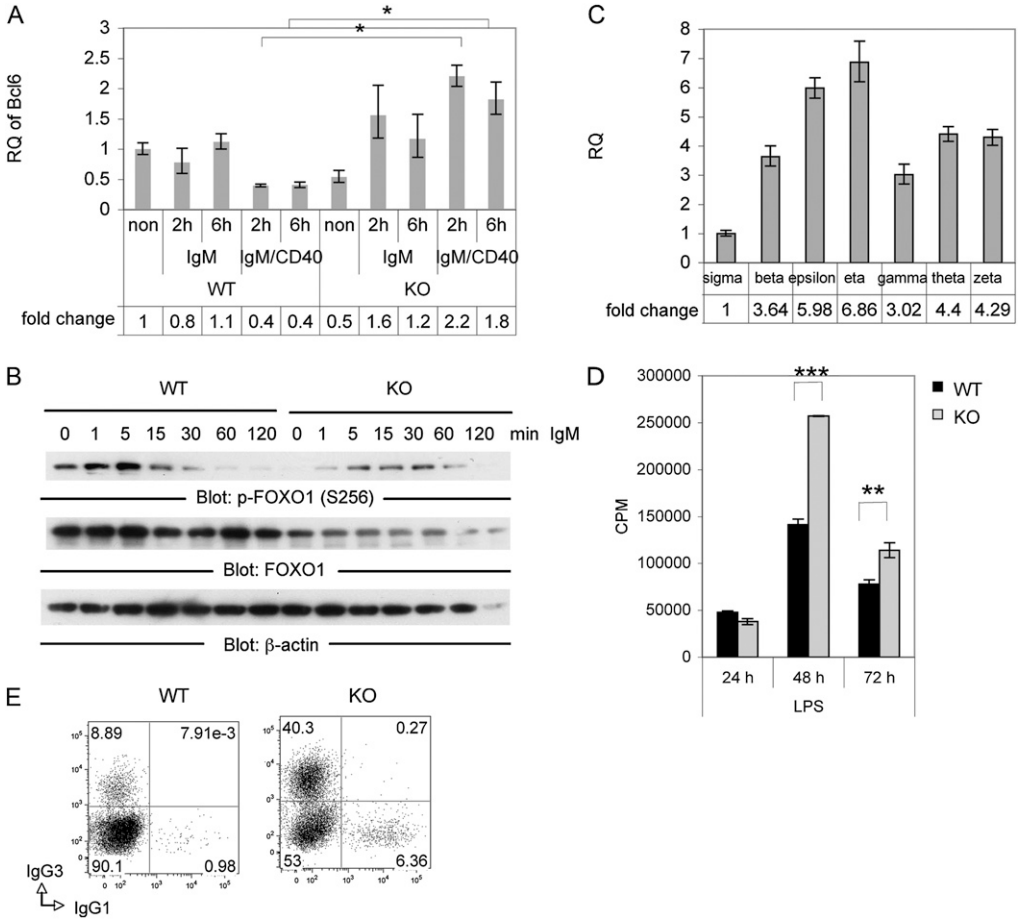

Fig. S7. Preliminary data on the effects of 14-3-3σ deficiency on Bcl-6 expression, FOXO1 phosphorylation, redundancy of 14-3-3 functions, and response to LPS stimulation. (*A*) Increased Bcl6 mRNA. Splenic B cells from WT and KO mice were left untreated (non) or stimulated for 2 or 6 h with 10 μg/mL anti-IgM (IgM) or 10 μg/mL anti-IgM plus 1 μg/mL CD40 ligand (IgM/CD40). Bcl6 mRNA levels were determined using qRT-PCR as for Fig. S6. (*B*) Reduced FOXO1 phosphorylation. Splenic purified B cells from WT and KO mice were left untreated (0) or stimulated for 1, 5, 15, 30, 60, and 120 min with 10 μg/mL anti-IgM (IgM). Cell lysates were subjected to Western blot analysis and developed by anti–p-FOXO1 (S256), anti-FOXO1, and anti–β-actin. (*C*) qRT-PCR analysis of seven 14-3-3 proteins in $B220^{+}IgM^{-}$ B-cell progenitors. Ct values were normalized to 14-3-3σ (fold change, 1) to determine the fold change in the other 14-3-3 proteins. (*D*) Enhanced [^{3}H]thymidine incorporation in response to LPS. Purified splenic B cells (1×10^{5}) from WT and KO mice were left untreated (medium control shown in Fig. 2*E*) or stimulated with 10 ng/mL LPS. At 15, 39, or 63 h after seeding, [^{3}H]thymidine (1 μCi) was added and cells were cultured for another 9 h. At 24, 48, or 72 h after seeding, [^{3}H]thymidine incorporation was assessed by scintillation counting. Data shown are the mean ± SD of triplicates (***$P < 0.0005$; **$P < 0.005$; *$P < 0.05$). (*E*) Accelerated CSR upon LPS treatment. Purified splenic B cells from WT and KO mice were treated with 25 ng/mL LPS and cultured for 5 d. $B220^{+}IgM^{-}$ lymphocytes were analyzed by FACS (IgG_3 vs. IgG_1).

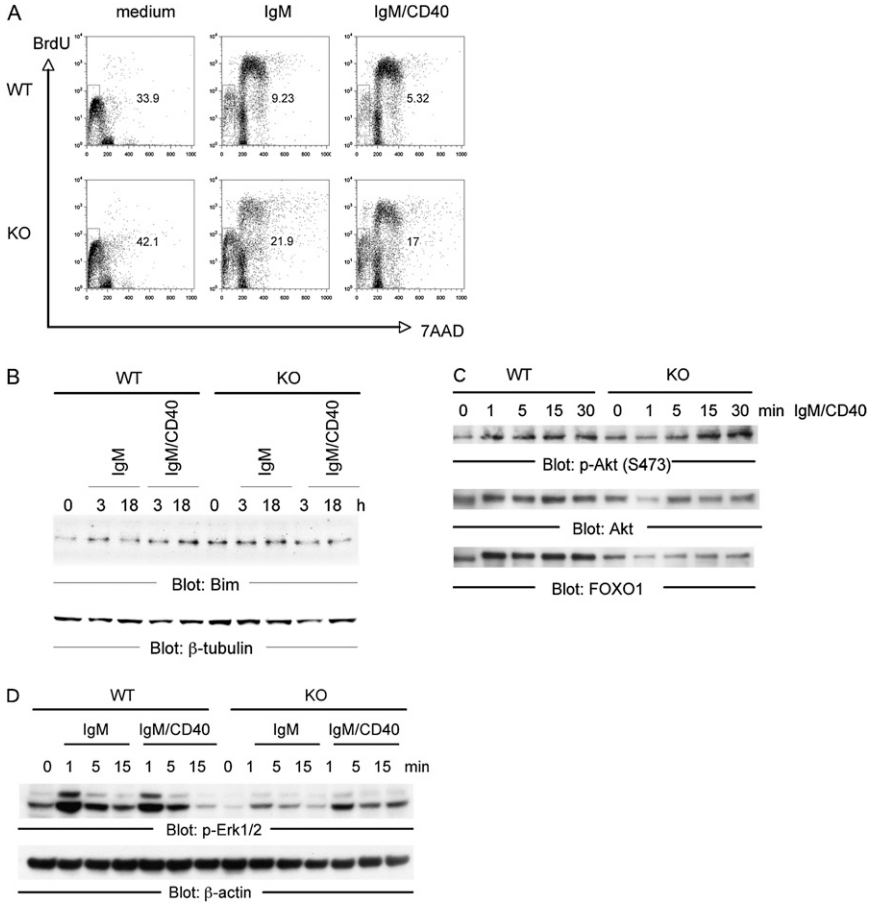

Fig. S8. IgM/CD40 costimulation in 14-3-3σ–deficient B cells compared with IgM stimulation. (*A*) IgM/CD40 signaling failed to correct apoptosis completely. Splenic purified B cells from WT and KO mice were left untreated (medium) or stimulated with 10 μg/mL anti-IgM (IgM) or 10 μg/mL anti-IgM plus 1 μg/mL CD40 ligand (IgM/CD40) for 40 h. Apoptotic cells were analyzed by intracellular FACS (BrdU vs. 7AAD). Quadrates show the percentage of apoptotic B cells relative to total B cells. (*B*) IgM/CD40 signaling did not induce an increased amount of Bim expression. Splenic purified B cells from WT and KO mice were left untreated (0), stimulated with 10 μg/mL anti-IgM (IgM) for 3 or 18 h, or stimulated with 10 μg/mL anti-IgM plus 1 μg/mL CD40 ligand (IgM/CD40) for 3 or 18 h. Cell lysates were subjected to Western blot analysis and developed by anti-Bim and anti–β-tubulin. (*C*) IgM/CD40 signaling induced a stronger Akt S473 phosphorylation than WT. Splenic purified B cells from WT and KO mice were left untreated (0) or stimulated with 10 μg/mL anti-IgM plus 1 μg/mL CD40 ligand (IgM/CD40) for 1, 5, 15, and 30 min. Cell lysates were subjected for Western blot analysis and developed by anti-p-Akt (S473), anti-Akt, and anti-FOXO1. (*D*) IgM/CD40 signaling did not rescue Erk phosphorylation to WT level. Splenic purified B cells from WT and KO mice were left untreated (0), stimulated with 10 μg/mL anti-IgM (IgM) for 1, 5, or 15 min, or stimulated with 10 μg/mL anti-IgM plus 1 μg/mL CD40 ligand (IgM/CD40) for 1, 5, or 15 min. Cell lysates were subjected to Western blot analysis and developed by anti–p-Erks (T202/Y204) and anti–β-actin.