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BCALM (AC099524.1) is a human B lymphocyte-specific lncRNA that modulates B cell receptor-mediated calcium signaling

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Abstract

Of the thousands of long non-coding RNAs (lncRNA) identified in lymphocytes, very few have defined functions. Here we report the discovery and functional elucidation of a human B-cell specific lncRNA with high levels of expression in three types of B-cell cancer and normal B cells. The *AC099524.1* gene is upstream of the gene encoding the B-cell specific phospholipase C gamma 2 (*PLCG2*), a B-cell specific enzyme that stimulates intracellular Ca^{2+} signaling in response to B cell receptor (BCR) activation. AC099524.1 (BCALM) transcripts are localized in the cytoplasm and, as expected, CRISPR/Cas9 knockout (KO) of *AC099524.1* did not affect *PLCG2* mRNA or protein expression. LncRNA interactome, RNA immunoprecipitation (RIP), and co-IP studies identified BCALM-interacting proteins in B cells, including phospholipase D 1 (PLD1), and kinase adaptor proteins AKAP9 (AKAP450) and AKAP13 (AKAP-Lbc). These two AKAP proteins form signaling complexes containing protein kinases A and C (PKA & PKC), which phosphorylate and activate PLD1 to produce phosphatidic acid (PA). BCR stimulation of BCALM-deficient B cells resulted in decreased PLD1 phosphorylation and increased intracellular Ca^{+} flux relative to wild-type cells. These results suggest that BCALM promotes negative feedback that down-modulates BCR-mediated Ca^{+} signaling by promoting phosphorylation of PLD1 by AKAP-associated kinases, enhancing production of PA. PA activates SHP-1, which negatively regulates BCR signaling. We propose the name BCALM for **B**-**C**ell **A**ssociated **L**ncRNA **M**odulator of BCR-mediated Ca^{+} signaling. Our findings suggest a new paradigm for lncRNA-mediated modulation of lymphocyte activation and signaling, with implications for B-cell immune response and BCR-dependent cancers.

INTRODUCTION

Appropriate response to environmental cues is critical for normal differentiation and cellular homeostasis and also serve as an essential firewall to prevent disease. This is true of innate and adaptive immune cells, which must differentiate self versus non-self antigens and

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respond accordingly. Binding of antigen to the B cell receptor (BCR) in B lymphocytes triggers assembly of the BCR signalosome and a cascade of cellular activity, including calcium flux, activation of transcription factors and changes in gene expression. We and others have demonstrated that B lymphocyte activation pathways and consequent gene expression changes promote survival, proliferation, and differentiation, but when deregulated can drive lymphomagenesis(1–3). However, the mechanisms that drive and sustain these changes remain incompletely defined.

Long non-coding RNAs (lncRNA) are involved in the regulation of gene transcription and signal transduction in normal and diseased cells, including cancer(4–8). LncRNA's functional versatility includes epigenetic modification, nuclear domain organization, transcriptional control, regulation of RNA splicing and translation, and modulation of protein signaling activity(9–12). While lncRNAs have emerged as key players in immune response and homeostasis, most reports concern innate or T cells, and little has been reported on the function of lncRNAs in human B lymphocytes(5, 11, 11, 13, 14). To address this gap, we previously developed a bioinformatic tool called PLAIDOH, which constructs a statistical model using chromatin, gene expression, subcellular localization, and genome architecture data coupled to biologically-based logic rules to predict lncRNA function. We validated its accuracy by comparison to published lncRNA functions, primarily in epithelial and myeloid tumors and cell lines(15).

To address the function of lncRNAs in B cells, we performed a global analysis of gene regulation in primary human B cell cancers and normal B cells from tonsil (chromatin immunoprecipitation [ChIP-] and RNA-seq). Our discovery analyses identified lncRNAs with high expression in Chronic Lymphocytic Lymphoma/Leukemia (CLL), Diffuse Large B Cell Lymphoma (DLBCL), and Follicular Lymphoma (FL). We used PLAIDOH to predict the function of these lncRNAs and then ranked them based on their potential role in B cell activation, survival, or signaling, based on B cell specificity, expression level, and the function of the genes or pathways likely targeted(15). These analyses identified AC099524.1 (RP11–960L18.1, ENSG00000261218), an intergenic multi-exon spliced lncRNA that is highly and specifically expressed in B cell lymphomas and normal B cells. The *AC099524.1* gene is located upstream of the gene for the B cell specific phospholipase C gamma 2 (PLCG2), which is recruited to the BCR signalosome upon antigen binding, resulting in its phosphorylation and activation. Activated PLCG2 hydrolyzes the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate second messengers diacylglycerol (DAG) and inositol-1,4,5,-triphosphate (IP_3) that then stimulate intracellular calcium flux and activate multiple downstream signaling pathways(16–18). Constitutional (germline) mutations in *PLCG2* cause the immune dysregulation syndrome PLAID, while acquired mutations confer resistance to Btk inhibitor therapy in patients with leukemia or lymphoma(19, 20). Because PLCG2 plays an essential role in B cell immune response, we sought to determine the molecular function of AC099524.1 (BCALM) in B lymphocytes.

We used lncRNA interactome (pull-down) assays, RNA-immunoprecipitation (RIP), co-immunoprecipitation, CRISPR knockout of AC099524.1, and B cell activation assays to elucidate the function of BCALM. These studies revealed that, though the *AC099524.1* gene coincides with enhancer regulatory elements, the transcript does not regulate the expression

level of neighboring genes *PLCG2* and *CMIP*. Instead, BCALM is localized to the cytoplasm and interacts with phospholipase D 1 (PLD1) and A-kinase proteins AKAP9 (AKAP450) and AKAP13 (AKAP-Lbc). AKAPs assemble kinase signaling complexes to ensure correct subcellular targeting, including protein kinases A and C (PKA and PKC), which phosphorylate and activate PLD1(21–30). PLD1 hydrolyzes lipid membrane component phosphatidylcholine (PC) to phosphatidic acid (PA), which activates the SHP-1 phosphatase(26, 31, 32), a negative regulator of BCR signaling(33, 34). Knockout of *AC099524.1* (BCALM) abrogated the association of PLD1 and AKAP9, attenuated phosphorylation of PLD1 and enhanced calcium flux after BCR stimulation. These results support a role for BCALM in promoting negative feedback modulation of BCR signaling by facilitating phosphorylation and activation of PLD1. Increased production of PA by activated PLD1 enhances SHP-1 phosphatase activity on BCR associated kinases, attenuating BCR signaling. Based on these findings, we suggest the name BCALM for **B C**ell **A**ssociated **L**ncRNA **M**odulator of BCR signaling. Taken together, these studies demonstrate a novel mechanism for negative feedback modulation of BCR signaling via a lncRNA.

MATERIALS AND METHODS

Lymphoma Samples and Normal B Cell RNA-seq Analysis

Lymph node biopsies, bone marrow, and peripheral blood were collected from patients with Follicular or Diffuse Large B Cell Lymphoma or Chronic Lymphocytic Leukemia/ Lymphoma seen at the Washington University (WU) Oncology Clinic. Patient characteristics are summarized in Table 1. Tonsils were collected from patients undergoing tonsillectomy at Barnes-Jewish and St. Louis Children's Hospitals. Peripheral blood from healthy donors was collected by the WU Volunteer for Health program. From these samples, $CD19^+$ B cells were purified and RNA extracted as described in(1, 15). RNA-seq was performed and analyzed as in(1, 15, 35). Briefly: RNA was isolated from 1–2×106 cells from each sample with a Qiagen RNeasy kit (Qiagen cat.# 74104). rRNA-depleted (Ribo-Zero, Epicentre, discontinued) libraries were prepared using TruSeq RNA Sample kits with indexed adaptors (Illumina) and subjected to 100 bp paired-end or 50 bp single-end sequencing on an Illumina Hi-Seq 2000 by the Washington University Genome Technology Access Center. Sequencing reads were aligned to hg38 using STAR (v2·5·3a)(36). RPKM normalized genome browser tracks were created with deepTools' (v3·1·0) bamCoverage utility and visualized on the UCSC genome browser. Read quantification was performed by Salmon (v0·11·0) using UCSC hg19 knownGene annotations(37), and differential gene expression analyses were performed with the DESeq2 R package (v1·20·0)(38). Genotify (v1·2·1) was used for manual gene curation(39). CPAT was used to exclude transcripts with coding potential(40).

PLAIDOH analyses

LncRNA analyses with PLAIDOH were performed as in(15) with the following changes. Subcellular localization of lncRNAs with expression detectable by RNAseq in our datasets (≥100 average normalized counts in at least one B cell cancer or normal group) was determined using the lncATLAS database (http://lncatlas.crg.eu/)(41). Seventy-two percent of lncRNAs detected (1270/1765) had localization data in lncATLAS. Localization RCI (relative concentration index) scores were downloaded for all lncRNAs in the database, then

the mean was calculated over all cell lines for each lncRNA. A lncRNA was determined to be nuclear if its mean RCI was less than −0.5, both nuclear and cytoplasmic if its mean RCI was between −0.5 and 0.5, and cytoplasmic if its mean RCI was greater than 0.5. RNA binding protein (RBP) subcellular localization was determined by location information in the Human Protein Atlas (http://www.proteinatlas.org)(42). An RBP was determined to be nuclear if it was annotated only as nuclear in the cell atlas or tissue atlas, cytoplasmic if annotated only as cytoplasmic in the cell atlas or tissue atlas, or both nuclear and cytoplasmic if shown to be located in the nucleus and cytoplasm by either the cell atlas or tissue atlas. If an RBP in the Encode eCLIP assay did not have localization data in the Human Protein Atlas, subcellular localization was determined using immunofluorescence as in (43). An RBP was considered to bind a site on a lncRNA if the same peak from the Encode eCLIP assay (defined as overlapping by at least 50% of its length) was present in at least two replicates (https://www.encodeproject.org/eclip/)(44, 45). AC099524.1 gene annotations from different genome databases are listed in Table 2.

Subcellular Fractionation and qRT-PCR

Sub-cellular fractionation of cytoplasm, nuclear total, nucleoplasm and chromatin for RNA isolation was performed as described in (46). cDNA was generated from whole cell lysate and cell fractions using the High Capacity RNA-to-cDNA Kit from ThermoFisher (4387406). qRT-PCR was performed using primers specific for PLCG2, AC099524.1, 18S, U1 snRNA, and GAPDH (Table 3) and the SYBR Green Real-Time PCR Master Mix. Expression was calculated relative to GAPDH using standard methods(15), except in for sub-cellular fractionation experiments where inverse log2 Ct values were calculated relative to the inverse log2 Ct values of the cytoplasmic fraction.

Luciferase reporter assays

Putative regulatory regions were PCR amplified from GM12878 B cell line gDNA (Table 3). Cloning into the luciferase reporter plasmid was performed and luciferase reporter assays performed as in Andrews et al(35) except that assays were performed in GM12878 B cells. All experiments were assayed in triplicate and performed at least twice. The average ratios between the firefly luciferase reporter plasmid containing a putative regulatory region and control (Nanoluc) luciferase readings were compared to the average ratio for the empty luciferase vector to determine relative luciferase activity.

Cell Culture, Genome Editing, and RNAi knockdown—OCI-Ly7 and U2932 cells were cultured in RPMI + 10% fetal bovine serum (FBS) or IMDM + 20% FBS. Knockdown with shRNA was performed as in(15). CRISPR knock out of *AC099524.1* in U2932 and OCI-Ly7 cells was performed as in(3) using target sequences in Table 3. Confirmation of genome editing was performed with PCR of genomic DNA and qRT-PCR using primers in Table 3.

RNA Pull Down—*In vitro* transcription from plasmids containing the sense (5’ to 3’) or anti-sense (3’ to 5’) *AC099524.1* (BCALM) cDNA (exons only) was performed using the T7 AmpliScribe kit (Lucigen AS3107) per manufacturer’s instruction with 10% of UTP replaced with biotinylated UTP. Sense and anti-sense RNA were heated to 65C for 10

minutes, then allowed to cool slowly to 4C over approximately 1 hour to allow secondary structure formation. Ten million cells were harvested per pull down and washed twice in cold PBS, spinning at 500xg in a 4C tabletop centrifuge for 5 minutes between washes. The final pellet was then resuspended in 1ml of supplemented RNA lysis buffer (0.025 M Tris, 0.28 M NaCl, 1% NP-40, 10% glycerol, pH 7.4 plus protease inhibitors, phosphatase inhibitors, PMSF all 1:100, 1:200 RNAse inhibitor, and 0.5mM DTT) and allowed to lyse on ice for 30 minutes. Lysates were then spun for 10 minutes at 13,000xg at 4C. The supernatant was moved to a fresh tube and supplemented with yeast tRNA. The lysate was diluted with supplemented RNA lysis buffer until the equivalent of 10 million cells per 750ul was achieved. 750ul of lysate was then aliquoted into separate Eppendorf tubes, one per condition. Five ug of cooled RNA was added per pull down and lysates were rotated for 4 hours at 4C. 50ul of Dynabeads MyOne Streptavidin C1 (65001) per condition were washed 3 times with unsupplemented RNA lysis buffer. Beads were then resuspended in their original volume of lysis buffer and 50ul were added to each pull down. Tubes were rotated an additional 45 minutes at 4C. Beads were collected on a Dynamag-2 magnet (12321D) and supernatant aspirated. The beads were then washed 5 times with RNA lysis buffer supplemented with PMSF and protease inhibitors, with 5 minute rotations at 4C in between washes. Finally, beads were resuspended in 50ul of loading dye, boiled for 10 minutes at 95C, separated on an SDS PAGE gel, silver stain was performed using the Pierce Silver stain kit (24612), and bands were excised from the gel and sent for mass spectrometry (Taplin Biological Mass Spectrometry Facility at Harvard Medical School, Cambridge, MA, USA or Danforth Center Proteomics & Mass Spectrometry Facility, St. Louis, MO, USA). Greater than 9 unique peptides were required for further analysis. List of proteins mapped to peptides detected by MS in Table S1. Data were analyzed using R packages dplyr and ggplot2.

RNA Immunoprecipitation (RIP)—10 million cells were harvested and washed twice in cold PBS, spun at 500xg in a 4C tabletop centrifuge for 5 minutes between washes. The final pellet was then resuspended in 1ml of RNA lysis buffer (0.025 M Tris, 0.28 M NaCl, 1% NP-40, 10% glycerol, pH 7.4 plus protease inhibitors, phosphatase inhibitors, PMSF all 1:100, 1:200 RNAse inhibitor, and 0.5mM DTT) and allowed to lyse on ice for 30 minutes. Lysates were then spun for 10 minutes at 13,000xg at 4C. The supernatant was moved to a fresh tube and 5ul reserved for Input. Antibody was added to the supernatant at recommended concentrations per manufacturer's instructions for Immunoprecipitation (antibodies used as in Western blot) and rotated overnight at 4C. Then 50 ul PureProteome Protein A/G Mix Magnetic Beads (MilliporeSigma) per condition were washed 3 times with RNA lysis buffer and added to each condition. After rotating for 4 hours beads were collected on a Dynamag-2 magnet (12321D) and supernatant aspirated. The beads were then washed 6 times with RNA lysis buffer supplemented with PMSF and protease inhibitors, with 5 minute rotations at 4C in between washes. Beads and Input tube were then resuspended in Proteinase K Buffer (10% SDS, 10mg/ml proteinase K in RIPA buffer) and incubated at 55C for 30 minutes with shaking. Tubes were then placed back on the magnet and the supernatant was removed and 250ul of RNA lysis buffer was added. One ml of trizol was added to each tube and RNA purified according to manufacturer's instructions. RNA was DNase-treated using ArticZymes Heat and Run gDNA removal kit (80200–250)

according to kit instructions. cDNA was made using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (ThermoFisher 4368814).

Co-immunoprecipitation (Co-IP)—Ten million cells per antibody were lysed by rotation at 4°C for 30 minutes in 0.025 M Tris, 0.15 M NaCl, 1% IGEPAL CA-630, 5% glycerol, pH 7.4 with protease and phosphatase inhibitors, spun at 13,000xg for 10 minutes at 4 °C, 10% of lysate supernatant was saved for input samples. Supernatants were incubated overnight at 4°C with 5 ul of AKAP9 antibody (Bethyl Laboratories A301–662A) or 3 ul of PLD1 antibody (Bethyl A305–241A). 40ul of Dynabeads Protein A (Thermo 10002D) per condition were washed 3 times with lysis buffer and incubated by rotation with antibody + supernatant for 3 hours at 4 °C. Beads were washed 3X with lysis buffer and collected using a Dynamag-2 magnet (Thermo 12321D). Proteins were eluted with Laemmli sample buffer.

Western Blot—Whole cells were lysed in RIPA buffer supplemented with protease inhibitors, an equal volume of loading dye was added, and lysates were boiled for 10 minutes at 95 °C. After transfer to nitrocellulose paper, blots were incubated in primary antibodies for AKAP9 (Bethyl Laboratories A301–662A), PLCG2 (Santa Cruz sc-407), phospho-PLCG2 (Tyr759, Cell Signaling Technologies, 3874), PLD1 (A305–241A) PLD1 (Santa Cruz 28314), phospho-PLD1 (Thr147, Fisher Scientific PA537688), DICER (Cell Signaling Technologies 3363S), DHX9 (Bethyl Laboratories A300–855A), or GAPDH (Abcam ab9485).

Calcium Flux Assays—Triplicate aliquots of 200,000 cells per condition were washed and resuspended in appropriate media plus 2% FBS. Cells were resuspended in Indo-1 working solution (1uM in media + 2% FBS) and incubated at 37C in the dark for 30–60 minutes. Cells were washed twice with media + 2% FBS. Calcium flux was measured by flow cytometry on a BD LSRFORTESSA X-20 according to Manufacturer's instructions. The emission of Indo-1 shifts from about 475 nm without Ca^{2+} (unbound) to about 400 nm with Ca^{2+} (Ca^{2+}-bound) when excited at about 350 nm. Baseline signal was acquired for 60 seconds, then 1ug/ml Anti-IgM antibody (Biolegend clone MHM-88, 314502) was added to stimulate B cell receptor signaling and Calcium flux, then signal was acquired for 4 additional minutes. The ratio of Ca^{2+}-bound/unbound Indo-1 signal was calculated and plotted over time and the confidence interval calculated from triplicates for each experiment using R (version 3.6.1, R packages dplyr, smooth, TTR, Mcomp, and ggplot2).

Statistical Analyses

All statistical tests were performed with GraphPad Prism (v8·1·1).

Data Accession

Sequencing data was deposited in GEO under accession numbers GSE62246 and GSE132053.

RESULTS

BCALM (AC099524.1) is a lncRNA upstream of PLCG2 that is specifically and highly expressed in malignant and normal B cells

To identify lncRNAs that may play roles in B cell lymphoma, we collected lymph node biopsies, bone marrow, and peripheral blood from patients with Follicular Lymphoma (FL), Diffuse Large B Cell Lymphoma (DLBCL), and Chronic Lymphocytic Leukemia/ Lymphoma (CLL). For non-malignant B cells, tonsils were collected from patients undergoing tonsillectomy and peripheral blood was collected from healthy donors. Patient and sample characteristics are summarized in Table 1. From these samples, $CD19^+$ B cells were purified, subjected to RNA extraction, and RNA-sequencing performed as previously described(1, 15, 35). LncRNAs have a range of cellular functions that are often initially categorized by subcellular localization. Consistent with this, lncRNAs expressed in B cells from CLL, DLBCL, FL and tonsil are localized primarily in the nucleus, with smaller proportions in the cytoplasm or both nucleus and cytoplasm (Fig. S1A). Overall, lncRNA expression levels are similar in cytoplasmic and nuclear fractions across the three types of B cell cancer and normal B cells (Fig. S1B). In addition, a subset of lncRNAs exhibits significant expression differences across these B cell cancer groups (Fig. S1C).

In our previous study, we identified an intergenic multi-exon spliced lncRNA highly expressed in FL, *AC099524.1* (ENSG00000261218), that is located 13kb upstream of *PLCG2*, which encodes the phospholipase C gamma 2 protein, a critical effector of downstream B-cell receptor (BCR) signaling(15). In this study, we found that *AC099524.1* (BCALM) is also expressed highly in DLBCL and CLL, as well as in normal B cells (Fig. 1A–B). Expression of BCALM is quite specific for B lymphocytes, as demonstrated by high expression in B lymphocytes and lymphocyte-rich tissues (tonsil, B cell lymphoma/ leukemia, Fig. 1A–B; spleen, small intestine, Fig. 1C), but has no detectable expression in T lymphocytes (Fig. 1A). We previously demonstrated that BCALM transcript is localized to the cytoplasm(15) and confirmed this in additional B cell lines (OCI-Ly7 and GM12878, Figs. 1D and S1D).

The functions of most lncRNAs have not been fully established, and prior to this study, the function of AC099524.1 (BCALM) was unknown. To address this knowledge gap, we previously developed a tool, PLAIDOH, to predict lncRNA function and to prioritize lncRNAs for experimental studies(15). PLAIDOH is a set of bioinformatic modules that analyzes and integrates expression, epigenome, proteome, and cellular localization data with biologically informed rules to predict the function of lncRNAs. BCALM transcript likely does not act as a transcriptional regulator since it is localized in the cytoplasm and its expression level does not significantly correlate with neighboring genes *CMIP* and *PLCG2* (Fig. 1A and (15)). To identify possible non-transcriptional functions, we previously used PLAIDOH to plot the interactions of lncRNAs with RNA-binding proteins (RBP) identified by enhanced cross-linking immunoprecipitation sequencing (eCLIP-seq)(44) by expression level, subcellular localization(41), and the number of RBP binding sites(15). However, this approach was inherently limited by the following: 1) the lncRNA-RBP interactions were identified by RIP of known RBPs, and thus no novel interacting proteins could be identified;

and 2) no B cell lines were used(43, 45). Therefore, we designed an experimental approach to elucidate the function of AC099524.1.

Genomic regulatory elements in the BCALM locus exhibit enhancer activity— BCALM (AC099524.1) transcript is localized primarily to the cytoplasm and shRNA knockdown of AC099524.1 does not alter the expression of neighboring genes *CMIP* or *PLCG2* (Figs. 1D, S1E and (15)), suggesting that cis-regulatory elements (e.g., enhancers), rather than the BCALM transcript, may control the expression of the genes in this locus. To address this question, we performed chromatin immunoprecipitation sequencing (ChIP-seq) in $CD19^+$ B cells isolated from FL, DLBCL, and CLL patient samples and tonsils. We specifically probed for H3K27ac, a chromatin mark associated with active enhancers, and H3K9/14ac (H3ac), a histone mark associated with active enhancers and promoters. Figure 2A shows enrichment of H3K27ac and H3ac in several regions that overlap and flank *AC099524.1* and its neighboring genes, *CMIP* and *PLCG2*. Coinciding with many of these regions are DNase hypersensitivity and binding sites for CTCF and EP300(47) in B cells, which mark sites of accessible chromatin, chromatin looping, and acetylation of chromatin, respectively. These epigenetic studies identified several putative enhancers in the region, as well as the promoter of *AC099524.1* (Fig. 2A). To test the transcriptional regulatory activity of these elements, we cloned them into luciferase reporter vectors and assayed them in a B cell line. We assayed the promoter of *AC099524.1* since lncRNA promoters often coincide with enhancer elements(5, 7, 48, 49), but not the promoter region of PLCG2 as it is a known gene promoter. Nearly all of the regions tested demonstrate transcriptional regulatory activity that is significantly higher than the empty vector control (Fig. 2B). The elements with the highest activity are located between *CMIP* and *AC099524.1*, in the first intron of *AC099524.1*, and in the second intron of *PLCG2*. These results confirm that genomic elements in this locus exhibit cis-regulatory activity and suggest that they may regulate the transcription of *PLCG2*, *CMIP*, and/or *AC099524.1*

Deletion of BCALM (AC099524.1) has no effect on PLCG2 protein expression or phosphorylation state

To generate a model with complete loss of BCALM, we designed CRISPR/Cas9 gene editing guides to delete the promoter and first exon or promoter and first two exons in two additional B cell lines (OCI-Ly7 and U2932, Figs. 3A and S2A–C). Heterozygous knockout reduced BCALM expression 25–50%, and homozygous knockout completely abrogated BCALM expression (Figs. 3B, D). As expected, there was no change in PLCG2 protein levels measured by western blot (Fig. 3C,E). PLCG2 is phosphorylated and activated by BCR-associated kinases upon binding of antigen to the B cell receptor (BCR)(16). Knockout of BCALM (AC099524.1) did not alter phospho-PLCG2 levels at multiple timepoints after BCR stimulation with anti-IgM treatment (Fig. 3C,E). These findings provide further evidence that BCALM transcript does not regulate RNA or protein levels of neighboring gene *PLCG2* in B cells.

BCALM interacts with signal transduction proteins in B cells

Some lncRNAs act as scaffolds to regulate protein/protein interactions or to directly inhibit or activate their protein targets in the cytoplasm(4, 5, 50–53). To identify BCALM

interacting proteins, we performed an RNA pulldown using *in-vitro* transcribed, biotinylated BCALM sense (experimental) and anti-sense (control) transcripts (Fig. 4A). Biotinylated sense and anti-sense transcripts were incubated with whole cell lysates from B lymphoma cell lines and streptavidin beads, washed, and bound proteins were eluted and separated by SDS-PAGE and visualized by silver stain. Figure 4B shows a representative silver stained gel comparing proteins bound by biotinylated sense or anti-sense BCALM transcript to input or streptavidin beads alone. Asterisks mark protein bands that are present in sense but not anti-sense lanes. We submitted sense and anti-sense lanes from a similar gel and from a high molecular weight gel for mass spectrometry (MS), which identified peptides and quantified signal intensity (Fig. 4C–D and Table S1). Some of these were previously reported to interact with BCALM by RNA binding protein immunoprecipitation and sequencing (eCLIP-seq) in myeloid and hepatic cell lines (15, 43–45). However, none of these coincided with the most enriched proteins in the RNA pull-down in B cells, suggesting that lncRNA-protein interactions may vary in different cell types.

Plotting by number of peptides and signal intensity demonstrates that several proteins were substantially enriched in sense BCALM pull-down compared to the anti-sense control (Fig. 4C). Several of those with the highest enrichment in sense BCALM pull-down have molecular weights that are consistent with bands in the silver stain gel, including DHX9 and PLD1 (Fig. 4C and Table S1). DHX9, a DEAH-containing RNA helicase, and DICER1, a DEXH-containing RNA helicase, both are known to bind RNA molecules, including lncRNA(54–58). PLD1, a phospholipase that hydrolyzes phosphatidylcholine (PC) to produce phosphatidic acid (PA), is a component of multiple signal-transduction pathways, including antigen receptor activation, and is a key player in tumorigenesis, but has not been previously reported to interact with RNA(24, 31, 32, 59–61). The high molecular weight bands were determined to be AKAP9 (AKAP450) and AKAP13 (AKAP-Lbc), scaffold proteins that assemble kinase complexes, including PKA and PKC(21–23, 29, 30), both of which phosphorylate and activate PLD1(27, 28, 31, 32, 62) (Fig. 4D). We confirmed the MS findings by Western blot, demonstrating that both PLD1 and DHX9 were enriched in sense compared to anti-sense AC099524.1 pull-down. DICER1 showed modest enrichment in sense AC099524.1 and as expected PLCG2 showed no enrichment (Fig. 4E). To validate the BCALM-protein interactions identified by RNA pull-down, we performed an orthogonal assay, RNA immunoprecipitation (RIP), in which antibodies against PLD1, DHX9, and DICER1 were used to assay interacting RNA transcripts. Results from the RIP assays show that BCALM transcript was significantly enriched compared to IgG control for each of the three proteins, with PLD1 and DICER1 having two-fold or greater enrichment, and DXH9 with 1.4-fold enrichment (Fig. 4F). Western blot confirmed immunoprecipitation of the target proteins (Fig. 4G). Taken together, these results identify interactions between BCALM and cytosolic signal transduction proteins, suggesting that BCALM may play a role in signaling pathways in B cells.

BCALM (AC099524.1) deficiency leads to increased calcium flux in response to BCR stimulation

Given its specificity for B cells, interaction with signaling pathway proteins, and proximity to PLCG2, we next assessed the effect of BCALM knockout on signaling downstream of

BCR activation. While PLD1's role in BCR signaling has not been previously examined, PLD1 is involved in TCR activation and chemotaxis in T cells and in antigen stimulation of mast cells(27, 28, 61, 63–67). We treated wild type (WT) and knockout (KO) B cell lines (OCI-Ly7) with anti-IgM to stimulate the BCR and collected at 0, 2, 5, 10, 30, 60, and 240 minutes post-stimulation. Figures 5A–B shows that PLD1 phosphorylation increases 2–3-fold over the time course in WT cells, while in KO cells, phosphorylation levels show minimal change at all timepoints. IgM stimulation did not alter the levels of BCALM transcript in WT or KO cells over the course of the experiment (Fig. S2D).

BCALM associates with PLD1 and AKAP9, a scaffold protein that associates with PKA and PKC, which phosphorylates PLD1. Since loss of BCALM resulted in reduced levels of phospho-PLD1, we hypothesized that BCALM may bring together PLD1 and an AKAP kinase complex, thereby promoting PLD1 phosphorylation. We tested this by immunoprecipitation of PLD1 in WT and KO B cell lines (OCI-Ly7). As shown in Fig. 5C, PLD1 co-precipitates AKAP9 in WT, but not in BCALM KO lymphoma cell lines, suggesting that BCALM is necessary for the association of PLD1 with the PKA/PKC kinase scaffold protein AKAP9.

We next assessed the effect of BCALM deficiency on calcium flux downstream of BCR stimulation. In these assays, anti-IgM stimulation of the BCR causes assembly of the BCR signalosome, in which BCR associated kinases activate PLCG2, which hydrolyzes PIP2 to DAG and IP3. IP3 stimulates IP3 receptor channels on endoplasmic reticulum to release calcium into the cytoplasm(33, 34). Indo-1 is a fluorescent calcium indicator with emission at 475nm when unbound that shifts to 400nm when bound to Ca^{+}, enabling accurate measurement of intracellular calcium concentrations with flow cytometry(68). For these studies, we preincubated WT, heterozygous or homozygous AC099524.1 (BCALM)-deficient cells with Indo-1 prior to BCR stimulation with anti-IgM. The resulting calcium release into the cytoplasm caused a shift in the ratio of bound:unbound Indo-1, which was detectable by flow cytometry. Compared to WT, heterozygous and homozygous AC099524.1 (BCALM)-deficient OCI-Ly7 lymphoma cells generated from two different gRNA pairs exhibited higher levels of calcium flux after BCR stimulation as shown by higher ratios of Ca^{+}-bound:unbound Indo-1 (Fig. 5D). A similar increase in calcium flux was observed in two AC099524.1 (BCALM) KO U2932 lymphoma cell lines compared to WT (Fig. S2E) and in a non-malignant B cell line treated with BCALM shRNAs (GM12878, Fig. S2F). Taken together, these data support a role for BCALM in calcium flux downstream of BCR stimulation, perhaps through modulation of PLD1 phosphorylation (summarized in Fig. 6; see Discussion).

DISCUSSION

In mature B lymphocytes, binding of antigen to the B cell receptor (BCR) activates signaling pathways and transcription factors that change gene expression, driving differentiation, survival, and proliferation(69, 70). Perturbation of these pathways is the hallmark of B cell cancers, but the underlying mechanisms remain incompletely defined(71–75). In normal hematopoietic cells and non-B cell cancers, long non-coding RNAs have emerged as key players in modulating gene expression, protein translation, and signaling pathways(4–6, 9,

11, 51, 53, 76–86), however little is known regarding their functional roles in B cell activation or lymphoma(6, 13, 87–90). To address this critical knowledge gap, we performed global epigenome and transcriptome studies in human B cell cancers and normal B lymphocytes and identified lncRNAs as potential actors in lymphocyte activation pathways.

We used an integrative bioinformatic tool we developed (PLAIDOH(15)) to selected one of these lncRNAs, AC099524.1, for functional experimental studies. AC099524.1(BCALM) had high potential for activity in B cells because of its specific and high expression in B cell cancers and normal B cells and its proximity to the gene that encodes PLCG2, a critical regulator of downstream BCR signaling. In addition, BCALM was intriguing due to its cytoplasmic localization, less common for lncRNAs but in agreement with PLAIDOH's prediction(15). Also in agreement with its functional prediction, BCALM transcript itself does not regulate the expression of neighboring genes *CMIP* or *PLCG2*, but enhancers in this locus are active and may regulate the expression of *AC099524.1*(BCALM*), CMIP* and/or *PLCG2*. These results are consistent with reports that, for some enhancer lncRNAs, the transcripts themselves are dispensable for "cis"-regulation of neighboring gene transcription and instead function in "trans" in other pathways. Indeed, we identified BCALM-interacting proteins that are involved in non-transcriptional cell processes, including signal transduction proteins PLD1 and AKAP9. Our initial RNA pull-down studies used an *in vitro* transcribed BCALM that might lack post-transcriptional modifications present on the endogenous lncRNA, and thus could have missed protein interactions that require such modifications. Nonetheless, subsequent RIP, co-IP, B cell activation, and calcium flux assays were performed in B cells and demonstrate that endogenous BCALM interacts with PLD1 and AKAP9 and modulates their activity.

Other cytosolic lncRNAs have been shown to modulate signaling pathways. For example, lncRNA AK023948 positively regulates AKT activity in breast cancer by stabilizing PI3K subunit p85 and is associated with poorer prognosis(52). In triple negative breast cancer, lncRNA LINK-A associates with BRK and promotes the phosphorylation and stabilization of HIFalpha, leading to activation of HIF1alpha transcriptional programs under normoxic conditions(53). In macrophages, LPS-induced lncRNA Mirt2 inhibits K63-linked ubiquitination of TRAF6, thereby attenuating NF-KB and MAPK pathways and providing negative feedback regulation of inflammation(77). Many other studies in cancer, immune cells and other organ systems also demonstrate diverse roles for cytoplasmic lncRNAs in metabolic, signal transduction and immune response pathways(4–7, 51, 53, 80, 85, 91–93).

Similar to the lncRNAs in the above studies, BCALM appears to act as a modulator of downstream BCR-mediated signaling and calcium flux. Figure 6 depicts a model for how this modulation may occur. Antigen binding to the BCR activates BCR-associated kinases and PLCG2, which hydrolyzes PIP2 to second messengers DAG and IP3. IP3 stimulates IP3 receptor Ca^{+} channels on the endoplasmic reticulum to release Ca^{+} into the cytoplasm, which, along with DAG, stimulates downstream signaling proteins, including PKC, which promote cell growth and survival in normal and lymphoma B cells(33, 34, 69, 70, 72–75, 94–96). PKC also phosphorylates and activates PLD1(27, 28, 31, 32). BCALM interacts with PLD1, AKAP9 (AKAP450) and AKAP13 (AKAP-Lbc). The AKAP proteins form signaling complexes with protein kinases A and C (PKA and PKC)(21–23, 29, 30), which

phosphorylate and activate PLD1(27, 28, 31, 32). PLD1 produces PA, which activates SHP-1(26, 97), a phosphatase that inhibits BCR-associated kinases and downregulates BCR signaling(71, 72, 75, 98, 99) (Fig. 6A). Without BCALM, interaction of PLD1 with the AKAP kinase complex is abrogated, resulting in decreased PLD1 phosphorylation and increased intracellular Ca^{+} concentrations in response to BCR stimulation, in a manner commensurate with BCALM transcript level (i.e., homozygous loss increased calcium flux more than heterozygous loss). These data suggest that BCALM acts as a scaffold and brings together PLD1 and AKAP-associated kinases to promote PLD1 phosphorylation and activation, resulting in increased SHP-1 activity and downregulation of BCR signaling and calcium flux in B cells. In the absence of BCALM, the interaction of PLD1 and AKAP9 is reduced and PLD1 phosphorylation is decreased, which reduces the PLD1 activity and PA production. Lower levels of PA would result in decreased SHP-1 activity and less inhibition of BCR signaling, thus higher levels of intracellular calcium (Fig. 6B). Together, these data suggest that BCALM transcript acts to promote negative feedback of BCR-mediated signaling and calcium flux in response to BCR stimulation.

Antigen receptor stimulation and downstream PLD and PKC signaling play essential roles in diverse immune response pathways. Phospholipase D is a critical component of lipid second messenger signaling, which is required for movement of cells (chemotaxis) and cellular components (e.g., secretory vesicles, lysosomes). In T lymphocytes, PLD1 deficiency impairs TCR-mediated signaling, proliferation, cell adhesion, and chemotaxis in autoimmune and infectious models(61, 64–66, 100). In mast cells, PLD1 is activated by antigen stimulation and is required for exocytosis of secretory granules(27, 28, 60, 101). In neutrophils and phagocytes, PLD1 is involved in chemotaxis, cell adhesion and migration, and its activation promotes antimicrobial defense by facilitating phago-lysosomal maturation(66, 67, 102, 103). There has been less study of PLD enzymes in B cells, however, reports in human CLL and FL highlight the importance of PLD1 in CXCL12-mediated adhesion and in mTOR activation downstream of the BCR-associated Syk kinase(104, 105). During BCR activation, PKC is activated by both DAG and Ca+, and in turn activates CARD11/MALT1/BCL-10 and NF-KB pro-survival and pro-growth pathways(75, 95, 96, 106–108). AKAP proteins assemble kinase-containing complexes, including PKA and PKC, and are involved in multiple signaling cascades in normal and malignant cells across organ systems(21, 23, 30, 109). Relevant to these studies, AKAP9 (AKAP450) plays a key role in T cells in immunological synapse formation during antigen-dependent T cell activation(110). In addition, AKAP9-deficient T cells exhibit decreased TCR recycling to the cell surface, resulting in reduced TCR re-activation and T cell retention in peripheral inflamed tissues(111). AKAP13 (AKAP-Lbc) forms a complex with the inhibitor of NF-KB b (IKKb) to control the production of proinflammatory cytokines in cardiac myocytes in response to adrenergic stimulation(23, 112). Taken together, these published studies and the work presented here suggest that BCR stimulation leads to phosphorylation and activation of PLD1 via AKAP-signaling kinase complexes, which is modulated by interaction with the lncRNA BCALM (AC099524.1). Further work is needed to determine whether loss of PLD1 or AKAP9 or AKAP13 in B cells similarly impacts BCR-mediated calcium flux, or functionally impacts BCR dynamics, immune synapse formation, or mTOR/NF-KB growth and survival pathways(95, 113, 114).

In summary, the results presented here suggest that the B cell specific lncRNA BCALM (AC099524.1) may play an important negative regulatory role in modulating calcium signaling downstream of BCR stimulation. BCALM thus represents a new paradigm for lncRNAs in B lymphocyte activation and signaling pathways, with implications for B cell development, immune response, and lymphoma pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Koues OI, Kowalewski RA, Chang L-W, Pyfrom SC, Schmidt JA, Luo H, Sandoval LE, Hughes TB, Bednarski JJ, Cashen AF, Payton JE, and Oltz EM. 2015 Enhancer sequence variants and transcription-factor deregulation synergize to construct pathogenic regulatory circuits in B-cell lymphoma. Immunity 42: 186–198. [PubMed: 25607463]
2. Cyster JG, and Allen CDC. 2019 B Cell Responses: Cell Interaction Dynamics and Decisions. Cell 177: 524–540. [PubMed: 31002794]
3. Huang Y, Koues OI, Zhao J, Liu R, Pyfrom SC, Payton JE, and Oltz EM. 2017 cis-Regulatory Circuits Regulating NEK6 Kinase Overexpression in Transformed B Cells Are Super-Enhancer Independent. Cell Rep. 18: 2918–2931. [PubMed: 28329684]
4. Huarte M 2015 The emerging role of lncRNAs in cancer. Nat. Med. 21: 1253–1261. [PubMed: 26540387]
5. Chen YG, Satpathy AT, and Chang HY. 2017 Gene regulation in the immune system by long noncoding RNAs. Nat. Immunol. 18: 962–972. [PubMed: 28829444]
6. Atianand MK, Caffrey DR, and Fitzgerald KA. 2017 Immunobiology of Long Noncoding RNAs. Annu. Rev. Immunol. 35: 177–198. [PubMed: 28125358]
7. Yao R-W, Wang Y, and Chen L-L. 2019 Cellular functions of long noncoding RNAs. Nat. Cell Biol. 21: 542–551. [PubMed: 31048766]
8. Fanucchi S, Fok ET, Dalla E, Shibayama Y, Börner K, Chang EY, Stoychev S, Imakaev M, Grimm D, Wang KC, Li G, Sung W-K, and Mhlanga MM. 2019 Immune genes are primed for robust transcription by proximal long noncoding RNAs located in nuclear compartments. Nat. Genet. 51: 138–150. [PubMed: 30531872]

9. Alvarez-Dominguez JR, and Lodish HF. 2017 Emerging mechanisms of long noncoding RNA function during normal and malignant hematopoiesis. Blood 130: 1965–1975. [PubMed: 28928124]
10. Batista PJ, and Chang HY. 2013 Long Noncoding RNAs: Cellular Address Codes in Development and Disease. Cell 152: 1298–1307. [PubMed: 23498938]
11. Mowel WK, Kotzin JJ, McCright SJ, Neal VD, and Henao-Mejia J. 2018 Control of Immune Cell Homeostasis and Function by lncRNAs. Trends Immunol. 39: 55–69. [PubMed: 28919048]
12. Clark BS, and Blackshaw S. 2017 Understanding the Role of lncRNAs in Nervous System Development In Long Non Coding RNA Biology. Advances in Experimental Medicine and Biology Springer, Singapore 253–282.
13. Verma A, Jiang Y, Du W, Fairchild L, Melnick A, and Elemento O. 2015 Transcriptome sequencing reveals thousands of novel long non-coding RNAs in B cell lymphoma. Genome Med. 7: 110. [PubMed: 26521025]
14. Agirre X, Meydan C, Jiang Y, Garate L, Doane AS, Li Z, Verma A, Paiva B, Martín-Subero JI, Elemento O, Mason CE, Prosper F, and Melnick A. 2019 Long non-coding RNAs discriminate the stages and gene regulatory states of human humoral immune response. Nat. Commun. 10: 821. [PubMed: 30778059]
15. Pyfrom SC, Luo H, and Payton JE. 2019 PLAIDOH: a novel method for functional prediction of long non-coding RNAs identifies cancer-specific LncRNA activities. BMC Genomics 20: 137. [PubMed: 30767760]
16. Weber M, Treanor B, Depoil D, Shinohara H, Harwood NE, Hikida M, Kurosaki T, and Batista FD. 2008 Phospholipase C-γ2 and Vav cooperate within signaling microclusters to propagate B cell spreading in response to membrane-bound antigen. J. Exp. Med. 205: 853–868. [PubMed: 18362175]
17. Wang J, Sohn H, Sun G, Milner JD, and Pierce SK. 2014 The autoinhibitory C-terminal SH2 domain of phospholipase C–γ2 stabilizes B cell receptor signalosome assembly. Sci Signal 7: ra89–ra89. [PubMed: 25227611]
18. Wang D, Feng J, Wen R, Marine JC, Sangster MY, Parganas E, Hoffmeyer A, Jackson CW, Cleveland JL, Murray PJ, and Ihle JN. 2000 Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity 13: 25–35. [PubMed: 10933392]
19. Woyach JA, Furman RR, Liu T-M, Ozer HG, Zapatka M, Ruppert AS, Xue L, Li DH-H, Steggerda SM, Versele M, Dave SS, Zhang J, Yilmaz AS, Jaglowski SM, Blum KA, Lozanski A, Lozanski G, James DF, Barrientos JC, Lichter P, Stilgenbauer S, Buggy JJ, Chang BY, Johnson AJ, and Byrd JC. 2014 Resistance Mechanisms for the Bruton's Tyrosine Kinase Inhibitor Ibrutinib. N. Engl. J. Med. 370: 2286–2294. [PubMed: 24869598]
20. Ombrello MJ, Remmers EF, Sun G, Freeman AF, Datta S, Torabi-Parizi P, Subramanian N, Bunney TD, Baxendale RW, Martins MS, Romberg N, Komarow H, Aksentijevich I, Kim HS, Ho J, Cruse G, Jung M-Y, Gilfillan AM, Metcalfe DD, Nelson C, O'Brien M, Wisch L, Stone K, Douek DC, Gandhi C, Wanderer AA, Lee H, Nelson SF, Shianna KV, Cirulli ET, Goldstein DB, Long EO, Moir S, Meffre E, Holland SM, Kastner DL, Katan M, Hoffman HM, and Milner JD. 2012 Cold urticaria, immunodeficiency, and autoimmunity related to PLCG2 deletions. N. Engl. J. Med. 366: 330–8. [PubMed: 22236196]
21. Diviani D, Reggi E, Arambasic M, Caso S, and Maric D. 2016 Emerging roles of A-kinase anchoring proteins in cardiovascular pathophysiology. Biochim. Biophys. Acta BBA - Mol. Cell Res. 1863: 1926–1936.
22. Diviani D, Osman H, and Reggi E. 2018 A-Kinase Anchoring Protein-Lbc: A Molecular Scaffold Involved in Cardiac Protection. J. Cardiovasc. Dev. Dis. 5: 12.
23. Esseltine JL, and Scott JD. 2013 AKAP signaling complexes: pointing towards the next generation of therapeutic targets? Trends Pharmacol. Sci. 34: 648–655. [PubMed: 24239028]
24. Exton JH 1999 Regulation of phospholipase D. Biochim. Biophys. Acta 1439: 121–133. [PubMed: 10425390]
25. Lopez CI, Pelletán LE, Suhaiman L, De Blas GA, Vitale N, Mayorga LS, and Belmonte SA. 2012 Diacylglycerol stimulates acrosomal exocytosis by feeding into a PKC- and PLD1-dependent positive loop that continuously supplies phosphatidylinositol 4,5-bisphosphate. Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids 1821: 1186–1199.

26. Park S-Y, and Han J-S. 2018 Phospholipase D1 Signaling: Essential Roles in Neural Stem Cell Differentiation. J. Mol. Neurosci. 64: 333–340. [PubMed: 29478139]
27. Chahdi A, Choi WS, Kim YM, Fraundorfer PF, and Beaven MA. 2002 Serine/threonine protein kinases synergistically regulate phospholipase D1 and 2 and secretion in RBL-2H3 mast cells. Mol. Immunol. 38: 1269–1276. [PubMed: 12217394]
28. Choi WS, Chahdi A, Kim YM, Fraundorfer PF, and Beaven MA. 2002 Regulation of phospholipase D and secretion in mast cells by protein kinase A and other protein kinases. Ann. N. Y. Acad. Sci. 968: 198–212. [PubMed: 12119277]
29. Carnegie GK, Smith FD, McConnachie G, Langeberg LK, and Scott JD. 2004 AKAP-Lbc Nucleates a Protein Kinase D Activation Scaffold. Mol. Cell 15: 889–899. [PubMed: 15383279]
30. Carnegie GK, and Scott JD. 2012 A-Kinase Anchoring Protein (AKAP) In Encyclopedia of Signaling Molecules Choi S, ed. Springer, New York, NY 89–96.
31. Su W, and Frohman MA. 2010 Chapter 144 - Phospholipase D In Handbook of Cell Signaling (Second Edition) Bradshaw RA, and Dennis EA, eds. Academic Press, San Diego 1167–1176.
32. Frohman MA 2015 The phospholipase D superfamily as therapeutic targets. Trends Pharmacol. Sci. 36: 137–144. [PubMed: 25661257]
33. Baba Y, and Kurosaki T. 2011 Impact of Ca2+ signaling on B cell function. Trends Immunol. 32: 589–94. [PubMed: 22000665]
34. Baba Y, and Kurosaki T. 2016 Role of Calcium Signaling in B Cell Activation and Biology In B Cell Receptor Signaling. Current Topics in Microbiology and Immunology Kurosaki T, and Wienands J, eds. Springer International Publishing, Cham 143–174.
35. Andrews JM, Schmidt JA, Carson KR, Musiek AC, Mehta-Shah N, and Payton JE. 2019 Novel cell adhesion/migration pathways are predictive markers of HDAC inhibitor resistance in cutaneous T cell lymphoma. EBioMedicine.
36. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, and Gingeras TR. 2013 STAR: ultrafast universal RNA-seq aligner. Bioinforma. Oxf. Engl. 29: 15–21.
37. Patro R, Duggal G, Love MI, Irizarry RA, and Kingsford C. 2017 Salmon provides fast and bias-aware quantification of transcript expression. Nat. Methods 14: 417–419. [PubMed: 28263959]
38. Love MI, Huber W, and Anders S. 2014 Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15.
39. Andrews J, El-Alawi M, and Payton J. 2018 Genotify: Fast, lightweight gene lookup and summarization. J. Open Source Softw. .
40. Wang L, Park HJ, Dasari S, Wang S, Kocher J-P, and Li W. 2013 CPAT: Coding-Potential Assessment Tool using an alignment-free logistic regression model. Nucleic Acids Res. 41: e74. [PubMed: 23335781]
41. Mas-Ponte D, Carlevaro-Fita J, Palumbo E, Hermoso Pulido T, Guigo R, and Johnson R. 2017 LncATLAS database for subcellular localization of long noncoding RNAs. RNA N. Y. N 23: 1080–1087.
42. Thul PJ, Åkesson L, Wiking M, Mahdessian D, Geladaki A, Blal HA, Alm T, Asplund A, Björk L, Breckels LM, Bäckström A, Danielsson F, Fagerberg L, Fall J, Gatto L, Gnann C, Hober S, Hjelmare M, Johansson F, Lee S, Lindskog C, Mulder J, Mulvey CM, Nilsson P, Oksvold P, Rockberg J, Schutten R, Schwenk JM, Sivertsson Å, Sjöstedt E, Skogs M, Stadler C, Sullivan DP, Tegel H, Winsnes C, Zhang C, Zwahlen M, Mardinoglu A, Pontén F, von Feilitzen K, Lilley KS, Uhlén M, and Lundberg E. 2017 A subcellular map of the human proteome. Science eaal3321. [PubMed: 28495876]
43. Sundararaman B, Zhan L, Blue SM, Stanton R, Elkins K, Olson S, Wei X, Van Nostrand EL, Pratt GA, Huelga SC, Smalec BM, Wang X, Hong EL, Davidson JM, Lécuyer E, Graveley BR, and Yeo GW. 2016 Resources for the Comprehensive Discovery of Functional RNA Elements. Mol. Cell 61: 903–913. [PubMed: 26990993]
44. Nostrand ELV, Pratt GA, Shishkin AA, Gelboin-Burkhart C, Fang MY, Sundararaman B, Blue SM, Nguyen TB, Surka C, Elkins K, Stanton R, Rigo F, Guttman M, and Yeo GW. 2016 Robust transcriptome-wide discovery of RNA-binding protein binding sites with enhanced CLIP (eCLIP). Nat. Methods 13: 508–514. [PubMed: 27018577]

45. Dominguez D, Freese P, Alexis MS, Su A, Hochman M, Palden T, Bazile C, Lambert NJ, Van Nostrand EL, Pratt GA, Yeo GW, Graveley BR, and Burge CB. 2018 Sequence, Structure, and Context Preferences of Human RNA Binding Proteins. Mol. Cell 70: 854–867.e9. [PubMed: 29883606]
46. Gagnon KT, Li L, Janowski BA, and Corey DR. 2014 Analysis of nuclear RNA interference in human cells by subcellular fractionation and Argonaute loading. Nat. Protoc. 9: 2045–60. [PubMed: 25079428]
47. ENCODE Project Consortium. 2012 An integrated encyclopedia of DNA elements in the human genome. Nature 489: 57–74. [PubMed: 22955616]
48. Cho SW, Xu J, Sun R, Mumbach MR, Carter AC, Chen YG, Yost KE, Kim J, He J, Nevins SA, Chin S-F, Caldas C, Liu SJ, Horlbeck MA, Lim DA, Weissman JS, Curtis C, and Chang HY. 2018 Promoter of lncRNA Gene PVT1 Is a Tumor-Suppressor DNA Boundary Element. Cell 173: 1398–1412.e22. [PubMed: 29731168]
49. Engreitz JM, Haines JE, Perez EM, Munson G, Chen J, Kane M, McDonel PE, Guttman M, and Lander ES. 2016 Local regulation of gene expression by lncRNA promoters, transcription and splicing. Nature 539: 452–455. [PubMed: 27783602]
50. Rashid F, Shah A, and Shan G. 2016 Long Non-coding RNAs in the Cytoplasm. Genomics Proteomics Bioinformatics 14: 73–80. [PubMed: 27163185]
51. Wang P, Xu J, Wang Y, and Cao X. 2017 An interferon-independent lncRNA promotes viral replication by modulating cellular metabolism. Science 358: 1051–1055. [PubMed: 29074580]
52. Koirala P, Huang J, Ho T-T, Wu F, Ding X, and Mo Y-Y. 2017 LncRNA AK023948 is a positive regulator of AKT. Nat. Commun. 8: 14422. [PubMed: 28176758]
53. Lin A, Li C, Xing Z, Hu Q, Liang K, Han L, Wang C, Hawke DH, Wang S, Zhang Y, Wei Y, Ma G, Park PK, Zhou J, Zhou YY, Hu Z, Zhou YY, Marks JR, Liang H, Hung M-C, Lin C, and Yang L. 2016 The LINK-A lncRNA activates normoxic HIF1α signalling in triple-negative breast cancer. Nat. Cell Biol. 18: 213–24. [PubMed: 26751287]
54. Aktaş T, Avşar Ilık İ, Maticzka D, Bhardwaj V, Pessoa Rodrigues C, Mittler G, Manke T, Backofen R, and Akhtar A. 2017 DHX9 suppresses RNA processing defects originating from the Alu invasion of the human genome. Nature 544: 115–119. [PubMed: 28355180]
55. Chakraborty P, Huang JTJ, and Hiom K. 2018 DHX9 helicase promotes R-loop formation in cells with impaired RNA splicing. Nat. Commun. 9: 4346. [PubMed: 30341290]
56. Fuller-Pace FV 2006 DExD/H box RNA helicases: multifunctional proteins with important roles in transcriptional regulation. Nucleic Acids Res. 34: 4206–4215. [PubMed: 16935882]
57. Zheng GXY, Do BT, Webster DE, Khavari PA, and Chang HY. 2014 Dicer-microRNA-Myc circuit promotes transcription of hundreds of long noncoding RNAs. Nat. Struct. Mol. Biol. 21: 585–590. [PubMed: 24929436]
58. Rybak-Wolf A, Jens M, Murakawa Y, Herzog M, Landthaler M, and Rajewsky N. 2014 A variety of dicer substrates in human and C. elegans. Cell 159: 1153–1167. [PubMed: 25416952]
59. Zhang Y, and Frohman MA. 2014 Cellular and Physiological Roles for Phospholipase D1 in Cancer. J. Biol. Chem. 289: 22567–22574. [PubMed: 24990946]
60. Choi WS, Kim YM, Combs C, Frohman MA, and Beaven MA. 2002. Phospholipases D1 and D2 regulate different phases of exocytosis in mast cells. J. Immunol. Baltim. Md 1950 168: 5682–5689.
61. Zhu M, Foreman DP, O'Brien SA, Jin Y, and Zhang W. 2018 Phospholipase D in TCR-Mediated Signaling and T Cell Activation. J. Immunol. Baltim. Md 1950 200: 2165–2173.
62. Jang M-J, Lee M-J, Park H-Y, Bae Y-S, Min DS, Ryu SH, and Kwak J-Y. 2004 Phosphorylation of phospholipase D1 and the modulation of its interaction with RhoA by cAMP-dependent protein kinase. Exp. Mol. Med. 36: 172–178. [PubMed: 15150446]
63. Bolomini-Vittori M, Montresor A, Giagulli C, Staunton D, Rossi B, Martinello M, Constantin G, and Laudanna C. 2009 Regulation of conformer-specific activation of the integrin LFA-1 by a chemokine-triggered Rho signaling module. Nat. Immunol. 10: 185–194. [PubMed: 19136961]
64. Mor A, Wynne JP, Ahearn IM, Dustin ML, Du G, and Philips MR. 2009 Phospholipase D1 regulates lymphocyte adhesion via upregulation of Rap1 at the plasma membrane. Mol. Cell. Biol. 29: 3297–3306. [PubMed: 19332557]

65. Göbel K, Schuhmann MK, Pankratz S, Stegner D, Herrmann AM, Braun A, Breuer J, Bittner S, Ruck T, Wiendl H, Kleinschnitz C, Nieswandt B, and Meuth SG. 2014 Phospholipase D1 mediates lymphocyte adhesion and migration in experimental autoimmune encephalomyelitis. Eur. J. Immunol. 44: 2295–2305. [PubMed: 24811005]

66. Lehman N, Fulvio MD, McCray N, Campos I, Tabatabaian F, and Gomez-Cambronero J. 2006 Phagocyte cell migration is mediated by phospholipases PLD1 and PLD2. Blood 108: 3564–3572. [PubMed: 16873675]

67. Iyer SS, Agrawal RS, Thompson CR, Thompson S, Barton JA, and Kusner DJ. 2006. Phospholipase D1 regulates phagocyte adhesion. J. Immunol. Baltim. Md 1950 176: 3686–3696.

68. Huang R, Cioffi J, Kimberly R, Edberg J, and Mayer L. 1995 B cell differentiation factor-induced human B cell maturation: stimulation of intracellular calcium release. Cell. Immunol. 164: 227–233. [PubMed: 7656331]

69. Wen Y, Jing Y, Yang L, Kang D, Jiang P, Li N, Cheng J, Li J, Li X, Peng Z, Sun X, Miller H, Sui Z, Gong Q, Ren B, Yin W, and Liu C. 2019 The regulators of BCR signaling during B cell activation. Blood Sci. 1: 119.

70. Shlomchik MJ, Luo W, and Weisel F. 2019 Linking signaling and selection in the germinal center. Immunol. Rev. 288: 49–63. [PubMed: 30874353]

71. Seda V, and Mraz M. 2015 B-cell receptor signalling and its crosstalk with other pathways in normal and malignant cells. Eur. J. Haematol. 94: 193–205. [PubMed: 25080849]

72. Davis RE, Ngo VN, Lenz G, Tolar P, Young RM, Romesser PB, Kohlhammer H, Lamy L, Zhao H, Yang Y, Xu W, Shaffer AL, Wright G, Xiao W, Powell J, Jiang JK, Thomas CJ, Rosenwald A, Ott G, Muller-Hermelink HK, Gascoyne RD, Connors JM, Johnson NA, Rimsza LM, Campo E, Jaffe ES, Wilson WH, Delabie J, Smeland EB, Fisher RI, Braziel RM, Tubbs RR, Cook JR, Weisenburger DD, Chan WC, Pierce SK, and Staudt LM. 2010 Chronic active B-cell-receptor signalling in diffuse large B-cell lymphoma. Nature 463: 88–92. [PubMed: 20054396]

73. Burger JA, and Chiorazzi N. 2013 B cell receptor signaling in chronic lymphocytic leukemia. Trends Immunol. 34: 592–601. [PubMed: 23928062]

74. Burger JA, and Wiestner A. 2018 Targeting B cell receptor signalling in cancer: preclinical and clinical advances. Nat. Rev. Cancer 18: 148–167. [PubMed: 29348577]

75. Young RM, Shaffer AL, Phelan JD, and Staudt LM. 2015 B cell Receptor Signaling in Diffuse Large B cell Lymphoma. Semin. Hematol. 52: 77–85. [PubMed: 25805587]

76. Valadkhan S, and Gunawardane LS. 2016 lncRNA-mediated regulation of the interferon response. Virus Res. 212: 127–136. [PubMed: 26474526]

77. Du M, Yuan L, Tan X, Huang D, Wang X, Zheng Z, Mao X, Li X, Yang L, Huang K, Zhang F, Wang Y, Luo X, Huang D, and Huang K. 2017 The LPS-inducible lncRNA Mirt2 is a negative regulator of inflammation. Nat. Commun. 8: 2049. [PubMed: 29230038]

78. Atianand MK, Hu W, Satpathy AT, Shen Y, Ricci EP, Alvarez-Dominguez JR, Bhatta A, Schattgen SA, McGowan JD, Blin J, Braun JE, Gandhi P, Moore MJ, Chang HY, Lodish HF, Caffrey DR, Fitzgerald KA, Atianand MK, Fitzgerald KA, Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ, Carpenter S, Aiello D, Atianand MK, Ricci EP, Gandhi P, Hall LL, Byron M, Monks B, Henry-Bezy M, Lawrence JB, et al., Castellanos-Rubio A, Fernandez-Jimenez N, Kratchmarov R, Luo X, Bhagat G, Green PH, Schneider R, Kiledjian M, Bilbao JR, Ghosh S, Chan J, Atianand M, Jiang Z, Carpenter S, Aiello D, Elling R, Fitzgerald KA, Caffrey DR, Chen GY, Nuñez G, Derrien T, Johnson R, Bussotti G, Tanzer A, Djebali S, Tilgner H, Guernec G, Martin D, Merkel A, Knowles DG, et al., Djebali S, Davis CA, Merkel A, Dobin A, Lassmann T, Mortazavi A, Tanzer A, Lagarde J, Lin W, Schlesinger F, et al., Engreitz JM, Sirokman K, McDonel P, Shishkin AA, Surka C, Russell P, Grossman SR, Chow AY, Guttman M, Lander ES, Giraud M, Jmari N, Du L, Carallis F, Nieland TJ, Perez-Campo FM, Bensaude O, Root DE, Hacohen N, Mathis D, Benoist C, Gomez JA, Wapinski OL, Yang YW, Bureau JF, Gopinath S, Monack DM, Chang HY, Brahic M, Kirkegaard K, Guttman M, Rinn JL, Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, Huarte M, Zuk O, Carey BW, Cassady JP, et al., Hacisuleyman E, Goff LA, Trapnell C, Williams A, Henao-Mejia J, Sun L, McClanahan P, Hendrickson DG, Sauvageau M, Kelley DR, et al., Hasegawa Y, Brockdorff N, Kawano S, Tsutui K, Tsutui K, Nakagawa S, Heyer EE, Ozadam H, Ricci EP, Cenik C, Moore MJ, Hu W, Yuan B, Flygare J, Lodish HF, Huang Y, Yao W. Li, X., Lin QJ, Yin JW, Liang Y, Heiner M, Tian B, Hui J,

Wang G, Huang W, Thomas B, Flynn RA, Gavzy SJ, Wu L, Kim SV, Hall JA, Miraldi ER, Ng CP, Rigo F, et al., Huarte M, Guttman M, Feldser D, Garber M, Koziol MJ, Kenzelmann-Broz D, Khalil AM, Zuk O, Amit I, Rabani M, et al., Janeway CA, Medzhitov R, Lamkanfi M, Dixit VM, Li Z, Chao TC, Chang KY, Lin N, Patil VS, Shimizu C, Head SR, Burns JC, Rana TM, Medzhitov R, Horng T, Mondal T, Subhash S, Vaid R, Enroth S, Uday S, Reinius B, Mitra S, Mohammed A, James AR, Hoberg E, et al., Morris KV, Mattick JS, O'Connell RM, Rao DS, Baltimore D, O'Leary VB, Ovsepian SV, Carrascosa LG, Buske FA, Radulovic V, Niyazi M, Moertl S, Trau M, Atkinson MJ, Anastasov N, Ray D, Kazan H, Cook KB, Weirauch MT, Najafabadi HS, Li X, Gueroussov S, Albu M, Zheng H, Yang A, et al., Schep AN, Buenrostro JD, Denny SK, Schwartz K, Sherlock G, Greenleaf WJ, Smale ST, Plevy SE, Weinmann AS, Zhou L, Ramirez-Carrozzi VR, Pope SD, Bhatt DM, Tong AJ, Ulitsky I, Bartel DP, West JA, Davis CP, Sunwoo H, Simon MD, Sadreyev RI, Wang PI, Tolstorukov MY, and Kingston RE. 2016 A Long Noncoding RNA lincRNA-EPS Acts as a Transcriptional Brake to Restrain Inflammation. Cell 165: 1672–1685. [PubMed: 27315481]

79. Hu G, Gong A-Y, Wang Y, Ma S, Chen X, Chen J, Su C-J, Shibata A, Strauss-Soukup JK, Drescher KM, and Chen X-M. 2016 LincRNA-Cox2 Promotes Late Inflammatory Gene Transcription in Macrophages through Modulating SWI/SNF-Mediated Chromatin Remodeling. J. Immunol. 196: 2799–2808. [PubMed: 26880762]
80. Jiang M, Zhang S, Yang Z, Lin H, Zhu J, Liu L, Wang W, Liu S, Liu W, Ma Y, Zhang L, and Cao X. 2018 Self-Recognition of an Inducible Host lncRNA by RIG-I Feedback Restricts Innate Immune Response. Cell 173: 906–919.e13. [PubMed: 29706547]
81. Roux BT, Heward JA, Donnelly LE, Jones SW, and Lindsay MA. 2017 Catalog of Differentially Expressed Long Non-Coding RNA following Activation of Human and Mouse Innate Immune Response. Front. Immunol 8. [PubMed: 28144241]
82. Alvarez-Dominguez JR, Hu W, Yuan B, Shi J, Park SS, Gromatzky AA, van Oudenaarden A, and Lodish HF. 2014 Global discovery of erythroid long noncoding RNAs reveals novel regulators of red cell maturation. Blood 123: 570–81. [PubMed: 24200680]
83. Alvarez-Dominguez JR, Knoll M, Gromatzky AA, and Lodish HF. 2017 The Super-Enhancer-Derived alncRNA-EC7/Bloodlinc Potentiates Red Blood Cell Development in trans. Cell Rep. 19: 2503–2514. [PubMed: 28636939]
84. Wang Z, Yang B, Zhang M, Guo W, Wu Z, Wang Y, Jia L, Li S, Cancer Genome Atlas Research Network, Xie W, and Yang D. 2018 lncRNA Epigenetic Landscape Analysis Identifies EPIC1 as an Oncogenic lncRNA that Interacts with MYC and Promotes Cell-Cycle Progression in Cancer. Cancer Cell 33: 706–720.e9. [PubMed: 29622465]
85. Chiu H-S, Somvanshi S, Patel E, Chen T-W, Singh VP, Zorman B, Patil SL, Pan Y, Chatterjee SS, Sood AK, Gunaratne PH, and Sumazin P. 2018 Pan-Cancer Analysis of lncRNA Regulation Supports Their Targeting of Cancer Genes in Each Tumor Context. Cell Rep. 23: 297–312.e12. [PubMed: 29617668]
86. Paralkar VR, Mishra T, Luan J, Yao Y, Kossenkov AV, Anderson SM, Dunagin M, Pimkin M, Gore M, Sun D, Konuthula N, Raj A, An X, Mohandas N, Bodine DM, Hardison RC, and Weiss MJ. 2014 Lineage and species-specific long noncoding RNAs during erythro-megakaryocytic development. Blood 123: 1927–37. [PubMed: 24497530]
87. Ronchetti D, Manzoni M, Agnelli L, Vinci C, Fabris S, Cutrona G, Matis S, Colombo M, Galletti S, Taiana E, Recchia AG, Bossio S, Gentile M, Musolino C, Raimondo FD, Grilli A, Bicciato S, Cortelezzi A, Tassone P, Morabito F, Ferrarini M, and Neri A. 2016 lncRNA profiling in early-stage chronic lymphocytic leukemia identifies transcriptional fingerprints with relevance in clinical outcome. Blood Cancer J. 6: e468. [PubMed: 27611921]
88. Sehgal L, Mathur R, Braun FK, Wise JF, Berkova Z, Neelapu S, Kwak LW, and Samaniego F. 2014 FAS-antisense 1 lncRNA and production of soluble versus membrane Fas in B-cell lymphoma. Leukemia 28: 2376–2387. [PubMed: 24811343]
89. Wang X, Sehgal L, Jain N, Khashab T, Mathur R, and Samaniego F. 2016 LncRNA MALAT1 promotes development of mantle cell lymphoma by associating with EZH2. J. Transl. Med 14: 346. [PubMed: 27998273]
90. Doose G, Haake A, Bernhart SH, López C, Duggimpudi S, Wojciech F, Bergmann AK, Borkhardt A, Burkhardt B, Claviez A, Dimitrova L, Haas S, Hoell JI, Hummel M, Karsch D, Klapper W,

Kleo K, Kretzmer H, Kreuz M, Küppers R, Lawerenz C, Lenze D, Loeffler M, Mantovani-Löffler L, Möller P, Ott G, Richter J, Rohde M, Rosenstiel P, Rosenwald A, Schilhabel M, Schneider M, Scholz I, Stilgenbauer S, Stunnenberg HG, Szczepanowski M, Trümper L, Weniger MA, Consortium IM-S, Hoffmann S, Siebert R, and Iaccarino I. 2015 MINCR is a MYC-induced lncRNA able to modulate MYC's transcriptional network in Burkitt lymphoma cells. Proc. Natl. Acad. Sci. 112: E5261–E5270. [PubMed: 26351698]

91. Lin H, Jiang M, Liu L, Yang Z, Ma Z, Liu S, Ma Y, Zhang L, and Cao X. 2019 The long noncoding RNA Lnczc3h7a promotes a TRIM25-mediated RIG-I antiviral innate immune response. Nat. Immunol. 1.
92. Zhang Y, Sun L, Xuan L, Pan Z, Hu X, Liu H, Bai Y, Jiao L, Li Z, Cui L, Wang X, Wang S, Yu T, Feng B, Guo Y, Liu Z, Meng W, Ren H, Zhu J, Zhao X, Yang C, Zhang Y, Xu C, Wang Z, Lu Y, Shan H, and Yang B. 2018 Long non-coding RNA CCRR controls cardiac conduction via regulating intercellular coupling. Nat. Commun. 9: 4176. [PubMed: 30301979]
93. Agliano F, Rathinam VA, Medvedev AE, Vanaja SK, and Vella AT. 2019 Long Noncoding RNAs in Host–Pathogen Interactions. Trends Immunol. 40: 492–510. [PubMed: 31053495]
94. Luo W, Weisel F, and Shlomchik MJ. 2018 B Cell Receptor and CD40 Signaling Are Rewired for Synergistic Induction of the c-Myc Transcription Factor in Germinal Center B Cells. Immunity 48: 313–326.e5. [PubMed: 29396161]
95. Phelan JD, Young RM, Webster DE, Roulland S, Wright GW, Kasbekar M, Shaffer AL, Ceribelli M, Wang JQ, Schmitz R, Nakagawa M, Bachy E, Huang DW, Ji Y, Chen L, Yang Y, Zhao H, Yu X, Xu W, Palisoc MM, Valadez RR, Davies-Hill T, Wilson WH, Chan WC, Jaffe ES, Gascoyne RD, Campo E, Rosenwald A, Ott G, Delabie J, Rimsza LM, Rodriguez FJ, Estephan F, Holdhoff M, Kruhlak MJ, Hewitt SM, Thomas CJ, Pittaluga S, Oellerich T, and Staudt LM. 2018 A multiprotein supercomplex controlling oncogenic signalling in lymphoma. Nature 560: 387–391. [PubMed: 29925955]
96. Young RM, and Staudt LM. 2013 Targeting pathological B cell receptor signalling in lymphoid malignancies. Nat. Rev. Drug Discov. 12: 229–243. [PubMed: 23449308]
97. Krishna S, and Zhong X-P. 2013 Regulation of Lipid Signaling by Diacylglycerol Kinases during T Cell Development and Function. In Front. Immunol.
98. Ravetch JV, and Lanier LL. 2000 Immune Inhibitory Receptors. Science 290: 84–89. [PubMed: 11021804]
99. Tsubata T 2018 Ligand Recognition Determines the Role of Inhibitory B Cell Co-receptors in the Regulation of B Cell Homeostasis and Autoimmunity. Front. Immunol. 9: 2276. [PubMed: 30333834]
100. Taylor HE, Simmons GE, Mathews TP, Khatua AK, Popik W, Lindsley CW, D'Aquila RT, and Brown HA. 2015 Phospholipase D1 Couples CD4+ T Cell Activation to c-Myc-Dependent Deoxyribonucleotide Pool Expansion and HIV-1 Replication. PLoS Pathog. 11: e1004864. [PubMed: 26020637]
101. Cockcroft S, Way G, O'Luanaigh N, Pardo R, Sarri E, and Fensome A. 2002 Signalling role for ARF and phospholipase D in mast cell exocytosis stimulated by crosslinking of the high affinity FcepsilonR1 receptor. Mol. Immunol. 38: 1277–1282. [PubMed: 12217395]
102. Koh H-J, Kim Y-R, Kim J-S, Yun J-S, Jang K, and Yang C-S. 2017 Toxoplasma gondii GRA7-Targeted ASC and PLD1 Promote Antibacterial Host Defense via PKCα. PLOS Pathog. 13: e1006126. [PubMed: 28125719]
103. Nishikimi A, Fukuhara H, Su W, Hongu T, Takasuga S, Mihara H, Cao Q, Sanematsu F, Kanai M, Hasegawa H, Tanaka Y, Shibasaki M, Kanaho Y, Sasaki T, Frohman MA, and Fukui Y. 2009 Sequential regulation of DOCK2 dynamics by two phospholipids during neutrophil chemotaxis. Science 324: 384–387. [PubMed: 19325080]
104. Montresor A, Bolomini-Vittori M, Simon SI, Rigo A, Vinante F, and Laudanna C. 2009 Comparative Analysis of Normal versus CLL B-Lymphocytes Reveals Patient-Specific Variability in Signaling Mechanisms Controlling LFA-1 Activation by Chemokines. Cancer Res. 69: 9281–9290. [PubMed: 19934331]
105. Leseux L, Hamdi SM, Al Saati T, Capilla F, Recher C, Laurent G, and Bezombes C. 2006 Syk-dependent mTOR activation in follicular lymphoma cells. Blood 108: 4156–4162. [PubMed: 16912221]

106. Blum KA 2015 B-cell receptor pathway modulators in NHL. Hematology 2015: 82–91. [PubMed: 26637705]

107. Limnander A, Depeille P, Freedman TS, Liou J, Leitges M, Kurosaki T, Roose JP, and Weiss A. 2011 STIM1, PKC-δ and RasGRP set a threshold for proapoptotic Erk signaling during B cell development. Nat. Immunol. 12: 425–433. [PubMed: 21441934]

108. Guo B, Su TT, and Rawlings DJ. 2004 Protein kinase C family functions in B-cell activation. Curr. Opin. Immunol. 16: 367–373. [PubMed: 15134787]

109. Reggi E, and Diviani D. 2017 The role of A-kinase anchoring proteins in cancer development. Cell. Signal. 40: 143–155. [PubMed: 28927666]

110. Robles-Valero J, Martín-Cófreces NB, Lamana A, Macdonald S, Volkov Y, and Sánchez-Madrid F. 2010 Integrin and CD3/TCR activation are regulated by the scaffold protein AKAP450. Blood 115: 4174–4184. [PubMed: 20231423]

111. Herter JM, Grabie N, Cullere X, Azcutia V, Rosetti F, Bennett P, Herter-Sprie GS, Elyaman W, Luscinskas FW, Lichtman AH, and Mayadas TN. 2015 AKAP9 regulates activation-induced retention of T lymphocytes at sites of inflammation. Nat. Commun. 6: 1–14.

112. del Vescovo CD, Cotecchia S, and Diviani D. 2013 A-kinase-anchoring protein-Lbc anchors IκB kinase β to support interleukin-6-mediated cardiomyocyte hypertrophy. Mol. Cell. Biol. 33: 14–27. [PubMed: 23090968]

113. He M, and Westerberg LS. 2019 Congenital Defects in Actin Dynamics of Germinal Center B Cells. Front. Immunol 10. [PubMed: 30723470]

114. Li J, Yin W, Jing Y, Kang D, Yang L, Cheng J, Yu Z, Peng Z, Li X, Wen Y, Sun X, Ren B, and Liu C. 2018 The Coordination Between B Cell Receptor Signaling and the Actin Cytoskeleton During B Cell Activation. Front. Immunol. 9: 3096. [PubMed: 30687315]

KEY POINTS

1. LncRNA BCALM (AC099524.1) is B-cell specific and highly expressed in human lymphomas
2. BCALM is necessary for the interaction of signal transduction proteins PLD1 and AKAP9
3. BCALM promotes negative feedback that down-modulates BCR-stimulated Ca^{+} signaling

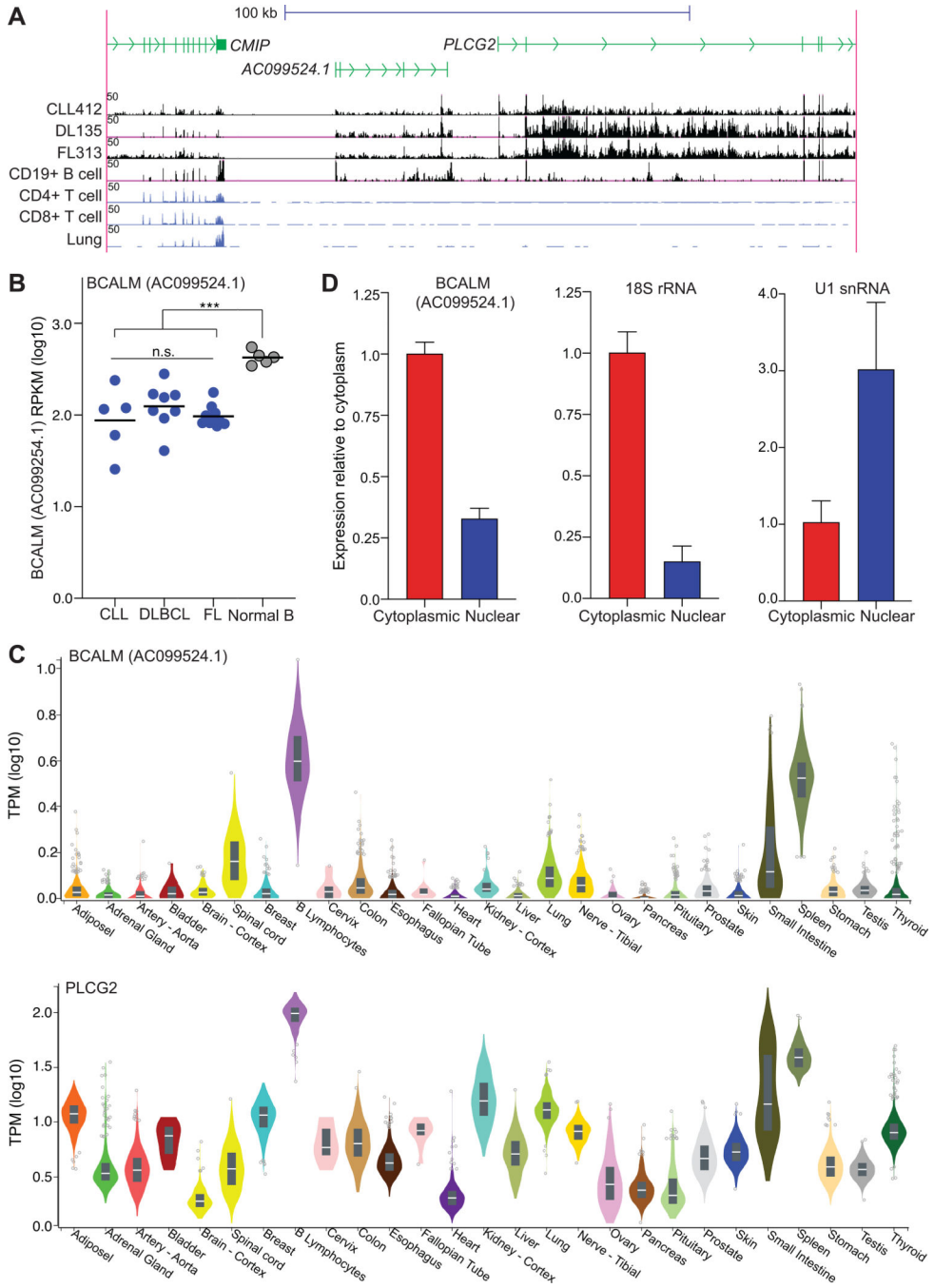

Figure 1. LncRNA BCALM (AC099524.1) is highly and specifically expressed in B lymphocytes.

A) UCSC Genome Browser screenshot shows the genomic locus containing *AC099524.1* and flanking genes. Tracks below show gene expression in representative samples of primary B cell cancers from WUSM patients (CLL412, DL135, FL313), non-malignant $CD19^+$ B and $CD4^+$ and $CD8^+$ T cells, and a lung adenocarcinoma cell line (A549) (RNA-seq, normalized transcripts per million). **B)** Expression of BCALM in purified B cells from WUSM B cell cancer primary samples and normal B cells (RNA-seq, RPKM = reads per million per kilobase, log10). (unpaired two-tailed t-test with Welch's correction, n.s., not

significant for any comparison between B cell cancer groups; *** $p < 0.001$) **C)** Violin plots show expression of BCALM and nearby coding gene PLCG2 across 27 different cell and tissue types (RNA-seq, TPM = transcripts per million, log10, gtexportal.org). **D)** Subcellular localization of RNA transcripts measured by qRT-PCR following fractionation of OCI-Ly7 lymphoma cells. (Fold change relative to same transcript in cytoplasm, mean ± SD). Data are representative of at least three independent experiments (D).

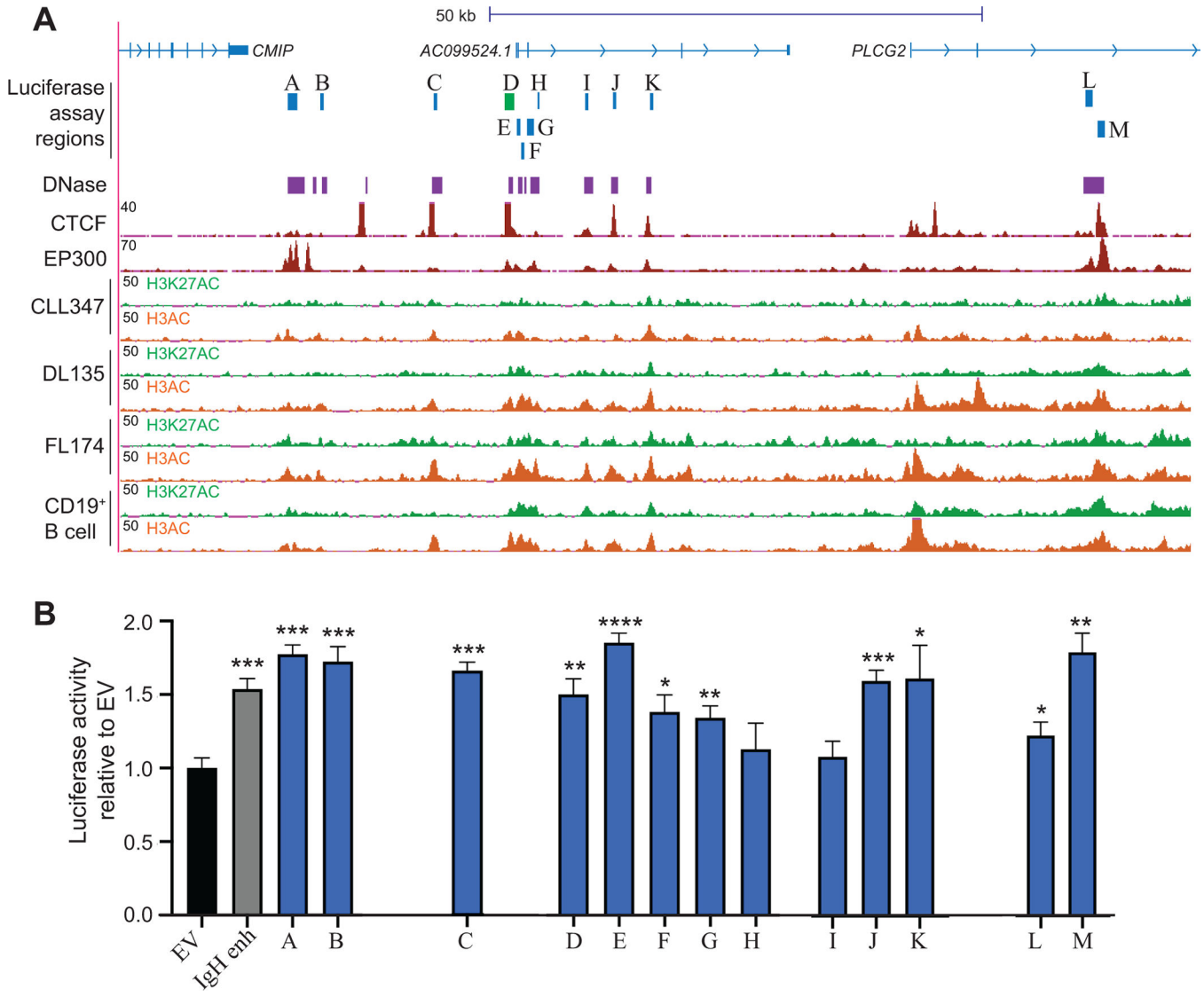

Figure 2. Active regulatory elements are distributed throughout the *CMIP/AC099524.1/PLCG2* locus.

A) UCSC Genome Browser screenshot shows the genomic locus containing *AC099524.1* and flanking genes. Putative regulatory regions assayed by luciferase reporter in (B) are labeled A-M and blue or green bars correspond to their size and location (blue: putative enhancer, green: promoter region of *AC099524.1*). Purple bars show peaks of DNase I hypersensitivity and tracks below show CTCF and EP300 peaks (GM12878 B cells, ENCODE(47)). Lower tracks show histone acetylation peaks (H3K27ac, H3ac) in representative WU lymphoma and non-malignant B cell samples (ChIP-seq, normalized reads per million). **B)** Bar graphs show luciferase reporter activity normalized to empty vector for each of the indicated regions in (A). (mean ± SD for 2–3 experiments; unpaired two-tailed t-test with Welch's correction, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

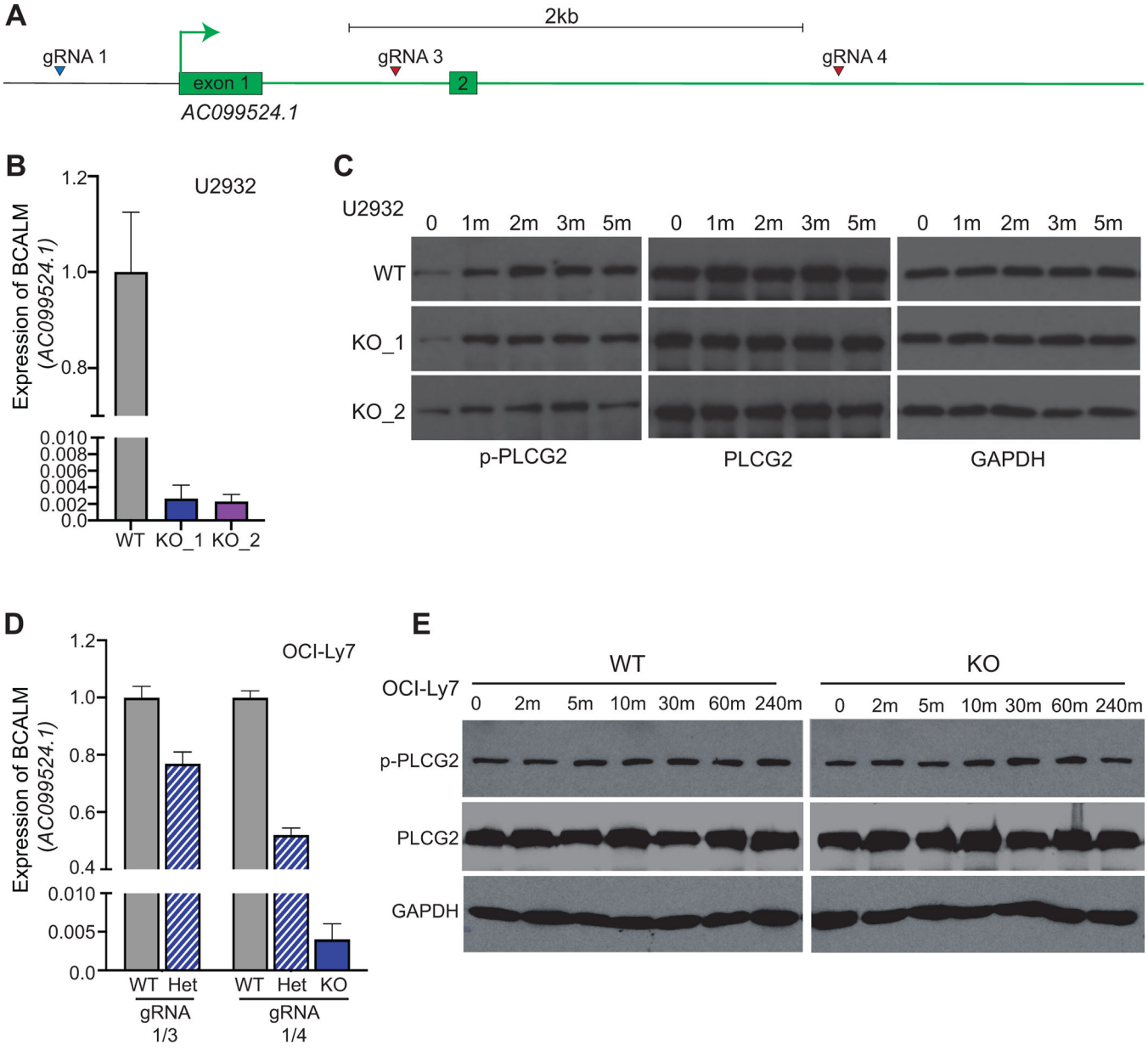

Figure 3. Knockout of BCALM (*AC099524.1*) does not affect PLCG2 expression or phosphorylation.

A) Diagram depicts the locations of CRISPR/Cas9 targeting gRNAs for gene editing of *AC099524.1* (red and blue triangles). **B)** Expression of *AC099524.1* in U2932 lymphoma cell line subclones post gene editing. Measured by qRT-PCR, normalized to GAPDH, and shown relative to Wild type (WT). gRNA pairs 1/3 and 1/4 were used for gene editing and are indicated. (mean ± SD; homozygous (KO); genomic PCR in Fig. S2A–C) **C)** Western blots show levels of phosphorylated and total PLCG2 protein after BCR stimulation with anti-IgM in U2932 WT and KO cells. GAPDH is a loading control. **D)** Expression of *AC099524.1* in OCI-Ly7 lymphoma cell line subclones post gene editing, as in B. (heterozygous (Het); genomic PCR in Fig. S2A–C). **E)** Western blots of OCI-Ly7 cells treated as in C. Representative of at least 2 independent experiments (B-E)

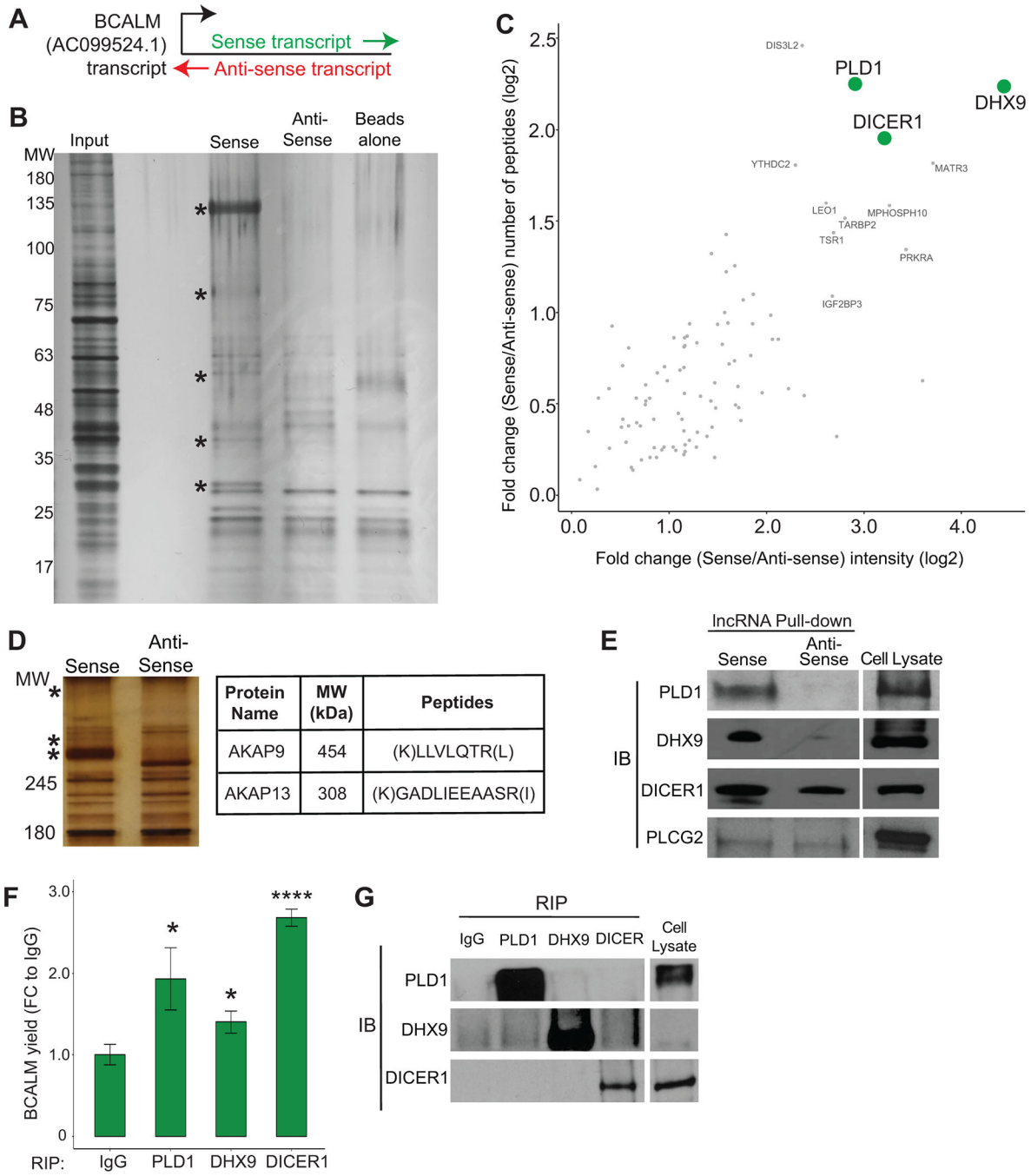

Figure 4. BCALM (AC099524.1) interacts with RNA binding and signaling transduction proteins.

A) Diagram of sense and anti-sense BCALM oligos used in B. **B)** Silver stained SDS-PAGE gel shows proteins in input whole cell lysate, sense and anti-sense BCALM pull-down, and beads alone control. Asterisks mark bands that are enriched in sense BCALM pull-down. **C)** Proteins identified by mass spectrometry after pull-down as in (B) and plotted by log2 fold change of sense:anti-sense BCALM for peptide number and signal intensity. **D)** (Left panel) Silver stain of high molecular weight gel showing bands enriched in sense AC099524.1 pull-

down compared to anti-sense (asterisks). (Right panel) Table of peptides identified by mass spectrometry from the highlighted gel bands. **E)** Western blots confirm BCALM pull-down of proteins highlighted in C. **F)** Bar graph shows relative amount of BCALM transcript pulled down by RIP of the indicated proteins. (qRT-PCR, unpaired two-tailed t-test * $p < 0.05$; **** $p < 0.0001$) **G)** Western blots confirm immunoprecipitation of indicated proteins from RIP in F. (Representative of at least 2 independent experiments, B-G.)

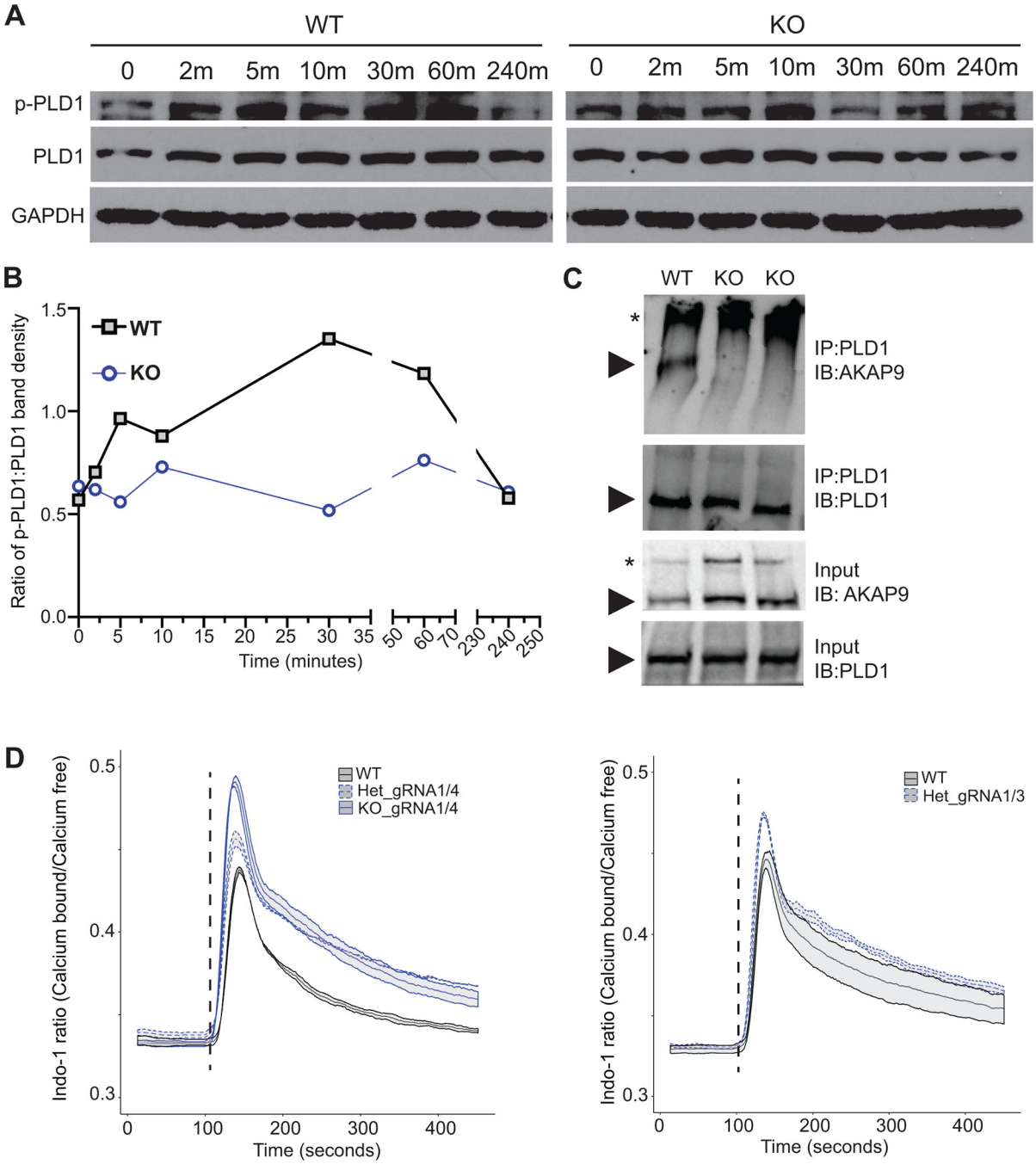

Figure 5. Loss of BCALM (AC099524.1) decreased PLD1 phosphorylation and increased calcium flux after BCR stimulation.

A) Western blots show levels of phosphorylated and total PLD1 protein after BCR stimulation with anti-IgM in OCI-Ly7 WT and KO cells. GAPDH is a loading control. **B)** Line graph shows the ratio of phospho-PLD1 to total PLD1 from A. **C)** Western blots show co-immunoprecipitation of AKAP9 with anti-PLD1 antibody in WT but not in BCALM KO OCI-Ly7 cells. (arrowheads: IB target protein bands, asterisks: non-specific bands) **D)** Indo1 ratio measures the calcium flux in OCI-Ly7 WT, Het, and KO cells after addition of anti-

IgM (vertical dotted line). Solid or dashed lines with intervening grey shading indicate the mean, 10^{th} and 90^{th} percentiles of 3 replicates. Representative of at least 2 independent experiments (A - D).

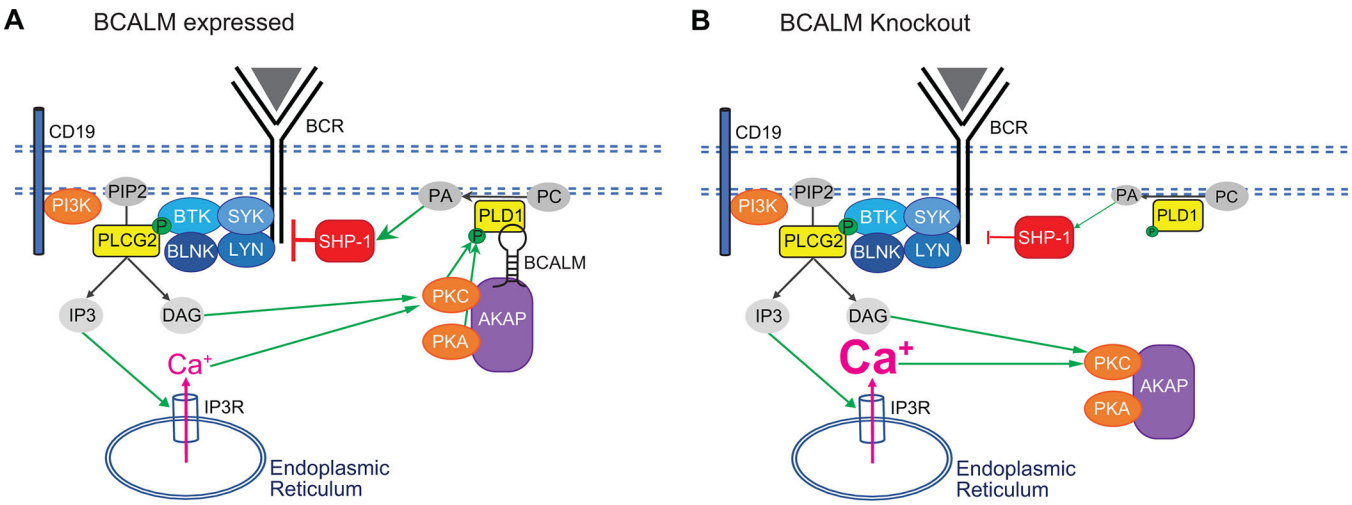

Figure 6. Model for BCALM transcript modulation of calcium flux after BCR stimulation.

A) After IgM-stimulation, BCR-associated kinases are activated and phosphorylate and activate PLCG2, which hydrolyzes PIP2 to DAG and IP3. IP3 stimulates Ca^{+} release from ER (endoplasmic reticulum) stores. DAG stimulates PKC, which associates with kinase-anchoring (AKAP) proteins. BCALM associates with AKAP proteins 9 and 13 and with PLD1, and thus may facilitate phosphorylation and activation of PLD1 by PKC and/or PKA. PA produced by activated PLD1 activates SHP-1, which dephosphorylates BCR-associated kinases, resulting in a downregulation of BCR signaling. **B)** Loss of BCALM decreased the association of PLD1 with AKAP9 and decreased PLD1 phosphorylation, the latter of which activates PLD1. Reduced PLD1 activity decreases PA production and results in less activation of SHP-1, decreasing the inhibition of BCR signaling. In sum, loss of BCALM could result in increased calcium flux via decreased feedback inhibition after BCR stimulation. (Green arrows = activation; red blunt-ended lines = inhibition; dark grey arrows = hydrolysis; pink arrows = passage through an ion channel.)

Table 1.

Patient Characteristics

Disease	Number	M/F	Age (Median/Range)
Chronic Lymphocytic Leukemia/Lymphoma (CLL)	19	9/10	60.5 (38 – 86)
Diffuse Large B Cell Lymphoma (DLBCL)	12	6/6	64.5 (50 – 77)
Activated B Cell (ABC) type	6		
Germinal Center B cell (GCB) type	5		
Grey Zone/Double Hit type	1		
Follicular Lymphoma (FL)	18	7/11	53 (35 – 78)
Stage 1–2	15		

Table 2.

AC099524.1 Genome annotations

HGNC gene symbol	RP11–960L18.1
Clone-based (Ensembl) gene name	AC099524.1
Ensembl Gene	ENSG00000261218
HAVANA manual id gene	OTTHUMG00000176531.2_5
Human (GRCh38.p13)	Chr16: 81,738,248–81,767,868, forward strand
GRCh37 location	Chr16:81,771,853–81,801,473

Table 3.

qRT-PCR and genomic DNA PCR primers

Gene mRNA target	Primer	Sequence
PLCG2	Forward	TCAATCCGTCCATGCCTCAG
PLCG2	Reverse	CCTCGACGTAGTTGGATGGG
AC099524.1	Forward	GTCACACAGCCAACTTGCG
AC099524.1	Reverse	AGCCTCTATCTGCTTACGTGC
U1	Forward	ATACTTACCTGGCAGGGGAGA
U1	Reverse	CAGGGGGAAAGCGCGAACGCA
18S	Forward	GTGTGTGGGTTGACTTCGGA
18S	Reverse	AAGGCTTTTCTCACCGAGGG
GAPDH	Forward	ACCCACTCCTCCACCTTTGAC
GAPDH	Reverse	TGTTGCTGTAGCCAAATTCGTT
Regulatory element region (luciferase) A	Forward	GCAGGACTGTCCACAACTGTC
Regulatory element region (luciferase) A	Reverse	GTGGTTACATACAAGAAGCCGTACA
Regulatory element region (luciferase) B	Forward	CGCAAACTGGGTGGCCTAAA
Regulatory element region (luciferase) B	Reverse	AAGTCCAGTGGCATGTGTCC
Regulatory element region (luciferase) C	Forward	AACGTTGTCATGGAGACCCG
Regulatory element region (luciferase) C	Reverse	CCATCCGCTACCCCAGGA
Regulatory element region (luciferase) D	Forward	GGCTGAGGCACAAGAATTGC
Regulatory element region (luciferase) D	Reverse	TATCCCAGCTCAGGGATGCT
Regulatory element region (luciferase) E	Forward	GCAGGCCCAGGAGTGATAAG
Regulatory element region (luciferase) E	Reverse	CAAGTTGGCTGTGTGACTGC
Regulatory element region (luciferase) F	Forward	GGAGCCCCCAGTATTTTCC
Regulatory element region (luciferase) F	Reverse	AACACCCTGTGCCTCCATT
Regulatory element region (luciferase) G	Forward	GGCAGGTACGCAGGTAGTA
Regulatory element region (luciferase) G	Reverse	TGTGGTCAGCATCCCTAGC
Regulatory element region (luciferase) H	Forward	CCTTCCTCCATAGACCAGCG
Regulatory element region (luciferase) H	Reverse	CAAAGCCCCAGCACAATACC
Regulatory element region (luciferase) I	Forward	CTCTCTGTAGGGGCGAGACT
Regulatory element region (luciferase) I	Reverse	GGGCGAAAACTCAAGCACTC
Regulatory element region (luciferase) J	Forward	CTGGCAGAGCTGGGATTGAA
Regulatory element region (luciferase) J	Reverse	CGGCATCCTAGACTGCTGTT
Regulatory element region (luciferase) K	Forward	AGAGGTGGTCCCCTTAGCTC
Regulatory element region (luciferase) K	Reverse	TACCGTGGTTGGTCTCCACT
Regulatory element region (luciferase) L	Forward	GGAGCCCCCAGTATTTTCC
Regulatory element region (luciferase) L	Reverse	AACACCCTGTGCCTCCATT
Regulatory element region (luciferase) M	Forward	GGCAGGTACGCAGGTAGTA
Regulatory element region (luciferase) M	Reverse	TGTGGTCAGCATCCCTAGC
CRISPR/Cas9 Target Upstream	gRNA 1	CCGCGGGGAGGTTTCGTACC

Gene mRNA target	Primer	Sequence
CRISPR/Cas9 Target Downstream 1	gRNA 3	TGCAATCACAGTTACTCGGG
CRISPR/Cas9 Target Downstream 2	gRNA 4	GAGCGCGTTCCTCCTACCTA
Genome editing intact site	Forward	CCCTGAAGGCTCTTGTCTGAC
Genome editing intact site	Reverse	CTCAGGAAGGGTTTCCAGATTTAGG
gRNA 1/3 deletion	Forward	CCCTGAAGGCTCTTGTCTGAC
gRNA 1/3 deletion	Reverse	GGTATGCACTGGCGCTG
gRNA 1/4 deletion	Forward	CCCTGAAGGCTCTTGTCTGAC
gRNA 1/4 deletion	Reverse	CCCACAATGGTAACTTTTGTGTGC
Control site	Forward	ACCCACTCCTCCACCTTTGA
Control site	Reverse	GTGGTCCAGGGGTCTTACTC
Intact site	Forward	GCTGCCCCTAAAAGGACAGATT
Intact site	Reverse	GGTATGCACTGGCGCTG