**Supplemental Methods**

**Overcoming Acquired Epigenetic Resistance to BTK Inhibitors**

**Shaffer, et al.**

*Cell culture*

Cell lines were obtained from the ATCC. Cell lines were grown at 37°C in the presence of 5% CO2 and maintained in Advanced RPMI (Gibco, 12633-012, LOT2187251 ) supplemented with 5% fetal bovine serum (Tet-tested, BioTechne, S10350, LOTJ19136) and 1% pen/strep with L-glutamine (Gibco, 10378-016), except for 293T/FT cells used to produce retro- and lentiviruses which were cultured in DMEM (Gibco, 11995-065, LOT2186868) supplemented with 20% fetal bovine serum and 1% pen/strep and 1% L-glutamine. All cell lines were regularly tested for mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza, LT07-218) and DNA fingerprinted by examining 16 regions of copy number variants (Jonathan Keats, personal communication). For induction of “tet-inducible” constructs, cultures were supplemented with 10ug/ml of doxycycline hyclate (Calbiochem, 2753889). Puromycin (Thermo-Fisher, A1113802) was used at 1ug/ml to select cells as indicated below.

*Drugs and basic molecular biology reagents*

These compounds were used at the concentrations indicated in individual figures and the text.

Ibrutinib, BTK inhibitor ((Selleckchem, S2680)

ABT-199, BCL2 inhibitor (Selleckchem, S8048)

EHT1864, RAC inhibitor (Selleckchem, S7482)

ABT199, BCL2 inhibitor (Selleckchem, S8048)

5-Azacytidine, DNA methylation inhibitor (Millipore-Sigma, A2385)

Givinostat, HDAC inhibitor (Selleckchem, S2170)

DMSO, vehicle for drugs (Sigma, D8418, Lot51K3486)

Ethanol, EtOH 200 proof, 1pint (The Warner-Graham Co., 64-17-5)

1x PBS, buffer base, (Gibco, 10010-023, Lot2257192)

*Sanger Sequencing of BTK/PLCG2 mutants*

DNA and RNA were prepared from pools or single cell clones (Qiagen, Allprep Mini kit, 80204). RNA was converted to cDNA (SuperScriptII Kit, ThermoFisher, 18064022) , and with DNA samples, was subjected to PCR and Sanger sequencing at the NCI CCR genomics core using the following primers (IDT, desalted, 25nM, see below), then sent to the NCI CCR Genomics core facility for BigDye Terminator (v1.1, 4337452) Sanger Sequencing and base calling.

See Supplemental Primer/Oligo Table A.

*Creation of lyt2 and gfp-expressing vectors for BTK/PLCG2 alleles and other genes*

For tracking and expression of various alleles of relevant signaling proteins, the retroviral vector pRSMX\_PuroGFP was used for doxycycline-inducible expression, as previously described [1]. A modified version of this vector, expressing truncated mouse CD8a (lyt2) in place of gfp, was also used in some experiments as described. Open reading frames were cloned into this vector’s MCS using Gibson technology and cloned cDNAs for each allele. For PLCG2 mutants, the QuikChange II (Agilent, 200523 ) site-directed mutagenesis kit was used to make single nucleotide changes recapitulating mutants observed by Sanger sequencing of ibrutinib resistant single cell clones.

*Virus production and transduction*

Lentiviruses were produced in 293FT cells by transfecting sgRNA vectors with packaging vectors pPAX2 (Addgene 12260) and pMD2.g (Addgene 12259) in a 4:3:1 ratio in serum-free Opti-MEM (ThermoFisher, 31985070). Trans-IT 293T (Mirus, MIR2700) was added and incubated for 15 minutes before adding dropwise to cells. Supernatants were harvested 24 and 48hours later, spun at 1000g to filtered to remove any virus producing cells and then incubated by spin-infection with target cells (1:1 volume viral supernatant:cell culture media) [2]. Viral supernatants were removed after the final spin-infection, and cells were given fresh media, then rested 24-48 hours before selection or use in experiments.

*Culture of cells in outgrowth experiments of IR cells and cells expressing mutant alleles of resistance genes*

For assessing the outgrowth of IR pools or IR clones, these cells were transduced as described above with gfp-expressing vectors and mixed at a ~1:10 ratio with control cells, typically gfp- parental lines. Cells were then challenged with vehicle (DMSO) or ibrutinib (10nM) over the given time course with media and drug refreshed every 2 days. GFP-positive cells were enumerated by flow cytometry on the indicated days as described below. Similar outgrowth experiments were performed with ibrutinib-sensitive parental cells engineered with control (empty) lyt2-expressing vectors, or those driving expression of ibrutinib-resistance alleles of PLCG2 and BTK. Lyt2+ (allele+) cells were tracked by flow cytometry (see below) over a time course of drug challenge as described.

*Flow cytometry for gfp+ and lyt2+ outgrowth experiments*

Cells were spun down (1800rpm 5min.) and washed with 1xPBS with 5% FBS plus 0.1% azide (FACS buffer). For gfp analyses, cells were resuspended in FACS buffer and subjected to flow cytometric analysis. To assess the expression of the lyt2 marker, cells were treated as above, stained with a 1:200 dilution of anti-lyt2 antibody (Biolegend, #100724) for 20 minutes at 4C, washed twice with 2 volumes of FACS buffer, then analyzed by flow cytometry. The percentage of viable cells was measured by flowcytometric gating on live cells by forward and side scatter, then the percent of gfp+ or lyt2+ cells was enumerated. Flowjo software (v10.7.1, Becton-Dickinson) was used to enumerate cells.

*Flow cytometry for live cell counting (calcein/EtBr)*

Carefully measured, equivalent volumes of well-mixed cells were spun down (1800rpm 5min.), media was carefully removed, and cells were resuspended in 1xPBS with a 1:100 dilution of calcein-AM vital dye (Invitrogen C3100MP) in DMSO with a 1:10,000 dilution of ethidium bromide (Sigma E1510-10ML ). Cells were protected from light and allowed to sit for 30 minutes before collecting equal volumes from each sample by flow cytometry. Calcein (FL1) high cells (EtBr, FL2 low) were counted as LIVE cells. Flowjo software was used to enumerate cells.

*Exome-SEQ*

As described fully in [3]. Briefly, DNA was extracted from cell cultures using the Qiagen AllPrep kit. Sequencing libraries were prepared using the Agilent SureSelectXT Human All Exon V5 target enrichment kit (Agilent, 5190-8872). Paired-end, 100- or 150bp sequencing was performed on an Illumina HiSeq 2500 or HiSeq3000 machine using Illumina TrueSeq V3 chemistry (Illumina, FC-401-3001). Variants, as compared to normal human controls, were called on the final alignment BAM file by VarScan2 software with cut-offs set at variant count >3 and variant frequency >10%.

*RNA-SEQ gene expression profiling*

RNA was extracted using the Qiagen AllPrep kit. Sequencing libraries for RNA‐sequencing were

prepared using the TruSeq RNA Library Prep Kit V2 (Illumina, RS-122-2001). Paired‐end 100 bp read sequencing was performed on a HiSeq 2500 system using Illumina TruSeq V3 chemistry.

Paired‐end reads were mapped to the human genome (NCBI build 37) using the gapped aligner STAR2.4.1, using the two‐pass method and parameters recommended by NCI Genomic Data Commons [4]. The alignment file was used for calculating the raw digital gene expression values by HTseqcount software 0.7.2, using the intersection‐nonempty model, which were further analyzed to provide normalized, Log2-transformed, digital gene expression values [3].

*Ibrutinib-response gene expression signature definition and gene expression signature enrichment*

From RNA-SEQ data obtained from the acute (24hr.) treatment of the HBL1 and TMD8 ABC DLBCL cell lines with ibrutinib (10nM), genes were identified whose expression decreased, on average in both lines, by Log2 <-0.4. These 574 repressed genes thus constituted the ‘ibrutinib-response’ signature referred to in the manuscript (Table S1C). To analyze this gene set and others throughout the manuscript, gene expression signature enrichment was performed as previously described [5]. Briefly, gene lists were tested for overlap with published gene signatures in a 2×2 contingency table using a Fisher’s exact test.

*Evolution of ibrutinib resistance- Barcode method*

One million HBL1 or TMD8 ABC DLBCL cells were transduced with a retroviral vector bearing a random 60mer barcode and a puromycin resistance gene (pRSMSpg, [1]), such that each cell received one unique barcode (MOI of 1:3). Cells were selected with puromycin (1ug/ml), expanded for 4 weeks, and a sample of cells was taken to establish the clonal (barcode) distribution at the start of the experiment. Duplicate cultures were then challenged with increasing concentrations of Ibrutinib (0.5nM week 1 to 10nM week 5) or cultured with DMSO alone. One million cells were harvested at weeks 1 and 5. DNA from each sample was purified (AllPrep kit, Qiagen cat80204), with an additional 80% ethanol wash. Libraries for high-throughput sequencing were created using PCR primers specific for the barcode vector (pRSMX, [1]), follows using primeSTAR HS DNA polymerase (Takara, #R010B).

PCR1:

2x master mix 25ul, 10ul forward primer 5’ GGTTAAGATCAAGGTCTTTTCACCTGGC (10uM, IDT de-salted), 10ul reverse primer 5’ CAGTGTGCCGGTCTCCGTTATCG (10uM, IDT de-salted), 4ul H2O, 2ug DNA in 1ul.

95C 2mins, then cycle 95C 20sec-58C 20sec-72C 20sec, 22 times and finish with 72C 3 mins.

Set up one reaction per 2ug DNA; use all genomic DNA across multiple reactions and pool for PCR2:

2x master mix 25ul, 1.5ul adapter forward primer D500 series (5uM, IDT de-salted), 1.5ul reverse primer D700 series (5uM, IDT de-salted), 10ul PCR1 from each sample in an individual reaction, water to 50ul total volume.

95C 2mins, then cycle 95C 20sec-58C 20sec-72C 30sec, 22 times and finish with 72C 3 mins.

D500/D700 series primers: See Supplemental Primer/Oligo Table B.

*Evolution of ibrutinib resistance- Expressed barcode method*

EBC vector- relevant unique region 18bp upstream of underlined sequence

pBA439 EBC vector

bp8241

CGATGCAATTTCCTCATTTTATTAGGAAAGGACAGTGGGAGTGGCACCTTCCAGGGTCAAGGAAGGCACGGGGGAGGGGCAAACAACAGATGGCTGGCAACTAGAAGGCACAGTCGAGGCTGATCAGCGGGTTTAAACGGGCCCTCTAGGC C -EBC insert-

🡨primer

CTAGG AATTCTTAATTAAGCTTGTGCCCCAGTTTGCTAGGGAGGTCGCAGTATC

TGGCCACTGCCACCTCGTGCTGCTCGACGTAGGT

Barcodes were amplified from DNA samples by next-gene sequencing as detailed above using custom primers, as follows using Takara ExTaq polymerase (Takara, #RR030A). See Supplemental Methods.

PCR1:

10x buffer 10ul, dNTPs 8ul, 5ul forward pLKO.1 BfuA1-F primer 5’ AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCG (5uM, IDT de-salted), 5ul reverse pLKO.1 BfuA1-R1.3 primer 5’ GTAATTCTTTAGTTTGTATGTCTGTTGCTATTATG (5uM, IDT de-salted), Ex-Taq 1.5ul, DNA up to 10ug in 20ul, H2O to 100ul final volume.

95C 5mins, then cycle 94C 30sec-65C 30sec-72C 20sec, 18 times and finish with 72C 5 mins.

Set up one reaction per ~10ug DNA; use all genomic DNA across multiple reactions and pool for PCR2, one reaction per original sample:

10x buffer 10ul, dNTPs 8ul, 5ul forward D500 series primer (5uM, IDT de-salted), 5ul reverse D700 series primer (5uM, IDT de-salted), Ex-Taq 1.5ul, 5ul PCR1 product H20 to 100ul.

95C 5mins, then cycle 94C 30sec-65C 30sec-72C 20sec, 18 times and finish with 72C 5 mins.

D500/D700 series primers:

See Supplemental Primer/Oligo Table C.

*STRING Analyses of gene sets*

Various sets of biologically-related genes were loaded onto the STRING website (https://string-db.org/,[6]) to identify known and inferred protein:protein interactions, and images were captured for subsequent display.

*Fast ATAC-Seq to identify regions of open chromatin*

HBL1 ABC DLBCL parental cells were cultured in media with DMSO (vehicle control) or 10nM ibrutinib for 24hrs. Three independent IR pools of HBL1, along with a naturally evolved genetically-resistant clone bearing the PLCG2 R665W allele, were cultured in parallel in 10nM ibrutinib. These lines were also treated with 5-Azacytidine (250nM) and Givinostat HDAC inhibitor (50nM) for 5 days and assayed as described below. Live cells were carefully counted by hemocytometer. If >20% are sick or dead, a MACS ‘dead cell’ removal kit (Miltenyi, 130-090-101) was used to clean up the culture. Between 5000-10000 cells were pelleted in a 1.5 ml microfuge tube, 4C 1500rpm 2mins. Supernatant was carefully removed so as not to disturb the (invisible) pellet. 50ul of transposase mix (Transposase mix (FC-121-1030 Illumina, G9441 Promega) was added, and the cell pellet was disrupted by pipetting 5 times.

Transposase Mix: 25ul 2xTD buffer, 2.5ul TDE, 0.5ul 1% digitonin, 22ul nuclease-free water.

Samples were incubated at 37C for 30 mins with agitation on an Eppendorf shaker (300rpm)

The reactions were purified with a QIAGEN MinElute Cleanup kit (Qiagen #28204), and eluted in 10ul of elution buffer. Samples were then amplified as follows:

10ul DNA, 10ul nuclease-free water, 5ul library primer pair (25uM, see (Ceribelli, 2016 #10116)), 25ul NEB 2x high fidelity PCR master mix.

PCR:1 cycle 5mins 72C, 30sec 98C, then 16 cycles: 10sec 98C, 30sec 63C, 1min 72C

Reactions were cleaned using a QIAGEN MinElute Cleanup kit (Qiagen #28204), and eluted in 40ul EB. A secondary purification using Agencourt Ampure Beads (A63881) was performed and library DNA was quantitated using a Qubit fluorometer and diluted for Illumina Sequencing, as described. For further details, see [5, 7].

*shRNA and sgRNA sequences*

shCTRL (SC4), [2, 8]

shTCF4, #10, [7]

shTLR9, #1, [8]

shMYD88, #1, [8]

|  |
| --- |
| shRAC2, #1124 5’- GATCCC**GGCCAGCTGCTCTAATATCGA**TTCAAGAGA**TCGATATTAGAGCAGCTGGCC**TTTTT  (IDT, desalted, cloned as described in [2] into a puro-gfp expressing, inducible shRNA vector) |

*CRISPR screening to determine essentiality of genes in parental cells and IR pools*

All of the following methods are detailed in their entirety in [8]. Inducible Cas9-expressing clones of the HBL1 and TMD8 ABC DLBCL lines were used to create independent IR pools as described above. These IR pools, and parent lines, were transduced with the Brunello sgRNA library, expanded, and induced for Cas9 expression for 21 days, with an initial starting sample of 40 million cells taken at day0 to provide the baseline sample for measuring sgRNA vector abundance. At day 21, cells were harvested, and DNA was prepared from all samples. sgRNA sequences were amplified, made into Illumina-compatible libraries, and sequenced to determine sgRNA abundance. To compare the change in abundance of sgRNAs targeting an individual gene, the normalize log2 read count of each guide was converted to a z-score of distributions at averaged across all guides for each gene. This metric is referred to as the CRISPR screen score, CSS, and indicated relative depletion (negative value = dropout, or gene is more essential) or enrichment (positive value = outgrowth, gene is less essential) of sgRNAs targeting a gene.

*Enumeration of cells with expression of RAC2 in single cell RNA-Seq samples above the parental average.*

The average expression of the RAC2 gene was determined for each parental cell line (HBL1, TMD8, DLBCL2) using the 10X genomics Loupe cell browser (V4.0). In t-SNE space, the number of cells from each population (parents and IR pools) with RAC2 expression higher than that average were enumerated, and plotted as shown.

*Creation and expression of human RAC2-BioID2*

As described for other human proteins [8], BioID2 (Addgene #80899) was appended to the C-terminus of human RAC2 using Gibson cloning technique, using the primers below (IDT, desalted) to bridge the C-terminus of RAC2 and add the BioID2 sequence. The RAC2-BioID2 cassette was cloned pBMN-MYD88L265P-VD-GFP as described with GFP removed by restriction digest with StuI and NotI and replaced with the LYT2 marker.

RAC2Cterm\_f1 5’ GCGGAGGCGGTGGATCGTACCAGGCCATGAAGTGTGTGGTG

RAC2Cterm\_r1 5’ TACGCGGCCGCCTCGAGTACTTACTAGAGGAGGCTGCAGGCGC

*Flow cytometry for intracellular proteins*

To detect intracellular proteins, specifically RAC2, cells were pelleted (5 mins. 1800rpm) in 96 well plates, washed with 1 volume of 1xPBS + 5%FBS, pelleted again and fixed with the addition of 200ul of 1% paraformaldehyde (EMS, 15710) in PBS for 10 minutes with gentle shaking. Cells were pelleted (5mins. 2200rpm), washed with 200ul 1xPBS +5%FBS +0.03% saponin (Sigma, 47036-250G-F, LotBCCC3326), and pelleted again. Cells were stained in 50ul of the preceding buffer with a 1:100 dilution of Alexa647 isotype control (Biolegend, 400130, LotB277417) or RAC2-Alexa647 antibody (NOVUS, NBP197947, LotA1223301-102218) for 2 hours at 4C with gentle shaking. Cells were pelleted, washed twice with 200ul of 1xPBS+5%FBS+0.03% saponin buffer, then resuspended in 150ul 1xPBS + 5%FBS, and subjected to flow cytometry. Average MFIs were measured in Flowjo, with isotype MFI being subtracted from each sample. MFIs were then normalized to the appropriate parental cell MFI for RAC2.

*Identification of the RAC2 interactome by mass spectrometry*

RAC2-BioID2 constructs were retrovirally transduced into DLBCL cell lines, as described above. Infected cells were enriched by positive selection with LYT2 magnetic beads (Invitrogen). As previously described [8], cells were then grown in SILAC media, containing amino acids labeled with stable isotopes or arginine and lysine, for 2 weeks prior to expansion to 100×106 cells. In certain cases, cells were treated with either 10nM ibrutinib or 200nM AZD2014 for 24 hours. 16 hours prior to lysis, biotin (Sigma) was added to a final concentration of 50μM to transduced cells. Cells were then lysed at 2.5 × 107 cells per ml in RIPA buffer modified for MS analysis (1% NP-40, 0.5% deoxycholate, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM Na3VO4, 5mM NaF, 1 mM AEBSF) for 10 min. on ice. Lysates were cleared by centrifugation at 14,000xg for 20 min. at 4°C. 35μl of Pre-washed streptavidin agarose beads were added to each sample; samples were then rotated at 4°C for 2 hours, then washed four times in 1X RIPA buffer, then solubilized with 4X LDS sample buffer (Invitrogen) with 1% β-mercaptoethanol, and boiled for 5 min. For MS analysis, proteins were separated by one-dimensional gel electrophoresis (4–12% NuPAGE Bis-Tris Gel, Invitrogen, USA) and the entire lane of a Coomassie blue-stained gel was cut into 23 slices. After tryptic digestion of the proteins the resulting peptides were resuspended in sample loading buffer (2% acetonitrile and 0.05% trifluoroacetic acid) and were separated by an UltiMate 3000 RSLCnano HPLC system (Thermo Fisher Scientific) coupled online to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). First, peptides were desalted on a reverse phase C18 precolumn (Dionex 5 mm length, 0.3 mm inner diameter) for 3 minutes. After 3 minutes the precolumn was switched online to the analytical column (30cm length, 75 mm inner diameter) prepared in-house using ReproSil-Pur C18 AQ 1.9 mm reversed phase resin (Dr. Maisch GmbH). Buffer A consisted of 0.1 % formic acid in H2O, and buffer B consisted of 80% acetonitrile and 0.1% formic acid in H2O. The peptides eluted from buffer B (5 to 42 % gradient) at a flow rate of 300 nl/min over 76 min. The temperature of the precolumn and the analytical column was set to 50°C during the chromatography. The mass spectrometer was operated in a TopN data-dependent mode, where the 30 most intense precursors from survey MS1 scans were selected with an isolation window of 1.6 Th for MS2 fragmentation under a normalized collision energy of 28. Only precursor ions with a charge state between 2 and 5 were selected. MS1 scans were acquired with a mass range from 350 to 1600 m/z at a resolution of 60,000 at 200 m/z. MS2 scans were acquired with a starting mass of 110 Th at a resolution of 15,000 at 200 m/z with maximum IT of 54ms. AGC targets for MS1 and MS2 scans were set to 1E6 and 1E5, respectively. Dynamic exclusion was set to 20 seconds. MS data analysis was performed using the software MaxQuant (version 1.6.0.1) linked to the UniProtKB/Swiss-Prot human database containing 155990 protein entries and supplemented with 245 frequently observed contaminants via the Andromeda search engine. Precursor and fragment ion mass tolerances were set to 6 and 20 ppm after initial recalibration, respectively. Protein biotinylation, N-terminal acetylation and methionine oxidation were allowed as variable modifications. Cysteine carbamidomethylation was defined as a fixed modification. Minimal peptide length was set to 7 amino acids, with a maximum of two missed cleavages. The false discovery rate (FDR) was set to 1% on both the peptide and the protein level using a forward-and-reverse concatenated decoy database approach. For SILAC quantification, multiplicity was set to two or three for double (Lys+0/Arg+0, Lys+8/Arg+10) or triple (Lys+0/Arg+0, Lys+4/Arg+6, Lys+8/Arg+10) labeling, respectively. At least two ratio counts were required for peptide quantification. The “re-quantify” option of MaxQuant was enabled. Data was filtered for low confidence peptides. Data were reported as the log2 normalized count of peptides for each protein.

*Proximity Ligation Assay for RAC2 interactions*

DLBCL cell lines or CLL cells were plated onto a 15 well μ-Slide Angiogenesis ibidiTreat chamber slide (Ibidi, 81506) and allowed to adhere to the surface for 30 min at 37°C. Cells were then fixed with 4% paraformaldehyde for 20 min at room temperature and then washed in PBS. Cellular membranes were labeled with 5 μg/ml wheat germ agglutinin (WGA) conjugated to Alexa Fluor 488 (Thermo Fisher Scientific, W11261) for 10 min at room temperature. Cells were permeabilized in cold methanol (Fisher, A412-4, Lot204026) for 10 min, washed in 1xPBS and then blocked in Duolink Blocking buffer (Sigma, DUO92102, DUO92104, DUO92106) for 30 min at room temperature. Primary antibodies were diluted in Duolink Antibody Diluent and incubated overnight at 4°C (see below). CLL cells were also counterstained with anti-IgM antibody to illuminate malignant cells (1:250 dilution, Biolegend, 314536, LotB288547). The following morning, cells were washed for 20 min in a large volume of 1xPBS with 1% BSA (Sigma, A7030-100G, LotSLCH1487), followed by addition of the appropriate Duolink secondary antibodies diluted and mixed according to the manufacturer’s instructions. Cells were incubated for 1 hour at 37°C, after which cells were washed in 1xTBS (10X, Quality Biological, 351-086-151, Lot723609) with 0.5% tween-20 (Sigma, P1379-100ML) for 10 min. Ligation and amplification steps of the PLA were performed using the Duolink in situ Detection Reagents Orange kit (Sigma, DUO92102, DUO92104, DUO92106) according to the manufacturer’s instructions. Following the PLA, cells were mounted in FluroShield mounting media with DAPI (abcam, ab104139, LotGR3221112-6). Images were acquired on a Zeiss LSM 880 Confocal microscope using Zeiss Zen Black version 2.3. PLA spots were counted in cell lines using Blobfinder version 3.2. PLA Score was determined by normalizing the number of PLA spots counted in each sample to the average number of PLA spots counted in the control sample.

PLA antibodies: Mouse anti-RAC2 (NOVUS, NBP197947, LotA1223301-102218), 1:500 dilution

with either Goat anti-IgM (Jackson, 109-005-129) 1:1000 dilution or Rabbit anti-PLCG2 (Cell Signaling, 55512S), 1:200 dilution

*High-throughput drug screening*

Cell lines and IR pools were expanded to ~100 million and screened using the MIPE5.0 library as described in [9, 10]. Briefly, 1,000 cells per well in 5μL of media were dispensed using a Multidrop Combi dispenser and a small cassette into barcoded 1,536 solid-bottom white Greiner Bio-one tissue culture-treated plates (catalog #789173-F). Advanced RPMI -1640 supplemented with 5% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and 2 mM glutamine was used, with 10nM ibrutinib maintained in IR pools. For the generation of standard 11-point dose–response curves, the cells were plated, followed by the immediate pintool addition of 23 nL of control compound (bortezomib) and library compounds using a Kalypsys pintool. For pre-plated matrix plates, the cells were added directly to the plates immediately after compounds were acoustically dispensed using an ATS-100 (EDC Biosystems). The plates were then covered with stainless steel cell culture Kalypsys lids and incubated at 37 °C with 5% CO2 under 95% humidity. For cell proliferation assays, the cells were incubated for 48 h and then 3 μL of CellTiter Glo luminescent cell viability assay reagent (Promega, G7570) was added using a Bioraptor Flying Reagent Dispenser (Aurora Discovery-BD). The plates were then incubated for 15 min at room temperature. The signal was measured using a 10-s exposure with a ViewLux (Perkin-Elmer) with a luminescent filter.

*ABC DLBCL xenografts- RAC and BCL2 inhibitors with/ without Ibrutinib*

All mouse experiments were approved by the National Cancer Institute Animal Care and Use Committee (NCI-ACUC) and were performed in accordance with NCI-ACUC guidelines and under approved protocols (METB054). Approved protocols allowed tumor growth below 20mm in any dimension; no animals had tumors which exceeded these limits. Female NSG (non-obese diabetic/severe combined immunodeficient/common gamma chain deficient) mice were obtained from NCI Fredrick Biological Testing Branch and used for the xenograft experiments between 6–8 weeks of age. HBL1 and DLBCL tumors were established by subcutaneous injection of 10 × 106 cells in a 1:1 Matrigel (Corning, 356235)/1xPBS suspension. Treatments were initiated when tumor volume reached a mean of 200mm3. Ibrutinib was prepared in 1xPBS with 50% (v/v) DMSO and administered i.p. once per day (5mg/kg/day for DLBCL2 and 50mg/kg/day for HBL1). EHT1864 Rac inhibitor was prepared in 1xPBS with 50% (v/v) DMSO and administered i.p. once per day (100mg/kg/day). ABT199 (Venetoclax) BCL2 was prepared in 1xPBS with 50% (v/v) DMSO and administered by gavage once per day (50mg/kg/day). Drugs were given at the same concentration and schedule as single agents. Tumor growth was monitored every other day by measuring tumor size in two orthogonal dimensions and tumor volume was calculated by the following equation: tumor volume = (length × width2)/2. Treatment randomization and experimenter blinding were not possible. Sample size was estimated based upon preliminary experiments.

1. Ngo, V.N., R.E. Davis, L. Lamy, X. Yu, H. Zhao, G. Lenz, et al., *A loss-of-function RNA interference screen for molecular targets in cancer.* Nature, 2006. **441**(7089): p. 106-10.

2. Yang, Y., A.L. Shaffer, 3rd, N.C. Emre, M. Ceribelli, M. Zhang, G. Wright, et al., *Exploiting synthetic lethality for the therapy of ABC diffuse large B cell lymphoma.* Cancer Cell, 2012. **21**(6): p. 723-37.

3. Schmitz, R., G.W. Wright, D.W. Huang, C.A. Johnson, J.D. Phelan, J.Q. Wang, et al., *Genetics and Pathogenesis of Diffuse Large B-Cell Lymphoma.* N Engl J Med, 2018. **378**(15): p. 1396-1407.

4. GDC, *NCI Genomic Data Commons RNA-Seq pipeline.* NCI GDC documentaton, 2018.[**https://docs.gdc.cancer.gov/Data/Bioinformatics\_Pipelines/Expression\_mRNA\_Pipeline/**](https://docs.gdc.cancer.gov/Data/Bioinformatics_Pipelines/Expression_mRNA_Pipeline/).

5. Ceribelli, M., P.N. Kelly, A.L. Shaffer, G.W. Wright, W. Xiao, Y. Yang, et al., *Blockade of oncogenic IkappaB kinase activity in diffuse large B-cell lymphoma by bromodomain and extraterminal domain protein inhibitors.* Proc Natl Acad Sci U S A, 2014. **111**(31): p. 11365-70.

6. Szklarczyk, D., A.L. Gable, D. Lyon, A. Junge, S. Wyder, J. Huerta-Cepas, et al., *STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets.* Nucleic Acids Res, 2019. **47**(D1): p. D607-D613.

7. Ceribelli, M., Z.E. Hou, P.N. Kelly, D.W. Huang, G. Wright, K. Ganapathi, et al., *A Druggable TCF4- and BRD4-Dependent Transcriptional Network Sustains Malignancy in Blastic Plasmacytoid Dendritic Cell Neoplasm.* Cancer Cell, 2016. **30**(5): p. 764-778.

8. Phelan, J.D., R.M. Young, D.E. Webster, S. Roulland, G.W. Wright, M. Kasbekar, et al., *A multiprotein supercomplex controlling oncogenic signalling in lymphoma.* Nature, 2018. **560**(7718): p. 387-391.

9. Lin, G.L., K.M. Wilson, M. Ceribelli, B.Z. Stanton, P.J. Woo, S. Kreimer, et al., *Therapeutic strategies for diffuse midline glioma from high-throughput combination drug screening.* Sci Transl Med, 2019. **11**(519).

10. Mathews Griner, L.A., R. Guha, P. Shinn, R.M. Young, J.M. Keller, D. Liu, et al., *High-throughput combinatorial screening identifies drugs that cooperate with ibrutinib to kill activated B-cell-like diffuse large B-cell lymphoma cells.* Proc Natl Acad Sci U S A, 2014. **111**(6): p. 2349-54.