MATERIALS AND METHODS

Immunization. Chicken γ globulin (CGG; Sigma-Aldrich) was haptenated with nitrophenyl (NP)-hydroxysuccinimide ester (Cambridge Research Biochemicals/Genosys). 7–12-wk-old B1-8 Tg mice (*9*), crossed onto the $J_H^{-/-}$ BALB/c background, which blocks expression of endogenous IgH genes (*28*), were immunized i.p. with 50 μg alum-precipitated NP_{30}-CGG. The Tg encodes a germline Vh186.2 rearrangement that, when combined with the 2-3% of Tg B cells expressing $V\lambda_1$, encodes an anti-nitrophenyl (NP) BCR.

Reagents. Antibodies (Abs) with the following specificities and conjugations were purchased from BD Biosciences: p-Syk-Alexa (Al)647 (Y352), p-Blnk-Al647 (Y84), p-Erk-Al647 (T202/Y204), p-p38-Al647 (T180/Y182), p-Akt-phycoerythrin (PE), (T308), p-Tyr-PE, and anti-mouse β-actin. Abs to the following were obtained from Cell Signaling: p-S6 ribosomal-Al488 (S235/236), p-Src family (Y416), SHP-1, SHIP-1, and p-SHIP-1 (Y1020). The following isotype controls were used with their relevant phospho-antibodies, mouse IgG1-Al647, mouse IgG1-Al488, mouse IgG1-PE, mouse IgG2b-Al647 and mouse IgG2b-PE (BD Biosciences); and Rabbit IgG-Al488 (Southern Biotech, conjugated in our lab). Anti-SHIP-1; (mouse mAb P1C1) and anti-SHP-1 (C-19 rabbit IgG) were obtained from Santa Cruz. Anti-p-Tyr (4G10 platinum) was from Millipore. Rabbit antibodies against the cytoplasmic domain of CD79a ("PRα") were produced as previously described (*29*). b.7-6 rat anti-mouse-IgM and HM79 anti-CD79b (*30*) were purified in our lab from serum-free medium cultures as described (*31*).

Spontaneous signaling in GC and resting B cells. We developed a technique to determine and compare basal levels of BCR associated phosphorylation by flow cytometry in gated populations

of GC and naïve B cells (fig. S1). Total splenocytes from naïve and d14 NP_{30}-CGG immunized mice were fixed by directly disrupting spleens into RPMI containing 1% paraformaldehyde (Electron Microscopy Sciences) and incubating for 15 minutes at room temperature. Cells were briefly incubated with ddH2O to wash out paraformaldehyde prior to ACK (Lonza) lysis of RBCs. Aliquots of fixed cells ($1.5x10^6$) were then permeabilized with ice-cold methanol (Electron Microscopy Sciences) and incubated for 30 minutes on ice. Cells were washed two times with PBS to remove methanol and then aliquots were either treated with phosphatase (calf intestinal phosphatase (CIP, NE BioLabs) or λ phosphatase (NE BioLabs), each giving similar results) or left untreated for 15 min at 37˚C. All aliquots were then incubated with phospho-specific antibodies for 30 min, combined with peanut agglutinin (PNA)-FITC (Vector Laboratories Inc.) and labeled goat anti-λ (Southern Biotech) and anti-B220 (RA3-6B2, BD Biosciences). Phosphorylation was measured in gated populations identified as GC ($B220^+\lambda^+PNA^+$) and non-GC / naive ($B220^+\lambda^+PNA^-$) B cells by flow cytometry using an LSRII (BD Biosciences). Data were analyzed and median fluorescence intensity (MFI) determined by FlowJo software (Tree Star).

Ex-vivo stimulation (fig. S2). Spleens from d13-14 NP_{30}-CGG immunized mice were processed into single cell suspensions in complete RPMI containing 10% FCS. Aliquots of $1.5x10^6$ cells were incubated 30 min to 1 h at 37˚C. Cells were then incubated with or without stimulation at 37˚C for various times as indicated in the figure legends. Reagents used for stimulation were prepared in RPMI. These included: hydrogen peroxide (H_2O_2) at 5 mM or 10 mM, anti-IgM (b.7-6) at 15 μg/ml, goat $F(ab')_2$ anti-IgM (Southern Biotech) 15 μg/ml, NP_{27}-BSA 5 μg/ml, 100 ng/ml Phorbol-12-myristate-13 acetate (PMA, Calibiochem)/ionomycin 1000 ng/ml. Reactions were stopped by adding paraformaldehyde to a 1.5% final concentration, incubating 15 min at

room temperature. Fixed cells were then permeabilized with ice-cold methanol and incubated for 30 min on ice. Cells were washed two times with PBS to remove methanol and then either treated with phosphatase or left untreated for 15 min at 37˚C. All aliquots were then incubated with phospho-specific antibodies for 30 min combined with peanut agglutinin (PNA, Vector Laboratories), goat anti-λ and anti-CD45/B220. FACS analysis was performed as above.

Intracellular Ca^{2+} mobilization. Total splenocytes at $3x10^6$ cells/ml were loaded with 5 μM Indo-1 acetoxymethyl (Indo-1 AM, Invitrogen) in Hank's balanced salt solution (HBSS, Gibco) containing 1% FCS at 37˚C for 30 min. Surface marker antibodies, PNA-FITC and anti-λ-PE were added for the last 15 min of incubation. Cells were washed twice and resuspended in HBSS/1% FCS. Ca^{2+} mobilization in gated GC and non-GC B cell populations was detected on the LSRII. After a baseline was established, cells were stimulated with 15 μg/ml anti-μ, 5 mM H_2O_2 or anti-μ+ H_2O_2 prepared in HBSS/1% FCS. Mean Ca^{2+} was determined by measurement of the fluorescence ratio of 440/40 nm to 530/30 nm emissions (violet to blue ratio). A positive control of ionomycin-stimulated cells was included in each experiment.

Cell sorting and purification. Single cell suspensions of splenocytes from NP_{30}-CGG immunized and naïve mice were washed and maintained in complete RPMI (Gibco) containing 10% FCS and kept on ice for the entire subsequent procedure to minimize any subsequent metabolic changes. Cells were stained with PNA and goat anti-λ, anti-CD45/B220 and ethidium monoazide (EMA, Invitrogen). GC ($EMA^-PNA^+\lambda^+$) and non-GC/naïve ($EMA^-PNA^-\lambda^+$) B cells were sorted simultaneously.

Immunoprecipitation and immunoblotting. Aliquots of sorted cells at $3x10^6$ cells/ml were left

unstimulated on ice or stimulated with anti-IgM (15µg/ml) for 2 minutes at 37°C and the reaction stopped with ice-cold TBS. The cells were subsequently lysed in non-denaturing TNT lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM EDTA, protease inhibitor (Complete, Roche) and phosphatase inhibitor (PhosStop, Roche) at 30µl/million cells. Lysates were centrifuged for 10 min at 10,000g at 4˚C. Cleared lysates were boiled with SDS reducing sample buffer for 5 min and separated by 4-15% SDS-PAGE (Bio-Rad) or subjected to immunoprecipitation. Lysates to be used for immuno-precipitation were precleared with protein G-sepharose beads (Sigma) by rotation for 1 h at 4°C, then incubated with a precipitating antibody overnight at 4°C. Immune complexes were isolated by adsorption to protein G-sepharose beads for 1 h at 4˚C. Beads were washed four times with 0.1% Triton X-100 lysis buffer then one time with PBS and boiled in 2x reducing sample buffer for 5 min. The samples were then fractionated by 4-15% gradient SDS PAGE. Gels were electrophoretically transfered to Immobilon-FL, polyvinylidene fluoride (PVDF, Millipore). Immunoblots were blocked with Odyssey blocking buffer (LI-COR Biosciences) for 1 h at room temperature, then incubated with mouse IgG2b anti-phosphotyrosine antibody 4G10 Platinum (Millipore) at 1/1000 dilution for 1h combined with antibody for the precipitated protein (isotype, rabbit IgG) for immunoprecipitation experiments and specific anti-phosphoprotein Abs for Western blots. The membrane were washed four times with TBS with Tween (TBST) then incubated with secondary antibodies IRDye 680 goat anti-rabbit and IRDye 800 conjugated goat anti-mouse at 1/10,000 dilution. The blots were then washed four times with TBST followed by one wash with PBS. Blots were scanned and labeled bands were detected, gated and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Enzymatic detection of tyrosine and serine/threonine phosphatases. Sorted GC, non-GC and

naïve B cells were lysed in a phosphatase storage buffer containing 25 mM Tris HCl (pH 7.4), 2 mM EDTA, 10 mM ß-mercaptoethanol and protease inhibitor (Complete, Roche) at 30 µl/ million cells. Phosphatase activity was detected using a phosphatase assay system kit (Promega). Phosphate contamination was removed from the lysates by Sephadex G-25 spin-columns supplied with the kit. Two tyrosine phosphopeptides, END(pY)INSAL and DADE(pY)LIPQQG and a serine/threonine phosphopeptide, RRA(pT)VA were used as substrates for tyrosine and serine/threonine phosphatase respectively. Each lysate was incubated in a 96 well plate with or without 100 µM substrate, 5 mM sodium vanadate (Calbiochem), 5µM okadaic acid (Calbiochem) and 50 mM sodium fluoride (Sigma Aldrich) at 30˚C for 30 minutes. A phosphate standard curve was generated for each assay. Free phosphate generated from the substrates was detected by molybdate dye solution, measured at 630 nm with a plate reader (Bio-Rad) and the amount was calculated from the standard curve.

Analysis of co-localization using imaging cytometry. Single cell suspensions were prepared as described above. Cells were stimulated for 2-30 minutes at 37˚C with b.7-6 at 15µg/ml. Cells were then fixed with 1.5% paraformaldehyde for 15 minutes and permeabilized with permwash buffer (0.1% Triton X-100, 3% calf serum, 0.05% sodium azide). Cells were next incubated with PE-conjugated primary antibodies to either SHP-1 (rabbit) or SHIP-1 (rat), mixed with donkey anti IgM-Al488, PNA-Al647 and goat anti-λ-Pacific blue in permwash buffer for 30 minutes at room temperature. Following washing in permwash buffer, cells were resuspended in PBS before analysis on the Amnis Imagestream X.

Colocalization of SHP-1 or SHIP-1 with BCR was analyzed in gated GC and non-GC populations using IDEAS software. The Bright Detail Similarity feature within IDEAS software

was used to mask localized brighter spots in the two input images (BCR and SHP-1) and Pearson's correlation coefficient was used to calculate similarity scores within the bright detail masks.

Cell cycle and phospho-flow analysis. Mice at d13-14 post-NP-CGG immunization were i.v.-injected once with 3 mg of 5-Bromo-2'-deoxyuridine (BrdU, Sigma Aldrich) and sacrificed 1h later. Splenocytes were processed in RPMI containing 10% FCS. Aliquots of 1.5 X 10^6 cells were incubated with or without 15 μg/ml b.7-6 for 5 min. Praformaldehyde fixation and methanol permeabilization were performed as described above. DNA was denatured by incubating cells in 100 Kunitz U/ml of DNAse/0.15 M NaCl/4.2 mM $MgCl_2$ for 30 min at room temperature. Anti-λ-PE, PNA-biotin, anti- p-Syk-Al647 and anti-BrdU-FITC were added for 30 min in PBS 1% BSA. 5μM of DAPI dilactate (Invitrogen) was added 10 min before analyzing the cells on an LSRII cytometer.

Cell cycle analysis for SHP-1 expression and BCR/SHP-1 colocalization. Mice at d13 post NP-CGG immunization were injected with BrdU for 1 hr to allow labeling of S phase dividing cells. Total splenocytes from d13 NP-CGG immunized mice were stimulated with b.7-6 (15μg/ml) for various time intervals as indicated. Cells were stained with anti-BrdU, and DAPI to identify phases of cell cycle. Images we obtained using the Amnis Imagestream X system, and analyzed to identify GC B cells in G1, S, G2 and M phases of the cell cycle. Colocalization was determined as described above. Data were analyzed with IDEAS software (Amnis)

Specific deletion of *Ptpn6* (SHP-1) in B cells

The generation of human CD20 bacterial artificial chromosome-derived Tg mice carrying an

IRES-ERT2-Cre ("TamCre") cassette in the 3' untranslated region of the CD20 locus is described in detail in fig. S8 and the legend to that figure. SHP-$1^{f/lfl}$ mice were crossed to hCD20-TamCre mice. SHP-$1^{fl/fl}$ mice either with or without the hCD20.TamCre allele were both injected i.p. with 3 daily doses of tamoxifen at d9-11 post immunization with NP-CGG. At d13 total splenocytes were stained with PNA-FITC, NIP-PE, EMA, anti-kappa Pacific Blue, and anti-B220 allophycocyanin-Cy7. Cells were then fixed with 1.5% paraformaldehyde for 15 minutes and permeabilized with BD permwash buffer. Cells were incubated with PE-conjugated primary antibody to SHP-1 in BD permwash buffer for 30 minutes at room temperature. Following washing, cells were resuspended in PBS/1% calf serum/0.05% sodium azide and analyzed on the LSRII flow cytometer.

SUPPLEMENTARY FIGURE LEGENDS

Fig. S1. Analysis of spontaneous signaling of GC and non-GC B cells directly ex-vivo. (A) Schematic diagram showing our procedure ("Instantfix") for rapidly fixing splenocytes with paraformaldehyde to detect spontaneous phosphorylation. (B) GC and non-GC B cells gates used to measure phosphorylation of signaling proteins based on PNA and Ig-λ_1 staining on gated $CD19^+$ B cells. We gated on Ag-specific (i.e. λ_1+) GC (peanut agglutinin, PNA^+) and "non-GC" (PNA^-) cells, by staining post-fixation. **(C)** Assessment of phosphorylation levels of p38, S6 ribosomal protein, and Akt in GC, non-GC and resting B cells. Staining and analysis was performed as described in Materials and Methods and the legend to Fig. 1. Histograms are representative results of Ser/Thr phosphorylation showing MFI from calf intestinal phosphatase (CIP)-treated (red line) and untreated (blue line) cells, overlaid for direct comparison. y-axes show relative cell number. Bar charts are net MFI indicating spontaneous phosphorylation, calculated by subtracting MFI of CIP treated from CIP untreated cells. Error bars are SEM from at least 5 independent experiments using cells pooled from spleens of at least two mice for each group. * $p < 0.05$ and ** $p < 0.01$ MFI of GC or non-GC compared to naïve.

Fig. S2. Flow cytometric Analysis of phosphorylation induced by stimulation of total splenocytes in gated GC and non-GC cells. (A) Schematic diagram showing our procedure of processing of splenocytes for ex-vivo stimulation. (B-D**)** Ex-vivo stimulation of GC, non-GC and naive B cells. Total splenocytes from d13 NP-CGG immunized mice were stimulated ex-vivo with $(Fab')_2$ anti-IgM (B), NP_{27}-BSA (C) or PMA/Ionomycin (D), then fixed and stained. Histograms depict distributions of phospho-protein staining. Figures are representative of 3 (B), 5 (C), or more than 5 (D) independent experiments. (B) Stimulation with $F(ab')_2$ anti-IgM (10μg/ml) for 1 minute (blue line) and 5 minutes (green line) compared to unstimulated

(red line). Amount of p-Syk in gated GC and non-GC B cells is shown. (C) Stimulation with NP_{27}-BSA for 5 minutes (blue line) and 15 minutes (green line) compared to unstimulated (red line). Histograms show staining for the indicated phospho-proteins in gated GC or non-GC cells. (D) Total splenocytes were stimulated with PMA (100 ng/ml) and ionomycin (1000 ng/ml). Erk and p38 phosphorylation were detected in gated GC and non-GC B cells following stimulation for the indicated times. p-Erk or p-p38 (red line) profiles are overlaid with irrelevant isotype-matched staining controls (grey line) for direct comparison.

Fig. S3. Differential signaling in GC and non-GC cells gated for equal expression of surface BCR. Splenocytes from day 13 immunized mice were isolated as in Supplemental Fig. 2 and treated with Al488-labeled b7.6 for simulataneous stimulation and BCR detection. Cells were then fixed and stained with PNA and anti-p-Syk. (A) Identification of BCR expression on GC (PNA^{hi}) and non-GC (PNA^{lo}) $CD19^{+}$ gated B cells. A narrow gate of equal levels of surface BCR expression was drawn for GC and non-GC cells**,** and expression of p-Syk was plotted (B) for cells falling into this gate. Syk phosphorylation was determined on gated GC (PNA^{hi}/IgM^{hi}, top row) and non-GC (PNA^{lo}/IgM^{hi}, bottom row) B cells at the indicated time (solid red) and plotted compared to isotype matched irrelevant Ab staining controls (dotted gray).

Fig. S4. Exclusion of trivial explanations for GC B cell non-responsiveness. (A) B cells remain viable after isolation and culture with anti-IgM. Total splenocytes from d13 NP-CGG immunized mice were incubated for 1 hr at 37˚C, then stimulated with anti-μ for 1, 5 or 15 minutes. They were then stained with live/dead violet dye (Invitrogen). Gates were drawn on dead cells, which exhibit high violet fluorescence and have lower FSC. Gates drawn indicate the low percentage of dead cells at all time points. (B) Activated naive B cells can signal through the

BCR. Naïve splenocytes were cultured for 48 hrs with CpG DNA (1 μg/ml, oligo 1826) or IL-4 (10ng/ml). Cells were washed and after 30 minutes incubation at 37^{o} C they were stimulated with anti-IgM (15μg/ml) for 5 minutes. Fresh spleen cells were similarly stimulated. Syk phosphorylation was determined in gated $\lambda^{+}B220^{+}$ B cells. p-Syk staining profiles (dark lines) from unstimulated (dotted lines) and stimulated cells (solid lines) were overlaid and compared to isotype control (grey lines).

Fig. S5. Spontaneous and induced phosphorylation of CD79. FACS-sorted GC and non-GC B cells from d13 immunized mice and naïve B cells were either left on ice unstimulated or stimulated with 15μg/ml anti-IgM for 2 minutes at 37˚C. CD79b was immunoprecipitated from lysates equivalent to $3x10^{6}$ cells using HM79, followed by Western blotting using: (A**)** anti-CD79a (PRα) or (B**)** anti-phosphotyrosine (4G10). Bar graphs **(**C**)** are median intensity of total CD79, p-CD79 and p-CD79 relative to total. Plots and bar graphs are representative of 4 independent trials with similar results.

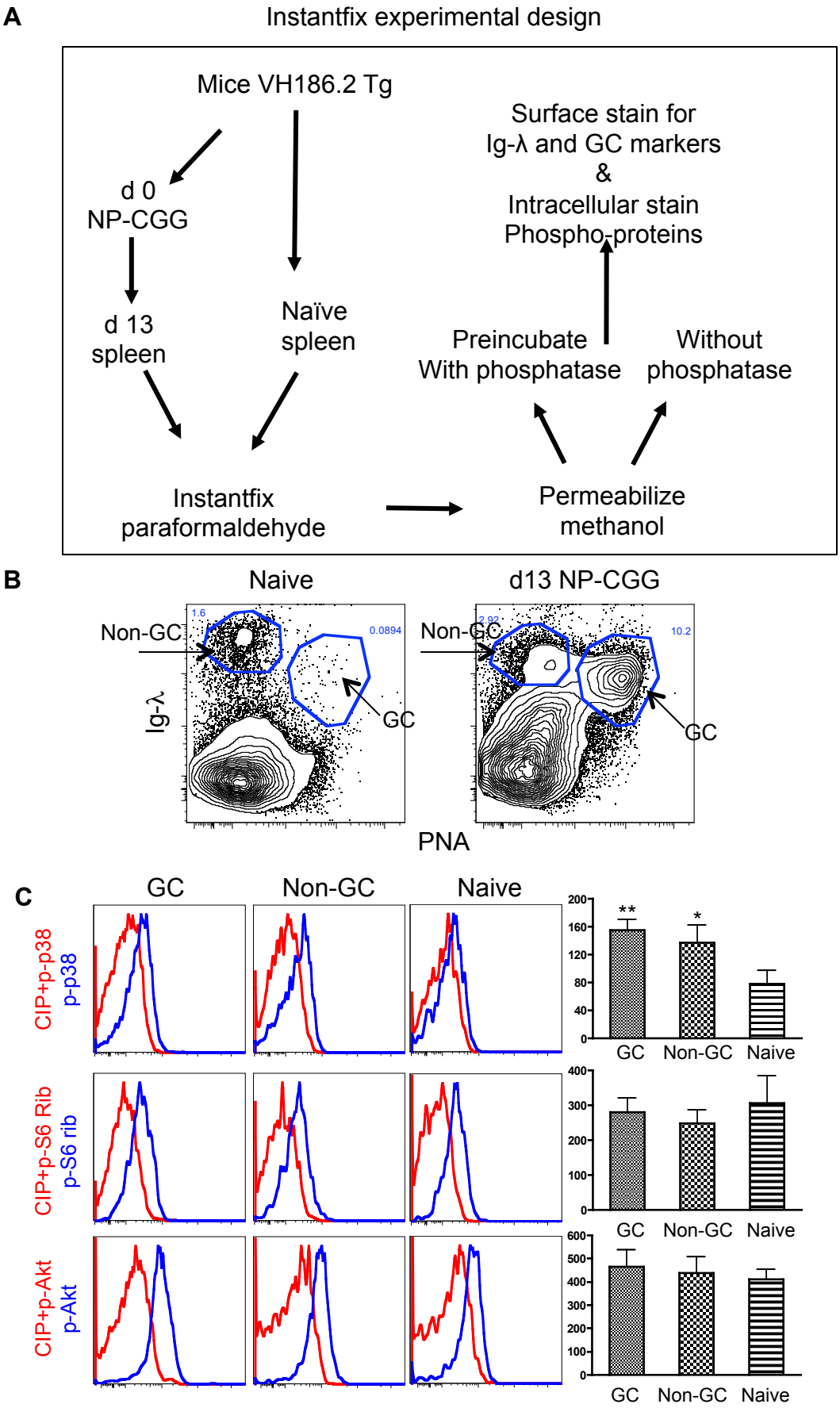
Fig. S6. Further analysis of phosphatase activity and signaling negative regulators in GC B cells. (A) Kinetics of response to tyrosine phosphatase inhibition. In vitro stimulation of total splenocytes from d13 NP-CGG immunized mice with 5mM H_2O_2 for the indicated times, followed by FACs detection of induced phosphorylation of Syk, Erk and tyrosine. Overlaid profiles of gated GC (open line) and non-GC (shaded areas) populations show the time course. (B) Greater tyrosine and serine/threonine phosphatase activity in GC vs non-GC or naïve B cells. Enzymatic activity (y-axis) was measured in phosphate-free cell lysates from sorted GC, non-GC and naïve B cells as described in Materials and Methods for each of the substrates: tyrosine phosphopeptide-1 (Tyr-1), tyrosine phosphopeptide-2 (Tyr-2) and a serine/threonine phosphopeptide (Ser/Thr). Specificity was assessed by incubating each reaction with or without the following inhibitors: sodium vanadate (Van), which totally inhibited phosphate release from Tyr-1 and Tyr-2 and partially inhibited release from Ser/Thr; and okadaic acid (OA) and sodium fluoride (NaF), which totally inhibited Ser/Thr phosphatase. Results are representative of 2 independent experiments with similar results. (C) Level of Src phosphorylation (Y416) was detected by Western blot of lysates from sorted GC and non-GC spleen B cells either unstimulated or stimulated with anti-IgM, with simultaneous detection of β-actin. Bar graph is MFI of p-SRC relative to β-actin representative of 2 similar experiments.

Fig. S7. Analysis of SHP-1 and SHIP-1 colocalization with BCR in stimulated GC and non-GC B cells. SHP-1 colocalization studies were performed as described in Fig. 4. (A) SHP-1/BCR co-localization scores (mean ± SEM) as a function of time in GC (squares) vs non-GC (triangles) cells. (B) Percentage of GC (squares) and non-GC (triangles) cells with similarity score of less than 1.2 (gated as in panel F) as a function of time. Note the different axes for the two cell types. (C) High throughput imaging cytometric analysis of SHIP-1/BCR association. Total splenocytes

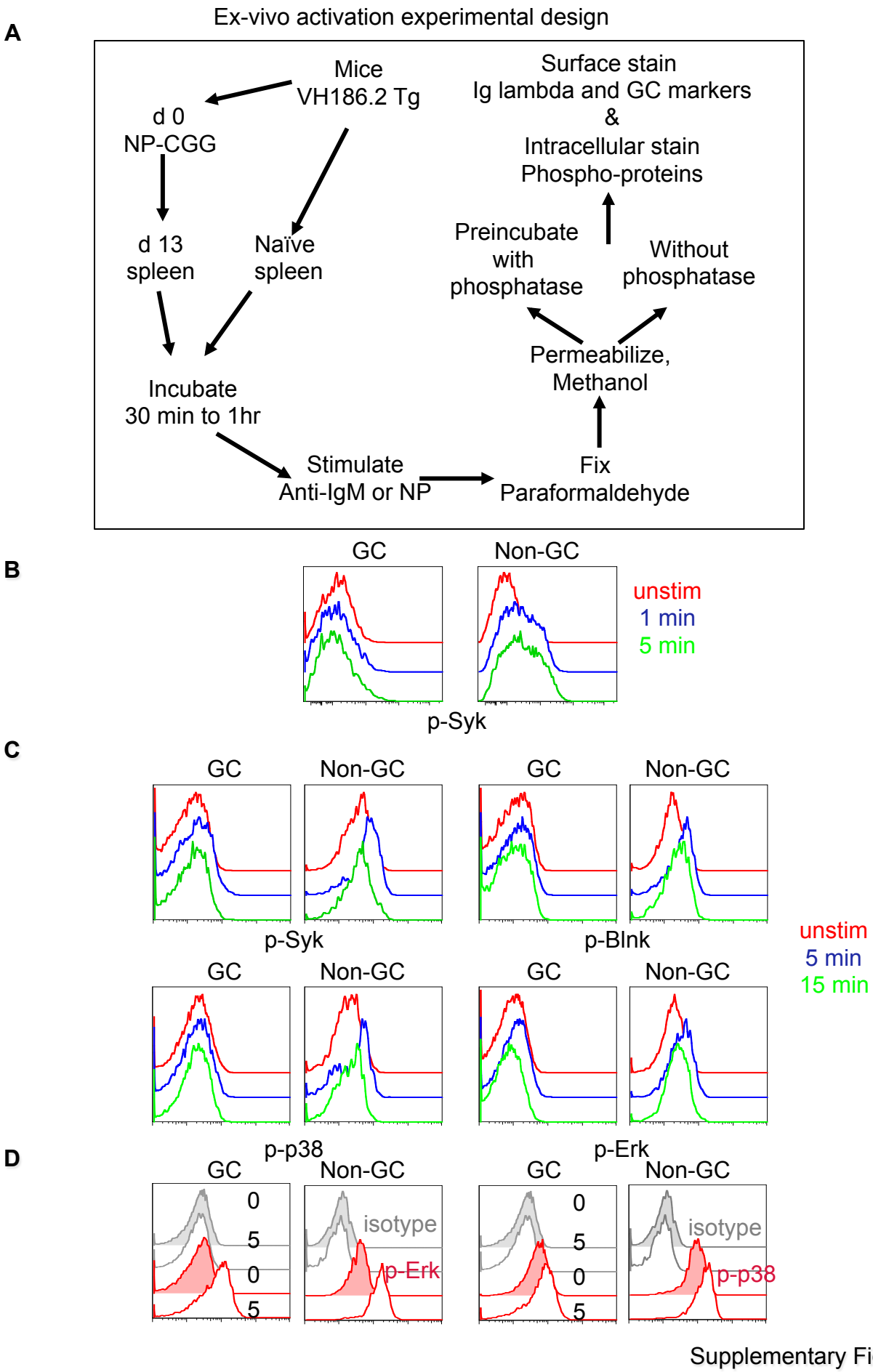
were either left unstimulated or stimulated with anti-IgM (b.7-6) for 2 and 5 minutes. Co-localization of SHIP-1 and BCR was measured in gated non-GC (λ_1^+ PNA^{lo}) and GC (λ_1^+ PNA^{hi}) B cells as described in Materials and Methods, and plotted as histograms. First column: GC (shaded area) and non-GC open line are overlaid, with three time points shown in the three rows. Second column: The distributions of SHIP/BCR similarity scores of unstimulated non-GC B cells (t=0 shaded area) overlaid with cells stimulated for 2 or 5 minutes (open line, rows 2 and 3). Third column: The distributions of SHIP/BCR similarity scores of unstimulated GC B cells (t=0, shaded area) are overlaid with cells stimulated for 2 or 5 minutes (open line, rows 2 and 3). (D) SHP-1 expression (median pixel intensity) in GC B cells by cell cycle phase as in Fig. 4C (***$p<0.0001$). (E) Summary of SHP-1/BCR colocalization scores with time in response to anti-IgM stimulation in GC B cells at different phases of cell cycle as described in Fig. 4E.

Fig. S8. Generation and validation of B cell specific inducible-Cre mice and their use in deletion of SHP-1 in $SHP\text{-}1^{fl/fl}$ mice. The bacterial artificial chromosome clone RP11-729B4 that was previously used to create human CD20 (hCD20) Tg mice that expressed efficiently and specifically in B cells (*32*) was modified by homologous recombination as described (*33*) to insert an internal ribosome entry site (IRES)-ErT2Cre (TamCre) cassette (*34*) in the 3' untranslated region of the hCD20 gene, as shown in (A). A 168kb insert of the modified RP11-729B4 clone was isolated and used to make Tg mice. This allele, which encodes expression of hCD20 as well as TamCre was intercrossed with mice carrying the rosa26-flox-STOP-YFP allele, which expresses YFP upon Cre action (*35*). Two Tg positive mice, one carrying the reporter allele and the other not, were treated with tamoxifen (0.15mg/g i.p. in olive oil) for 3 days. PBL was analyzed on day 0, prior to treatment and day 4, one day post-treatment. (A) FACS plots of staining for B220 and hCD20in Tg (top row) and non-Tg

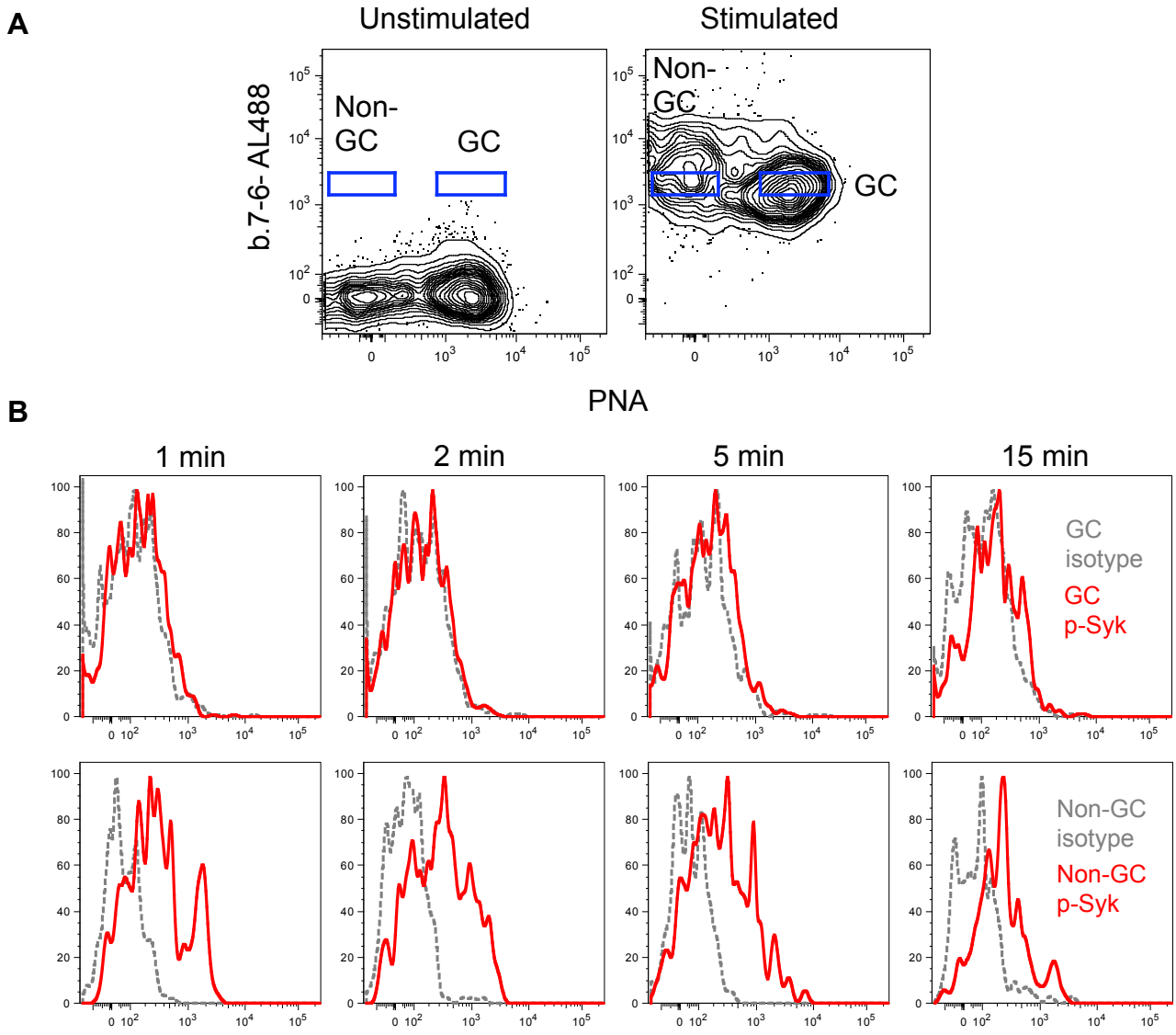
mice. Note the uniform staining with hCD20 on B cells only in the Tg but not littermate mouse. (B) Expression of YFP (x-axis) before and after tamoxifen treatment. YFP expression is only seen after tamoxifen treatment (b, d) and only in the mouse carrying the hCD20-TamCre allele (b). It was only observed in the hCD20+ B cells (87.7% frequency) but not in other cell types, demonstrating the inducibility, specificity and efficiency of the Tg. Results are representative of more than 5 mice tested. (C) SHP-1 is specifically deleted in B cells of SHP-$1^{fl/fl}$ hCD20.TamCre mice. The hCD20-TamCre Tg allele was backcrossed for more than 14 generations to B6 before being intercrossed with SHP-$1^{fl/fl}$ mice and then backcrossed to obtain Tg positive or negative littermates that were homozygous for the floxed allele. Left panel is representative histograms of SHP-$1^{f/f}$ hCD20 Cre (red line) and SHP-$1^{f/f}$ hCD20 Non-Cre control (blue line). Administration of 3 doses of tamoxifen at day 9-12 of NP-CGG immunization led to a uniform and marked reduction SHP-1 expression only in splenic B cells (top panel) but not in non-B cells (bottom panel) of Cre mice analyzed at day 14. Right panel shows SHP-1 mean fluorescence intensity (MFI) from 3 independent experiments showing significant inhibition of SHP-1 in B cells $p<0.0001$ (top) but not in non-B cells (bottom) in hCD20-TamCre (squares) as compared to non-Tg control mice (triangles).



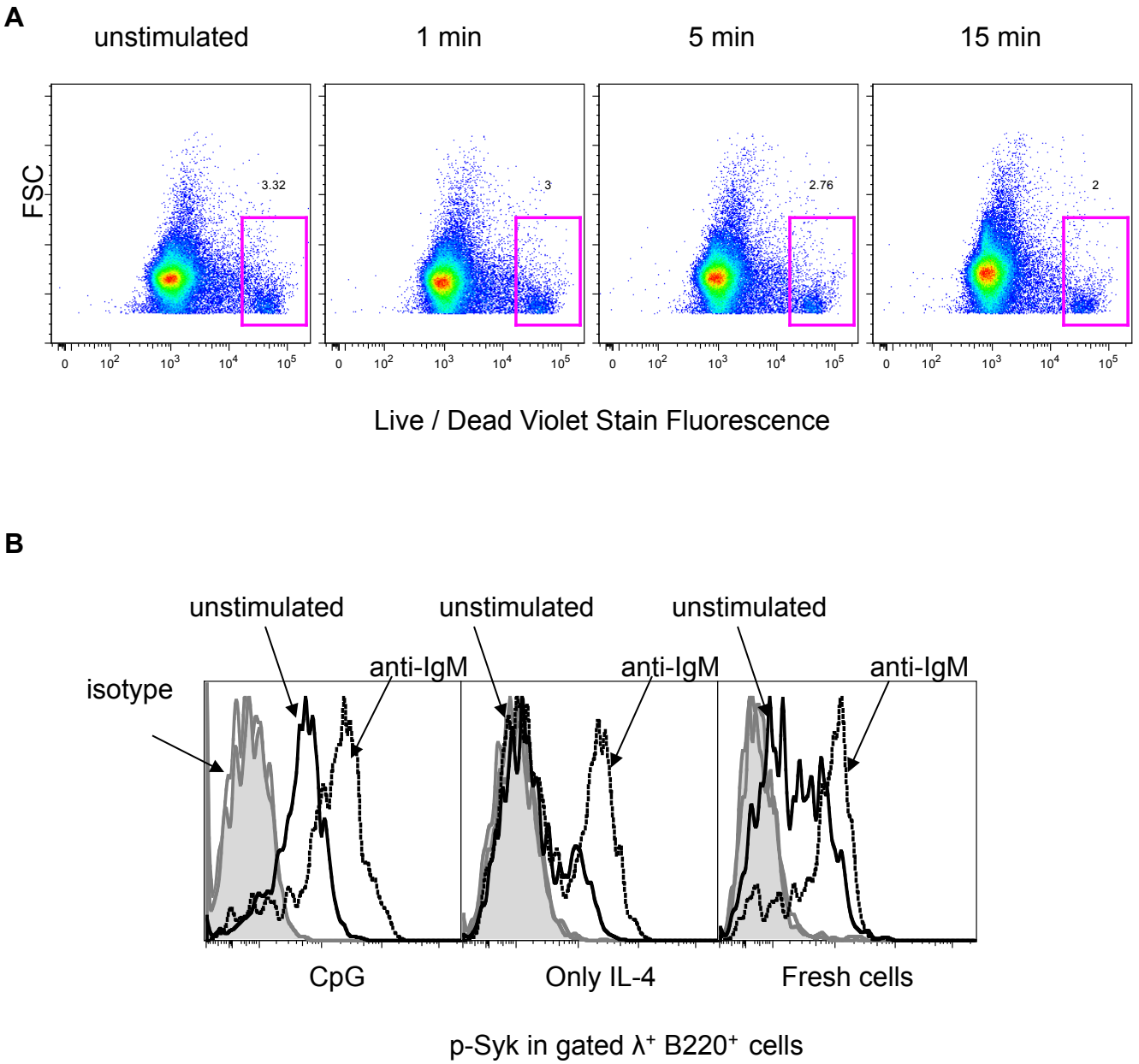
Supplementary Figure 1



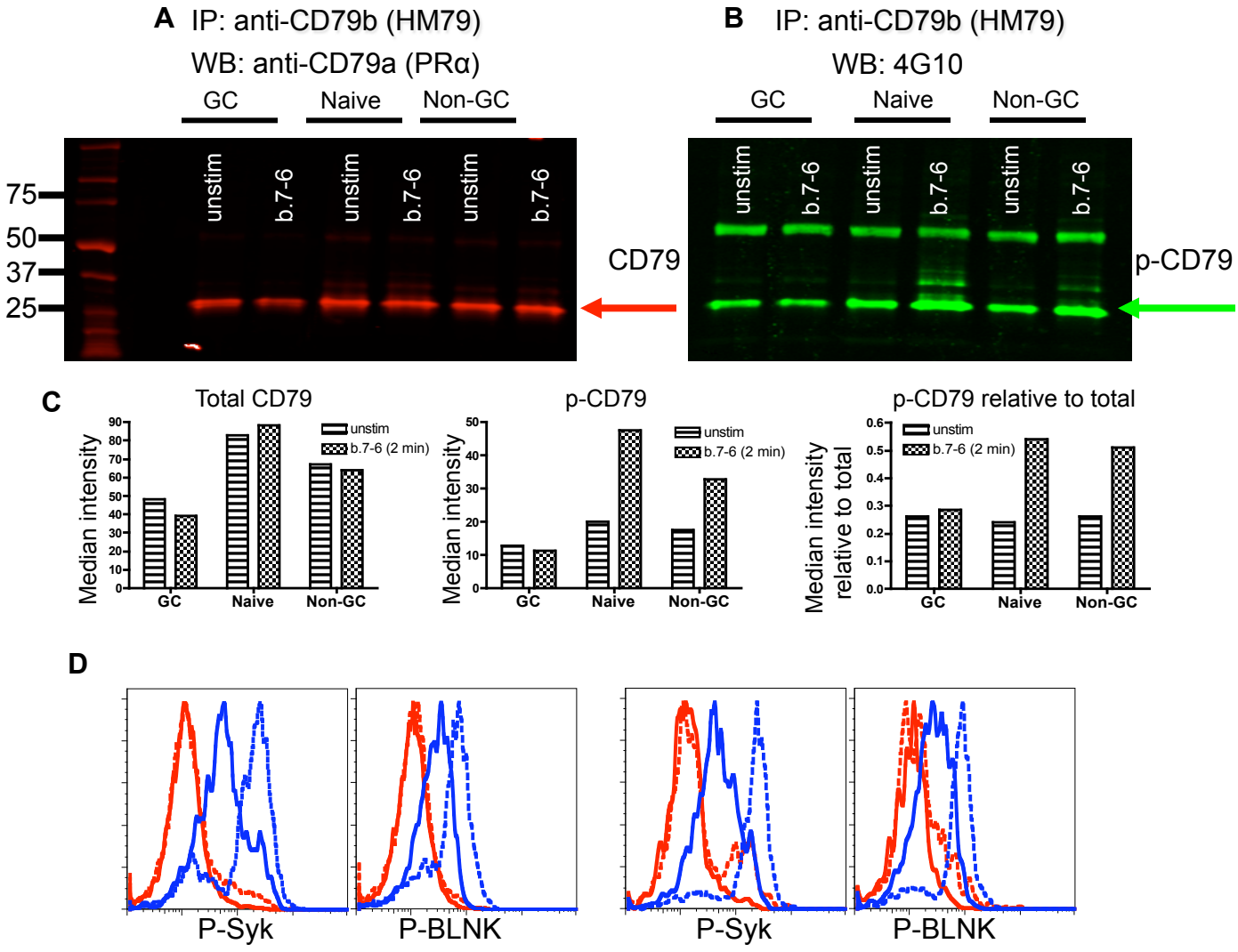
Supplementary Figure 2



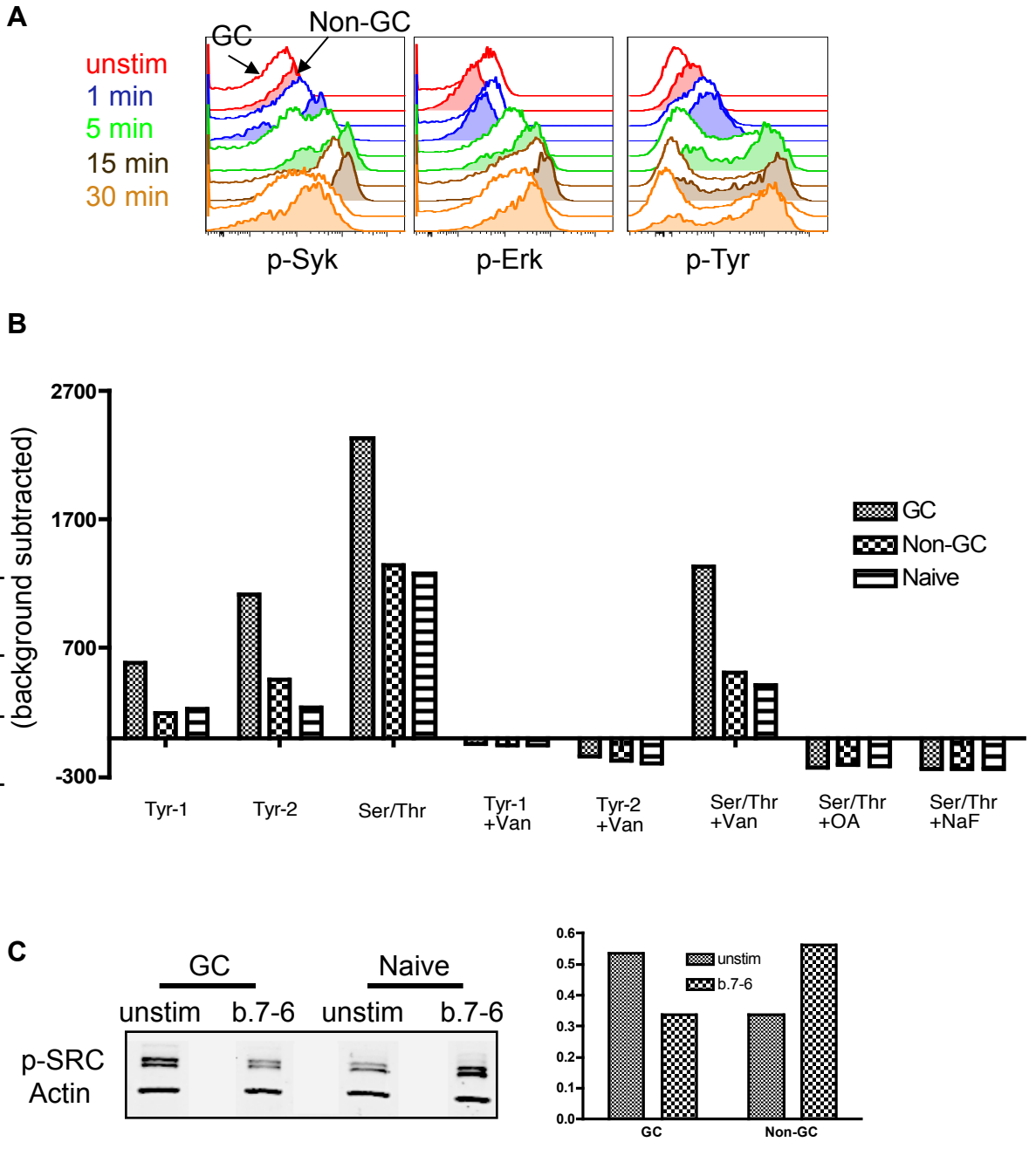
Supplementary Figure 3



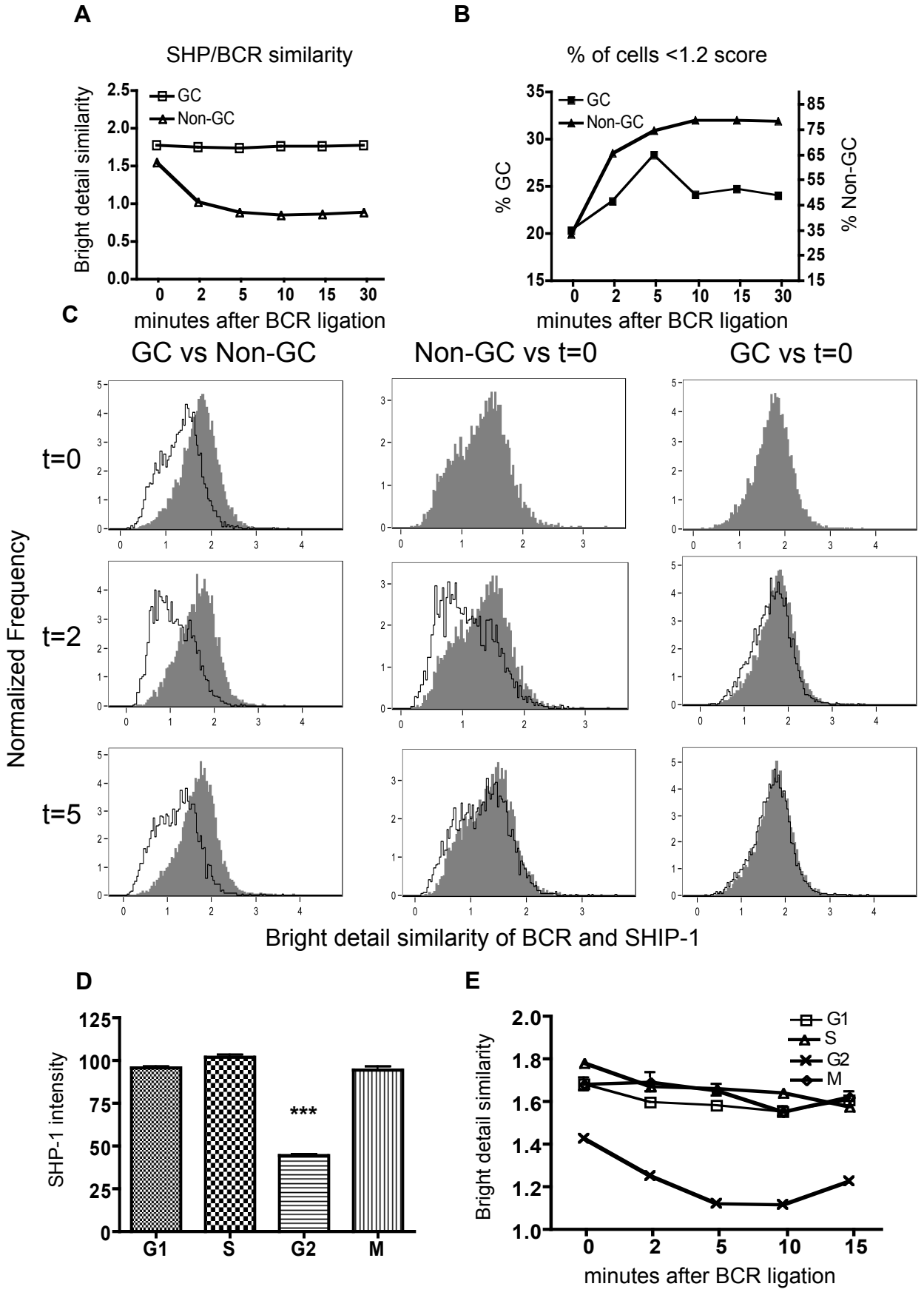
Supplementary Figure 4



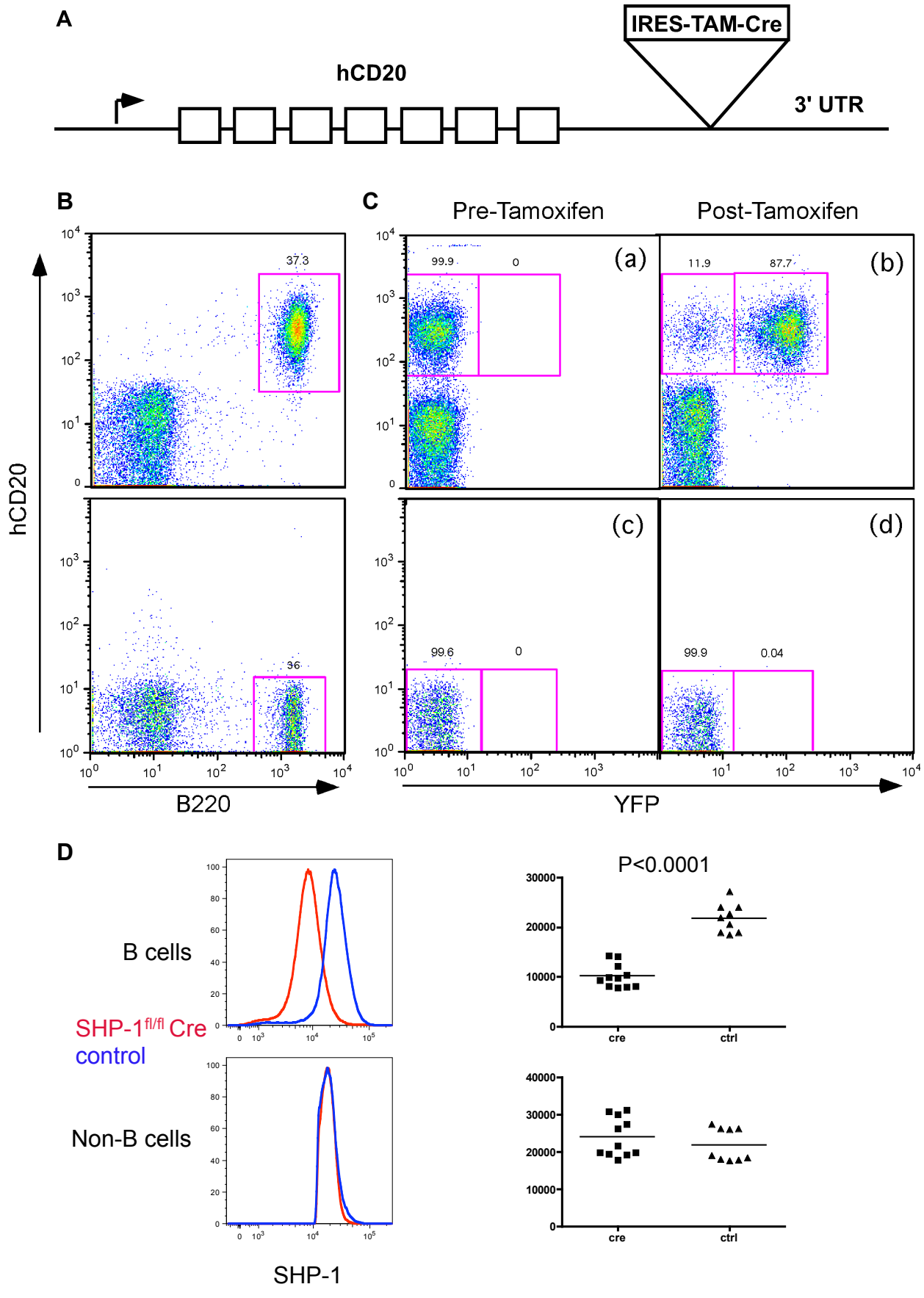
Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7



Supplementary Figure 8