Supporting Information

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SI Materials and Methods

Cell Culture, BCR Stimulation, and Cell Lysis. All cell lines were cultured in RPMI medium (Invitrogen) supplemented with 10–20% (vol/vol) heat-inactivated FBS (Invitrogen), penicillin/streptomycin (Invitrogen), and L-glutamine (Invitrogen) at 37 °C and 5% CO_2.

SILAC labeling of DG75 and Daudi cells was performed by culturing the cells in SILAC-RPMI medium 1640 devoid of arginine and lysine (Pierce) supplemented with 10% (vol/vol) dialyzed FCS (PAA) and the respective SILAC amino acids (all from Cambridge Isotopes). "Light" (L) SILAC medium contained [$^{12}C_6{}^{14}N_4$]-L-arginine and [$^{12}C_6{}^{14}N_2$]-L-lysine; "intermediate" (M) SILAC medium contained [$^{13}C_6{}^{14}N_4$]-L-arginine and 4,4,5,5-d_4-L-lysine; "heavy" (H) SILAC medium contained [$^{13}C_6{}^{15}N_4$]-L-arginine and [$^{13}C_6{}^{15}N_2$]-L-lysine.

For BCR stimulation, DG75 and Daudi cells were starved in RPMI without supplements for 15 min, and subsequently BCR were stimulated for the indicated durations with 10 μg/mL $F(ab)_2$ anti-human IgM antibodies (Dianova) at 37 °C. For phosphoproteomic and protein expression profiling of DG75 cells, the L-labeled batches were left untreated whereas the M-labeled batches were BCR-stimulated for 2 min and the H-labeled batches for 5 min. In a second experimental part, the L-batches were again left untreated whereas the M- and H-batches were stimulated for 10 and 20 min, respectively. L-labeled Daudi batches were left unstimulated (0 min) whereas M-labeled cells were BCR-stimulated for 2 min and the H-labeled cells for 10 min. For analysis of tonic BCR signaling, L-batches were treated with shRNAs targeting CD79a or DMSO and H-batches were treated with either unspecific shRNAs or the SYK inhibitor PRT-062607.

Cell lysis for SILAC-labeled and label-free cells was performed as described in Oellerich et al. (41), except that the phosphatase inhibitor mixtures 2 and 3 (Sigma) were added according to the manufacturer's instructions. Cell lysis for proteomic profiling of tyrosine phosphorylation was performed according to the manufacturer's instructions by using a urea-based lysis buffer (PTMScan Kit; Cell Signaling Technology).

Antibodies, Compounds, Plasmids, and Reagents. Antibodies against the following proteins were used: SLP65, pSLP65, PLCγ2, pPLCγ2, ERK, pERK, SYK, pSYK, BTK, pBTK, CD79a, pCD79a, CBL, pCBL, ACTN4, actin (all from Cell Signaling Technology), pTyr (4G10; Millipore) and ARFGEF2 (Abcam). Tyrosine-phosphorylated peptides were enriched by using the PTMscan Phosphotyrosine Rabbit mAb P-Tyr-1000 kit (Cell Signaling Technology).

PRT-062607 was obtained from Selleck Chemicals, dissolved in DMSO at 10 mM, used in a final concentration of 250 nM, and stored at –80 °C.

For lentiviral knockdown of CD79a, ARFGEF2, and ACTN4, we used the inducible retroviral vector described by Fellmann et al. (43).

shRNA-Mediated Knockdown Experiments. Cells were transduced with retroviral vectors coexpressing inducible unspecific shRNAs and GFP or shRNAs targeting the 3′ UTR of CD79a and GFP. Transduced cells were enriched by FACS sorting so that ∼50% of cells were GFP-positive. At this point, shRNA expression was induced using 1 μg/mL doxycycline, and the cell fraction positive for GFP was monitored over time by flow cytometry as described in Schmitz et al. (4). The same experimental approach was performed with shRNAs targeting ARFGEF2 and ACTN4.

Immunohistochemistry. Each tissue sample was fixed in 4% (vol/vol) buffered formalin and embedded in paraffin. Informed consent from all patients at the University Medical Center Göttingen and a corresponding ethics vote was obtained, which allows for the utilization of biomaterial for biobanking and scientific purposes.

For the immunohistochemical analysis, 2-μm tissue sections were incubated in EnVision Flex Target Retrieval Solution, pH low for ACTN4 and ARFEF2 staining (both from Dako), followed by incubation with primary antibodies against ACTN4 diluted at 1:1,000 (Atlas Antibodies) or against ARFGEF2 (Abcam) for 40 min at room temperature. Polymeric secondary antibodies coupled to horseradish peroxidase (EnVision Flex+; Dako) and DAB (Dako) were applied to visualize the sites of immunoprecipitations. Tissue samples were analyzed by light microscopy after counterstaining with Meyer's hematoxylin.

Two independent pathologists evaluated all tissue sections using a three-stage staining score: 0, negative; 1, moderate intensity of staining; 2, strong intensity of staining.

Apoptosis Studies. Daudi and BL41 cells were treated with doxycycline for 4 d to express shRNAs specific for (*i*) ARFGEF2 or (*ii*) ACTN4 or unspecific shRNAs. Annexin V APC/7-AAD staining was performed with the Annexin V-APC/7-AAD Apoptosis Detection Kit (Biolegend). Cells were analyzed by flow cytometry with a FACSCanto II flow cytometer (Becton Dickinson).

Flow Cytometry. For detection of BCR cell surface expression, 10^6 B cells were fixed in 2% (vol/vol) formaldehyde. Afterward, Fc receptor blocking reagent (Miltenyi Biotec) was applied according to the manufacturer's instructions before staining of IgM-BCR by APC-labeled anti-IgM (eBioscience). The APC signal was detected by flow cytometry (LSR Fortessa; BD Biosciences).

Pathway Enrichment Analysis and Functional Annotation. Pathway enrichment analysis was performed using R 3.2.1 and the R/Bioconductor package ReactomePA v1.14.4 [Yu and He (44); bioconductor.org/packages/release/bioc/html/ReactomePA.html]. *P* values were adjusted for multiple comparisons using the Benjamini–Hochberg procedure.

Gene ontology functional annotations were retrieved using the mygene package (version 1.6.0). Only terms supported by experimental evidence codes (EXP, IDA, IPI, IMP, IGI, and IEP) were considered for the analysis.

Full Proteome Analysis. For protein expression analysis, light-, medium-, and heavy-labeled DG75 cell lysates were mixed in a 1:1:1 ratio. A total of 150 μg of protein was separated by SDS/PAGE using precast Bis-Tris minigels (NuPAGE Novex 4–12%; Life Technologies) and visualized by staining with Coomassie Brilliant Blue (Serva). Each lane was cut into 23 slices, reduced with DTT (Sigma-Aldrich), and alkylated with iodoacetamide (IAM; Sigma-Aldrich), digested in-gel with trypsin (Serva), extracted, and analyzed by mass spectrometry.

Protein Digestion and Phosphopeptide Enrichment for SILAC-Based Quantitative Global Phosphoproteomic Analysis. For investigation of phosphorylation dynamics, equal amounts of SILAC-labeled cell lysates were mixed, treated with Benzonase (Novagen) for 1 h at 37 °C, and precipitated with acetone. The precipitate was dissolved in 1% RapiGest Surfactant (Waters) in 25 mM ammonium bicarbonate (Sigma-Aldrich), reduced with 10 mM DTT for 1 h at 65 °C, and alkylated by IAM at a final concentration of 20 mM for 1 h at 37 °C. Proteins were digested with

trypsin (Promega) at a 1:20–1:50 (wt/wt) trypsin:protein ratio in the presence of 0.1% RapiGest at 37 °C overnight. The digest was acidified to 1% formic acid and cleared of precipitated material by centrifugation at maximum speed for 30 min; the supernatant was then evaporated to dryness in a SpeedVac concentrator (Thermo Scientific). For samples of DG75 BCR stimulations, peptides were subsequently fractionated by strong cation exchange (SCX) chromatography (BioBasic SCX 50 × 2.1 mm; Thermo Fisher) on an FPLC system (SMART; Pharmacia) with a salt concentration gradient. Buffer A contained 10 mM ammonium formate (Sigma-Aldrich) and buffer B 500 mM ammonium formate, both at pH 2.65 and containing 30% (vol/vol) acetonitrile (ACN). Twenty fractions were collected over a 50-min gradient at a flow rate of 100 $\mu L \cdot min^{-1}$. Fractions 1–12 were dried, resuspended in a solution of 200 mg/mL 2,5-dihydroxybenzoic acid (DHB; Sigma-Aldrich) in 80% (vol/vol) ACN and 5% (vol/vol) TFA, and loaded on in-house made TiO_2 (GL Science) spin columns as described previously (1). TiO_2 beads were washed three times with 200 $mg \cdot mL^{-1}$ DHB in 80% (vol/vol) ACN, 5% (vol/vol) TFA, and five times with 80% (vol/vol) ACN, 5% (vol/vol) TFA. Phosphopeptides were eluted with 0.3 M NH_4OH (pH ≥ 10.5), dried in a SpeedVac concentrator, and analyzed by mass spectrometry.

For all other samples, phosphopeptides were enriched directly from cell lysates before prefractionation. In brief, digested peptides were resuspended in incubation buffer [80% (vol/vol) ACN, 5% (vol/vol) TFA, 5% (vol/vol) glycerol] and incubated with TiO_2 beads (10 μm; GL Science) at a 1:8 peptide:bead ratio (wt/wt) with end-over-end rotation at 37 °C for 20 min. The peptide concentration was maintained at around 2 mg/mL during the incubation. After incubation, all TiO_2 beads were loaded onto an empty spin column (5 μm frit; Hoefer Inc.) and washed three times each with incubation buffer, 80% (vol/vol) ACN, 5% (vol/vol) TFA and 60% (vol/vol) ACN, 0.1% FA. Phosphopeptides were then eluted with 1 M 2,2'-(propane-1,3-diyldiimino)bis[2-(hydroxymethyl)propane-1,3-diol] in 0.5 M NH_4OH (pH ≥10.5) and acidified immediately with 10% (vol/vol) FA. The eluate was desalted on a C18 spin column and fractionated using basic pH reverse phase chromatography (XBridge C18 3.5 μm, 150 × 1.0 mm; Waters).

Phosphopeptide Enrichment for pYome Analysis. Antibody-based enrichment for tyrosine-phosphorylated peptides was done with the Phospho-Tyrosine (P-Tyr-1000) Rabbit mAb Kit (Cell Signaling Technology). SILAC-labeled DG75 and Daudi cells were lysed in 8 M urea (2 M thiourea) buffer, mixed 1:1:1 (or 1:1) according to cell number/protein concentration and prepared as described in the manufacturer's instructions (Cell Signaling Technology).

LC-MS/MS Analysis and Data Processing. Peptide fractions of the global phospho-proteome and technical replicates of the pYome of BCR-stimulated DG75 cells were analyzed on a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ Orbitrap Velos; Thermo Scientific) coupled to a nanoflow liquid chromatography system (Agilent 1100 series; Agilent). Samples were preconcentrated and desalted on a trap column (ReproSil-Pur 120 C18-AQ, 5 μm; 20 × 0.100 mm, packed in-house; Dr. Maisch GmbH) at 5 $\mu L/min^{-1}$ in loading buffer [2% (vol/vol) acetonitrile, 0.1% formic acid in water]. Peptides were separated on an analytical column [ReproSil-Pur 120 C18-AQ, 3 μm, (Dr. Maisch GmbH); 200 × 0.075 mm, packed in-house into a PF360-75-15-N picofrit capillary, (New Objective)] using a 105-min linear gradient from 5% to 40% (vol/vol) acetonitrile containing 0.1% formic acid at a flow rate of 300 $nL \cdot min^{-1}$. The mass spectrometer was operated in data-dependent acquisition (DDA) mode, automatically switching between MS and MS/MS acquisitions. Survey spectra from *m/z* 350–1,600 were acquired in the Orbitrap at a resolution setting of 60,000 FWHM at *m/z* 400. Product-ion spectra of the 10 most abundant precursors with charge states 2+ to 5+ per cycle were acquired using collision-induced dissociation (CID) in the linear ion trap at a normalized collision energy (NCE) of 35% and an isolation width of 2 *m/z*. Automatic gain control (AGC) target values and maximum injection times for MS and MS/MS were 1×10^6 in 500 ms and 5×10^4 in 100 ms, respectively.

DG75 pYome samples were measured on an EASY n-LC 1000 (Thermo Scientific) coupled to a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive; Thermo Scientific). The samples were preconcentrated and desalted on a trap column (20 × 0.1 mm, ReproSil-Pur 120 C18-AQ, 5 μm, packed in-house; Dr. Maisch GmbH) at 5 $\mu L \cdot min^{-1}$ in loading buffer [2% (vol/vol) ACN, 0.1% FA]. Peptides were separated on an analytical column (200 × 0.075 mm, ReproSil-Pur 120 C18-AQ, 3 μm, packed in-house; Dr. Maisch GmbH) using an 80-min linear gradient from 4% to 34% buffer B [95% (vol/vol) ACN, 0.1% FA] versus a decreasing concentration of buffer A (0.1% FA) at a flow rate of 300 nL/min. The Q Exactive was operated in a DDA selecting the top 12 most abundant precursors for higher energy collisional dissociation (HCD) in the collision cell with an isolation width of 2 *m/z* and an NCE setting of 28%. Survey spectra from *m/z* 350–1600 were acquired with an MS resolution setting of 70,000 FWHM at *m/z* 200 and product ion spectra with a MS/MS resolution of 17,500 in the Orbitrap. AGC target values and maximum injection times for MS and MS/MS were set to 1×10^6 in 60 ms and 2×10^5 in 60 ms, respectively.

For all other samples, phosphopeptides were analyzed on a Q Exactive HF mass spectrometer (Thermo Fisher) coupled with an Ultimate 3000 RSLC (Dionex). Phosphopeptides were separated on a self-made capillary column (ReproSil-Pur 120 C18-AQ, 3 μm, 350 × 0.075 mm; Dr. Maisch GmbH) with a 70-min linear gradient of 2–40% buffer B [80% (vol/vol) ACN, 0.1% FA] and versus buffer A (0.1% FA in water) at a constant flow rate of 300 $nL \cdot min^{-1}$. The mass spectrometer was operated in DDA mode using a top 20 method with a survey scan resolution setting of 120,000 FWHM and an MS/MS resolution setting of 35,000 FWHM at 200 *m/z*. HCD was performed with an NCE setting of 28% and an isolation width of 1.4 *m/z*. AGC target values and maximum ion injection times for MS and MS/MS were set 1×10^6 in 40 ms and 1×10^5 in 64 ms, respectively.

All raw files were processed using MaxQuant software (v1.5.2.8, MPI for Biochemistry) (45). MS/MS spectra were searched against a UniProtKB/Swiss-Prot human database containing 88,993 protein entries (downloaded July 2014) supplemented with 245 frequently observed contaminants via the Andromeda search engine (46). Precursor and fragment ion mass tolerances were set to 6 and 20 ppm after initial recalibration, respectively. STY phosphorylation, protein N-terminal acetylation, and methionine oxidation were allowed as variable modifications. Cysteine carbamidomethylation was defined as a fixed modification. Minimal peptide length was set to seven amino acids, with a maximum of two missed cleavages. The false discovery rate (FDR) was set to 1% on both the peptide and the protein level using a forward-and-reverse concatenated decoy database approach.

For SILAC quantitation, multiplicity was set to two or three for double (Lys+0/Arg+0, Lys+8/Arg+10) or triple (Lys+0/Arg+0, Lys+4/Arg+6, Lys+8/Arg+10) labeling, respectively. At least two ratio counts were required for peptide quantitation. Both the "match between runs" and "re-quantify" options of MaxQuant were enabled.

All of the raw files and MaxQuant search results have been deposited to the ProteomeXchange Consortium (proteomecentral.proteomexchange.org/cgi/GetDataset) via the PRIDE partner repository (47) with the dataset identifier PXD003492.

Data Evaluation and Bioinformatics. Data analysis was conducted with Perseus software (v1.5.2.4, MPI for Biochemistry). After removing decoy and contaminant entries, identified phosphosites

with a localization probability lower than <0.75 were filtered out. SILAC ratios and peptide intensities were logarithmized (log2 and log10, respectively). For quality control, multiscatter plots displaying Pearson's correlation coefficients were generated (Fig. S2). P-sites with SILAC ratios showing Z scores >2 or <−2 in at least one time point in each dataset were defined as significantly regulated. Unsupervised clustering analysis (row clustering with Euclidean distance, average linkage, preprocessed with *k*-means, number of clusters 300) was performed in Perseus using only regulated common p-sites after normalization as described by Deshmukh et al. (48).

Additional network visualization of quantitative phosphoproteomic datasets was performed with the Cytoscape (v3.2.1) application PhosphoPath (v1.1) (cytoscape.org) [Shannon et al. (49)]. Protein–protein interaction information was retrieved from the BioGRID database (www.thebiogrid.org), and kinase–substrate information from PhosphoSitePlus (www.phosphosite.org/homeAction.action). The quantitative networks were prepared as described by Raaijmakers et al. (50).

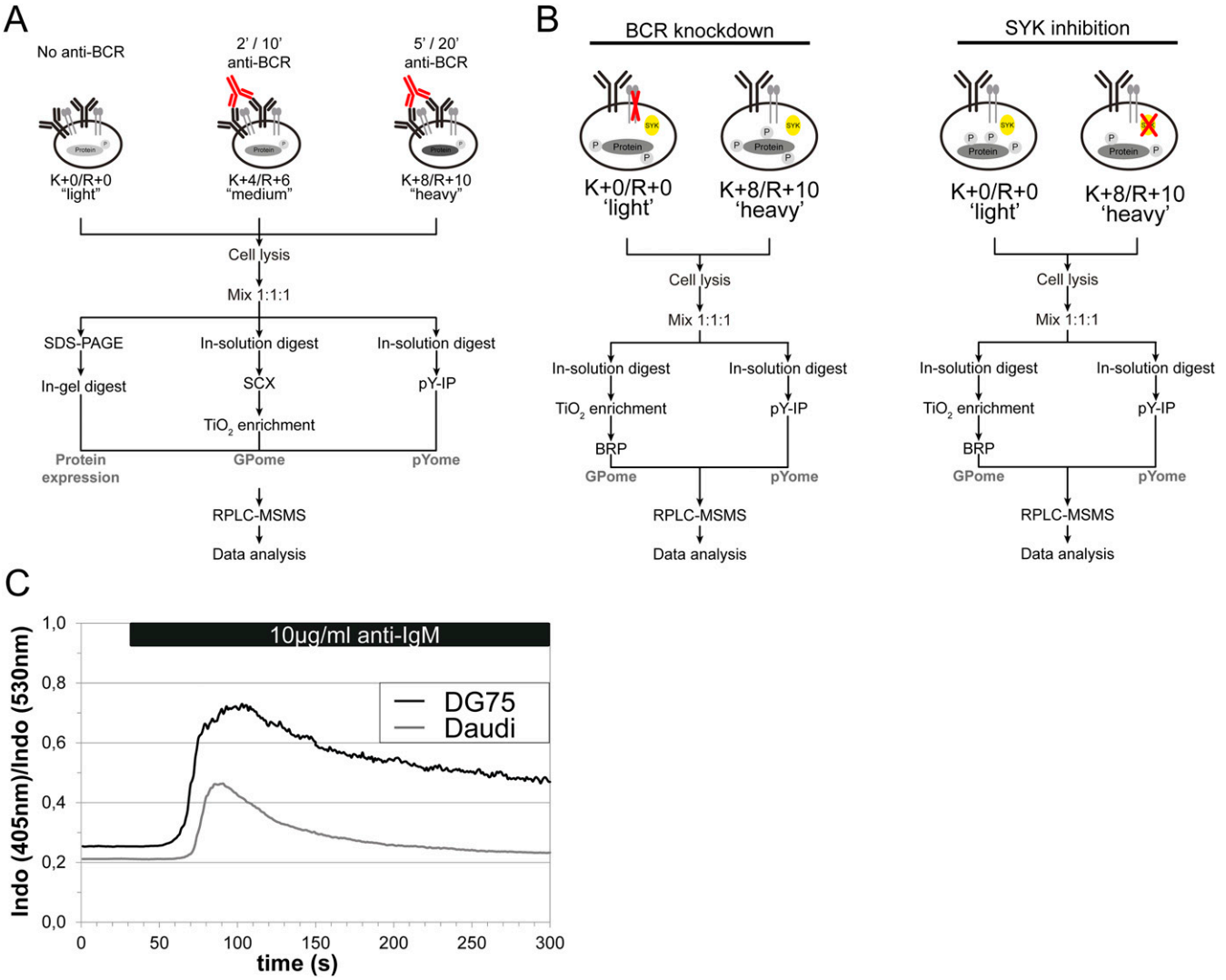
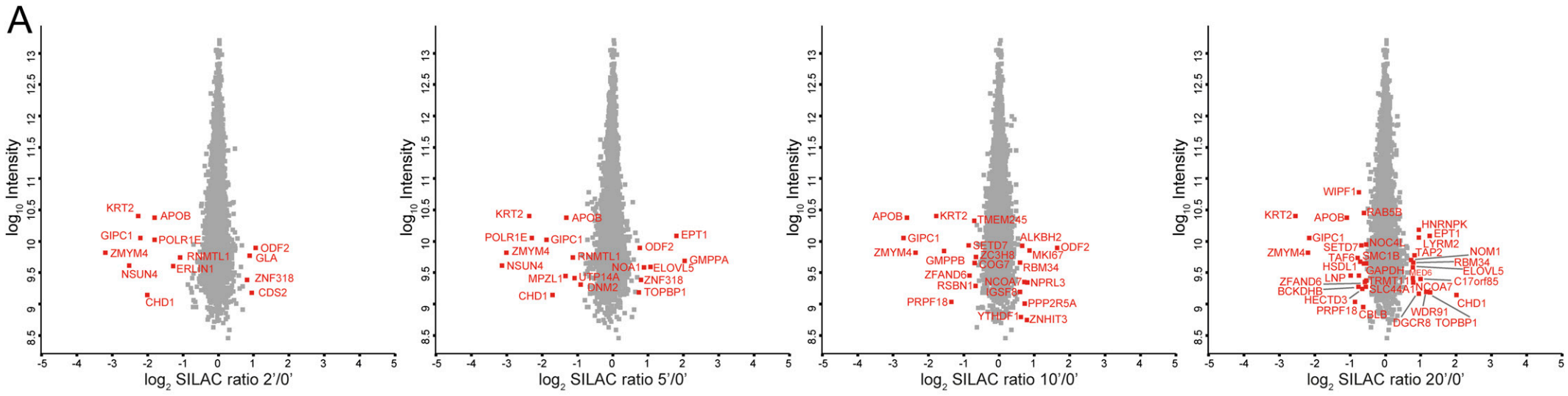


Fig. S1. (*A*) Schematic representation of a 3-plex SILAC approach for profiling phosphorylation dynamics in resting and BCR-stimulated DG75 cells. DG75 cells were cultured in SILAC medium as indicated and were left untreated, or were BCR-stimulated, for 2, 5, 10, or 20 min. Daudi cells were stimulated for 2 and 10 min. Lysates were mixed in a 1:1:1 ratio and digested with trypsin. Resulting phosphopeptides were enriched by either SCX/TiO_2 chromatography (global phosphoproteome analysis; GPome) or phosphotyrosine immunoprecipitation (pY-IP; pYome analysis), and analyzed by LC-MS/MS. For analysis of protein expression levels, proteins were separated by 1D-PAGE, digested with trypsin, and analyzed by LC-MS/MS (see *SI Materials and Methods* for details). (*B*) Schematic representation of a 2-plex SILAC approach for profiling phosphorylation changes upon inducible CD79a knockdown or upon SYK inhibition. DG75 cells were cultured in SILAC medium and treated as indicated. Lysates were processed as described in *A*. (*C*) DG75 and Daudi cells were loaded with the ratiometric Ca^{2+}-chelator INDO-1-AM and subjected to BCR-induced Ca^{2+} flux analysis by flow cytometry.



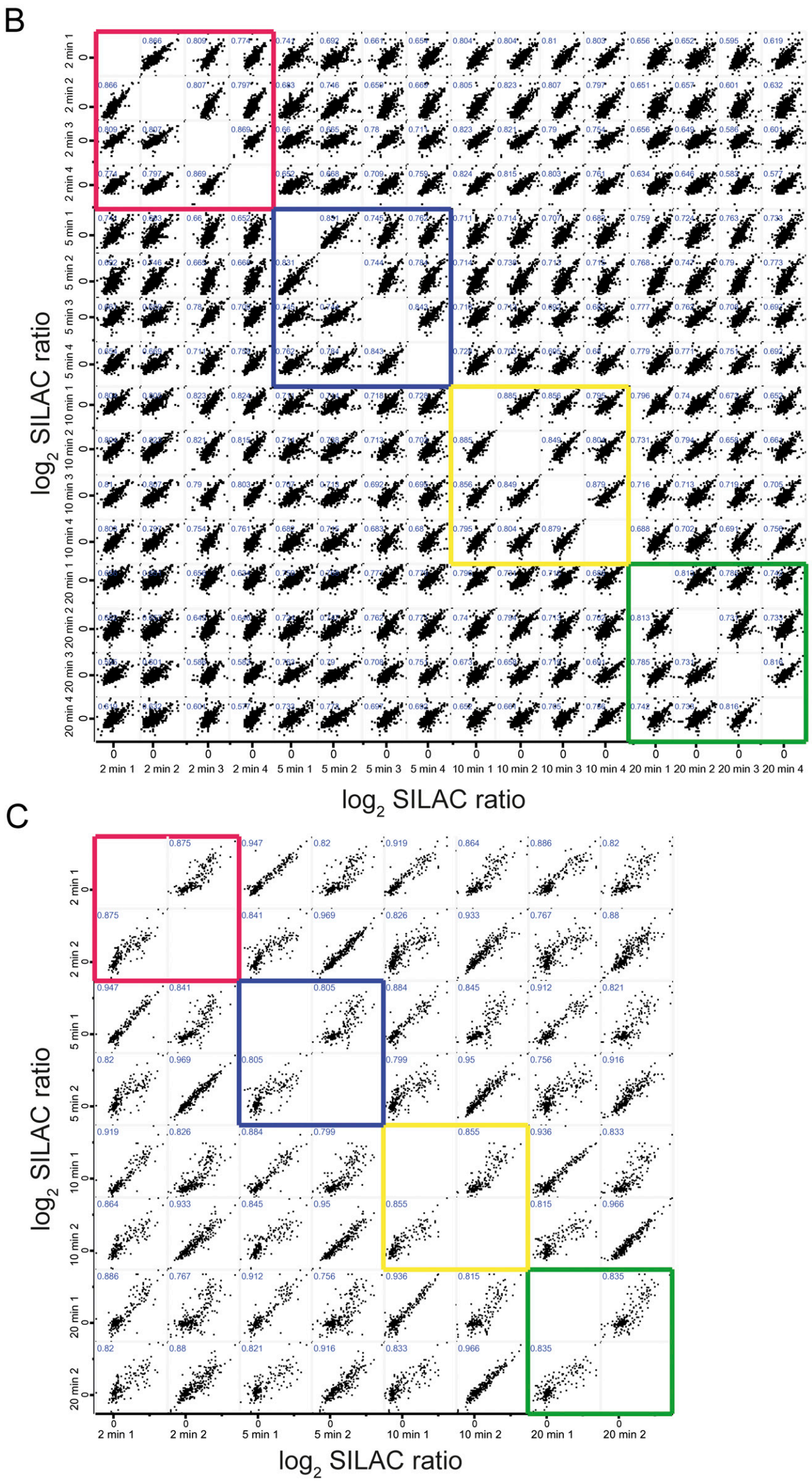


Fig. S2. (*A*) Protein expression profiling: Scatter plots of normalized "protein groups" SILAC ratios (log_2) versus intensity (log_{10}) for 2, 5, 10, and 20 min after BCR stimulation. Outlier proteins are marked red, and the respective gene names are shown. (*B*) Pearson's correlation analysis of normalized SILAC ratios (log_2) of all p-sites quantified in four biological replicates (indicated by 1, 2, 3, and 4) from the global phosphoproteome experiment of DG75 cells. Pearson's correlation coefficient is indicated in blue in the upper left corner of each scatter plot. The pink box highlights the correlation of the biological replicates of 2 min, blue 5 min, yellow 10 min, and green 20 min BCR stimulation. (*C*) Pearson's correlation analysis of normalized SILAC ratios (log_2) of biological replicates (indicated by 1 and 2) of all p-sites derived from the pYome experiment. Pearson's correlation coefficient is shown in blue in the upper left corner of each scatter plot. The pink box highlights the correlation of the biological replicates of 2 min, blue 5 min, yellow 10 min, and green 20 min BCR stimulation.

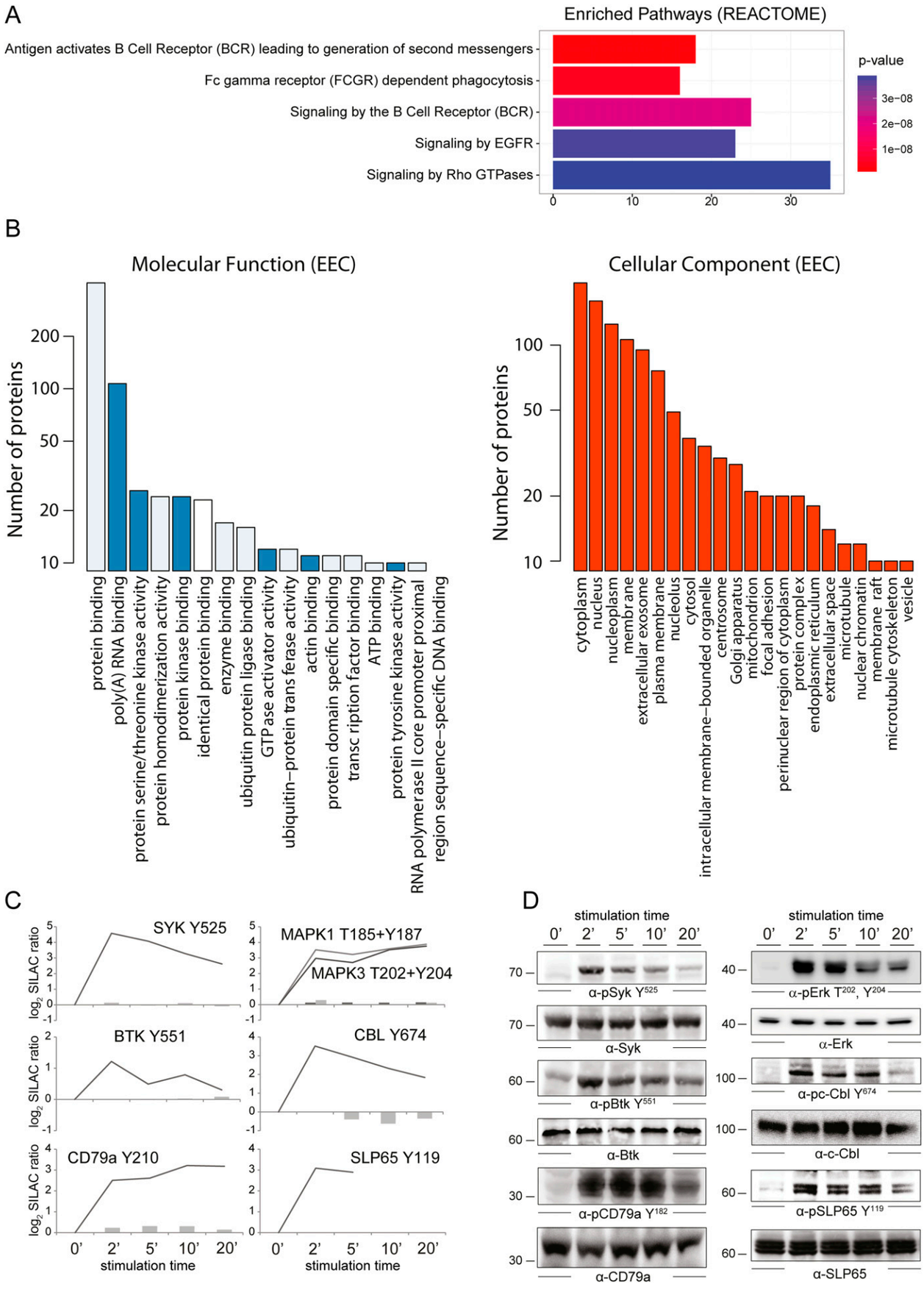


Fig. S3. (*A*) Pathway enrichment analysis for BCR effectors identified upon BCR stimulation of DG75 cells. (*B*) Experimentally validated molecular function and cellular component ontology terms annotated to BCR effector proteins identified in DG75 cells. Bars representing the numbers of cytoskeleton and transcriptional regulators, kinases, and RNA-binding proteins are marked in dark blue. See *SI Materials and Methods* for details. (*C*) Mass-spectrometric quantification of expression levels (grey bars) and p-sites (line graphs) of the indicated BCR signaling effectors. (*D*) Phosphorylation dynamics and respective expression levels of proteins analyzed in *C* were monitored by immunoblotting.

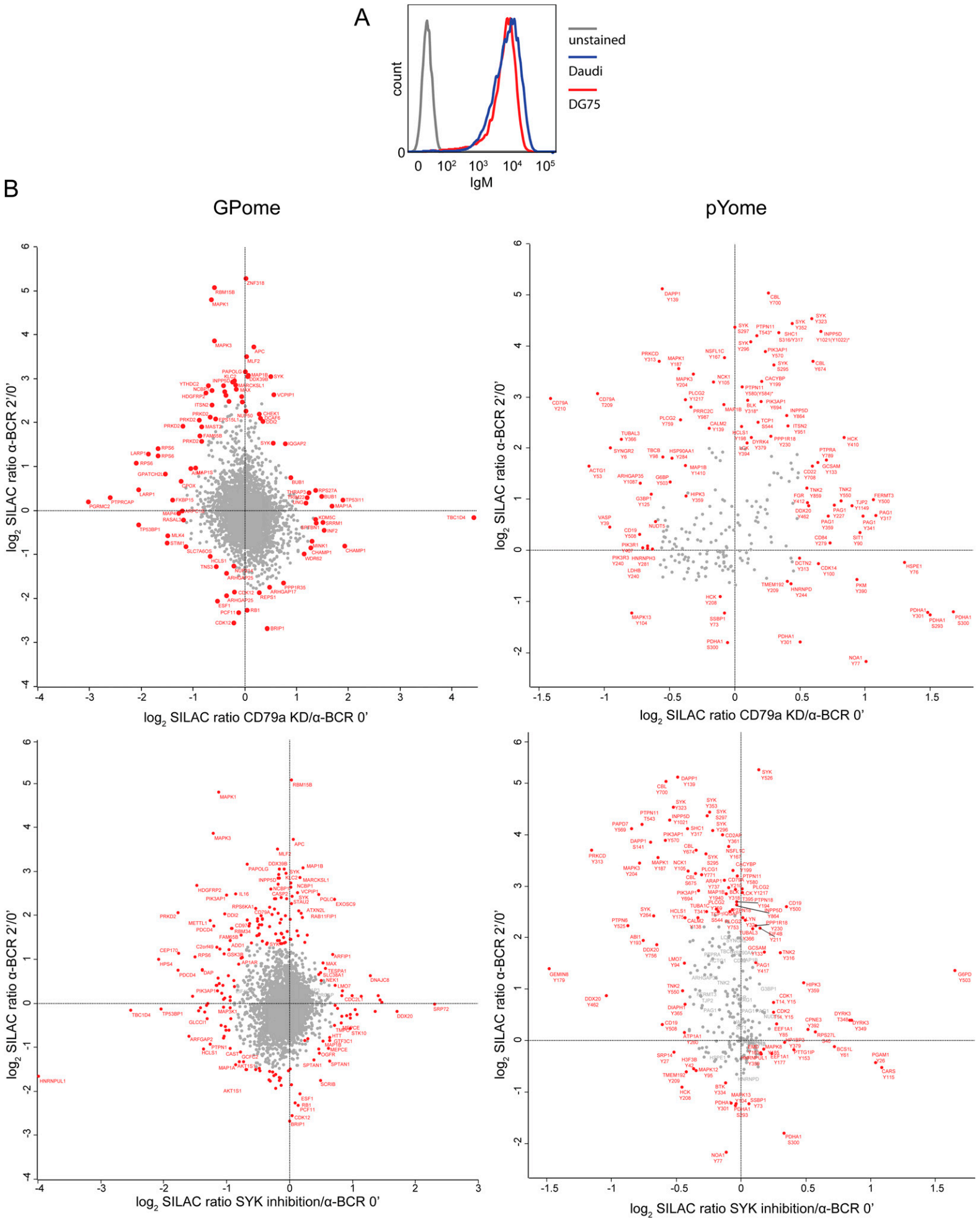


Fig. S4. (*A*) BCR cell surface expression was monitored by flow cytometry (red line, DG75; blue line, Daudi). (*B*) Scatter plots showing the fold-change of p-sites on mainly serines/threonines (GPome, *Left*) and mainly tyrosines (pYome, *Right*) as determined by quantitative MS upon BCR stimulation versus CD79a knockdown and SYK inhibition. Selected phosphorylated proteins and p-sites are highlighted.

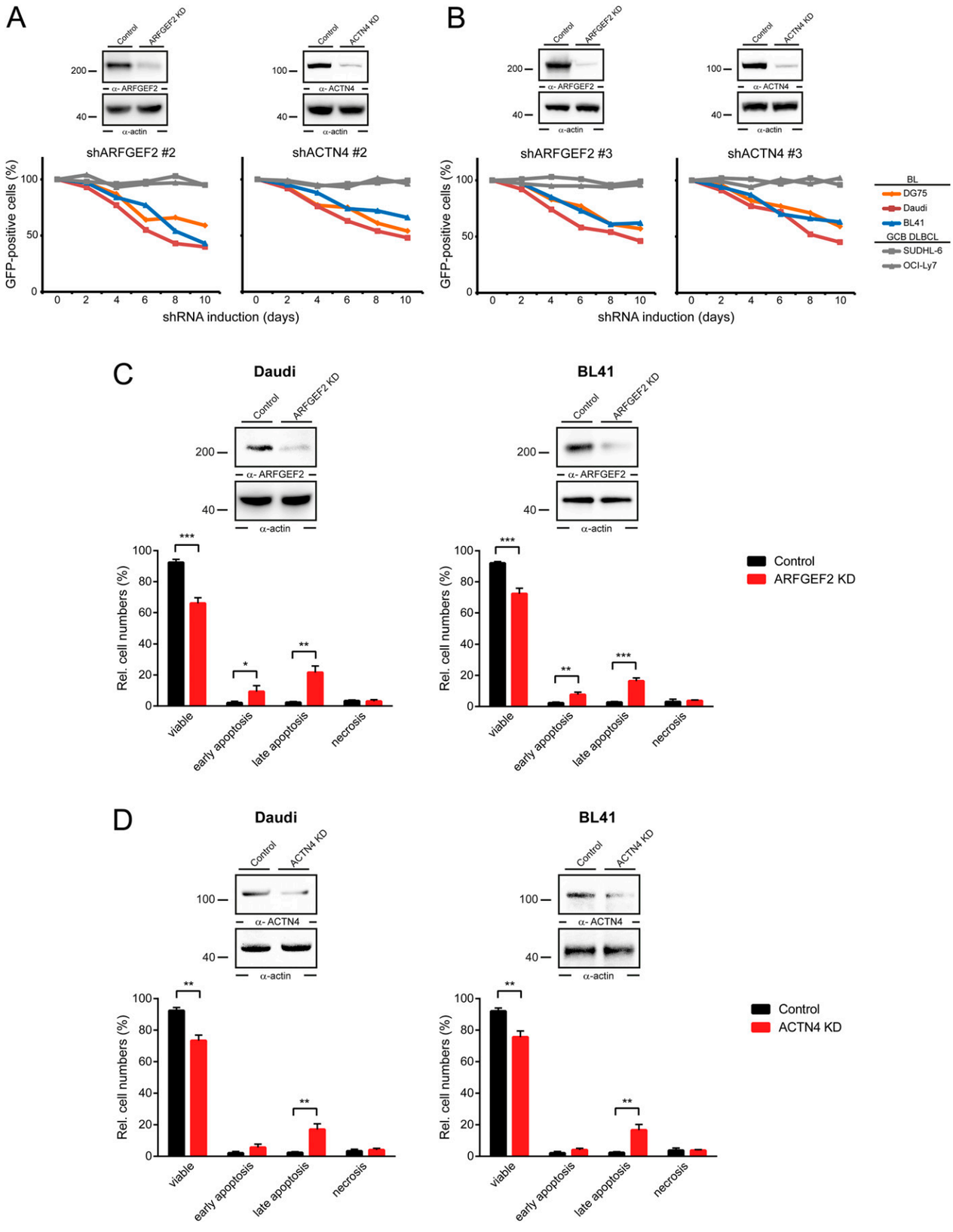


Fig. S5. (*A* and *B*) Immunoblots of cleared cellular lysates derived from control cells that were treated with unspecific shRNAs and the respective knockdown cells with antibodies against ARFGEF2 and ACTN4 (*Upper*). Protein loading was monitored by anti-actin immunoblotting (*Lower*). ARFGEF2 and ACTN4 shRNAs are toxic for BL. The figure shows the fraction of GFP-positive, shRNA-expressing cells relative to the GFP-negative, shRNA-negative fraction at the indicated times (normalized to the day 0 values). Data represent three experiments. (*C* and *D*) Immunoblots showing reduction of ARFGEF2 (*C*) and ACTN4 (*D*) expression levels in Daudi and BL41 cells upon 24 h doxycycline treatment whereas induction of nonspecific control shRNAs showed no reduction of ARFGEF2 and ACTN4 expression levels. Actin is shown as a loading control (*Upper*). Flow cytometric Annexin V/7-AAD–based apoptosis analysis of Daudi and BL41 cells 4 d after induction of ARFGEF2 and ACTN4-specific shRNAs or unspecific control shRNAs (*Lower*). Statistical significance: $*P < 0.05$, $**P < 0.01$ using Student's *t* test, $***P < 0.001$.

Dataset S1. Quantified class I phosphorylation sites in BCR-stimulated DG75 and Daudi cells

Dataset S1

All scores and localization probabilities were calculated by MaxQuant. The column of multiplicity indicates the number of phosphate groups identified in a peptide. Significance was defined as described in *SI Materials and Methods*.

Dataset S2. Quantified class I phosphorylation sites in DG75 cells upon SYK inhibition and CD79a knockdown

Dataset S2

All scores and localization probabilities were calculated by MaxQuant. The column of multiplicity indicates the number of phosphate groups identified in a peptide. Significance was defined as described in *SI Materials and Methods*.

Dataset S3. Quantified class I phosphorylation sites in Daudi cells upon BCR stimulation and SYK inhibition

Dataset S3

All scores and localization probabilities were calculated by MaxQuant. The column of multiplicity indicates the number of phosphate groups identified in a peptide. Significance was defined as described in *SI Materials and Methods*.

Dataset S4. shRNA sequences

Dataset S4