

Inhibition of B-Cell Receptor signaling disrupts cell adhesion in mantle cell lymphoma via RAC2

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Supplementary Methods

Cell metabolic activity and cell growth assays

MCL cell lines were treated with various concentrations of ibrutinib. The metabolic activities of cells were measured with MTT assay following 72 hrs ibrutinib treatment per manufacturer's instructions (Roche Applied Science, Indianapolis, IN, USA). Viable cell numbers were determined on the Muse Cell Analyzer (EMD Millipore, Billerica, MA, USA). The half maximal inhibitory concentration (IC₅₀) and cell growth curve were generated using Prism 5 (GraphPad, La Jolla, CA, USA).

Cell adhesion assay

The cell adhesion assay was performed as described, with modifications [21, 22]. Briefly, NKTert stromal cells (10⁴) were seeded in flat-bottom 96-well plates in a volume of 100 μL and cultured overnight. MCL cell lines or primary MCL tumors (10⁴/100 μL) were then seeded and co-cultured with NKTert cells with simultaneous addition of 400 nM of ibr or DMSO. After 24 hr of cell culture, plates were then washed twice by gently pipetting 200 μL pre-warmed PBS buffer to remove the non-adhesive MCL cells. Attached cells were then fixed with 4% neutral paraformaldehyde for 10 minutes, stained with 1 ug/ml Alexa Fluor® 790 goat anti-human IgM for 20 minutes, and scanned on Odyssey® CLx Imaging System (LI-COR biosciences, Lincoln, NE, USA). MCL cells that had adhered to the NKTert cell stromal cells were captured. The integrated fluorescence density (IFD) was analyzed by Image Studio™ Software (LI-COR biosciences, Lincoln, NE, USA). This assay was performed in six replicate reactions. The relative cell adhesion percentage was defined as the ratio of IFD in an individual well to the highest IFD across the entire group.

The adhesion assay with the MD Anderson cohort of primary MCL cells was performed using the following protocol: HS-5 stromal cells (1x10⁴) were seeded in flat-bottom 96-well plates in a volume of 100 μL and cultured overnight. MCL primary tumor cells (10⁵/100 μL) were then seeded and co-cultured with HS-5 cells with simultaneous addition of 400 nM of ibr or DMSO. After 24 hr of culture, plates were washed twice with 200 μL pre-warmed PBS buffer to remove the non-adhesive MCL cells. Cells remained were lysed with Cell Titer-Glo Luminescent Cell Viability Assay Reagent (Promega, Madison, WI, USA), and luminescence was quantified using the BioTek Synergy HTX Multi-mode Micro Plate Reader (Winooski, VT, USA).

Stable expression of td-Tomato-hCD40L-BMF

Transduction of BMF cells was performed according to manufacturer's instructions (VectorBuilder). BMF cells were seeded overnight at 30-50% confluence. Lentivirus was added to cells along with polybrene (5 µg/ml) and incubated overnight. Fresh media containing antibiotics (neomycin for hCD40L and puromycin for td-Tomato) for selection was added one day after transduction. After selection (2-10 days), expression was confirmed by fluorescence microscopy and flow cytometry.

RNA Sequencing and Data Analyses

Biological triplicate experiments were conducted according to published recommendations [24]. Total RNA was isolated from cells with RNA mini kit (Qiagen, Valencia, CA, USA). Library preparation for single-end RNA-seq was performed using the TruSeq Stranded RNA Sample Preparation kit (Illumina, San Diego, CA, USA) per the manufacturer's protocol. The quality of the library was assessed on Agilent DNA 1000 chips and quantity measured by real-time PCR. The libraries were then sequenced on Illumina HiSeq 2500 with single-end sequencing of 50 cycles. The resulting 18-30 million reads per sample were aligned using Spliced Transcripts Alignment to a Reference (STAR), converted to BAM file with Picard tools and finally transformed into counts with FeatureCounts. Counts normalization and differential gene expression between DMSO and ibrutinib treatment conditions were conducted using DeSeq2 [24]. Gene Set Enrichment Analysis (GSEA) was conducted to recover the gene sets of differentially expressed genes [25], and significant enrichment was defined as both $p < 0.05$ and false discovery rate $q\text{-value} < 0.25$. Lymphoma gene set signatures were curated and defined by Staudt's group [27] and applied previously [28, 29].

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 4 µm sections cut from FFPE blocks. Antigen was retrieved with heat-induction in EDTA (pH 8.0), followed with overnight incubation at 4°C with RAC2 antibody at 1:200 dilution. Envision two-step kit (Dako, Glostrup, Denmark) was used to visualize the positive signaling. The calculation of IHC score was conducted as previously described [26].

Immunoblotting and Co-Immunoprecipitation

Immunoblotting and co-immunoprecipitation were done according to standard procedures. Cells were lysed in RIPA or co-immunoprecipitation (co-IP) specific lysis buffer (Thermo Fisher Scientific, Waltham, MA USA). Immunoprecipitation was performed using Dynabeads® Protein G (Thermo Fisher Scientific, Waltham, MA USA) according to manufacturer's instructions. Protein extracts or co-IP products were separated with 4%-15% gradient SDS-PAGE gels (Bio-rad, Hercules, California, USA), and visualized on Odyssey® CLx Imaging System (LI-COR biosciences, Lincoln, NE, USA). Gel bands were scanned and quantified with Gel-Pro Analyzer V4.0 software (Media Cybernetics, Rockville, MD, USA).

Immunofluorescence and Confocal Microscopy

Immunofluorescence and confocal microscopy were done as previously described [23]. Td-Tomato-hCD40L-BMF were seeded overnight on coverlips in 24-well plates (60,000 cells/well). MCL cells (100,000 cells/well) were labeled with CFSE and then were co-cultured with the BMF. The co-cultures were treated with 400 nM ibrutinib or DMSO for 24 hrs. The cells were gently washed with PBS to remove any non-adherent MCL cells and then fixed with 4% paraformaldehyde for 10 mins. After fixation, the cells were stained with DAPI for 5 mins, and then the coverslips were mounted onto slides using permount. All coverslips were imaged on the Marianas Yokogawa type spinning disk confocal microscope (Zeiss) with a Plan-Apochromat 40×/1.3 oil objective lens. A 3x3 montage with z-stack was performed for 4 random fields per treatment. Images were captured using two Evolve back-thinned, air chilled (-85°C) CCD cameras (512x512 16 µm pixels). All images were acquired using the volume viewer tool on SlideBook. The assay was repeated twice in duplicate. The image analysis was performed using the cell counter plugin in ImageJ. The ratio of MCL (green) to BMF (red) was determined for each field.

For BLNK and RAC2 confocal analysis, JeKo-1 or MAVER-1 cells were pre-treated with 400 nM of ibrutinib for 6 hrs followed by anti-IgM (20 µg/mL) stimulation for 15 min at 37 °C. Cells were seeded on Shadon Single Cytoslides at 4°C for 20 mins (5991056, Thermo scientific) and were then fixed with 4% paraformaldehyde for 20 mins and permeabilized with 0.1% triton X-100 for 15 mins at room temperature. After permeabilization, cells were blocked with 1% of bovine serum albumin and then incubated with polyclonal goat anti-human RAC2 antibody (PA1-9091, Thermo scientific) or polyclonal rabbit anti-human phospho-BLNK (Y96) antibody (PA5-105018, Thermo scientific) overnight at 4 °C. Cells were then washed three times with PBS and incubated with Alexa Flour 488 chicken anti-rabbit IgG (A-21441, Thermo scientific), Alexa Flour 594 donkey anti-goat IgG (A-11058) and DAPI for 2 hrs at room temperature. After immunostaining, cells were washed three times with PBS and sealed with fluoromount-G anti-fade (0100-35, SouthernBiotech). All cytoslides were imaged on Leica SP8 laser scanning confocal microscope using 63x HC PL APO CS2 oil lens, NA 1.40. Argon laser (488nm) with appropriate detection windows was used to visualize Alexa Flour 488, solid state lasers were used to visualize DAPI (405nm) and Alexa Flour 594. The images were analyzed by ImageJ (Version 1.53c).

RAC2 plasmid construction, siRNA and nucleofection

Human full length RAC2 cDNA was cloned from HBL-1 cell cDNA library by PCR with the following primers: Forward: ATG CAG GCC ATC AAG TGT GTG GT; Reverse: GAG GAG GCT GCA GGC GC. The PCR product was then cloned into pENTER™/SD/D-TOPO vector (Invitrogen, Carlsbad, CA USA) following manufacturer's instructions. Subsequently, the RAC2 cDNA was shuffled into pcDNA™-DEST40 Gateway™ expression vector containing 6x His-tag (Invitrogen, Carlsbad, CA USA) through BP-LR reaction with Gateway™ LR Clonase (Invitrogen, Carlsbad, CA USA). The sequence was confirmed by Sanger's sequencing. A pool of

three RAC2 target-specific siRNAs and non-targeting control siRNA were purchased from Santa Cruz (Santa Cruz, CA, USA). Transfection of the RAC2 expression vector and siRNA into Mino cells were conducted using Nucleofector Kit V on Amaxa Nucleofector 2b Device (Lonza, Allendale, NJ, USA) according to the manufacturer's protocol using the T-001 program. Cells were then allowed to recover for 48 hrs before further experiments.

CRISPR-Cas9

The RNP-based CRISPR/Cas9 delivery method was applied to generate RAC2 knockouts in Granta-519 cells [47]. Briefly, the two pre-designed single guide RNAs (sgRNA) targeting the human RAC2 gene were ordered from IDT (Coralville, IA): sgRAC2#1 5'- AGGAGGACTACGACCGTCTC-3' and sgRAC2#2 5'- CGGACGTTCTCATAAGAGGC-3'. Cas9-sgRNA RNP complexes were then generated by incubating 7.5 µg of Alt-R s.p. HiFi Cas9 Nuclease v3 (IDT, USA) with 5 µg of the sgRNA for 30 minutes at room temperature. Control was generated with Cas9 alone in the absence of sgRNA. For electroporation, the RNP complex was added to 1×10^6 cells suspended in 100 µL of cell line Nucleofector Buffer T, and electroporated using Amaxa Nucleofector 2b device (Lonza, Allendale, NJ, USA) program O-017. Immunoblot analysis and cell adhesion were performed 72 hrs after electroporation.

Xenograft study

Female SCID mice (18–22 g) from Charles River Labs (Beijing, China) were used for this study. Tumor growth was initiated by subcutaneous injection of 1×10^7 Mino cells in a 1:1 mixture of serum-free growth medium and Matrigel (BD Bioscience, San Jose, CA, USA) in the right flank of each subject animal. When tumor volume reached approximately 150 mm³ in size, after removing animals with extreme tumor size (<100 or >300 mm³) and body weight (<19 or >23g), the remaining mice were randomized into treatment and control groups (6 animals in each group). Either 0.5% methylcellulose or ibrutinib at 50 mg/kg of body weight was administered by oral gavage daily. The dose 50 mg/kg was selected according to the pharmacology review of ibrutinib published by the FDA Center for Drug Evaluation and Research accessible at https://www.accessdata.fda.gov/drugsatfda_docs/nda/2013/205552Orig1s000PharmR.pdf (p28). Tumor size and body weight were recorded twice a week. Animals were euthanized on Day 21 after treatment, and tumor tissues were removed and embedded in paraffin.

A

Cell lines	IC ₅₀ (μmol/L) for Ibr	Sensitivity
JeKo-1	0.60	Sensitive
Mino	1.13	Intermediate
REC-1	1.26	Intermediate
JVM-2	1.46	Intermediate
MAVER-1	2.69	Resistant
Granta519	8.26	Resistant

B

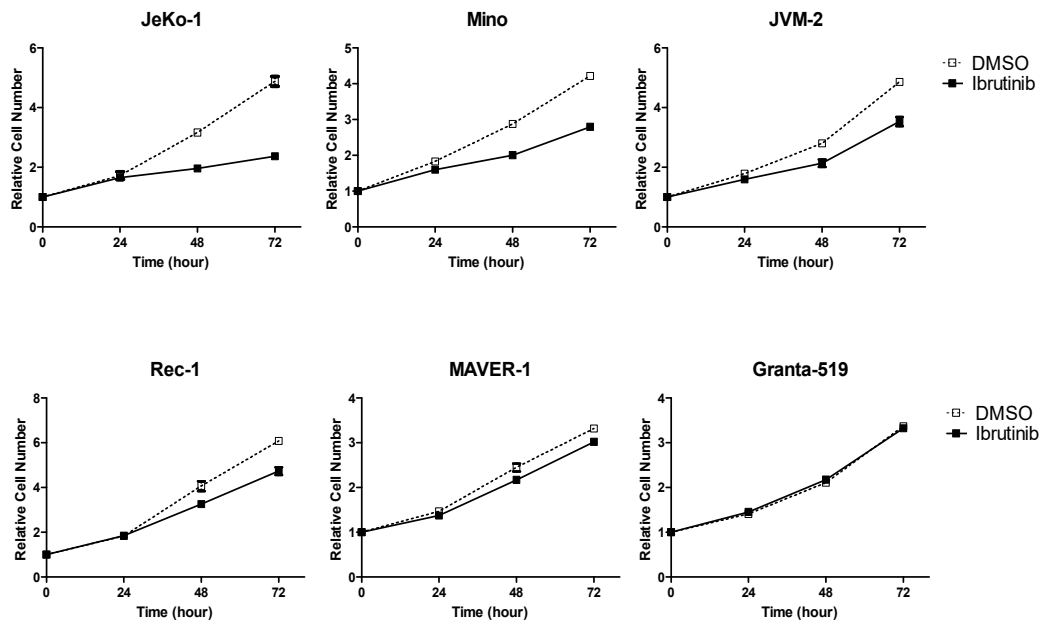


Fig. S1. : MCL cell lines have variable intrinsic sensitivity to ibrutinib. (A) IC₅₀ values of ibrutinib in a panel of six MCL cell lines determined by MTT assay. Cells were treated with varying doses of ibrutinib for 72 hrs. Results represent the average of three experiments in which six replicate reactions were performed. IC₅₀ values were derived using GraphPad Prism 6. Please note that data shown here were published previously (Ming et. al. Mol Cancer Ther. 2018;17(12):2564-2574). **(B)** Growth of MCL cell lines was variably inhibited by ibrutinib, and was correlated with the MTT assay. Cells were treated with or without 400 nM of ibrutinib for 72 hrs. The experiment was performed three times in triplicate. The average of the triplicates was used as single data point and average values of three experiments were plotted here. Numbers were normalized to initial plating cell number at time zero. Error bars represent mean±SEM.

Figure S2

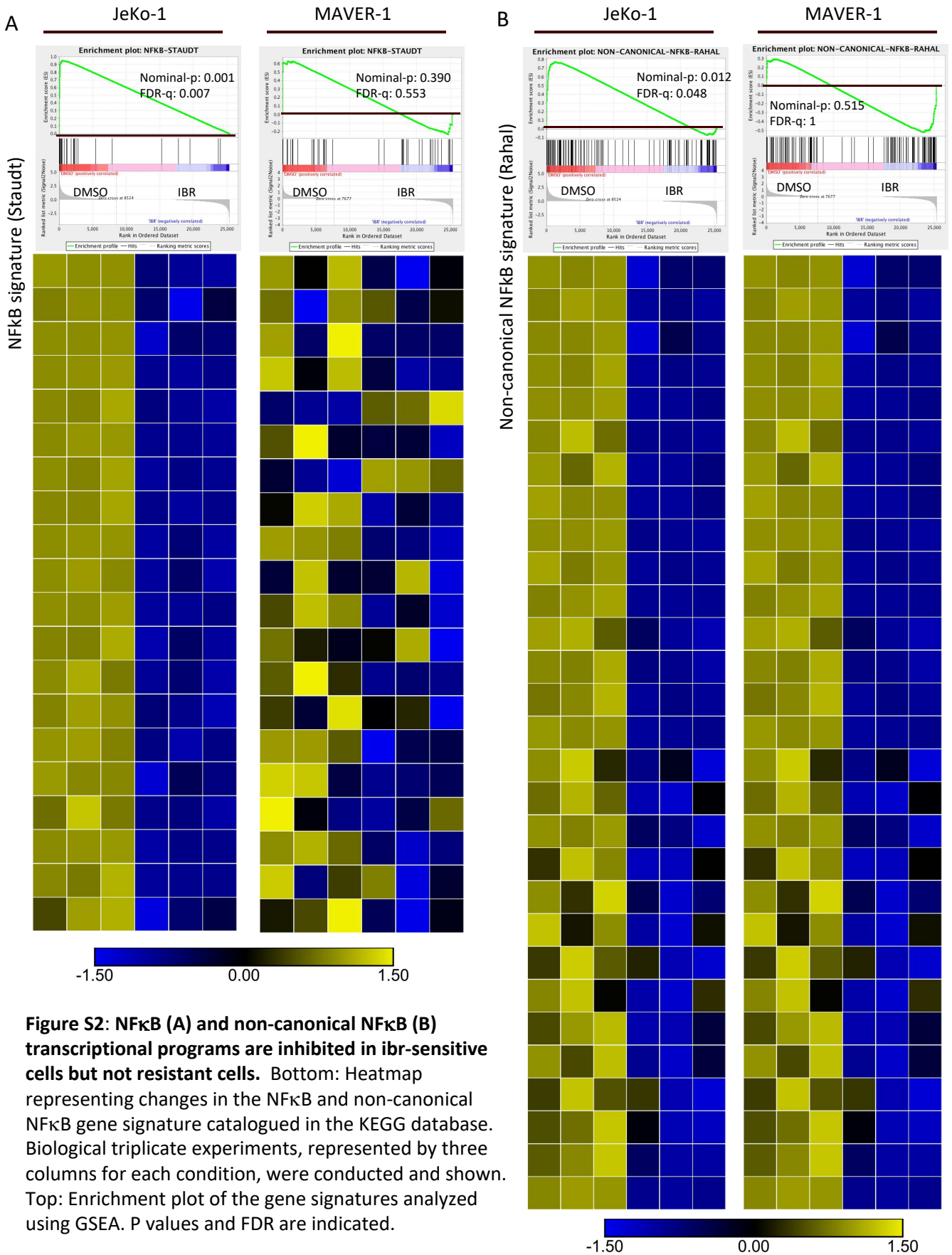


Figure S3

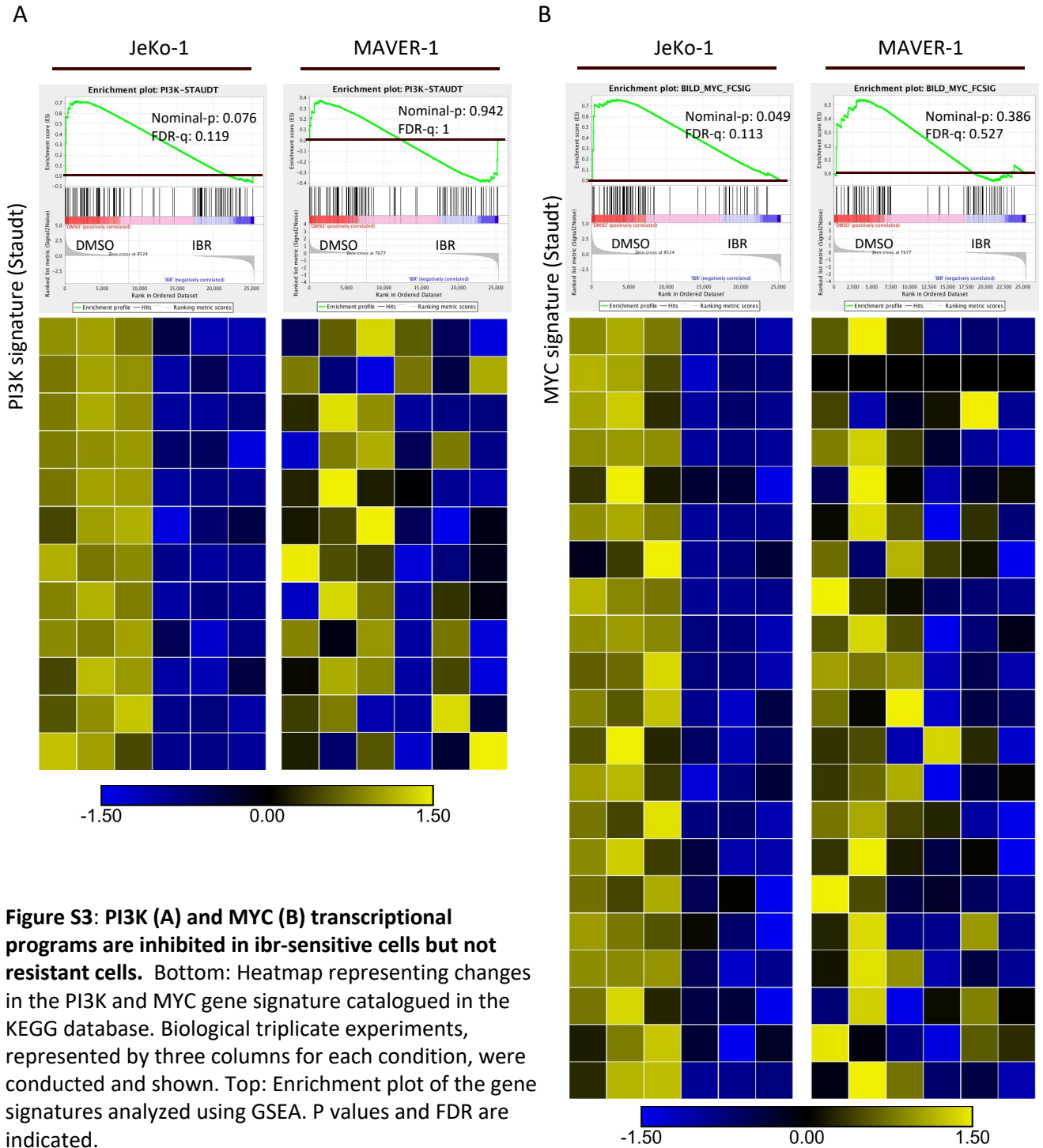


Figure S3: PI3K (A) and MYC (B) transcriptional programs are inhibited in ibr-sensitive cells but not resistant cells. Bottom: Heatmap representing changes in the PI3K and MYC gene signature catalogued in the KEGG database. Biological triplicate experiments, represented by three columns for each condition, were conducted and shown. Top: Enrichment plot of the gene signatures analyzed using GSEA. P values and FDR are indicated.

Figure S4

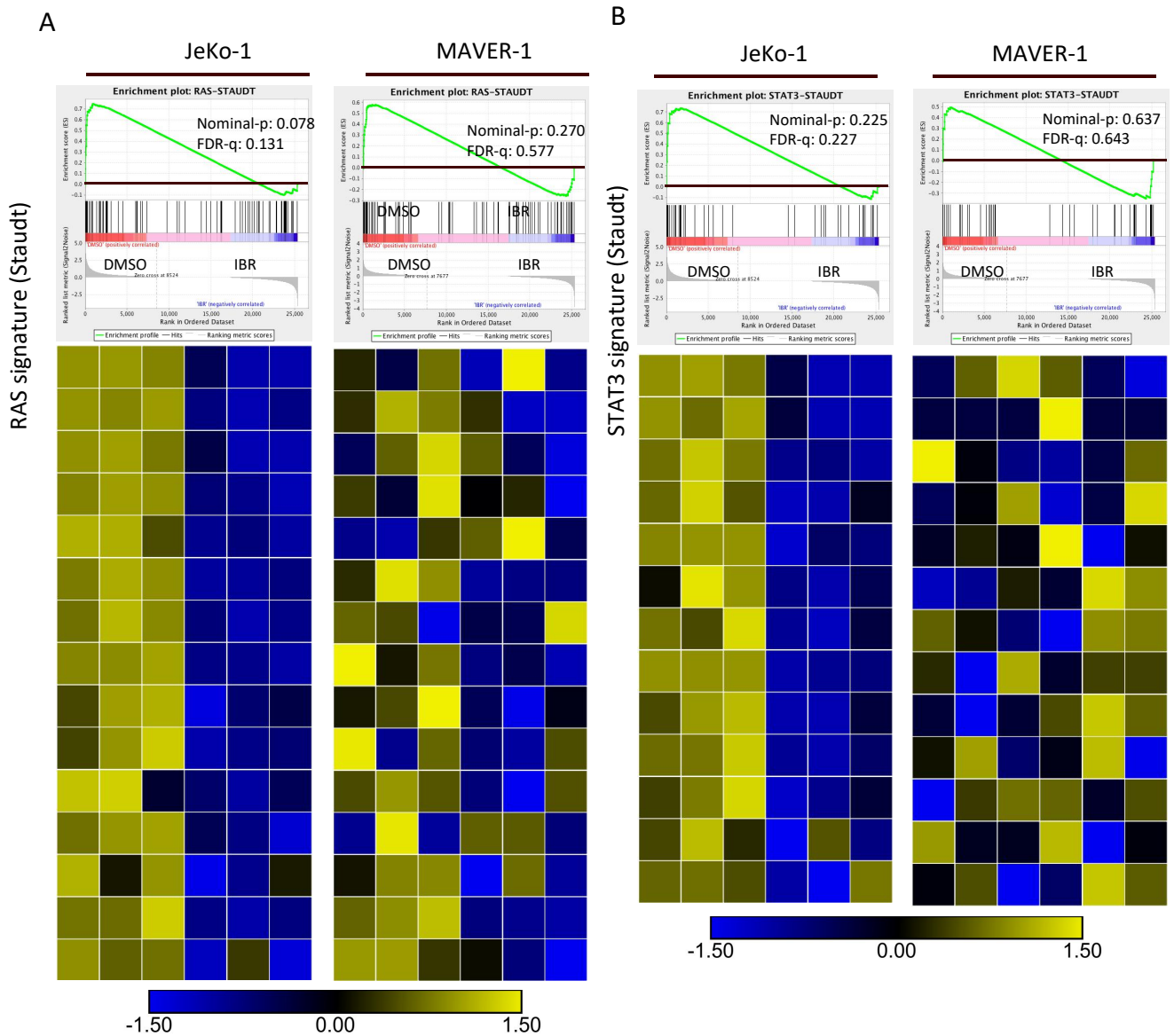


Figure S4: RAS (A) and STAT3 (B) transcriptional programs are not affected by ibrutinib in MCL cells. Bottom: Heatmap representing changes in the RAS and STAT3 gene signature catalogued in the KEGG database. Biological triplicate experiments, represented by three columns for each condition, were conducted and shown. Top: Enrichment plot of the gene signatures analyzed using GSEA. P values and FDR are indicated.

Figure S5

	JeKo-1	MAVER-1
Cellular response to IBR		
	Sensitive	Resistant
Transcriptomic response to IBR		
Gene signature	IBR vs DMSO	IBR vs DMSO
NFkB		
NC-NFkB		
PI3K		
MYC		
RAS		
STAT3		
BCR		
CAM		

Down-regulated

No change

Figure S5. Summary of Gene Set Enrichment Analysis (GSEA) of oncogenic pathways. These include NFkB(A), non-canonical NFkB(B), PI3K(C), MYC(D), RAS(E), STAT3(F). The leading genes enriched in ibrutinib-treated JeKo-1 were used to generate heatmaps and used as a baseline to compare with MAVER-1. The GSEA results are summarized in heatmap format(G), with pathways that are down regulated shown in green, and pathways without significant changes shown in grey. Cut-off P=0.05 and FDR=0.25. These data were previously described in Lee et. al. (Blood Adv. 2018 Aug 28;2(16):2039-2051).

Figure S6

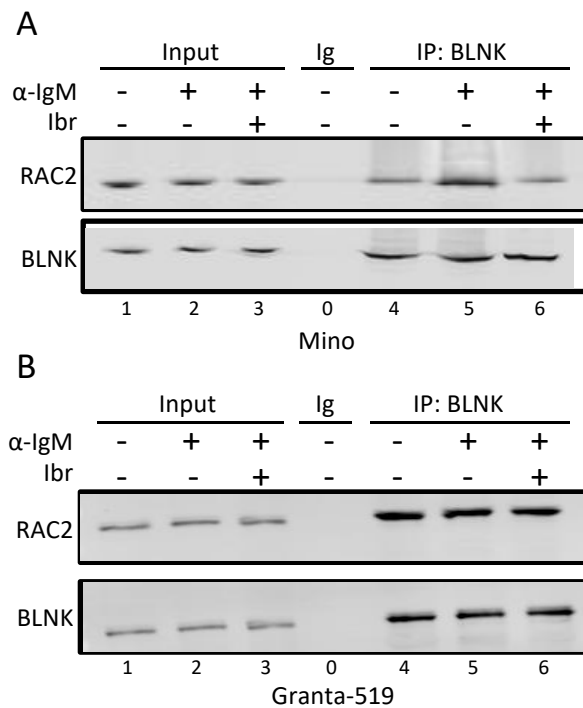


Figure S6. Expression and BLNK association of RAC2 are down-regulated by ibrutinib in sensitive cells, but not in resistant cells. Immunoblotting of Co-IP products. Cells were pre-incubated with or without 400 nM of ibr for an hour and stimulated with or without anti-IgM antibody for 15min before cell lysis. Immunoblotting was performed for least three times using either whole cell extracts (Input) or anti-BLNK pull-downs in Mino (A) and Granta-519 (B).

Figure S7

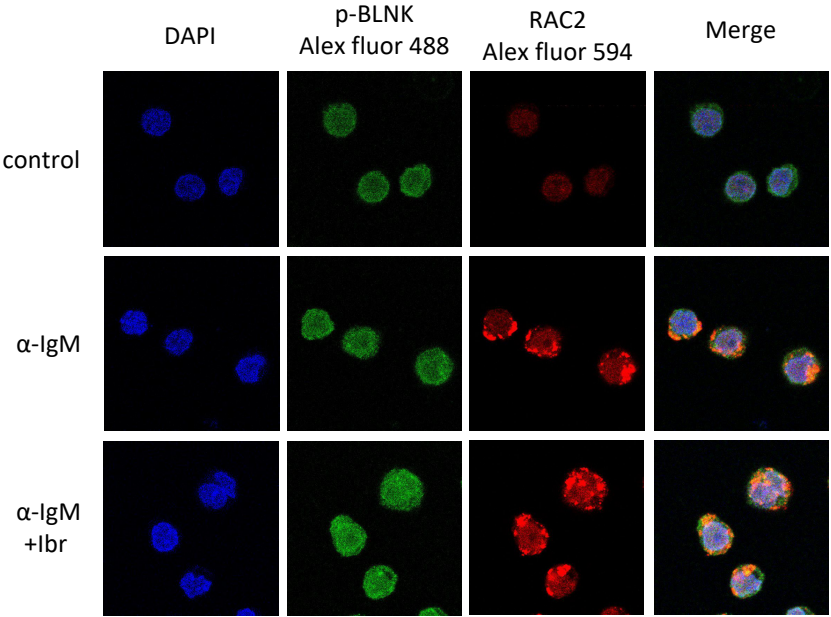
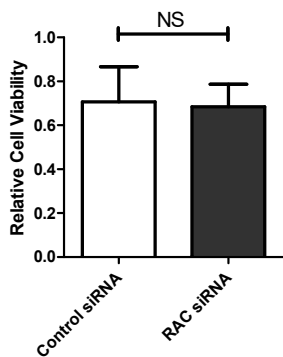


Figure S7. Lack of p-BLNK and RAC2 colocalization in MAVER-1 cells by Confocal analysis. Cells were pre-incubated with or without 400 nM of ibr for six hours and stimulated with or without anti-IgM. Cells were fixed, permeabilized and stained with anti-pBLNK and anti-RAC2. p-BLNK membrane association was unchanged upon α -IgM stimulation.

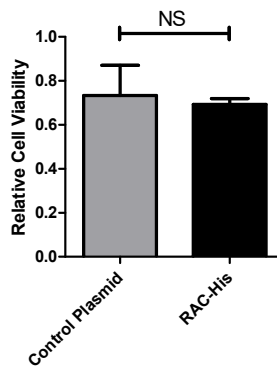
Figure S8

JeKo-1

A

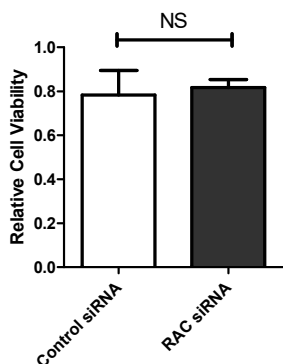


B



Mino

C



D

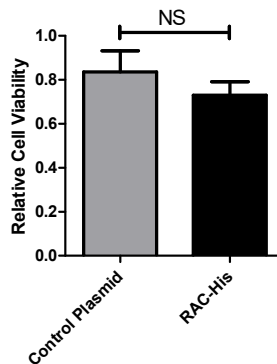


Figure S8. Cell viability was not significantly affected by transfection of siRNA or RAC2 expression vector. Cell viability, measured simultaneously, was not significantly affected by the transfection of siRNA (A and C) or RAC2 expression vectors (B and D) in JeKo-1 (A and B) or Mino cells (C and D). Error bar represents mean \pm SEM of six replicate reactions. NS: no significance.

Figure S9

		Ki67	Blastoid
U Chicago	Patient 1	N/A	Yes
	Patient 2	N/A	No
	Patient 3	High	Yes
	Patient 4	Low	No
	Patient 5	N/A	No
	Patient 6	Low	No
MD Anderson	Patient 7	10-20%	Yes
	Patient 8	15%	No
	Patient 9	20%	No
	Patient 10	80%	No
	Patient 11	90%	Yes
	Patient 12	70-80%	No

Figure S9. Ki67 and Blastoid morphology of primary MCL samples.
These samples were used in cell adhesion assays in Fig. 6 B-E.