Overcoming Acquired Epigenetic Resistance to BTK Inhibitors

Arthur L. Shaffer III\textsuperscript{1,7}, James D. Phelan\textsuperscript{1}, James Q. Wang\textsuperscript{1}, DaWei Huang\textsuperscript{1}, George W. Wright\textsuperscript{2}, Monica Kasbekar\textsuperscript{1}, Jaewoo Choi\textsuperscript{1}, Ryan M. Young\textsuperscript{1}, Daniel E. Webster\textsuperscript{1}, Yandan Yang\textsuperscript{1}, Hong Zhao\textsuperscript{1}, Xin Yu\textsuperscript{1}, Weihong Xu\textsuperscript{1}, Sandrine Roulland\textsuperscript{3}, Michele Ceribelli\textsuperscript{1,3}, Xiaohu Zhang\textsuperscript{3}, Kelli M. Wilson\textsuperscript{3}, Lu Chen\textsuperscript{3}, Crystal McKnight\textsuperscript{3}, Carleen Klumpp-Thomas\textsuperscript{3}, Craig J. Thomas\textsuperscript{1,3}, Björn Häupl\textsuperscript{4}, Thomas Oellerich\textsuperscript{4}, Zachary Rae\textsuperscript{5}, Michael C. Kelly\textsuperscript{5}, Inhye E. Ahn\textsuperscript{6}, Clare Sun\textsuperscript{6}, Erika M. Gaglione\textsuperscript{6}, Wyndham H. Wilson\textsuperscript{1}, Adrian Wiestner\textsuperscript{6}, Louis M. Staudt\textsuperscript{1,7}

\textsuperscript{1}Lymphoid Malignancies Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892 USA
\textsuperscript{2}Biometric Research Program, Division of Cancer Diagnosis and Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD, 20892, USA
\textsuperscript{3}Division of Preclinical Innovation, National Center for Advancing Translational Sciences, National Institutes of Health, Bethesda, Maryland 20892, USA
\textsuperscript{4}Department of Medicine II, Hematology/Oncology, Goethe University, 60590 Frankfurt, Germany; German Cancer Consortium/German Cancer Research Center, 69120 Heidelberg, Germany; and Department of Molecular Diagnostics and Translational Proteomics, Frankfurt Cancer Institute, 60596 Frankfurt, Germany
\textsuperscript{5}Cancer Research Technology Program, Single Cell Analysis Facility, Frederick National Laboratory for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892 USA
\textsuperscript{6}Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892 USA
\textsuperscript{7}Corresponding authors; address correspondence to lstaudt@mail.nih.gov or arthurlshaffer3@gmail.com.

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Summary
The use of Bruton tyrosine kinase (BTK) inhibitors to block B cell receptor (BCR)-dependent NF-kB activation in lymphoid malignancies has been a major clinical advance, yet acquired therapeutic resistance is a recurring problem. We modeled the development of resistance to the BTK inhibitor ibrutinib in the ABC subtype of diffuse large B cell lymphoma, which relies on chronic active BCR signaling for survival. The primary mode of resistance was epigenetic, driven in part by the transcription factor TCF4. The resultant phenotypic shift altered BCR signaling such that the GTPase RAC2 substituted for BTK in the activation of phospholipase Cγ2, thereby sustaining NF-κB activity. The interaction of RAC2 with phospholipase Cγ2 was also increased in chronic lymphocytic leukemia cells from patients with persistent or progressive disease on BTK inhibitor treatment. We identified clinically available drugs that can treat epigenetic ibrutinib resistance, suggesting combination therapeutic strategies.

Statement of Significance
In diffuse large B cell lymphoma, we show that primary resistance to BTK inhibitors is due to epigenetic rather than genetic changes that circumvent the BTK blockade. We also observed this resistance mechanism in chronic lymphocytic leukemia, suggesting that epigenetic alterations may contribute more to BTK inhibitor resistance than currently thought.
Introduction

The importance of B cell receptor (BCR) signaling for the survival of lymphoma cells was discovered using functional genomic screens for essential genes in cell line models of the ABC subtype of diffuse large B cell lymphoma (DLBCL) [1]. ABC DLBCL cells have a “chronic active” form of BCR signaling that constitutively triggers a signaling cascade that activates the pro-survival NF-κB pathway [1]. These functional studies were bolstered by the discovery of recurrent gain-of-function mutations targeting the BCR subunits CD79A and CD79B in ABC DLBCL tumors [1]. These mutations augment ongoing BCR receptor signaling in ABC DLBCL that is triggered by the binding of the BCR to diverse self-antigens [2]. The kinase BTK is an essential component of the BCR signaling pathway that phosphorylates and activates phospholipase Cγ2 (PLCγ2, PLCG2), triggering molecular processes that culminate in the nuclear translocation of NF-κB. Accordingly, the covalent BTK inhibitor ibrutinib was selectively toxic for ABC DLBCL models, offering a means to therapeutically interdict chronic active BCR signaling in this aggressive lymphoma [1].

Ibrutinib and other BTK inhibitors (e.g. acalabrutinib, zanabrutinib) have been transformative in the treatment of several indolent B cell cancers, including chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), marginal zone lymphoma, and Waldenstrom macroglobulinemia (reviewed in [3]). In DLBCL, BTK inhibitors have shown significant clinical activity but have not produced lasting remissions in most patients. In the first phase II trial of ibrutinib monotherapy in relapsed/refractory DLBCL, objective responses were observed in 39% of patients with ABC DLBCL, translating into significantly longer overall survival in ABC DLBCL patients. Nonetheless, median progression-free survival in ABC DLBCL was very short (2 months), indicating that BTK monotherapy failed to irradicate the malignant cells in most patients.

Genetic analysis of biopsy samples from patients on the ibrutinib monotherapy trial revealed that patients with ABC DLBCL whose tumors harbored both a CD79B mutation and a MYD88L265P mutation had an 80% response rate while the response rate in the remaining ABC cases was only a 30% [4]. This observation revealed a functional cooperation between the BCR and MYD88 pathways [4], leading to the discovery of a supramolecular complex termed the “My-T-BCR”, consisting of the BCR, TLR9 and MYD88L265P along with many other proteins involved in NF-κB activation, including CARD11, BCL10, MALT1 and IkB kinase (IKK) [5]. ABC DLBCLs that form a My-T-BCR complex are “addicted” to chronic active BCR signaling, explaining their exceptional response to ibrutinib monotherapy.

The co-occurrence of CD79B and MYD88L265P mutations is a common genetic trait in primary extranodal lymphomas, including primary central nervous system lymphoma (PCNSL). In fact, PCNSL and other primary extranodal lymphomas belong to the recently described “MCD” genetic subtype, which is defined by a distinctive genetic signature that sets these tumors apart from other DLBCLs [6, 7]. As predicted from this genetic profile and pre-clinical studies of ibrutinib in MCD cell line models [1], ibrutinib monotherapy produced an 77-89% objective response rate in PCNSL [8, 9]. However, continuous administration of ibrutinib in PCNSL was associated with rapid disease progression in most patients [8], as had been observed in ABC DLBCL. Together, these clinical observations suggest that resistance to BTK
inhibition occurs rapidly in DLBCL, even in tumors that are addicted to BCR-dependent NF-κB activation.

Since its clinical approval, ibrutinib has been administered to thousands of patients with MCL and CLL. While initial response rates are high, resistance to ibrutinib monotherapy is an increasing problem [10-13]. The best-characterized form of ibrutinib resistance is genetic, with mutations targeting BTK and its downstream substrate PLCγ2. BTK mutations most often modify the cysteine residue (C481) to which ibrutinib covalently attaches. Mutations targeting PLCγ2 confer ibrutinib resistance by disrupting the enzyme’s autoinhibitory interactions, creating constitutively active alleles that propagate signals to downstream effectors of NF-κB activation [13-15].

The timing and frequency of these ibrutinib resistance mutations indicate that they may not be the initial drivers of ibrutinib resistance in CLL. In a recent analysis of CLL patients in stable remission on ibrutinib, roughly half had a readily detectable CLL clone in the peripheral blood (>50,000 cells/ml) [16]. In 43% (13/30) of patients, no BTK or PLCG2 mutation was detected, and among those with mutations, more than one quarter had VAFs under 10%, consistent with sub-clonality. In another study, CLL patients on ibrutinib acquired BTK resistance mutations after a median time on treatment of 35.1 months [15]. These studies suggest that non-genetic mechanisms of resistance may occur in some or all CLL patients treated with a BTK inhibitor and precede the acquisition of BTK or PLCG2 resistance mutations.

To date, BTK resistance mutations have been reported in two patients with relapsed/refractory DLBCL treated with ibrutinib monotherapy [17]. In circulating tumor DNA, BTK mutations only became readily detectable after 5-19 months, suggesting that they may have arisen from a pool of lymphoma cells that was resistant to ibrutinib, perhaps by a non-genetic mechanism.

In the present study, we explored the development and mechanistic basis of ibrutinib resistance in multiple cell line models of ABC DLBCL, using genetic, functional genomic and proteomic tools. Based on the mechanisms we uncovered, we identified therapeutic vulnerabilities in ABC DLBCL cells that could be exploited to overcome resistance to BTK inhibitors.
Epigenetic Ibrutinib Resistance in ABC DLBCL Lines

To create models of ibrutinib-resistant ABC DLBCL, we used HBL1, TMD8, DLBCL2, and U2932 cell lines, which recapitulate essential aspects of ABC genetics and biology [5] and are sensitive to ibrutinib in the nanomolar range [1, 18] as measured by the Growth Rate Inhibition 50% value (GR50; [1, 23]). Lines were single-cell cloned to limit genetic and epigenetic variation, and then exposed to ibrutinib at concentrations increasing weekly from ~10-fold below to ~2-fold above the GR50 for one week (Fig. 1A). The resulting cultures were maintained in ibrutinib (10nM) and will be referred to as ibrutinib-resistant (IR) pools. Of note, the cell cultures retained high viability throughout this process, suggesting that the outgrowth of rare subclones was not required for ibrutinib resistance under these conditions.

We subjected some IR pools to single cell cloning and will refer to these as IR clones (Fig. 1A). Targeted sequencing revealed that several, but not all, IR clones had acquired mutations in \( BTK \) and \( PLCG2 \) that have been shown to confer ibrutinib resistance [13, 15, 17, 19] (Fig. S1A). The \( BTK^{C481F} \) mutation, which prevents ibrutinib binding, was detected in multiple independent TMD8 IR clones. Two independent U2932 IR clones acquired a \( BTK^{T471I} \) mutation, which targets an active site threonine that stabilizes ibrutinib binding. Several IR clones of HBL1 and U2932 had \( PLCG2^{R665W} \) or \( PLCG2^{A708P} \) mutations that confer relative ibrutinib resistance [15]. Whether endogenously or ectopically expressed, these mutant isoforms mediated ibrutinib resistance but did not provide any survival advantage in the absence of ibrutinib (Figs. 1B, S1B, C). None of these mutations was observed at >10% VAF in the IR pools after six weeks of culture, but after more than one year in culture, the \( BTK^{C481Y} \) mutation was detected in one TMD8 IR pool while the HBL1 IR pools remained devoid of \( BTK \) and \( PLCG2 \) mutations. Whole-exome sequencing of more than twenty independently derived IR pools provided no evidence of mutations that could account for ibrutinib resistance (Tables S1A and S1B). Together, these findings suggested that non-genetic mechanisms could account for the rapid acquisition of ibrutinib resistance by ABC lines.

To investigate the stability of ibrutinib resistance phenotype, we cultured HBL1 and TMD8 IR pools in the absence of ibrutinib for one week. Upon ibrutinib re-challenge, each IR pool maintained its resistance as did IR clones with ibrutinib resistance mutations (Fig. 1B). Remarkably, the stability of the IR resistant phenotype was maintained for up to 4 months (Fig. 1C). We also investigated whether short term ibrutinib exposure was sufficient to create stable ibrutinib resistance. HBL1 cells were exposed to twice the GR50 value (10nM) of ibrutinib for one or 8 weeks, cultured for 7 days without ibrutinib and then re-challenged with ibrutinib (10nM). The 8-week cultures generated a stable resistance phenotype whereas the 1-week cultures did not (Fig. S1D).

To characterize the IR phenotype further, we profiled gene expression in the IR pools and parental ABC lines by RNA sequencing (RNA-seq). To generate an ibrutinib response signature, we also acutely exposed parental cells to ibrutinib (10nM, 24 hours) and identified genes that decreased in expression (log2 fold change <-0.4) relative to pre-treatment levels (Table S1C, D). This ibrutinib response signature overlapped significantly with previously defined signatures of BCR, MYD88, and NF-κB activity in ABC DLBCL cells as well as signatures of cellular proliferation (Table S1E) [20]. The IR clones with \( BTK \) and \( PLCG2 \) mutations expressed
the ibrutinib response signature at levels comparable to those in the untreated parental line (Fig. 1D, Fig. S1E), as expected. By contrast, IR pools (Fig. 1D) and IR clones lacking resistance mutations (Fig. S1E, Table S1D, F) expressed the ibrutinib response signature at levels that were intermediate between the levels in acutely treated parental lines and genetically mutant IR clones. This suggested that the mechanisms by which the IR pools persist in ibrutinib might be distinct from those of IR clones bearing resistance mutations.

To support the possibility that the ibrutinib resistance of IR pools was epigenetic, we treated the IR pools and the parental ABC lines with the DNA methylation inhibitor 5-azacytidine and the histone deacetylase inhibitor (HDAC) givinostat, a combination that can alter the epigenome of cancer cells [21, 22]. While this drug combination had little or no effect on the ibrutinib sensitivity of parental lines or the ibrutinib resistance of genetically mutant IR clones, this treatment increased the ibrutinib sensitivity of the IR pools (Fig. 1E). In fact, HDAC inhibition alone re-sensitized the IR pools to ibrutinib (Fig. S1F). These data suggest that the rapid development of ibrutinib resistance in ABC models is generated by non-genetic mechanisms that can be reversed by epigenetic modification.

Tracking the Evolution of Ibrutinib Resistance in ABC DLBCL Lines

We next used single cell barcoding to track the development of ibrutinib resistance. We transduced HBL1 cells with lentiviruses bearing unique 60-base pair DNA barcodes such that each cell was tagged with one barcode. We considered three general cell fates in the transduced population: death of “sensitive” cells, expansion of “resistant” cells, and maintenance without expansion of “persistent” cells (Fig. 2A). As expected, the distribution of barcodes among cells treated with DMSO did not change after five weeks in culture (Fig. 2B). By contrast, cells challenged with ibrutinib exhibited all three cell fates. Many barcoded cells were undetectable or decreased more than 10-fold after five weeks of ibrutinib treatment and were deemed sensitive. Of the remaining barcoded cells, ~5% were classified as resistant since their prevalence increased >10-fold over and consequently constituted ~30% of all cells in the ibrutinib-treated cultures at week 5. The remaining cells the cultures changed little in frequency during ibrutinib treatment and were designated persistent. Similar results were observed using the TMD8 line (Fig. 2B, Fig. S2A, Table S2A).

To explore the phenotypic differences between the sensitive, persistent and resistant cells, we used lentiviruses to introduce a barcode that is transcribed into mRNA, allowing it to be detected by both DNA sequencing and by single-cell RNA sequencing (scRNA-seq), as described [23]. Because scRNA-seq only interrogates a few thousand cells, we limited the starting cell pools to ~300 uniquely barcoded cells, allowing us to sample multiple offspring from each cell in the final analysis. Cells with expressed barcodes were sampled immediately prior to ibrutinib selection and then treated with increasing concentrations of ibrutinib or with DMSO over 5 weeks. By bulk DNA sequencing, we defined barcodes representing resistant (n=6) and persistent (n=7) cells, with all other barcodes representing sensitive cells. We identified the resistant and persistent barcodes in the scRNA-seq data and observed that the relative read counts of these barcodes from RNA and DNA sequencing were linearly correlated, as expected (Fig. 2C).

Figure 2D displays the t-SNE plots of gene expression in each cell in the ibrutinib and DMSO cultures (Figs. 2D, S2B). Ibrutinib profoundly altered the gene expression landscape of
persistent and resistant cells and confirmed the depletion of the sensitive cells from the ibrutinib cultures. In the t-SNE plot from the DMSO culture, ibrutinib sensitive cells were intermingled with those destined to become persistent and resistant cells when exposed to ibrutinib, suggesting that the persistent and resistant cell states may not pre-exist in the starting population.

While the gene expression of persistent and resistant cells cultured in ibrutinib was markedly distinct from the same barcoded cells in DMSO, these two subpopulations were not homogeneously intermingled in the ibrutinib clusters suggesting that they might have distinct phenotypes (Figs. 2D, S2B). First we identified genes that were more highly expressed in both the persistent and resistant cells compared with cells growing in DMSO (>0.25 log₂ fold change; Tables S2B, C) and used a database of gene signatures relevant to normal and malignant B cell biology [20] to identify enriched signatures (Table S2D, p<0.01; Fisher’s exact test). Persistent and resistant cells upregulated genes in pan-B cell and germinal center B cell signatures, genes encoding MHC class II subunits, and genes that are transactivated by IRF4 and TCF4 (see below), which play pivotal roles in the ABC DLBCL biology [24, 25] (Fig. 2E, Table S2C,D). Compared with resistant cells, persistent cells had higher expression of genes characteristic of the ABC subtype, including regulators of survival (BCL2), proliferation (CCND2) and oncogenic signaling (STAT3). Conversely, resistant cells had higher expression of genes that are upregulated in proliferating cells, including genes directly transactivated by MYC, which is epigenetically overexpressed in the ABC subtype [20, 26] (Fig. 2E, Table S2D). This finding likely explains the expansion of the resistant population relative to the persistent population. Expressed barcode experiments in the TMD8 line yielded similar phenotypic differences among the IR subpopulations (Fig. S2C, Table S2E).

In separate experiments, we used scRNA-seq to identify convergent gene expression programs in ibrutinib-resistant cells generated from 3 ABC cell lines (HBL1, TMD8, DLBCL2) (Fig. 2F). Among genes upregulated in the ibrutinib-resistant pools, 195 were identified in at least 2 of the ABC lines (Fig. 2G, Tables S2F,G). Of these, 52 of which were also upregulated in ibrutinib persistent and resistant HBL1 cells in the expressed barcode experiment described above. Functional organization of these 52 genes using STRING [27] revealed subsets involved in BCR signaling, antigen presentation and protein translation (Fig. 2H). The BCR subset included the BCR signaling subunits CD79A and CD79B, the adapter BLNK that promotes the phosphorylation of PLCγ2 by BTK [28, 29], and the small GTPase RAC2, which contributes to BCR-activated calcium mobilization by PLCγ2 [30].

**Epigenetic Retuning of Oncogenic Signaling in Ibrutinib Resistance**

To identify chromatin changes associated with epigenetic resistance, we performed ATAC-seq [31], comparing the parental line HBL1, an HBL1 IR clone with PLCG2^R665W, three HBL1 IR pools, and the ibrutinib-sensitive revertants of these pools following exposure to 5-azacytidine plus givinostat (Fig. 3A). We identified 37,348 ATAC-seq peaks (regions of open chromatin), of which 7,308 were more accessible in the IR pools than in the PLCG2^R665W IR clone and parental cells (>0.3 log₂ increase in read count), and 1215 that were less accessible following 5-azacytidine plus givinostat treatment (>0.3 log₂ decrease in read count, Tables S3A, B).
Using the GREAT genomic mapping tool [32], we identified the two most proximal genes to each ATAC-seq peak (within 100 kb). The subset of peaks that were enriched in the IR pools were often observed in or near genes encoding components of ABC DLBCL oncogenic signaling pathways, essential ABC DLBCL transcription factors, and anti-apoptotic BCL2 family members (Fig. 3A, Table S3B). Peaks associated with many of these genes were quantitatively diminished upon treatment of the IR pools with 5-azacytidine plus givinostat, including peaks associated with genes encoding proximal BCR components (LYN, SYK, BTK, RAC2, PLCG2) and the downstream NF-κB activation pathway (PKCB2, CARD11, BCL10, MALT1, REL) (Fig. 3B, Table S3B).

To identify transcription factors responsible for the profound epigenetic shift in the IR pools, we performed DNA binding motif enrichment analysis of ATAC-seq peaks (Fig. 3C; Table S3C) [33]. Open chromatin regions in the parental line were significantly enriched in binding sites for transcription factors essential to the pathogenesis of ABC DLBCL, including the IRF4-SPIB heterodimer, which binds ETS/IRF sites (e-value=1.1E-14) [25] and Oct-2 (POU2F2), which binds octamer sites (e-value=2.2E-9) [34]. While ATAC-seq regions in the parental and IR pools were equivalently enriched in these two binding motifs, those in the IR pools uniquely enriched in binding sites for the E-box factors TCF3 and TCF4 (e-value=2.8E-7). Whereas TCF3 is essential in normal and malignant germinal center B cells [6, 35], TCF4 is uniquely essential in ABC DLBCL cells [24]. We therefore hypothesized that TCF4 might be responsible, in part, for the epigenetic shift in ibrutinib resistant ABC DLBCL cells.

We performed TCF4 ChIP-seq analysis using HBL1 cells engineered to express the bacterial biotinylase BirA along with a TCF4 isoform tagged with a domain that is biotinylated by BirA, thereby enabling streptavidin-mediated capture of TCF4-bound DNA fragments [36]. The intersection of ATAC-seq peaks with TCF4 bound peaks revealed direct TCF4 target genes, including many of the genes involved in oncogenic survival pathways in ABC DLBCL (Table S3D). Figure 3D displays TCF4-bound ATAC-seq peaks in the RAC2, TLR9 and BCL2 loci, which coincided with DNaseI hypersensitivity peaks and with the presence of H3K27 acetylated chromatin, indicative of enhancer function. Each of these peaks was more pronounced in IR pools than in the either the parental line or the PLCG2^{R665W} IR clone. While knockdown of TCF4 was toxic in the HBL1 and TMD8 parental lines, knockdown of this factor was consistently more deleterious in the IR pools (Fig. 3E). Together, these observations suggest that the transcriptional regulatory network in IR pools was skewed towards greater dependency on TCF4 and its targets.

**Altered Dependencies in Ibrutinib-resistant ABC DLBCL**

To uncover potential new therapeutic vulnerabilities in ibrutinib-resistant cells, we performed genome-wide CRISPR-Cas9 screens for essential genes in two parental ABC cell lines (HBL1, n=4; TMD8, n=6) and 5 independently derived IR pools (HBL1 IR pools, n=2; TMD8 IR pools, n=3) [37]. As described [5], we calculated a “CRISPR screen score” (CSS) for each gene, which is a Z-score metric for the degree of dropout or enrichment of single guide RNAs (sgRNAs) targeting that gene compared to other sgRNAs in the library. Of the targeted genes, 3,398 scored as essential in the IR pools (average log₂ CSS < -0.5) while 229 scored as tumor suppressors (average log₂ CSS > 0.5). Of the essential genes, 1,758 (51.7%) were dubbed
“common essential” by the DepMap Project [38], the vast majority of which were equivalently essential in the parental lines and IR pools (1,675; 95.3%) (Table S4).

We identified 701 genes that were more essential in the IR pools than in their parents (average ΔCSS < -0.5) and 1000 genes that were less essential in IR pools (average ΔCSS > 0.5) (Fig. 4A). Importantly, IR pools differed profoundly from the parental lines in their dependency on genes encoding signaling and regulatory proteins that promote survival of ABC DLBCL cells (Fig. 4A, Table S4). Figure 4B arranges these proteins into regulatory pathways, with the left half of each protein icon depicting its essentiality (CSS) in the IR pools and the right half depicting its essentiality in IR pools relative to parental cell lines (ΔCSS).

Unexpectedly, the IR pools depended on the BCR subunits CD79A and CD79B as well as SYK to an equal or greater extent than the parental lines, despite the inhibition of BTK by ibrutinib. Also unexpected was the increased dependence of the IR pools on NF-κB pathway components, including the α and β subunits of IKK (CHUK and IKBKB, respectively), and the chaperone HSP90 (HSP90B1), which is an integral component of this kinase [39]. The IR pools were also more dependent on the NF-κB subunit p65 (RELA) and IkBζ (NFKBIZ), a modulator of transactivation by NF-κB. Conversely, inactivation of negative regulators of BCR signaling (LYN, PTPN6) and NF-κB (TNIP1, NFKBIE) promoted greater outgrowth in the IR pools than in the parental lines, consistent with an enhanced reliance of the IR pools on BCR-dependent NF-κB activation.

Together, these data suggested that the BCR-dependent NF-κB pathway may be reconfigured in the IR pools to bypass the inhibition of BTK kinase activity by ibrutinib. As expected, BTK was less essential in the IR pools than in the parental lines. Nonetheless, knockout of BTK was toxic for the IR pools, suggesting a kinase-independent function of BTK. A primary function of BTK in the BCR pathway is phosphorylation and activation of PLCγ2. While a catalytically inactive BTK isoform is capable of stimulating certain mutant PLCγ2 isoforms [40], the continued dependence of the IR pools on wild type PLCγ2 suggested that this enzyme may be activated by another mechanism in these cells. It was notable in this regard that the IR pools were more dependent than the parental lines on RAC2, a Rho-family GTPase that when bound to GTP, interacts directly with PLCγ2, stimulating its enzymatic activity [41-43]. Also notable was the increased dependence of the IR pools on VAV1, a guanine nucleotide exchange factor for RAC2 that activates RAC2 following BCR stimulation [44]. These observations suggest that RAC2 could function as a bypass mechanism to activate PLCγ2, thereby overcoming the inhibition of BTK kinase activity by ibrutinib.

The enzymatic products of PLCγ2 function as second messengers to activate protein kinase C β (PKCβ, PRKCB), which phosphorylates CARD11, triggering assembly of the multiprotein “CBM” adapter complex that activates NF-κB. The IR pools were dependent on PKCβ, and the CBM components CARD11, BCL10, and MALT1, albeit to a lesser degree than in the parental lines, consistent with their dependence on PLCγ2. Conversely, the IR pools were more dependent than the parental lines on a signaling module consisting of TLR9, MYD88 and IRAK1, as well as on UNC93B1, a chaperone necessary for trafficking of TLR9 to endolysosomes (Fig. 4B). These findings suggest that the IR pools partially shifted the burden of NF-κB activation from the CBM module to the TLR9/MYD88 module.
Also notable was the increased reliance of the IR pools on BCL2, which cooperates with NF-κB to sustain the survival of ABC DLBCL cells [18], and on several nuclear regulatory factors that influence gene expression genome-wide, including MYC and BRD4.

**RAC2 as a Mediator of Epigenetic Ibrutinib Resistance**

Given the increased dependence of IR pools on RAC2 and its altered chromatin structure in IR pools, we further explored the role of RAC2 in mediating ibrutinib resistance. Confirming the CRISPR screen results, knockdown of RAC2 was significantly more toxic in the IR pools than in parental lines (Fig. 5A). scRNA-seq analysis revealed that IR pools (n=9) had more cells with high RAC2 than the parental lines (Fig. 5B). The IR pools had correspondingly higher levels of RAC2 protein as assessed by intracellular flow cytometry (Fig. 5C), consistent with increased chromatin accessibility at the RAC2 locus and specific binding of TCF4 to a putative enhancer 3’ of the RAC2 locus (Fig. 3A, 3B, 3D). Accordingly, treatment of 3 independent HBL1 IR pools with 5-azacytidine plus givinostat reduced RAC2 protein expression to the level in the parental line (Fig. 5D).

To gain insight into the increased essentiality of RAC2 in the IR pools, we profiled gene expression changes by RNA-seq following knockdown of RAC2 in parental TMD8 cells and in a derived IR pool (Fig. 5E, F and Table S5A, B). In the IR pool but not the parental line, RAC2 knockdown significantly decreased expression of signatures of ABC DLBCL identity (ABCDLBCL-1), B cell-specific genes repressed by Blimp-1 (Blimp-1), and genes induced by NF-κB (NFκB-2), STAT3 (STAT3Up-1), and IRF4 (IRF4Up-9) (Fig. 5F, Table S5B). These signatures included genes encoding multiple proximal BCR signaling components including CD79B, mediators of BCR-dependent NF-κB signaling (BLNK, BTK, PLCγ2), and VAV1, which links proximal BCR signaling to RAC2 activation (Fig. 5E, F). Also downregulated were genes encoding components of other pro-survival pathways in ABC DLBCL, including PI3 kinase (PIK3CD, PIK3AP1), TLR9 signaling (MYD88, IRAK1, TRAF6), IL-10 receptor signaling (TYK2, STAT3), and BCL2. Finally, RAC2 knockdown decreased expression of genes encoding several master regulatory transcription factors that define the ABC DLBCL phenotype (IRF4, SPIB, BATF, TCF4) and B cell identity (PAX5, EBF1) as well as the MYC heterodimeric partner MAX and the NF-κB subunit p50 (NFKB1). These data show that RAC2 is a pleiotropic regulator of the epigenetic landscape of the IR pools, in keeping with its essential role in these cells.

To understand how RAC2 contributes to ibrutinib resistance in ABC DLBCL cells mechanistically, we defined a RAC2 “interactome” by ectopically expressing a fusion protein in which RAC2 was joined to a promiscuous biotin ligase, BioID2 [45], which biotinylates any protein within a ~10-30 nM radius. We used SILAC quantitative mass spectrometry of streptavidin-captured proteins to determine the enrichment of each protein identified in RAC2-BioID2-expressing cells compared to control cells expressing just BioID2 [5]. The majority of biotinylated proteins (n=368) were similarly enriched in lysates from parental lines and IR pools, demonstrating that the RAC2 interactome is an inherent feature of ABC DLBCL (Table S6A, S6B).

RAC2-proximal proteins were grouped into functional categories by STRING analysis [27], revealing associations with several core cytoplasmic processes [46] (Fig. 6A). The proximity of RAC2 to a large number of actin polymerization regulators fits with its known regulation of lamellipodia formation and membrane ruffling [47]. RAC2 was also associated with vesicular trafficking proteins, consistent with its role in endocytosis and phagocytosis [48].
In normal B cells, RAC2 is essential for the formation of an immune synapse, in which the BCRs cluster as they engage membrane-bound antigens and are ringed by the integrin LFA-1 bound to ICAM on the opposing membrane [44]. Accordingly, RAC2-BioD2 associated with two subunits of LFA-1 – CD11a (ITGAL) and CD18 (ITGB2) – as well as CD11d (ITGAD), which heterodimerizes with CD11a, and two integrin regulators, FERMT3 and CKAP4. RAC2 was also associated with RHOQ, which is required in B cells for optimal germinal center responses [49], and a number of GAP proteins and exchange factors for RHO family members. As expected, RAC2 also interacted with PAK1 and PAK2, thereby triggering these kinases to phosphorylate many targets, including ERK MAP kinase [50].

Strikingly, RAC2 interacted with multiple proteins involved in proximal BCR signaling (Fig. 6A). RAC2 associated with the BCR itself (CD79A, CD79B), Src-family kinases (LYN, FYN, LCK), and mediators of downstream BCR-dependent NF-κB activation (BLNK, BTK). RAC2 was strongly associated with PLCγ2, as previously reported [41, 42], but not with more distal components of the BCR pathway, except CARD11. RAC2 was also associated with other B cell-restricted membrane proteins, including CD20 (MS4A1), CD22, and CD72, each of which negatively regulates proximal BCR signaling [51-53].

To verify the proteomic association of RAC2 with proximal BCR signaling components, we developed proximity ligation assays (PLA) [54] to visualize the subcellular locations where RAC2 interacts with PLCγ2 and the BCR. In HBL1 and TMD8 cells, both RAC2/PLCγ2 PLA and RAC2/IgM PLA revealed bright foci of interaction in the cytoplasm and the plasma membrane. These RAC2 interaction foci were notably more numerous in IR pools than in the parental lines (Figs. 6B, S3A). These puncta were specific for RAC2 since they were quantitatively decreased by RNA interference-mediated knockdown of RAC2 (Fig. 6C). Moreover, the RAC2/PLCγ2 and RAC2/IgM PLA signals were corelated among the 6 IR pools, further supporting the specificity of these assays (Fig. 6C).

These results suggested that a RAC2/PLCγ2 PLA could be used to identify epigenetic resistance to BTK inhibitors in patient samples. To test this hypothesis, we turned to CLL, a disease more amenable than DLBCL to serial sample collection during their course of BTK inhibitor treatment. We assembled pre-treatment peripheral blood leukemic samples from CLL patients (n=4) as well as on-treatment samples from patients with persistent leukemia (n=4) or progressive disease with acquired sub-clonal mutations in BTK (n=2) or PLCG2 (n=2) (Table S6C). We performed a RAC2/PLCγ2 PLA of these CLL samples and enumerated the fluorescent puncta in a blinded fashion. After unblinding, we observed a significant, >2-fold increase in RAC2/PLCγ2 interaction in the persistent and progressive disease samples compared with pre-treatment samples (p=0.011) (Fig. 6D, Table S6C, D). In paired samples, the patient’s on-treatment samples had more numerous RAC2/PLCγ2 PLA puncta than patient’s pre-treatment leukemic cells (Fig. 6D). The increased RAC2/PLCγ2 interaction in ibrutinib-resistant CLL and ABC DLBCL cells suggests that epigenetic upregulation of RAC2 is a biomarker of ibrutinib resistance in diverse B cell malignancies.

**Targeting epigenetic resistance to BTK inhibitor treatment in ABC DLBCL**

To identify therapeutic approaches for BTK inhibitor-resistant B cell malignancies, we performed a high-throughput drug screen of 3 parental ABC lines and 8 independently derived
IR pools using a library of approved or investigational anti-cancer agents (n>2400) [18, 55]. As expected, IR pools were less sensitive than parental cells to the BTK inhibitors in this library (Fig. 7A, Tables S7A, B). The IR pools were also more sensitive to inhibitors of several regulatory pathways that sustain ABC DLBCL viability in addition to NF-κB (Fig. 1D). Specifically, the IR pools were hypersensitive to AKT inhibitors (AZD-26, ipatasertib, Akt inhibitor VIII) that block the PI3 kinase pathway, STAT3 inhibitors (niclosamide, cryptotanshinone) that block autocrine cytokine signaling through JAK1, and BCL2 family inhibitors (venetoclax, navitoclax, obatoclax), which promote apoptosis. Drugs in the immunomodulatory (Imid) class (pomalidomide, lenalidomide) were also more toxic for IR pools, in keeping with their ability to decrease expression of IRF4, a master regulatory transcription factor that controls ABC DLBCL viability [25]. IR pools were more sensitive to BET inhibitors (RVX-208, GSK-525768, I-BET762), which target the chromatin regulator BRD4, most likely because they have been shown to decrease IKK activation of NF-κB in ABC DLBCL cells [56]. The increased sensitivity of the IR pools to HDAC inhibitors (Mocetinostat, MC-1586) was expected given their ability to reverse epigenetic ibrutinib resistance (Fig. S1E). Finally, several glucocorticoids (e.g. dexamethosone, prednisolone) were more toxic for the IR pools by an unknown mechanism, although this observation is consistent with the known synergy between ibrutinib and these drugs in killing ABC DLBCL cells [18].

Notably, a small molecule inhibitor of RAC1 and RAC2 (NSC-23766) was among the most differentially toxic drugs in this screen (Fig. 7A), consistent with the greater sensitivity of IR pools to RAC2 knockdown (Fig. 4B, 5A). Subsequent experiments confirmed the added sensitivity of HBL1 IR pools to a similar RAC inhibitor, EHT1864, and demonstrated that the combination of ibrutinib and EHT1864 decreased the viability of HBL1 cells in a super-additive fashion (Fig. 7B). To demonstrate that this drug was hitting its target in ABC cells, we measured PLCγ2 enzymatic activity, which is augmented by RAC2 [41-43]. Ibrutinib decreased PLCγ2 activity in parental HBL1 cells as expected while EHT1864 had no effect in these cells (Fig. 7C). By contrast, EHT1864 decreased PLCγ2 activity in HBL1 IR pools as well as in an HBL1 clone with a PLCG2 mutation, indicating that RAC2 regulates PLCγ2 in cells with either genetic or epigenetic resistance to ibrutinib (Fig. 7C).

Finally, we evaluated whether drugs that can overcome ibrutinib resistance in vitro can also control in vivo growth of xenografts generated from IR pools. We established IR pools from the HBL1 and DLBCL2 lines as subcutaneous tumors in immunodeficient mice, and, as a control, we also engrafted the ibrutinib-sensitive parental lines. In the parental lines, the BCL2 inhibitor venetoclax was minimally effective as a single agent but was able to dampen tumor growth significantly in combination with ibrutinib (Fig. 7D). By contrast, venetoclax was active as a single agent in xenografts of the IR pools and the addition of ibrutinib did not substantially improve tumor control. Alone, the RAC inhibitor EHT1864 had little activity against parental ABC DLBCL xenograft growth but it substantially augmented the ability of ibrutinib to control growth of these tumors (Fig. 7E). By contrast, EHT1864 administration as a single agent significantly suppressed the growth of IR pool xenografts, with little added benefit from ibrutinib addition. Together, these experiments demonstrate that epigenetic ibrutinib resistance can be overcome by drugs with distinct mechanisms of action at concentrations that can be safely achieved in vivo.
Discussion

The advent of BTK inhibitors has transformed the care of patients with lymphoid malignancies, but resistance to monotherapy arises frequently, especially in DLBCL [4]. While BTK and PLCG2 mutations promote genetic resistance to ibrutinib in CLL [11, 13], MCL [19] and occasionally DLBCL [17], our analysis indicates that B cell malignancies can readily evade BTK inhibitors, most likely through an epigenetic mechanism. In multiple models of ABC DLBCL, continuous ibrutinib exposure induced a stable state of resistance driven, in part, by the action of the transcription factor TCF4. The profound changes in gene expression in ibrutinib-resistant ABC cells effectively “re-wired” the BCR-dependent NF-κB signaling mechanism to evade the blockade imposed by ibrutinib. Specifically, the small GTPase RAC2 substituted for BTK in the activation of PLCγ2 in ibrutinib-resistant cells, thereby sustaining NF-κB activity and cell viability. Likewise, in ibrutinib-resistant CLL cells from patients with persistent or progressive disease on BTK inhibitor treatment, the interaction of RAC2 and PLCγ2 was increased, suggesting that the epigenetic mechanisms we defined in ABC DLBCL models may also be relevant to other B cell neoplasias. Finally, we identified multiple drugs that can overcome epigenetic ibrutinib resistance, including small molecule inhibitors of RAC2 and BCL2 that were effective against ibrutinib-resistant ABC DLBCL models, both in vitro and in vivo.

The ease with which ABC DLBCL cells evaded BTK inhibition in vitro suggests that the same could be true in patients treated with BTK inhibitors. We hypothesize that epigenetic changes in the tumor phenotype may be the initial mechanism by which tumor cells adapt to treatment with BTK inhibitors, akin to the “persistent” cells that were the predominant subpopulation in the IR pools. These persistent cells may set the stage for the subsequent emergence of tumor subclones bearing BTK or PLCG2 mutations that genetically “hard wire” resistance. Though molecular data from ibrutinib-treated DLBCL patients are limited, one study reported a patient with two independent BTKC481S mutant alleles that were detected in circulating tumor DNA after a long latency on treatment [17]. Since these BTK mutations do not confer a selective advantage in the absence ibrutinib, these two mutant alleles may have arisen independently from a reservoir of epigenetically resistant cells that persisted while the patient was in clinical remission.

The development of resistance to BTK inhibitors has been studied in greater depth in CLL, and the available evidence also supports a role for epigenetic resistance. In one study of 61 CLL patients treated with ibrutinib, the mean reduction in absolute lymphocyte count after 6 months on therapy was 64.6%, meaning that a substantial population of malignant B cells persisted despite BTK inhibition [57]. Among patients with persistent or progressive CLL on ibrutinib, 15-43% lacked detectable BTK and PLCG2 mutations in their leukemic cells [11, 16, 58]. While there may be other genetic mechanisms of ibrutinib resistance in some cases [59], these studies suggest that epigenetic mechanisms may well contribute. Our analysis of 8 BTK inhibitor-treated patients with persistent or progressive CLL supports this view: the leukemic cells had greater RAC2/PLCγ2 association than pre-treatment samples, concordant with our observations in ABC cells with epigenetic ibrutinib resistance.

Our studies uncovered several targeted drugs that could overcome epigenetic resistance in ABC DLBCL, many of which are currently approved for use in lymphoid malignancies. The
BCL2 inhibitor venetoclax was more toxic for the IR pools than the parental ABC lines, in keeping with a greater dependence on BCL2 in the IR pools than in the parental lines. Accordingly, venetoclax was more effective against ibrutinib-resistant xenografts than ibrutinib-sensitive parental ABC xenografts. Lenalidomide and other Imid drugs were also more active against the ibrutinib-resistant pools, suggesting that co-administration of lenalidomide with ibrutinib could overcome ibrutinib-resistance. Consistent with this, the combination of ibrutinib, lenalidomide, and rituximab produced a high objective response rate (65%) and complete response rate (41%) in patients with relapsed/refractory non-GCB DLBCL [60].

Another drug class, the histone deacetylase inhibitors, were also more toxic for the ibrutinib-resistant pools than for the parental lines, in keeping with their ability to reverse the epigenetic alterations responsible for ibrutinib resistance, either alone or together with 5-azacytidine. These findings also fit with high throughput drug screens in which multiple histone deacetylase inhibitors synergized with ibrutinib in killing ABC DLBCL cells [18].

Our data nominate RAC2 as a promising new drug target for the treatment of ABC DLBCL tumors that rely upon chronic active BCR signaling. The small molecule RAC inhibitor EHT1864 had greater toxicity for ibrutinib-resistant ABC cells than for their ibrutinib-sensitive parents, in keeping with the heightened sensitivity of ibrutinib-resistant ABC cells to CRISPR-mediated inactivation of RAC2. EHT1864 had single agent efficacy against xenografts of ibrutinib-resistant ABC cells, and its combination with ibrutinib inhibited the growth of ibrutinib-sensitive parental ABC DLBCL xenografts in a super-additive fashion. RAC2 knockout mice are grossly normal without evident defects in major organ function, although they have impaired neutrophil chemotaxis due to defective regulation of the actin cytoskeleton [61]. RAC2-deficient B cells developed normally except for a significant defect in the generation of B-1 B cells and marginal zone B cells, a phenotype shared by mice deficient in other components of proximal BCR signaling [30]. Together, these data suggest that therapeutic targeting of RAC2 might be achievable with an acceptable safety profile.

Finally, our study has implications for the use of BTK inhibitors in combination with other agents for the treatment of lymphoid malignancies. Continuous dosing with a BTK inhibitor would likely generate epigenetic resistance, but in our ABC models, short term (1 week) ibrutinib treatment was insufficient to establish durable epigenetic resistance. Furthermore, continuous ibrutinib treatment can also have serious adverse effects, including susceptibility to invasive fungal diseases, most likely due to the necessary role of BTK in innate immune responses [9, 62, 63]. In the recent “Phoenix” phase III clinical trial, addition of ibrutinib to R-CHOP chemotherapy provided a significant survival benefit to younger patients (age<60) [64]. However, in older individuals, ibrutinib addition was associated with more serious side effects than R-CHOP alone. In this trial, ibrutinib was administered continuously, which our work suggests would foster epigenetic resistance. While ibrutinib monotherapy infrequently produces long-term remissions in DLBCL [4], ibrutinib synergizes with chemotherapy agents in killing ABC DLBCL cells, presumably by lowering the apoptotic threshold [18]. Therefore, it would be rational to consider an intermittent dosing schedule in which a BTK inhibitor is given only during the first week of the three-week R-CHOP chemotherapy cycle. Such intermittent dosing might allow the BTK inhibitor to still synergize with the chemotherapy agents in killing lymphoma cells while minimizing both the toxicity of ibrutinib and the development of epigenetic resistance.
Materials and Methods

Experimental design and statistics
The experiments presented have been repeated and results reproduced. Error bars and/or p-values are shown to indicate statistical significance. Statistical tests are documented in the text and/or the figure legends. Data presented without error bars are exemplar experiments, representing one of a series of independent biological replicates. See Supplemental Methods for details.

Genomic datasets
All genomic data sets can be found here:
SRA/NCBI BioProject
and here
https://lymphochip.nih.gov/local/Staudt_Ibrutinib_resistance_BCD/

Cell culture
Cell lines were obtained from the ATCC (www.atcc.org). Parental cell lines were passage for 10-30 generations before thawing a fresh vial (under 20 previous generations). Lines were tested for mycoplasma using the MycoAlert Mycoplasma Detection Kit (Lonza, LT07-218) and identity was confirmed by DNA fingerprinting/PCR which examines 16 regions of copy number variants (Jonathan Keats, personal communication).

GR50 calculation
The growth rate inhibition metric was calculated by growing cells in media with serial dilutions of ibrutinib (Selleckchem, S2680) from 0.001nM through 1000nM [65, 66].

Creation and maintenance of ibrutinb-resistant (IR) pools and ibrutinb-resistant (IR) single cell clones (SCC)
See manuscript text. Resistant pools were maintained by splitting (1:3) and re-feeding every 2-3 days with fresh media containing ibrutinib. Single cell clones were isolated by limiting dilution (1 cell / 3 wells in a 96 well plate) from resistant pools.

Drugs and basic molecular biology reagents
See Supplemental Methods

Sanger Sequencing of BTK/PLCG2 mutants
See Supplemental Methods

Creation of lyt2 and gfp-expressing vectors for BTK/PLCG2 alleles and other genes
See Supplemental Methods

Virus production and transduction
See Supplemental Methods
Culture of cells in outgrowth experiments of IR cells and cells expressing mutant alleles of resistance genes
See Supplemental Methods

Flow cytometry for gfp+ and lyt2+ outgrowth experiments
See Supplemental Methods

Flow cytometry for live cell counting (calcein/EtBr)
See Supplemental Methods

Exome-SEQ
See Supplemental Methods

RNA-SEQ gene expression profiling
See Supplemental Methods

Ibrutinib-response gene expression signature definition and gene expression signature enrichment
See Supplemental Methods

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, [67]), 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 #80204), with an additional 80% ethanol wash. Libraries for high-throughput sequencing were created using PCR primers specific for the barcode vector (pRSMX, [67]), using primeSTAR HS DNA polymerase (Takara, #R010B). See Supplemental Methods. Library DNA was purified and quantitated as in [5]. Paired-end 150 bp-read sequencing was performed on a Next-Seq 500 system using Illumina TruSeq V3 chemistry for identification and counting of barcodes (Table S2A). Barcodes were identified from the first 18bp of unique sequence after the MCS from the vector. Relative depletion or enrichment of each barcode was calculated, compared to the starting population.

Evolution of ibrutinib resistance- Expressed barcode (EBC) method
To capture single cell gene expression, 300 ABC DLBCL cells (HBL1 or TMD8) were transduced with the empty Perturb-Seq base vector library [23] and selected, such that each cell received one unique barcode. Cultures were expanded to ~20 million cells, starting samples were taken for DNA and RNA preparation (AllPrep, Qiagen) to establish clonal distribution, and cultures were split for subsequent treatment with DMSO, or ibrutinib as described above. Five million
cells were captured from cultures at week 5 for DNA analysis of barcodes, while simultaneously, from the same cultures ~6000 cells were captured for single cell analyses.

Single cell suspensions were loaded on the 10x Genomics Chromium platform at a concentration to capture ~5000 cells (10X Genomics, 1000127, 1000269, 1000215, 1000190), and single cell RNA-Seq libraries were generated using 3' Gene Expression v2 chemistry following the user guide. Full-length cDNA was reserved for targeted amplification to enrich for expressed barcodes within the cDNA libraries. Expressed barcodes were enriched by PCR amplification and directly indexed with a primer containing the P5 adaptor and partial TruSeq Read1 sequence (AATGATACGGACACCACCTCTACACGCTCTTCCCTACACGACGCTTCTCTTCTCTTGAGACTGTCAAGAGTCGTACGGCGACCACCCAGATCTACACTCTTTCCCTACACGACGCTCTCGCTTA- GTGACTGAGTTCAAGAGTCGTACGGCGACCACCCAGATCTACACTCTTTCCCTACACGACGCTCTC). Gene expression libraries and expressed barcode libraries were pooled and sequenced on a NextSeq 500, with a Read1 of 26 cycles, Index1 of 8 cycles, and Read2 of 98 cycles. Fastq files were demultiplexed using the Cell Ranger v3.0.1 pipeline (10x Genomics) with –barcode-mismatch=0 setting to avoid potential index collisions between expressed barcodes library indices and gene expression indices. Demultiplexed reads from the expressed barcode libraries were processed using Bowtie. After expressed barcodes were corrected for 1bp mismatches, the list of identified cell barcodes was filtered to cell barcodes that existed in the corresponding filtered gene expression matrix, which represents the profiled cells in the dataset. For each sample, the top observed expressed barcodes based on highest UMI count was determined to be that individual cell’s expressed barcode. Samples representing part of the same culture, but at different timepoints were combined and only expressed barcodes observed in at least ten cells in the entire dataset were retained for further analysis. Cells were assigned resistance phenotypes (Table S2B) as determined by both DNA and RNA analyses from the same populations. EBC gene expression was displayed as a t-SNE projection using the 10X Genomics Loupe Browser (V4.0). See Supplemental Methods for further details.

10X single cell RNA-Seq transcriptomics

Single cell suspensions were loaded on the 10x Genomics Chromium platform at a target capture number of ~5000 cells, and single cell RNA-Seq libraries generated using the 3' Gene Expression v2 chemistry following the user guide according to the manufacturer’s instructions. Sequencing of libraries was performed on an Illumina NOVA-Seq. FAST-Q files were processed through the 10X Cell Ranger program and visualized via the 10X Loupe program (V4.0).

STRING Analyses of gene sets

See Supplemental Methods

Fast ATAC-Seq to identify regions of open chromatin

See Supplemental Methods

ATAC-Seq Regions-of-open chromatin ‘peak’ calling, gene associations and motif analysis
Fastq files from the Illumina Sequencing run were mapped to the human genome (hg19, Bowtie). ‘Peaks’ of open chromatin were called as follows: Reads were aligned to hg19 by Bowtie2 with default parameters. The genome was divided into bins of 100bp, and for each experiment the number of bins that overlapped were counted. Peak regions were defined by bins which had more than 100 counts separated by consecutive bins having more than 50 counts in at least one experiment. Bin counts for each sample were normalized to match the HBL1 DMSO-treated parent line based on the total number of mapping reads derived from Bowtie2. All sample’s signal values were scaled to total HBL1 parental ATAC signal across the entire genome. The signal value for a peak in a sample was equal to the average normalized count for all bins in that peak’s region scaled as follows:

Peak count value = # of reads in the sample peak * (parental total counts/sample total).

This allowed direct, mathematical comparison of peak values across all ATAC-Seq samples. Bed/WIG files were generated and used to generate images in the Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway). Public access at: “hg19_IBR finale 061521”.

For DNAse hypersensitivity and ChIP-SEQ of TCF4 binding in HBL1, methods are detailed in [36, 56]. H3K27Ac tracks are publicly available [68].

To assign genes to regions of open chromatin, chromosomal coordinates of ATAC-Seq ‘peaks’ were uploaded to GREAT (http://bejerano.stanford.edu/great/public/html/), and the two most proximal genes (within -/+ 100kb) were identified and associated with that ‘peak’. Transcription factor motif enrichment was performed by inputting chromosomal ‘peak’ coordinates were into GALAXY (https://usegalaxy.org/) to capture .fasta files of genomic sequences, which were then loaded into RSAT (https://rsat01.biologie.ens.fr/rsa-tools/peak-motifs_form.cgi) for motif enrichment analysis using the Jaspar and ENCODE transcription factor databases. Enriched motifs for TCF4 could be mapped back to ATAC-seq regions from the RSAT output.

Chromosomal regions from TCF4 ChIP-Seq and ATAC-Seq with at least 50bp of overlap were identified by overlap analysis in GALAXY.

**shRNA and sgRNA sequences**
See Supplemental Methods

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

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

**Flow cytometry for intracellular proteins**
See Supplemental Methods

**Creation and expression of human RAC2-BioID2**
See Supplemental Methods

**Identification of the RAC2 interactome by mass spectrometry**
See Supplemental Methods

**CLL Patient samples.**
All patients provided written informed consent under an NIH, NHLBI IRB-approved protocol (04-H-0012) that allows collection of samples for research. Samples from patients treated with acalabrutinib (NCT02337829) were collected before treatment and on treatment while still having persistent lymphocytosis. Samples from patients on ibrutinib (NCT01500733) were collected at the time of clinical progression. Clinical data and outcome of therapy have been described previously (Ref: PMID: 32202637 and PMID: 9483101, respectively). PBMCs were isolated by density gradient centrifugation using Ficoll (Millipore Sigma, F4375). PBMCs were then washed twice, resuspended in FBS containing 10% DMSO and stored in a liquid nitrogen freezer.

**Proximity Ligation Assay for RAC2 interactions**
See Supplemental Methods

**High-throughput drug screening**
See Supplemental Methods

**PLCG activity assay**
The day prior to the assay 5 million cells in 8mls of media in T25 flasks (ThermoFisher, 169900), were prepared with vehicle or the drugs as indicated. The next morning, aliquots of 300ul of each culture were plated in duplicate in separate 96-well flat bottom plates (Corning, COSTAR3595). One plate was used to assess cell viability as described above using the Calcein/EtBr method. The second plate was used to make cell lysates to assess PLC activity by competitive ELISA (Cisbio72IP1PEA). Values for PLC activity were measured by absorbance on a TECAN infinite M200PRO plate reader, and were normalized by the live cell number in each culture.

**ABC DLBCL xenografts- RAC and BCL2 inhibitors with/ without Ibrutinib**
All animal studies were performed in compliance with NIH and NCI guidelines and regulations, as monitored by the Office of Animal Care and Use (https://oacu.oir.nih.gov): NIHProtocol # METB054. See Supplemental Methods for details.

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Author Contributions

ALSIII: Conceptualization of the study, Methodology design, development, Software programming/development/execution, Validation/verification of results and analysis, Formal analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization, Resources

JDP: Conceptualization of the study, Methodology design, development, Validation/verification of results and analysis, Formal analysis, Investigation, Data Curation, Writing – Review & Editing, Visualization, Resources

JQW: Conceptualization of the study, Methodology design, development, Validation/verification of results and analysis, Formal analysis, Investigation

MK: Conceptualization of the study, Methodology design, development, Validation/verification of results and analysis, Formal analysis, Investigation

DWH: Conceptualization of the study, Methodology design, development, Software programming/development/execution, Validation/verification of results and analysis, Formal analysis, Investigation, Data Curation

GW: Methodology design, development, Software programming/development/execution, Validation/verification of results and analysis, Formal analysis, Data Curation

JC: Conceptualization of the study, Methodology design, development, Validation/verification of results and analysis, Formal analysis, Investigation

RMY: Conceptualization of the study, Methodology design, development, Writing – Review & Editing, Resources

DHW: Methodology design, development, Software programming/development/execution, Validation/verification of results and analysis, Formal analysis, Data Curation

YY: Methodology design, development, Validation/verification of results and analysis, Investigation, Data Curation, Resources

HZ: Methodology design, development, Investigation, Resources

XY: Methodology design, development, Validation, Investigation, Resources

WX: Methodology design, development, Resources

SR: Methodology design, development, Validation/verification of results and analysis, Investigation

MC: Conceptualization of the study, Methodology design, development, Validation/verification of results and analysis, Formal analysis, Investigation, Data Curation, Resources

XZ: Methodology design, development, Validation/verification of results and analysis

KW: Methodology design, development, Validation/verification of results and analysis

LC: Methodology design, development, Validation/verification of results and analysis

CM: Methodology design, development, Validation/verification of results and analysis

CK-T: Methodology design, development, Validation/verification of results and analysis

CT: Conceptualization of the study, Methodology design, development, Validation/verification of results and analysis, Supervision Oversight, Project Administration

BH: Methodology design, development, Software programming/development/execution, Validation/verification of results and analysis, Formal analysis, Data Curation

TO: Conceptualization of the study, Methodology design, development, Validation/verification of results and analysis, Supervision Oversight, Project Administration
ZR: Methodology design, development, Validation/verification of results and analysis, Investigation, Data Curation
MCK: Supervision Oversight, Project Administration, Conceptualization of the study, Methodology design, development, Software programming/development/execution, Validation/verification of results and analysis, Formal analysis, Investigation, Data Curation, Resources
IEA: Conceptualization of the study, Methodology design, development, Investigation, Data Curation, Resources
CS: Conceptualization of the study, Methodology design, development, Investigation, Data Curation, Resources
EMG: Conceptualization of the study, Methodology design, development, Investigation, Data Curation, Resources
WW: Supervision Oversight, Project Administration, Writing – Review & Editing
AW: Supervision Oversight, Project Administration, Resources, Conceptualization of the study, Investigation, Resources
LMS: Supervision Oversight, Project Administration, Funding Acquisition, Conceptualization of the study, Methodology design, development, Formal analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization, Resources
References


FIGURE LEGENDS

Figure 1. Epigenetic Ibrutinib Resistance in ABC DLBCL lines. A. Schematic of the creation of ibrutinib-resistant (IR) pools (pink) and selection of ibrutinib-resistant (IR) single cell clones (blue) from ibrutinib-sensitive ABC DLBCL parental cell lines. B. Ibrutinib-sensitive ABC DLBCL cells, or IR pools and clones, maintained in drug or grown without drug for 1 week were marked by transduction with a GFP-expressing retrovirus (GFP+), mixed with drug-sensitive (GFP-) parental cells, and challenged with 10nM ibrutinib or vehicle (DMSO). Competitive outgrowth was measured by using the log$_2$ ratio of the percent of GFP+ IR cells in ibrutinib vs. DMSO is plotted over 12 days in culture. C. Parental HBL1 cells, an IR clone with a PLCg2 resistance mutation (R665W), and 3 independent IR pools were grown in the absence of ibrutinib for the indicated days, in duplicate wells, challenged with 10nM ibrutinib or DMSO for 4 days, and live cells were counted by flow cytometry (calcein+, EtBr-). Cell numbers are normalized to no drug (DMSO) controls (error bars = s.e.m.). D. Log$_2$ of the average expression of a signature of genes down-regulated by acute ibrutinib treatment (log$_2$<0.4, 24hrs, Table S1C), plotted for 2 ABC DLBCL cell lines as well for IR clones and IR pools of those lines, normalized to ibrutinib-sensitive parent cells treated with vehicle (DMSO). Significance was determined by ANOVA testing. E. Live cells were counted by flow cytometry from cultures of 2 ABC DLBCL cell lines, as well as IR clones and IR pools of those lines, from duplicate wells, with and without serial treatment with ‘epigenetic’ drugs 5-azacytidine (250nM, 5 days, AZAC) followed by HDAC inhibitor Givinostat (50nM, 5 days, HDACi). Cell numbers are normalized to DMSO-treated vehicle, controls (error bars = s.e.m.). See also FigS1, TableS1.

Figure 2. Tracking the evolution of ibrutinib resistance phenotypes
Creating single, barcoded cells to track the evolution of ibrutinib resistance. B. One million HBL1 ABC DLBCL cells were transduced (MOI of 1:3) such that each cell received one unique barcode. Cells were expanded, and a sample of cells was taken to establish the clonal (barcode) distribution of cells 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 vehicle alone. One million cells were harvested at week1 and week5. DNA from each sample was subjected to high-throughput sequencing for identification and counting of barcodes (Table S2A). Relative depletion or enrichment of each barcode (clone) was calculated, compared to the starting population, and binned with the number of barcodes/bin plotted versus the relative change in clonal frequency (ibrutinib/DMSO). The designated resistance phenotype (sensitive, persistent, resistant) is depicted above the graph. Error bars represent s.d. of counts in each bin for the duplicate samples. C. A similar barcode experiment was carried out on two independently-derived, expanded populations of 300 HBL1 cells (A and B), each with unique expressed barcodes (EBC) that could be detected by both next-generation DNA sequencing (NGS) and single-cell RNA-sequencing (scRNA-Seq) at week 5 following DMSO or ibrutinib (10nM) treatment versus initial starting populations. Clones with correlated barcodes, DNA vs. RNA clonal frequency, were designated as persistent or resistant phenotypes as shown. D. scRNA-Seq t-SNE plots of the cells with barcodes from C. under control (DMSO) or ibrutinib (10nM) challenge conditions: persistent cells, blue, resistant cells, red, sensitive cells, green. E. Summary of gene expression signature enrichment (Table S2B,C,D) from scRNA-Seq genes up in both persistent and resistant cells selected with ibrutinib (gray) versus DMSO treated samples; signatures of genes higher in persistent cells than resistant cells selected in ibrutinib (pink) and vice versa (purple). Significance of the enrichment is shown with a description of the signature. F. scRNA-seq tSNE plots of parental ABC DLBCL lines and 3 independently derived IR pools of each line (Table S2E). G. Overlaps in genes upregulated in IR cells. The upper panel depicts the genes commonly upregulated in scRNA-seq of IR pools vs. parental lines (Table S2E, S2F). The lower panel depicts the overlap in genes upregulated in >= 2 of 3 cell line IR pools (F.) versus genes upregulated in persistent and resistant cells in ibrutinib versus DMSO samples from the EBC experiment (D.). H. The functionally annotated STRING depiction of the intersection genes from the lower part of G. showing genes upregulated in >= 2 of 3 cell line IR pools from scRNA-Seq and genes higher in persisters and resisters in ibrutinib vs. DMSO from the EBC experiment. Unlinked proteins and lincRNAs were removed from this depiction. See also FigS2, TableS2.
Figure 3. Epigenetic retuning of oncogenic signaling in ibrutinib resistance
Schematic of samples, and subsequent treatments, upon which ATAC-Seq was performed in the HBL1 ABC DLBCL line to identify regions of open chromatin. Normalized (to the parental HBL1 control) ATAC-Seq peak counts near important ABC DLBCL genes are shown for both IR pools (pink, average of 3 independent pools) and an IR mutant (PLCG2 R665W) clone (blue), normalized to the open chromatin of the parental HBL1 line, with the peak start site mapped to the human genome (hg19) and the extent of the open region indicted (kB) (Table S3B). B. Differences in regions of open chromatin near critical ABC DLBCL signaling and survival pathway genes. Chromatin regions more accessible on average in IR pools than in parental HBL1 cells or the PLCG2 resistant mutant (PLCG2 R665W) are indicated (yellow, left side, log₂ difference >=0.3) with a decrease in accessibility of the same region in IR pools when treated with AZAC/HDACi (log₂ change <=-0.3, Table S3B) indicated in blue (right side) C. Percent of regions of open chromatin from the HBL1 parent line or IR pools that have at least one binding motif for the indicated factor(s) based on RSAT analysis (top ENCODE motifs e-value <0.01) (Table S3C). D. ATAC-Seq regions of open chromatin near selected genes that are more accessible on average in IR pools (pink) than the parental line (gray) or genetic resistant mutant (dark blue). Also shown: open chromatin determined by DNase hypersensitivity (black bar, denoting peak region), along with TCF4 transcription factor binding in the parental line (aqua, ChIP-Seq), H3K27Ac histone ChIP-Seq from HBL1 [68], and fold increase in peak size comparing the average peak signal from IR pools to the parent line (Table S3B). E. Measuring the viability of parental ABC DLBCL cells (HBL1 and TMD8) or IR pools, as assessed by flow cytometry, after transduction with retroviruses bearing doxycycline-inducible shRNAs, specific for TCF4 or a control transcript, and a co-expressed GFP marker, over a time course of induction. These represent biological repeats on the parental lines and independently derived IR pools performed at different times. Error bars represent s.d. from the mean. See also TableS3.

Figure 4. Altered dependencies in ibrutinib resistant ABC DLBCL
Plot of the average difference in CRISPR screen score ((log₂ ΔCSS), Table S4A) between IR pools and parental lines from CRISPR drop-out screens for all genes in the ‘Brunello’ sgRNA library. Negative ΔCSS value = relative depletion vs. parent (more toxicity with gene loss); positive (ΔCSS) value = relative enrichment vs. parent (less toxicity with gene loss). Genes involved in ABC DLBCL pathobiology are shaded red. B. Summary of CRISPR ‘drop-out’ screens using the ‘Brunello’ library mapped on to essential pathways controlling ABC DLBCL survival, comparing ibrutinib sensitive parental lines to IR pools. Average CRISPR screen score (CSS, log₂ scale) was calculated for each gene targeted by guide RNAs in the ‘Brunello’ library (Table S4A) and compared between populations. Blue/yellow shading, left side, relative depletion or outgrowth of cells with sgRNA targeting that gene in IR pools. Purple/orange shading, right side, relative essentiality of a gene in the IR pools as compared to the ibrutinib-sensitive parent cell line. See also TableS4.

Figure 5. RAC2 as a mediator of epigenetic ibrutinib resistance
Viability of ABC DLBCL parent lines (HBL1, TMD8) and IR pools after the knockdown of RAC2, as assessed by flow cytometry of cells transduced with retroviruses bearing doxycycline-inducible shRNAs (CTRL transcript or RAC2-targeting) that also constitutively express a GFP marker compared to un-transduced cells in the same cultures. These represent triplicate biological repeats on the parental lines and independently derived IR pools performed at different times. Error bars represent s.d. from the mean. B. RAC2 mRNA expression, as determined by scRNA-Seq (Fig. 2F) for 3 independently derived IR pools of each ABC DLBCL line, plotted as the percent of cells in each population having higher RAC2 expression than the parental line average. C. RAC2 protein expression (MFI) as measured by intracellular flow cytometry in parent lines and 3 independent IR pools, normalized to the parental line RAC2 mean fluorescent index (MFI). D. RAC2 protein expression (MFI) as measured by intracellular flow cytometry in HBL1 parent and IR pools treated with 5-AZAC plus HDACi for 4 days. MFI is normalized to the DMSO (control) treated parental line. E. The expression of critical ABC DLBCL genes is shown from TMD8 parent cells and an IR pool expressing a doxycycline-inducible shRNA targeting RAC2 compared to cells expressing a control shRNA. Note, shRNA induction is partially leaky resulting in a decrease in RAC2 mRNA at day0. F. Signature enrichment within the set of genes down-regulated upon shRNA knockdown of RAC2 in an IR pool (Table S5A, B). See also TableS5.
Figure 6. RAC2 protein interactions are a marker of epigenetic ibrutinib resistance
A. Enrichment of RAC2-interacting proteins in ABC DLBCL cells after RAC2-bioID2 expression followed by quantitative mass spectrometry of streptavidin captured proteins as analyzed by STRING (Tables S6A, B). B. Proximity ligation assay (PLA, red puncta) for interaction between RAC2 and PLCG2 (upper panels) or RAC2 and IgM (lower panels) in HBL1 parental cells and an IR pool. Nuclei are stained blue (DAPI) and cell membranes are green (wheat germ agglutinin Alexa488). C. Quantitation of PLA puncta (RAC2/PLCG2 and RAC2/IgM) in IR pools (pink box), normalized to parent lines. Puncta values from HBL1 bearing an shRNA against RAC2 is also shown as a PLA specificity control. D. Quantitation of RAC2/PLCG2 puncta per IgM+ cell for each CLL patient category (n=4/category, from patients pre-treatment, during treatment with acalabrutinib while still manifesting lymphocytosis (persistent disease), or on ibrutinib at the time of progressive disease) and for matched pre/on-treatment samples from the same patients, with representative images from these CLL patient samples (showing 1 of 4 cases in each category): RAC2/PLCG2 PLA in red, nuclei in blue. Error bars represent s.e.m., p-values = Student’s T-test (Table S6C,D). See also FigS3, TableS6.

Figure 7. Targeting epigenetic resistance to BTK inhibitor treatment in ABC DLBCL
Summary of the log2 maximum drug response (MAXRESP, dose (μM) at which a maximal inhibitory response is achieved, Table S7A, B) differences between IR pools and parental ABC DLBCL lines for several classes of drugs with the number of drugs in each class also shown. Positive numbers indicate resistance in IR pools; negative numbers indicate increased sensitivity in IR pools. Values were averaged across parents and IR pools for individual drugs and further averaged across drugs classes that were represented by more than one drug. Error bars – s.e.m. B. Ibrutinib-sensitive HBL parent and IR pools cells were treated with DMSO, RAC inhibitor EHT1864 (μM) and/or ibrutinib (nM) for 4 days and live cells (calcein+, EtBr-) were enumerated by flow cytometry from duplicate wells. Error bars – s.d. C. Ibrutinib-sensitive HBL parent, the PLCG2 R665W genetic resistance mutant and independent IR pools cells were treated with DMSO, RAC inhibitor EHT1864 (8.5μM) or ibrutinib (10nM) overnight and PLCG activity was assessed by ELISA in duplicate wells. Each cell type was normalized to its own untreated DMSO control. Error bars – s.d. D. Monitoring the growth of xenografts, by tumor volume, of ABC DLBCL parent lines (HBL1, DLBCL2) or derived IR pools in NOD/SCID mice (n=5/treatment) treated with vehicle (DMSO), the BCL2 inhibitor venetoclax, or both drugs combination. E. Monitoring the growth of xenografts, by tumor volume, of ABC DLBCL parent lines (HBL1, DLBCL2) or derived IR pools in NOD/SCID mice (n=5/treatment) treated with vehicle (DMSO), the BTK inhibitor ibrutinib, the RAC inhibitor EHT1864, or both drugs in combination. See also TableS7.
SUPPLEMENTAL INFORMATION

Three Supplemental Figures with legends, seven Supplemental Tables with legends, keyed to Figures in the main manuscript, and Supplemental Methods with Supplemental Primer Tables can be downloaded separately.
Figure 1. Epigenetic Ibrutinib Resistance in ABC DLBCL lines

A

B

C

D

E

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Figure 2. Tracking the evolution of ibrutinib resistance phenotypes

A) Single cell cloned, ibrutinib-sensitive parental ABC line.
   - Time, sample
   - Expands
   - Barcoded lentivirus (1 barcode/cell)
   - ibrutinib

B) Duration of treatment (weeks):
   - Depleted
   - Persistent
   - Resistant

C) DNA barcode count log2(ibrutinib/time)

D) Comparing DMSO and ibrutinib treatments:
   - Expressed barcode type:
     - ibrutinib sensitive
     - ibrutinib persistent
     - ibrutinib resistant

E) Gene expression changes:
   - Signature
   - Enrichment p-value
   - Phenoype

F) Gene expression in TMD8, HBL1, and DLBCL IR pools.

G) Upregulated genes in IR pools:
   - TMD8
   - DLBCL
   - HBL1

H) Protein translation and presentation.

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Figure 3. Epigenetic retuning of oncogenic signaling in ibritinib resistance

A

B

C

D

E

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Figure 4. Altered dependencies in ibrutinib resistant ABC DLBCL

A

Figure 4A: 
- Gene expression data showing Log2 Average ΔCSS (IR pools - parent cell lines) for various genes.
- Key genes include VAV1, CD79A, IRAK1, MAP3K7, IKBK, AKT2, IRF4, RELA, SYK, CDK6, NFkB, NFkB, MYD88, TRD4, TYK2, MYC, IRAK1, UNC93B1, HSP90B1, PLCG2, BLNK, TLR9, PRKCB, MALT1, LYN, TNIP1, BTK, BCL10, CARD11, NFkBE PTPN2, PTPN6.

B

Figure 4B: 
- Network diagram illustrating the interactions between various pathways:
  - BCR proximal
  - PI3K pathway
  - NF-κB pathway
  - TLR pathway
  - JAKSTAT pathway
- Outgrowth of knockouts in IR pools indicated by color gradient.
- Gene expression analysis showing less essential genes in IR pools than parent.
Figure 6. RAC2 protein interactions are a marker of epigenetic ibrutinib resistance

A

B

C

D

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Figure 7. Targeting epigenetic resistance to BTK inhibitor treatment in ABC DLBCL
Overcoming Acquired Epigenetic Resistance to BTK Inhibitors


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