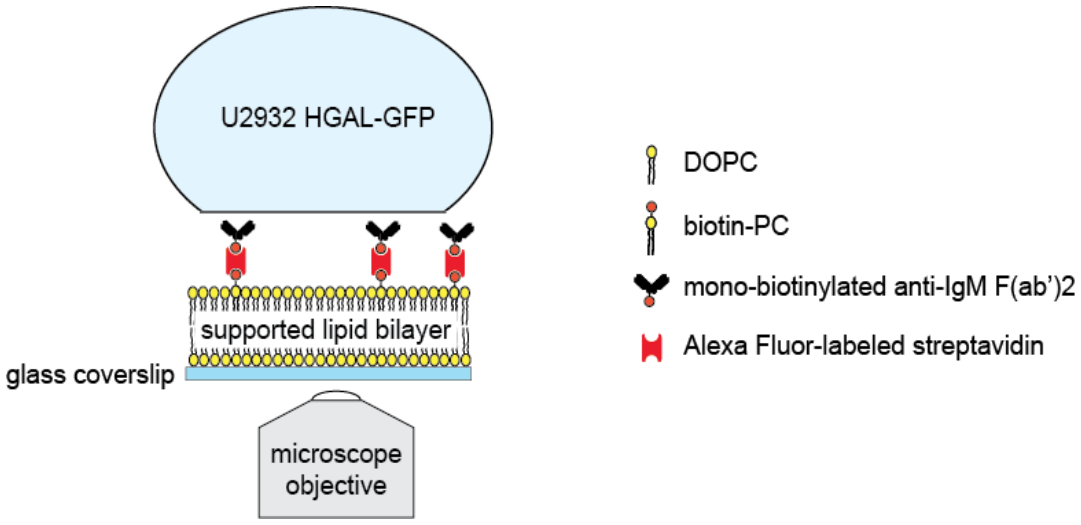
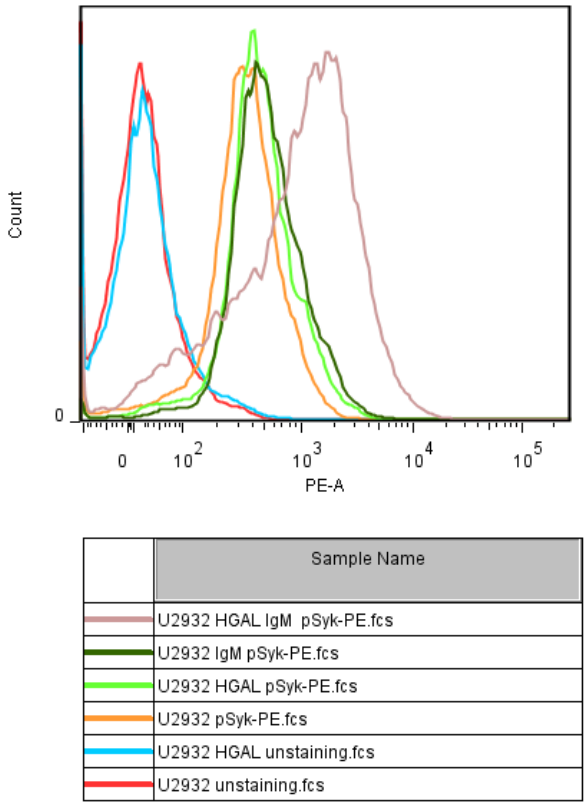
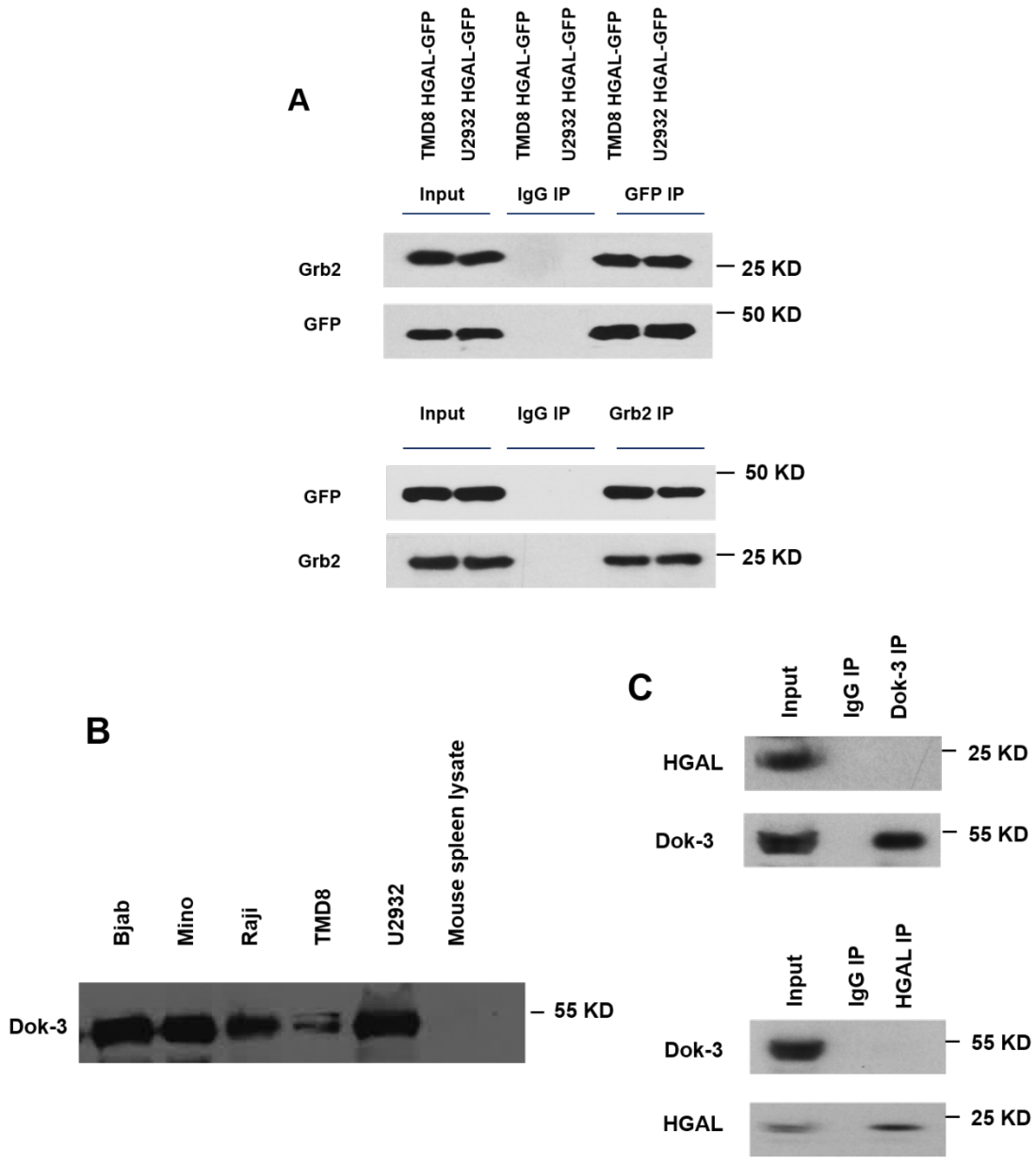
Supplemental Figures



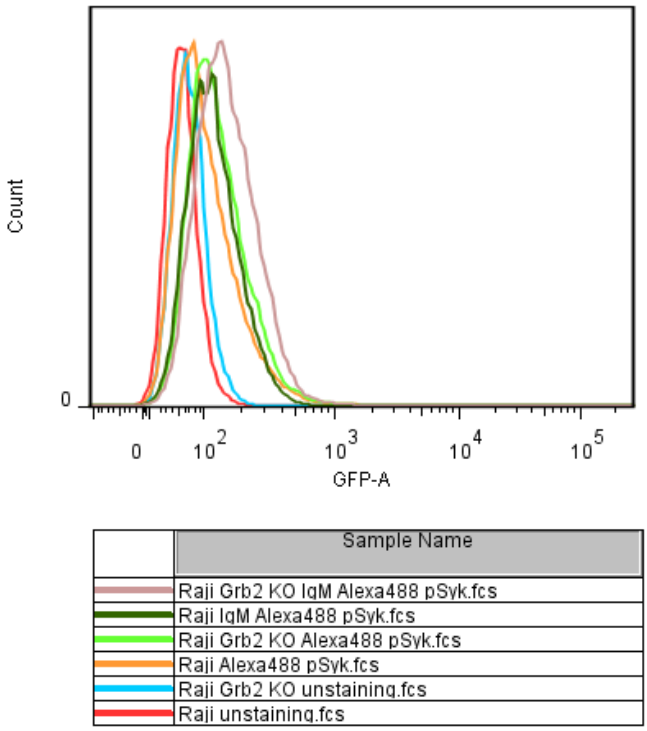
Supplementary Figure 1. Schematic representation of an experimental design. Schematic representation of an experimental system that employs confocal microscopy to visualize the redistribution of protein following the activation of a B cell by a supported lipid bilayer functionalized with anti-IgM F(ab')$_2$. Abbreviation: 1,2-dioleoylsn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl) (biotin-PC).



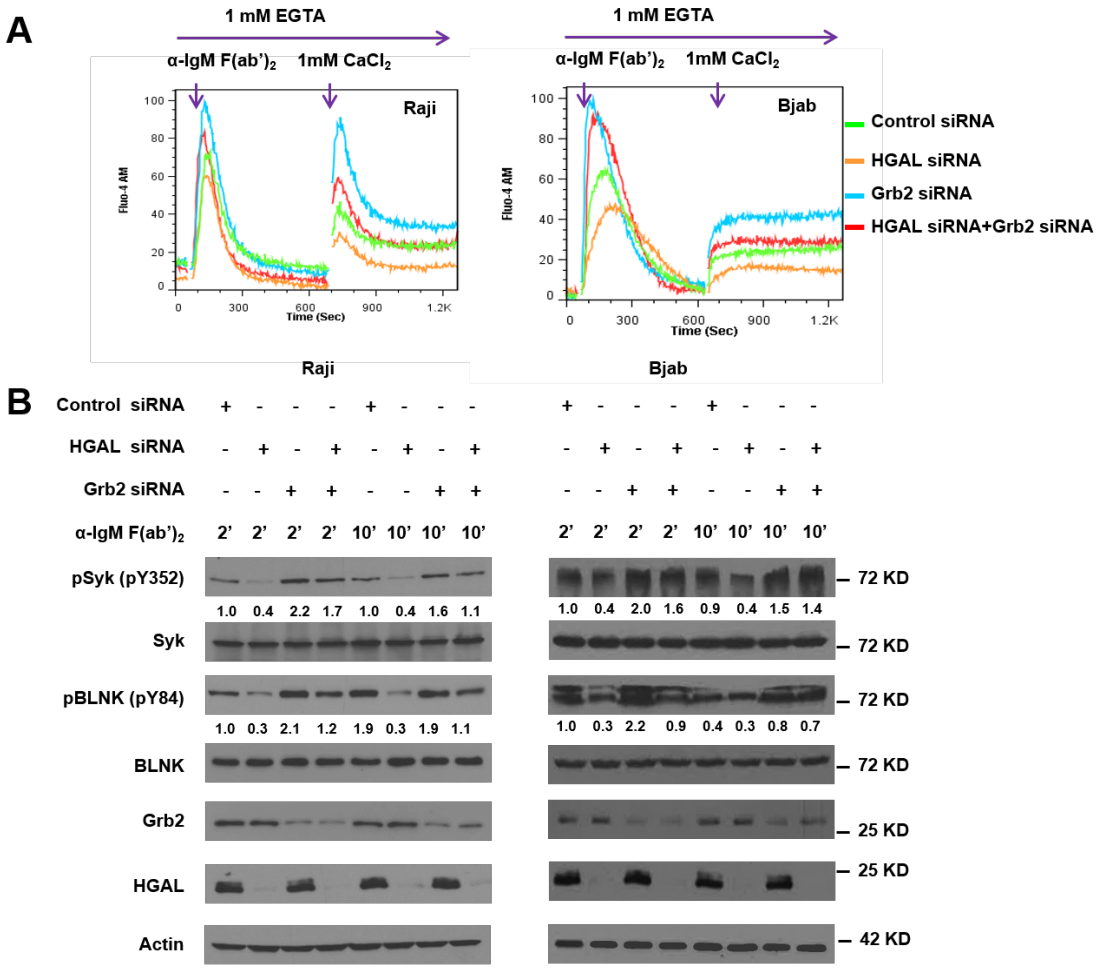
Supplementary Figure 2. pSyk(Y352) staining in U2932 cells. Phospho-flow cytometry was used to assess intracellular pSyk(Y352) expression in the presence or absence of BCR crosslinking in U2932 and U2932 HGAL cell lines. The X-axis denotes expression (log scale) and Y-axis indicates cell number.



Supplementary Figure 3. A) Reciprocal Co-IP of HGAL-GFP and Grb2 in TMD8 HGAL-GFP and U2932 HGAL-GFP lymphoma cells stably transfected with HGAL-GFP; B) Dok-3 expression in Bjab, Mino, Raji, TMD8, U2932 and mouse spleen. Protein extracts were prepared from whole-cell lysates and blotted with anti Dok-3 antibody; C) Reciprocal Co-IP experiment of HGAL and Dok-3 proteins in Raji lymphoma cells.

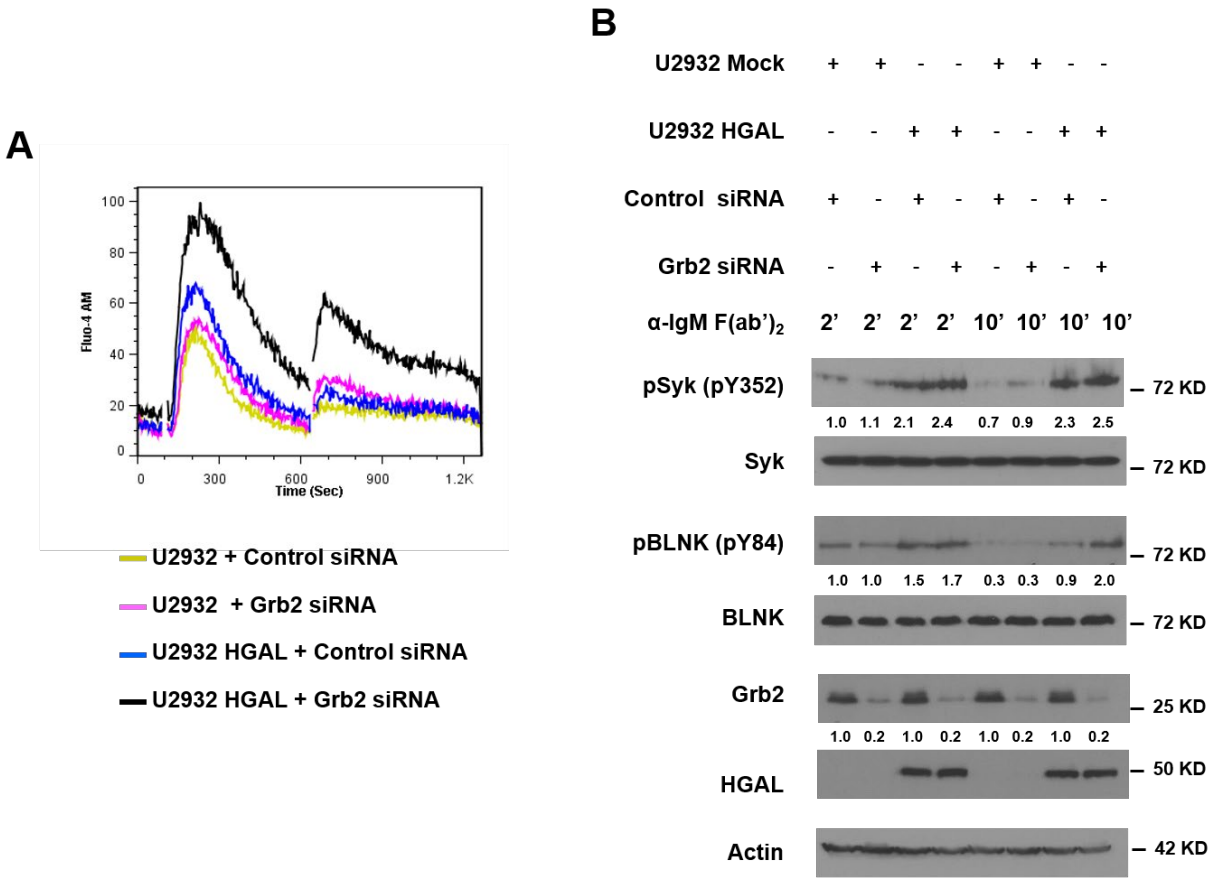


Supplementary Figure 4. pSyk(Y352) staining in U2932 cells. Phospho-flow cytometry was used to assess intracellular pSyk(Y352) expression in the presence or absence of BCR crosslinking in Raji and Raji Grb2 knockout cell lines. The X-axis denotes expression (log scale) and Y-axis indicates cell number.

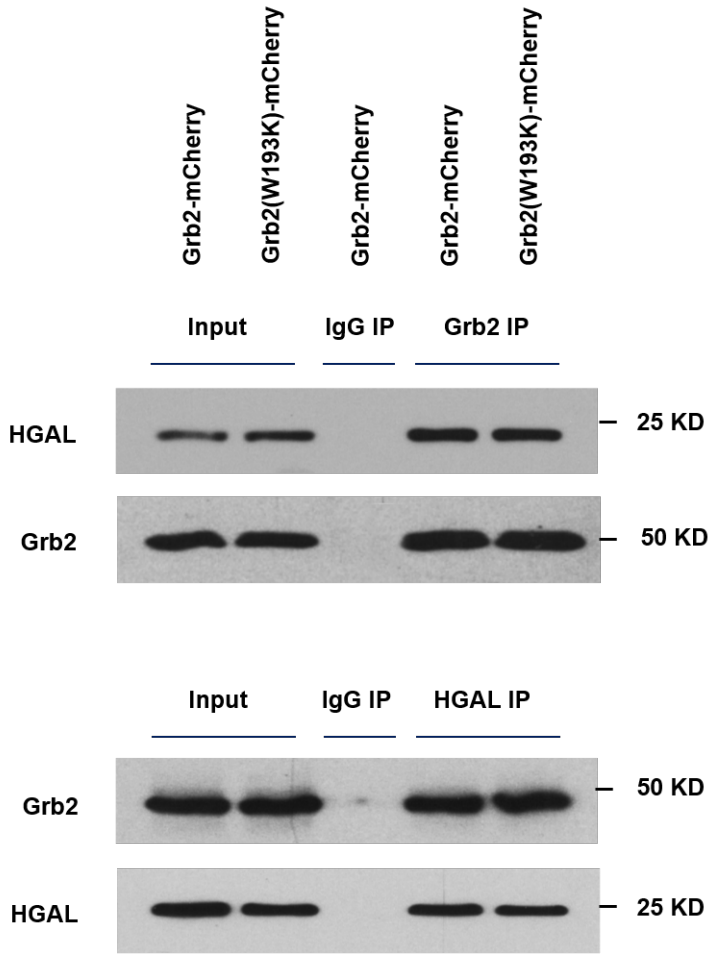


Supplementary Figure 5. Opposite effects of HGAL and Grb2 on BCR-induced intracellular signaling. A) BCR-induced intra- and extracellular Ca^{2+} mobilization of the indicated Raji and Bjab cells recorded by flow cytometry. Lines represent Raji and Bjab cells transfected with control siRNA (green), HGAL siRNA (orange), Grb2 siRNA (blue) and a combination of HGAL and Grb2 siRNAs (red); B) Raji and Bjab cells utilized in (A) were stimulated with anti-human IgM $F(ab')_2$ for 2 and 10 minutes. Whole-cell lysates were prepared, separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies. Actin was blotted to demonstrate equal loading. Densitometry was measured for phosphorylated Syk and BLNK normalized for actin content. The value 1 was assigned to each protein in Raji and Bjab cells, respectively, transfected with control siRNA and stimulated for 2

minutes and 10 minutes respectively. No phosphorylation is observed in unstimulated cells (not shown).

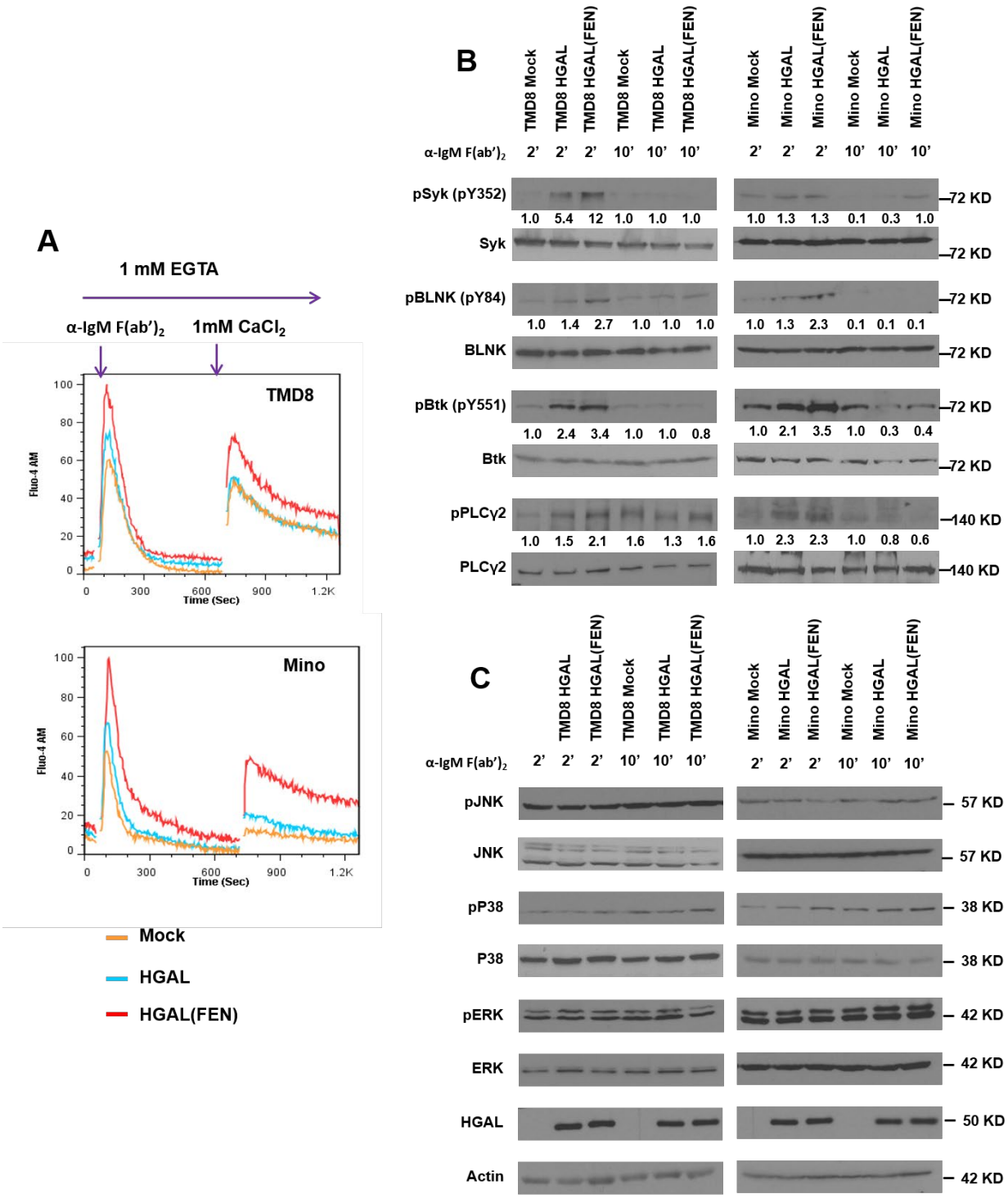


Supplementary Figure 6 Opposite effects of HGAL and Grb2 on BCR-induced intracellular signaling. A) BCR-induced intra- and extracellular Ca^{2+} mobilization of the indicated U2932 cells recorded by flow cytometry. Lines represent U2932 cells transfected with control siRNA (mustard), Grb2 siRNA (pink), plasmid encoding HGAL (blue) and a combination of plasmid encoding HGAL and Grb2 siRNAs (black); B) U2932 cells utilized in (A) were stimulated with anti-human IgM F(ab′)$_2$ for 2 and 10 minutes. Whole-cell lysates were prepared, separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies. Actin was blotted to demonstrate equal loading. Densitometry was measured for phosphorylated Syk and BLNK and normalized for actin content. The value 1 was assigned to each protein in U2932 cells transfected with control siRNA and stimulated for 2 minutes and 10 minutes respectively.



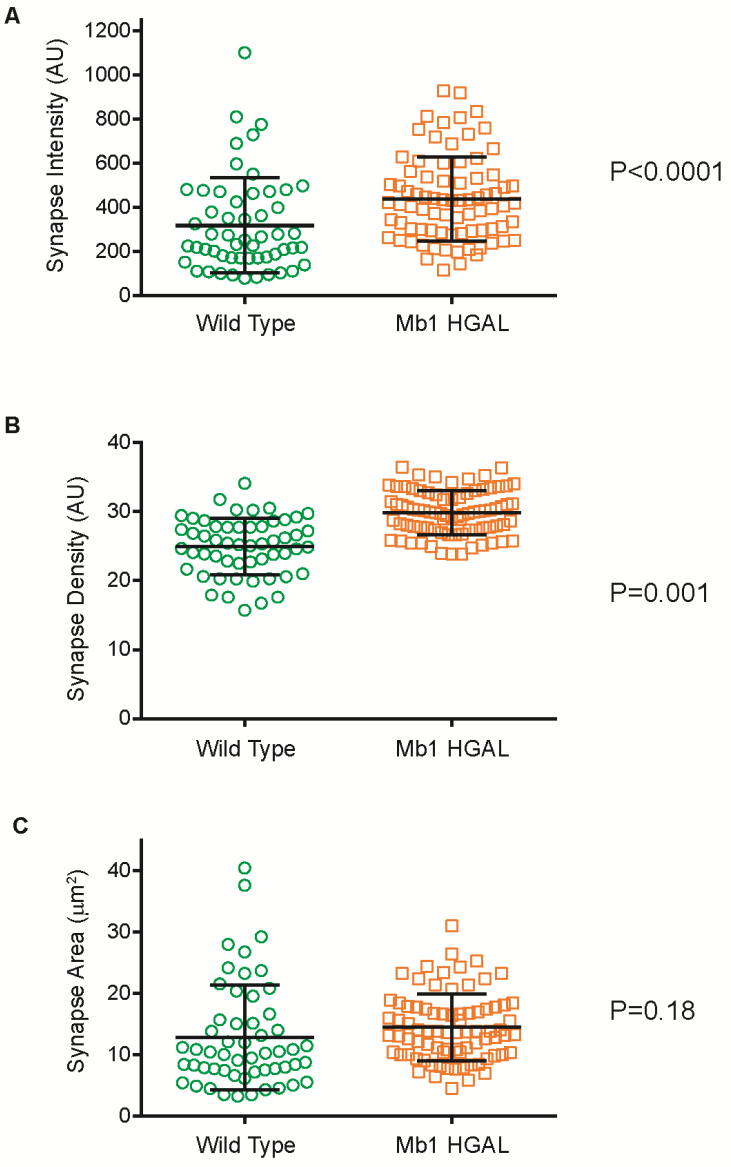
Supplementary Figure 7. Both Grb2 and its mutant Grb2(W193K) interact with HGAL

Reciprocal Co-IP of HGAL and Grb2 or Grb2(W193K) from Raji cells transient transfected with Grb2-mCherry or Grb2(W193K)-mCherry.

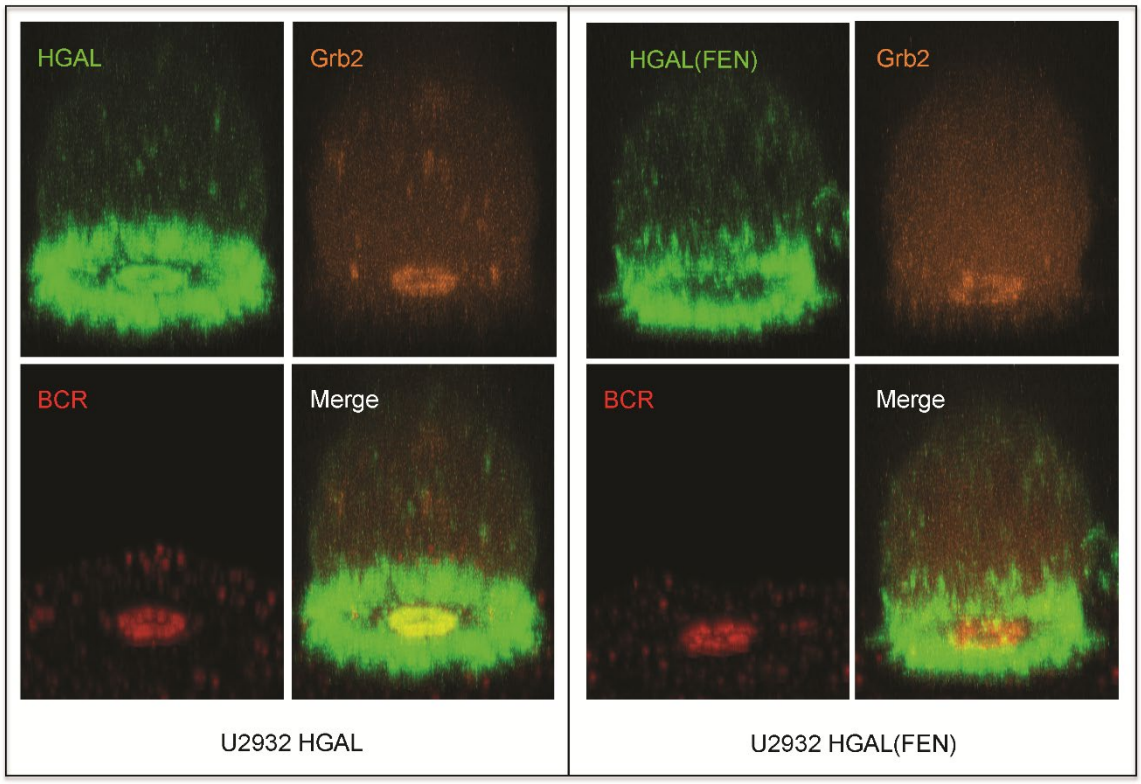


Supplementary Figures 8. HGAL (FEN) mutant not interacting with the Grb2 protein exhibits enhanced effects on BCR signaling compared to the wild-type HGAL in TMD8 and MINO cells. A) BCR-induced intra- and extracellular Ca^{2+} mobilization of the indicated

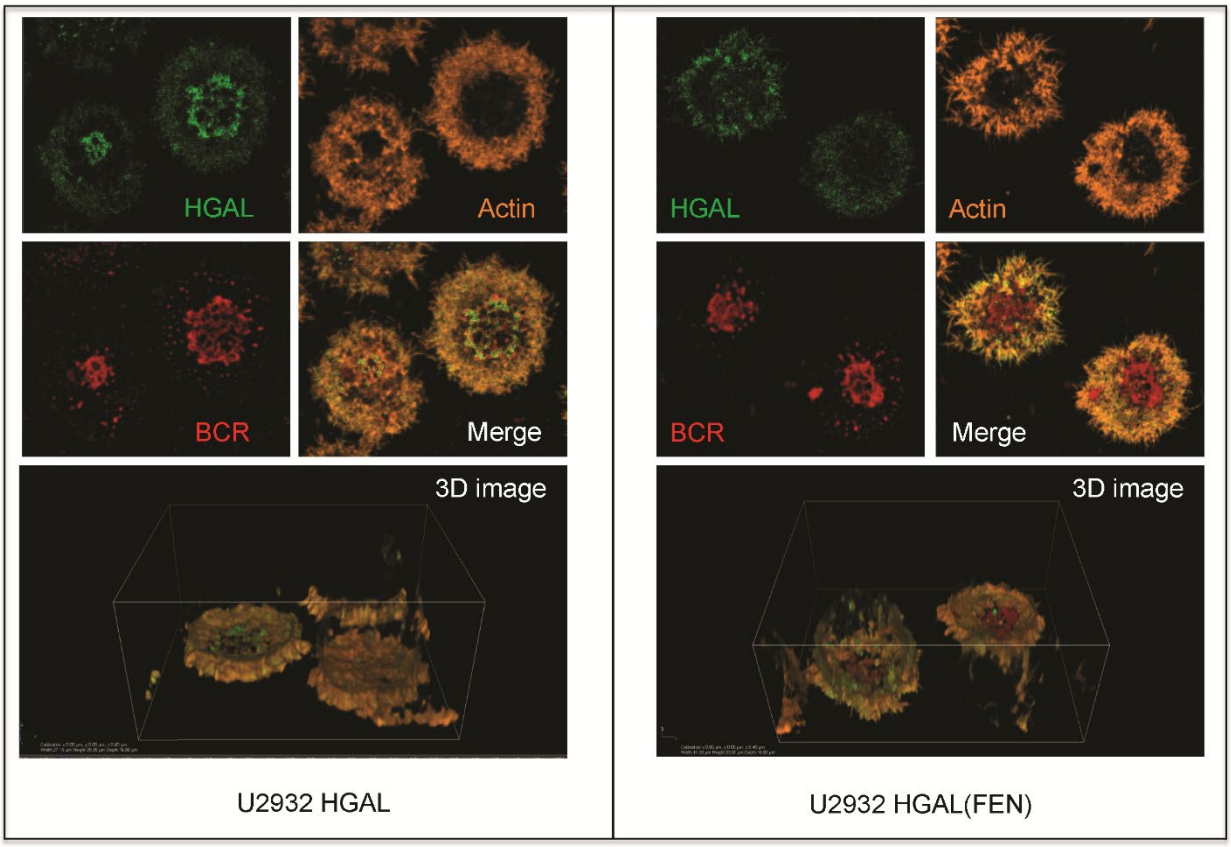
TMD8 and Mino cells recorded by flow cytometry. Lines represent wild-type TMD8 or Mino cells transfected with the following plasmids: mock (orange), HGAL (blue), HGAL (FEN) (red); B and C) TMD8 (B) and Mino (C) cells utilized in (A) were stimulated with anti-human IgM $F(ab')_2$ for 2 and 10 minutes. Whole-cell lysates were prepared, separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with the indicated antibodies. Actin was blotted to demonstrate equal loading. Densitometry was measured for phosphorylated Syk, BLNK, BTK and PLCγ2 normalized for actin content. The value 1 was assigned to for each protein in TMD8 or Mino cells transfected with mock plasmid and stimulated for 2 minutes.



Supplementary Figure 9. HGAL expression increases B cell synapse intensity in *Rosa26*^{HGAL}/Mb1-Cre mice. Spleen B cells were isolated from the *Rosa26*^{HGAL}/Mb1-Cre and littermate wild type mice and were seeded on a lipid bilayer functionalized with anti-IgM F(ab')$_2$ for 5 minutes, Confocal images were acquired to analyze B cell synapse intensity, density and area. A) Synapse intensity; B) Synapse density; C) Synapse area. Experiments were repeated 2 times. 54 B cells in wild-type and 79 B cells in *Rosa26*^{HGAL}/Mb1-Cre mice were used for the analysis. A Student *t*-test was used for statistical analysis.



Supplementary Figure 10. 3D images of U2932 cells transfected with HGAL-GFP and Grb2-mCherry or HGAL(FEN)-GFP and Grb2-mCherry. Cells were seeded on a planar lipid bilayer coated with anti-human IgM $F(ab')_2$ for 30 minutes at 37C, fixed with 4% paraformaldehyde, and imaged by confocal microscopy. Z-stacks were generated from 0.5-μM thick serial sections. 3D images construction and rendering were performed using Nikon's NIS-Elements image analysis.



Supplementary Figure 11. HGAL colocalizes with actin at pSMAC in planar lipid bilayer model. A) Upon antigen stimulation of wild-type HGAL expressing U2932 cells, BCR microclusters formed and eventually accumulated in a central Super Molecular Activation Clusters (cSMAC) at 30 min. HGAL colocalizes with BCR in central SMAC; actin redistributes and colocalizes with HGAL in peripheral SMAC (pSMAC) (top left panel); In HGAL (FEN) mutant expressing cells, the mutant exhibits only pSMAC distribution and colocalizes with actin (top right panel); 3D images are shown in the lower panel.

Supplemental Materials and Methods

Cell transfection, virus packaging, infection and cell sorting

Amaxa Nucleofector Kits (Amaxa Inc, Gaiphersburg, MD) were used for transfection of plasmids or siRNA into lymphoma cell lines according to the manufacturer's instructions, as previously reported by us[1,2]. Briefly, 3×10^6 of lymphoma cells per Nucleofector sample were centrifuged for 5 minutes (min) at 200g and re-suspended in 100µl of the Nucleofector Solution B at room temperature. A total of 2 µg of siRNA or plasmids were added to the cell suspensions and the mixtures were transferred to the Amaxa certified cuvettes. Nucleofection was performed using M013 for Raji and X005 for U2932, Bjab, Mino and TMD8 cells, respectively.

Lentivirus plasmids were packaged into virus particles and used to infect TMD8 and Mino cells. Briefly, Ampho pack 293T cells were cultured on p100 plates in DMEM (10% FBS with antibiotics) to 70-80% confluence. Lentivirus, VPR and VSVG plasmids at a ratio of 4:3:1 (e.g. 2µg:1.5µg:0.5µg) were added and mixed into 500 µl of Opti-MEM® I Reduced Serum Media (Gibco) to which 24 µl of Plus Reagent (Invitrogen) was added. This mixture (Mixture I) was incubated at room temperature for 10-15 minutes and then 12 µl of Lipofectamine 2000 (Invitrogen) in 500 µl of Opti-MEM (Mixture II) was added, mixed and left at room temperature for 20 minutes (Mixture III). Ampho pack 293T cells were washed once with 3 ml of serum free DMEM and then 3ml of Opti-MEM and prepared Mixture III was added to the plates, which were gently shacked to make sure that the mixture is distributed evenly. The plates were incubated for 4-5 hours at 37°C with 5% CO_2. The suspension mixture fluid was then discarded and 10 ml of full DMEM media (10% FBS with antibiotics) was added. The plates were left in the incubator for additional an 48-72 hours and supernatant containing viral particles was collected into 15ml tubes, centrifuged at 8000g for 10 minutes and used for cell infection. To this end, $1x10^6$ cells were spun down, re-suspended with 1 ml of virus particles to which protamine sulfate at a final concentration of 2 µg/ml was added.

After plasmids transfection or virus infection, cells were incubated for 48 hours in humidified 37°C/5% CO_2 incubator before utilization in subsequent experiments. GFP or mCherry positive cells were sorted using the Aria II instrument (BD Biosciences, San Jose, CA).

Western blotting and immunoprecipitation

A total of 5×10^6 cells were homogenized in NP40 buffer (1 x phosphate-buffered saline [PBS], 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 10mM phenylmethylsulfonyl fluoride, 1μg/mL aprotinin, and 100mM sodium orthovanadate) on ice for 15 min. Cell lysates were centrifuged at 14,000*g* for 5 min at 4°C to remove insoluble material. Protein concentration of lysates was determined using Coomassie protein assay reagent (Thermo Scientific, Rockford, IL) and samples were adjusted to equal protein concentrations. For Western blotting, 20 μg of whole-cell lysates were separated on a 12% SDS-PAGE, transferred to polyvinylidene difluoride membranes (BioRad Laboratories, Hercules, CA), blocked with 5% non-fat milk in PBS with 0.1% Tween 20 (PBST) for 1hour, and immunoblotted with the specified primary antibodies for 1 hour at room temperature or at 4 °C overnight. The membranes were washed three times in PBST for 5 min and then immunoblotted with appropriate secondary antibodies conjugated with horseradish peroxidase and visualized by western blotting luminol reagent (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

For immunoprecipitation (IP), 400 μg of protein was precipitated for 1 to 2 hours with the indicated antibody at 4 °C with rotation. Protein G-agarose (Invitrogen, Carlsbad, CA) were added and the mixture was rotated for an additional 1 hour. Precipitated complexes were washed four times in NP-40 buffer, boiled in protein loading buffer (2 x concentrate: 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris HCl (pH 6.8)), separated on a 12% SDS-PAGE gel and immunoblotted with the indicated antibodies.

Phosphatase, [^{32}P]-ATP Lyn and Syk kinase treatment, GST pull down assay and Mass-spectrometry

A total of 1.0x10^7 of Raji cells were lysed with NP40 buffer and cell lysates was incubated with the λ protein phosphatase reaction buffer (50 mM HEPES, 100 mM NaCl, 2 mM DTT, 0.01% Brij, 1$MnCL_2$) in a water bath at 30°C for 30 min. This reaction mixture was used for immunoprecipitation experiments.

Purified recombinant Lyn or Syk kinases and Trx-HGAL recombinant proteins were added to the [^{32}P]-ATP assay cocktail (25 mM MOPS, 12.5 mM β-glycerolphosphate, 25mM $MnCL_2$, 25 mM $MgCl_2$, 5 mM EGTA, 2 mM EDTA, 0.25 mM DTT, 250mM ATP, [32P]-ATP (1 mCi/100 µL). The mixture was incubated in a water bath at 30°C for 30 min. The reaction was terminated by adding SDS sample buffer (final concentration 62.5 mM Tris-HCl pH 6.8, 2.5 % SDS, 0.002 % Bromophenol Blue, 0.7135 M (5%) β-mercaptoethanol, 10 % glycerol) and samples were heated at 95°C for 5 minutes and loaded on 10% SDS PAGE gel. The gel was stained with Gelcode Blue Stain Reagent (Thermo Fisher Scientific Inc, Grand Island, NY) and de-stained with water. The gel was dried and exposed to X-ray film.

For GST pull down assays, kinase or phosphatase treated recombinant Trx-HGAL (1µg) was incubated with GST-Grb2, or GST (1µg, each) for 12 hours at 4°C with gentle rotation in 1.5 ml microcentrifuge tubes containing 300 µl of pre-equilibrated glutathione Sepharose 4B beads in PBS. The mixtures were centrifuged at 600×*g* for 5 min and washed five times with 1ml of PBS. Proteins bound to the beads were eluted with 50 mM Tris–HCl, pH 8.0, 10 mM reduced glutathione and resolved on a 12% SDS polyacrylamide gel. The proteins were subsequently analyzed by Western blot using anti-GST (Santa Cruz Biotech) or anti-HGAL antibodies.

For microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (µLC/MS/MS), precipitated Trx-HGAL, pre-treated with Lyn or Syk kinases, was run on 10% SDS-PAGE gel and detected by Coomassie brilliant blue staining. Bands of Trx-HGAL size

were cut out and submitted for analysis to the Taplin Biological Mass Spectrometry Facility, Harvard Medical School (Boston, MA).

Preparation of human Grb2 SH2 domain (residues 56-155)

The SH2 domain (residues 56-155) of human Grb2 was cloned into pET30 bacterial expression vector with an N-terminal His-tag using Novagen LIC technology. The recombinant protein was subsequently expressed in Escherichia coli BL21*(DE3) bacterial strain (Invitrogen) and purified on a Ni-NTA affinity column (Invitrogen, Carlsbad, CA) using standard procedures. Briefly, bacterial cells were grown at 20°C in Terrific Broth to 1.0 OD_{600} (optical density unit at 600nm) prior to induction with 0.5mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (EMD, Billerica, MA). The bacterial culture was further grown overnight at 20°C and the cells were subsequently harvested and disrupted using a BeadBeater.(Biospec, Bartlesville, OK). After separation of cell debris using high-speed centrifugation, the cell lysate was loaded onto a Ni-NTA column and washed extensively with 20mM imidazole to remove non-specific binding of bacterial proteins to the column. The protein was subsequently eluted with 200mM imidazole and dialyzed against an appropriate buffer to remove excess imidazole. Further treatment on a Hiload Superdex 200 size-exclusion chromatography (SEC) column (GE, Milwaukee, WI) coupled in-line with GE Akta FPLC system (GE, Milwaukee, WI) led to purification of SH2 domain to apparent homogeneity, as judged by SDS-PAGE analysis. The final yield was typically between 10-20 mg proteins of apparent homogeneity per liter of bacterial culture. Protein concentration was determined spectrophotometrically on the basis of an extinction coefficient of 13,980 $M^{-1}cm^{-1}$ calculated using the online software ProtParam at ExPasy Server (http://web.expasy.org/protparam/)[3]

Peptide synthesis

12-mer phosphorylated (pYEN) and unphosphorylated (YEN) peptides derived from the Grb2 binding site in HGAL (residues 103-114) were commercially obtained from GenScript Corporation.

The amino acid sequence of these peptides is as follows: pYEN 103-AEEYpYENVPCKA-104 and YEN 103-AEEYYENVPCKA-104. The peptide concentrations were measured gravimetrically.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were performed on a Microcal VP-ITC instrument.(Northampton, MA) Briefly, the SH2 domain of Grb2 and HGAL peptides were dialyzed in 50mM Sodium phosphate, 1mM EDTA and 5mM β-mercaptoethanol at pH 7.0. All experiments were initiated by injecting 25 x 10μl aliquots of 1-2 mM of each HGAL peptide from the syringe into the calorimetric cell containing 1.46ml of 50-100 μM of SH2 domain of Grb2 at 25 °C. The change in thermal power as a function of each injection was automatically recorded using the ORIGIN software[4] (Originlab, Northampton, MA) and the raw data were further processed to yield binding isotherms of heat release per injection as a function of molar ratio of each HGAL peptide to SH2 domain. The heats of mixing and dilution were subtracted from the heat of binding per injection by carrying out a control experiment in which the same buffer in the calorimetric cell was titrated against each HGAL peptide in an identical manner. To extract the equilibrium dissociation constant (K_d) and the enthalpic change (ΔH) associated with binding, the ITC isotherms were iteratively fit to a one-site model by non-linear least squares regression analysis using the integrated ORIGIN software[4]. The free energy change (ΔG) upon peptide binding was calculated from the relationship: $\Delta G = RT\ln K_d$ where R is the universal molar gas constant (1.99 cal/K/mol) and T is the absolute temperature. The entropic contribution ($T\Delta S$) to the free energy of binding was calculated from the relationship: $T\Delta S = \Delta H - \Delta G$ where ΔH and ΔG are as defined above.

Calcium mobilization measurement

Human lymphoma cells ($5x10^6$) were twice washed with RPMI 1640 medium and stained with 2 μM of Fluo-4 AM (Invitrogen, Carlsbad, CA) for 30 min at 37°C. The cells were again washed twice with the RPMI 1640 medium and stimulated with goat $F(ab')_2$ anti-human IgM.

Ca^{2+} mobilization was measured in real-time by LSR-Fortessa-HTS (BD Biosciences, San Jose, CA) with a laser tuned at 488 nm. Release of intracellular Ca^{2+} was measured for 6 minutes in the presence of 1mM EGTA. Subsequently, extracellular Ca^{2+} was restored to 1mM $CaCl_2$ in order to monitor Ca^{2+} entry across the plasma membrane. Raw data files were transferred to FlowJo 7.6.4 software (Ashland, OR) and are presented as a median and in comparative overlay analyses.

Luciferase reporter assay

U2932 cells were co-transfected in triplicate with firefly luciferase reporter plasmid pNFκB-Luc (4 µg) and constitutively active *Renilla reniformis* luciferase-producing vector pRL-TK (20 ng; Promega, Madison, WI) using the Amaxa Nucleofector Kit. Firefly (*Photinus pyralis*) and *R reniformis* luciferase activities were detected with the Dual Luciferase assay kit according to manufacturer's instructions (Promega, Madison, WI). Data are presented as an average ± standard error of the mean.

Syk activity assay

Syk kinase activity was measured as we previous reported[5] using Omnia kinase assay kit according to the manufacturer's instructions (Invitrogen, Carisbad, CA). Briefly, Syk was immunoprecipitated from Raji cells or Raji cells cross linked with goat $F(ab')_2$ anti-human IgM antibodies for 5 min and divided into equal fractions, to which purified HGAL protein, Grb2 protein, or both were added. Immunoprecipitates with control antibody and beads only, purified HGAL alone, Grb2 alone and water alone were used as negative controls. Following addition of tyrosine reaction buffer, the SpectraMax M5 (Molecular Devices Inc, Sunnyvale, CA) instrument was used to record every 30 seconds for 40 minutes fluorescence in kinetic mode at 30°C with excitation and emission wavelengths of 360 and 485 nm, respectively.

Confocal microscopy measurements of synapse formation

To investigate the contribution of HGAL to B cell synapse formation, we characterized the rate of BCR cluster formation for the different lymphoma cell lines. In these experiments, cells were introduced to the anti-IgM $F(ab')_2$ functionalized planar membrane formed within a paralleled flow chamber. The cells were in contact with the planar membrane within seconds after they were injected into the thin (40 μm) flow chamber. Cells were fixed with 4% paraformaldehyde (Thermo Scientific, Waltham, MA) at specified times to terminate BCR redistribution. Confocal images of the cells coupled to the planar bilayer were acquired on a Nikon A1R scanning laser confocal microscope with a 60X oil immersion objective (N.A. 1.4) at an acquisition rate of 2 seconds per frame. Maximum projection images were derived from five 0.5 μm spaced sectional confocal images acquired in vicinity of the glass coverslip surface and were analyzed by NIH ImageJ to determine the changes in BCR cluster formation by lymphoma cells following contact with the anti-IgM $F(ab')_2$ functionalized planar membrane. There were 5 to 25 cells in each of the acquired images. Analyses were carried out on a minimum of 25 cells for each set of measurements. BCR clusters formed by the individual cells were identified manually by the accumulation of fluorescence signals from Alexa Fluor 555 labeled streptavidin used to couple the $F(ab')_2$ to planar membrane. From within the manually selected Region-of-interest (ROI), a top 25% fluorescence intensity cutoff was used to identify the BCR clusters. The fluorescence intensity of BCR clusters was normalized using the background fluorescence of the planar membrane. Two-way analysis of variance (ANOVA) was carried out in Graphpad Prism to determine whether differences in measurements acquired with different U2932 cell lines are statistically significant.

Animal Studies

All animal work has been conducted in accordance with national and international guidelines on animal care and was approved by the Bioethics Committee of University of Salamanca and by the Bioethics Subcommittee of Consejo Superior de Investigaciones

Cientificas (CSIC). The Rosa26-Lmo2 vector was generated as follows: The HGAL cDNA clone was subcloned into the Rosa26UA plasmid using the AscI site. This plasmid contained the Loxp-pgk-Neo-tPA-Loxp- AscI-FheI-IRES-EGFP within the Rosa26 homology arms, and the final targeting construct contained Loxp-pgk-Neo-tPA-Loxp-HGAL-IRES-EGFP. The G4 mouse ES cell line, a generous gift of Dr. Andras Nagy and Dr. Marina Gertsenstein, was used to modify the Rosa26 locus by homologous recombination at the Servicio de Transgénesis CNB-CBMSO UAM/CSIC (Madrid), where chimeric mice were also generated. Heterozygous Rosa26-HGAL animals were obtained by mating chimera with C57BL/6J females. The Rosa26-HGAL mice were bred to Mb1-Cre mice to generate Rosa26-HGAL+Mb1-Cre mice. Spleen B cells were isolated from the *Rosa26*^{HGAL}/Mb1-Cre and littermate wild-type mice, as previously reported by us[5]m and were seeded on a lipid bilayer functionalized with anti-IgM F(ab')$_2$ for 5 minutes. Synapse intensity and density of B cell synapse were determined by analysis of acquired confocal images using NIH ImageJ. Experiments were repeated 2 times. 100 B cells in wild-type and 150 B cells in *Rosa26*^{HGAL}/Mb1-Cre mice were used for the analysis. t-test was used for statistical analysis.

Statistical analysis and densitometry measurement

A 2-tailed Student *t* test was used, and $p < .05$ was considered statistically significant. Densitometry analyses were done using Image J (National Institutes of Health).

References:

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Supplemental Movies:

1. Three dimensional distribution of HGAL, Grb2 and BCR in cells expressing wilt-type HGAL.
2. Three dimensional distribution of HGAL, Grb2 and BCR in cells expressing HGAL(FEN) mutant.
3. Kinetics of BCR relocalization following BCR stimulation in cells expressing wilt-type HGAL.
4. Kinetics of BCR relocalization following BCR stimulation in cells expressing HGAL(FEN) mutant.