Overcoming Acquired Epigenetic Resistance to BTK Inhibitors

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The use of Bruton tyrosine kinase (BTK) inhibitors to block B-cell receptor (BCR)-dependent NF-κB 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 activated B-cell (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 Cy2, thereby sustaining NF-κB activity. The interaction of RAC2 with phospholipase Cy2 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.

**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.

*See related commentary by Pasqualucci, p. 555.*

**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 activated B-cell (ABC) subtype of diffuse large B-cell lymphoma (DLBCL; ref. 1). ABC DLBCL cells have a “chronic active” form of BCR signaling that constitutively triggers a signaling cascade that activates the prosurvival 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 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 Cy2 (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 and 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 ref. 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 patients with ABC DLBCL. Nonetheless, median progression-free survival in ABC DLBCL was very short (2 months), indicating that BTK monotherapy failed to eradicate 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, whereas the response rate in the remaining ABC cases was only 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 BCR, TLR9, and MYD88L265P along with many other proteins involved in NF-κB activation, including CARD11, BCL10, MALT1, and 1κB kinase (IKK; ref. 5). ABC DLBCLs that form an 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 preclinical studies of ibrutinib in MCD cell line models (1), ibrutinib monotherapy produced a 77% to 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. Although initial response rates are high, resistance to ibrutinib monotherapy is an increasing problem (10–13). The best-characterized form of resistance in ABC DLBCL cells is associated with BTK mutations only became readily detectable after 5 to 19 months, suggesting that they may have arisen from a pool of cells with ibrutinib resistance that 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, 20; Supplementary Fig. S1A). The BTKR665W mutation, which prevents ibrutinib binding, was detected in multiple independent TMD8 IR clones. Two independent U2932 IR clones acquired a BTKT471I mutation, which targets an active site threonine that stabilizes ibrutinib binding. Several IR clones of HBL1 and U2932 had PLCG2R665W or PLCG2T471R 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 (Fig. 1B; Supplementary Fig. S1B and S1C). None of these mutations were observed at >10% VAF in the IR pools after 6 weeks of culture, but after more than 1 year in culture, the BTKR665W mutation was detected in one TMD8 IR pool whereas the HBL1 IR pools remained devoid of BTK and PLCG2 mutations. Whole-exome sequencing of more than 20 independently derived IR pools provided no evidence of mutations that could account for ibrutinib resistance (Supplementary Table S1A and S1B). Together, these findings suggested that nongenetic mechanisms could account for the rapid acquisition of ibrutinib resistance by ABC lines.

To investigate the stability of ibrutinib resistance, we cultured HBL1 and TMD8 IR pools in the absence of ibrutinib for 1 week. Upon rechallenge, each IR pool maintained its resistance, as did IR clones with ibrutinib resistance mutations (Fig. 1B). Remarkably, the stability of the IR 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 (10 nmol/L) of ibrutinib for 1 or 8 weeks, cultured for 7 days without ibrutinib, and then rechallenged with ibrutinib (10 nmol/L). The 8-week cultures generated a stable resistance phenotype, whereas the 1-week cultures did not (Supplementary 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 (10 nmol/L, 24 hours) and identified genes that decreased in expression (log2 fold change < −0.4) relative to pretreatment levels (Supplementary Table S1C and S1D). 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 (Supplementary Table S1E; ref. 21). The IR clones with 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; refs. 1, 19). 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 1 week (Fig. 1A). The resulting cultures were maintained in ibrutinib (10 nmol/L) 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.

RESULTS
Epigenetic Ibrutinib Resistance in ABC DLBCL Lines
To create models of IR ABC DLBCL, we used HBL1, TMD8, DLBCL2, and U2932 cell lines, which recapitulate essential
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BTK and PLCG2 mutations expressed the ibrutinib-response signature at levels comparable with those in the untreated parental line (Fig. 1D; Supplementary Fig. S1E) as expected. By contrast, IR pools (Fig. 1D) and IR clones lacking resistance mutations (Supplementary Fig. S1E; Supplementary Table S1D and S1F) 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 (22, 23). Although 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 reexpressed the IR pools to ibrutinib (Supplementary Fig. S1F). These data suggest that the rapid development of ibrutinib resistance in ABC models is generated by nongenetic 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

**Figure 1.** Epigenetic ibrutinib resistance in ABC DLBCL lines. A, Schematic of the creation of ibrutinib-resistant (IR) pools (pink) and selection of 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-). (Error bars, SEM.) See also Supplementary Fig. S1 and Supplementary Table S1.
with lentiviruses bearing unique 60-base-pair (bp) 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 5 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 5 weeks of ibrutinib treatment and were deemed sensitive. Of the remaining barcoded cells, ∼5% were classified
as resistant because their prevalence increased >10-fold over and consequently constituted ~30% of all cells in the ibrutinib-treated cultures at week 5. Barcoded cells that changed little in frequency during ibrutinib treatment were designated persistent. Similar results were observed using the TMD8 line (Fig. 2B; Supplementary Fig. S2A; Supplementary 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-seq (scRNA-seq) as described (19). Because scRNA-seq interrogates only 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-distributed Stochastic Neighbor Embedding (t-SNE) plots of gene expression in each cell in the ibrutinib and DMSO cultures (Fig. 2D; Supplementary Fig. 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 preexist in the starting population.

Although 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 (Fig. 2D; Supplementary Fig. 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; Supplementary Table S2B and S2C) and used a database of gene signatures relevant to normal and malignant B-cell biology (21) to identify enriched signatures (Supplementary Table S2D; P < 0.01, Fisher 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 (refs. 24, 25; Fig. 2E; Supplementary Table S2C and S2D). 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 (refs. 21, 26; Fig. 2E; Supplementary 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 (Supplementary Fig. S2C; Supplementary Table S2E).

In separate experiments, we used scRNA-seq to identify convergent gene-expression programs in IR cells generated from three ABC cell lines (HBL1, TMD8, and DLBCL2; Fig. 2F). Among genes upregulated in the IR pools, 195 were identified in at least 2 of the ABC lines (Fig. 2G; Supplementary Table S2F and S2G). Of these, 52 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 subunit 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 assay for transposable-accessible chromatin sequencing (ATAC-seq; ref. 31), comparing the parental line HBL1, an HBL1 IR clone with PLCγ2R665W, 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 PLCγ2R665W IR clone and parental cells (≥0.3 log2 increase in read count), and 1,215 that were less accessible following 5-azacytidine plus givinostat treatment (≥0.3 log2 decrease in read count; Supplementary Table S3A and S3B).

Using the GREAT (Genomic Regions Enrichment of Annotations Tool) 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 antiapoptotic BCL2 family members (Fig. 3A; Supplementary 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, and PLCγ2) and the downstream NF-κB activation pathway (PKCβ2, CARD11, BCL10, MAL1, and REL; Fig. 3B; Supplementary 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; Supplementary Table S3C; ref. 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-SP1B heterodimer, which binds ETS/IRF sites (e-value = 1.1E–14; ref. 25) and Oct-2 (POU2F2), which binds octamer sites (e-value = 2.2E–9; ref. 34). Although ATAC-seq regions in the parental and IR pools were equivalently enriched in these two binding
Figure 3. Epigenetic retuning of oncogenic signaling in ibrutinib resistance. A, 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 three independent pools) and an IR mutant (PLCG2 R665W) clone (blue), with the peak start site mapped to the human genome (hg19) and the extent of the open region indicated (kb; Supplementary Table S3B). AZAC/HDACi, 5-azacytidine/HDAC inhibitor; UTR, untranslated region.

B, ATAC-seq regions of open chromatin near selected genes that are more accessible on average in IR pools (pink) than the parental line (black) 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 (Supplementary Table S3B). C, 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 short hairpin RNAs (shRNA), specific for TCF4 or a control transcript, and a coexpressed 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, SD from the mean. See also Supplementary Table S3.
motifs, those in the IR pools were 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 IR ABC DLBCL cells.

We performed TCF4 chromatin immunoprecipitation sequencing (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 (Supplementary Table S3D). Figure 3D displays TCF4-bound ATAC-seq peaks in the RAC2, TLR9, and BCL2 loci, which coincided with DNA-seq 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 either the parental line or the PLCγ2ΔIR clone. Although 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 toward greater dependency on TCF4 and its targets.

Altered Dependencies in IR ABC DLBCL

To uncover potential new therapeutic vulnerabilities in IR cells, we performed genome-wide CRISPR-Cas9 screens for essential genes in two parental ABC cell lines (HBL1, n = 4; TMD8, n = 6) and five independently derived IR pools (HBL1 IR pools, n = 2; TMD8 IR pools, n = 3; ref. 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 (sgRNA) targeting that gene compared with other sgRNAs in the library. Of the targeted genes, 3,398 scored as essential in the IR pools (average log₂ CSS < −0.5), whereas 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%; Supplementary Table S4).

We identified 701 genes that were more essential in the IR pools than in their parents (average ΔCSS < −0.5) and 1,000 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; Supplementary Table S4). Figure 4B arranges these proteins into regulatory pathways, with the left half of each protein icon depicting its essentiality in the IR pools (CSS) 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 IκBζ (NFKBIZ), a modulator of transactivation by NF-κB. Conversely, inactivation of negative regulators of BCR signaling (LYN, PTEN) 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 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. Although 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 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 multi-protein “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 endosomes (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, knockout of RAC2 was significantly more toxic in the IR pools than in parental

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Figure 4. Altered dependencies in IR ABC DLBCL. A, Plot of the average difference in CRISPR screen score ([log2 ΔCSS]; Supplementary Table S4A) between IR pools and parental lines from CRISPR dropout screens for all genes in the “Brunello” sgRNA library. Negative ΔCSS value = relative depletion versus parent (more toxicity with gene loss); positive ΔCSS value = relative enrichment versus parent (less toxicity with gene loss). Genes involved in ABC DLBCL pathobiology are shaded red. B, Summary of CRISPR “dropout” screens using the “Brunello” library mapped on to essential pathways controlling ABC DLBCL survival, comparing ibrutinib-sensitive parental lines to IR pools. Average CSS (log2 scale) was calculated for each gene targeted by guide RNAs in the “Brunello” library (Supplementary 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 with the ibrutinib-sensitive parent cell line. See also Supplementary Table S4.
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Figure 5. RAC2 as a mediator of epigenetic ibrutinib resistance. A, Viability of ABC DLBCL parent lines (HBL1 and TMD8) and IR pools after the knockdown of RAC2 as assessed by flow cytometry of cells transduced with retroviruses bearing doxycycline-inducible short hairpin RNAs (shRNA; control transcript or RAC2 targeting) that also constitutively express a GFP marker compared with untransduced 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, SD from the mean.

B, RAC2 mRNA expression as determined by scRNA-seq following knockdown of RAC2 in parental TMD8 cells (Fig. 5A) for three independently derived IR pools of each ABC DLBCL line, plotted as the percentage of cells in each population having higher RAC2 expression than the parental line average. C, RAC2 protein expression (mean fluorescent index, MFI) as measured by intracellular flow cytometry in parent lines and three independent IR pools, normalized to the parental line RAC2 MFI. D, RAC2 protein expression (MFI) as measured by intracellular flow cytometry in HBL1 parent and IR pools treated with 5-azacytidine plus HDAC inhibitor (AZAC/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 (Supplementary Table S5A and S5B).

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 and F; Supplementary Table S5A and S5B). In the IR pool but not the parental line, RAC2 knockdown significantly decreased expression of ABC DLBCL identity (ABC DLBCL-1); B-cell–specific genes repressed by Blimp-1 (Blimp-1); and genes induced by NF-kB (NFkB-2), STAT3 (STAT3Up-1), and IRF4 (IRF4Up-9; Fig. 5F; Supplementary Table S5B). These signatures included genes encoding multiple proximal BCR signaling components including CD79B, mediators of BCR-dependent NF-kB signaling (BLNK, BTK, PLCγ2), and VAV1, which links proximal BCR signaling to RAC2 activation (Fig. 5E and F). Also downregulated were genes encoding components of other prosurvival pathways in ABC DLBCL, including PI3 kinase (PIK3CD, PIK3AP1), TLR signaling (MYD88, IRAK1, TRAF6), IL10 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, and TCF4) and B-cell identity (PAX5 and EBF1) as well as the MYC heterodimeric partner MAX and the NF-kB 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 “interaction” 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 to 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 with control cells expressing just BioID2 (S). 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 (Supplementary Table S6A and S6B).

RAC2-proximal proteins were grouped into functional categories by STRING analysis (27), revealing associations with several core cytoplasmic processes (ref. 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 (ITGAD)—as well as CD11d (ITGAD), which heterodimerizes with CD11a, and two integrin regulators, FERM3 and CKA4. 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 PKA1 and PKA2, 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 and CD79B), Src family kinases (LYN, FYN, LCK), and mediators of downstream BCR-dependent NF-κB activation (BLNK and 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).

Figure 6. RAC2 protein interactions are a marker of epigenetic ibrutinib resistance. A, Enrichment of RAC2-interacting proteins in ABC DLBCL cells after RAC2-BioD2 expression followed by quantitative mass spectrometry of streptavidin-captured proteins as analyzed by STRING (Supplementary Table S6A and S6B). B, PLA (red puncta) for interaction between RAC2 and PLCG2 (top) or RAC2 and IgM (bottom) 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 a short hairpin RNA against RAC2 are 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 pretreatment, during treatment with acalabrutinib although 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 one of four cases in each category). RAC2/PLCG2 PLA in red; nuclei in blue. Error bars, SEM; P values = Student t test (Supplementary Table S6C and S6D). See also Supplementary Fig. 5S and Supplementary Table S6.
To verify the proteomic association of RAC2 with proximal BCR signaling components, we developed proximity ligation assays (PLA; ref. 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 (Fig. 6B; Supplementary Fig. S3A). These puncta were specific for RAC2 because they were quantitatively decreased by RNA interference–mediated knockdown of RAC2 (Fig. 6C). Moreover, the RAC2/PLCγ2 and RAC2/IGM PLA signals were correlated among the six 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 the course of BTK inhibitor treatment. We assembled pretreatment peripheral blood leukemic samples from patients with CLL (n = 4) as well as on-treatment samples from patients with persistent leukemia (n = 4) or progressive disease with acquired subclonal mutations in BTK (n = 2) or PLCG2 (n = 2; Supplementary 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 pretreatment samples (P = 0.011; Fig. 6D; Supplementary Table S6C and S6D). In paired samples, the on-treatment samples had more numerous RAC2/PLCγ2 PLA puncta than pretreatment leukemic cells (Fig. 6D). The increased RAC2/PLCγ2 interaction in IR 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 three parental ABC lines and eight independently derived IR pools using a library of approved or investigational anticancer agents (n > 2,400; refs. 18, 55). As expected, IR pools were less sensitive than parental cells to the BTK inhibitors in this library (Fig. 7A; Supplementary Table S7A and S7B). 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, and Akt inhibitor VIII) that block the PI3 kinase pathway, STAT3 inhibitors (niclosamide and cryptotanshinone) that block autocrine cytokine signaling through JAK1, and BCL2 family inhibitors (venetoclax, navitoclax, and obatoclax), which promote apoptosis. Drugs in the immunomodulatory (Imid) class (pomalidomide and lenalidomide) were also more toxic for IR pools, in keeping with their ability to decrease expression of IRE4, a master regulatory transcription factor that controls ABC DLBCL viability (25). IR pools were more sensitive to BET inhibitors (RVX-208, GSK-525768, and 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 and MC-1586) was expected given their ability to reverse epigenetic ibrutinib resistance (Supplementary Fig. S1E). Finally, several glucocorticoids (e.g., dexamethasone and 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 (Figs. 4B and 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 superadditive 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, whereas 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). Although **BTK and PLCG2** mutations promote genetic resistance to ibrutinib in CLL (11, 13), MCL (20), 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
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**Figure 7.** Targeting epigenetic resistance to BTK inhibitor treatment in ABC DLBCL. A, Summary of the log maximum drug response [dose (μmol/L)] at which a maximal inhibitory response is achieved: Supplementary Table S7A and S7B] 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 drug classes that were represented by more than one drug. Error bars, SEM. Imid, immunomodulatory. B, Ibrutinib-sensitive HBL parent and IR pool cells were treated with DMSO, RAC inhibitor EHT1864 (8.5 μmol/L), and/or ibrutinib (nmol/L) for 4 days, and live cells (calcein AM) were enumerated by flow cytometry from duplicate wells. Error bars, SD. Ibrutinib-sensitive HBL parent, the PLCG2 R665W genetic resistance mutant, and independent IR pool cells were treated with vehicle (DMSO), RAC inhibitor EHT1864 (8.5 μmol/L), or ibrutinib (10 nmol/L) overnight, and PLCG activity was assessed by ELISA in duplicate wells. Each cell type was normalized to its own untreated DMSO control. Error bars, SD. D, Monitoring the growth of xenografts, by tumor volume, of ABC DLBCL parent lines (HBL1 and DLBCL2) or derived IR pools in NOD/SCID mice (n = 5/treatment) treated with vehicle (DMSO), the BCL2 inhibitor venetoclax, or the combination of both drugs. E, Monitoring the growth of xenografts, by tumor volume, of ABC DLBCL parent lines (HBL1 and 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 Supplementary Table S7.

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 IR 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 IR cells, thereby sustaining NF-κB activity and cell viability. Likewise, in IR 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 IR 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 patients with DLBCL are limited, one study reported a patient with two independent \( BTK^{C481S} \) mutant alleles that were detected in circulating tumor DNA after a long latency on treatment (17). Because these \( BTK \) mutations do not confer a selective advantage in the absence of 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 patients with CLL 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% to 43% lacked detectable \( BTK \) and \( PLCG2 \) mutations in their leukemic cells (11, 16, 58). Although 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 eight BTK inhibitor–treated patients with persistent or progressive CLL supports this view: The leukemic cells had greater RAC2/PLCγ2 association than pretreatment 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 IR xenografts than ibrutinib-sensitive parental ABC xenografts. Lenalidomide and other ImiD drugs were also more active against the IR pools, suggesting that coadministration 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–germinal center B-cell DLBCL (60). Another drug class, the HDAC inhibitors, was also more toxic for the IR 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 HDAC 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 IR ABC cells than for their ibrutinib-sensitive parents, in keeping with the heightened sensitivity of IR ABC cells to CRISPR-mediated inactivation of RAC2. EHT1864 had single-agent efficacy against xenografts of IR ABC cells, and its combination with ibrutinib inhibited the growth of ibrutinib-sensitive parental ABC DLBCL xenografts in a superadditive 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, the addition of ibrutinib to R-CHOP (rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin, and prednisone) chemotherapy provided a significant survival benefit to younger patients (age <60; ref. 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. Although 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 3-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.

**METHODS**

**Experimental Design and Statistical Analysis**

The experiments presented have been repeated, and results are 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 Supplementary Methods for details.

**Genomic Data Sets**

All genomic data sets can be found via the SRA/NCBI BioProject (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA750745), accession PRJNA750745, and the Staudt Lab (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 passaged for 10 to 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 (J. 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 0.001 nmol/L through 1,000 nmol/L [65, 66].

Creation and Maintenance of IR Pools and IR Single-Cell Clones

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 1 week (Fig. IA). The resulting cultures were maintained in ibrutinib (10 nmol/L) and will be referred to as IR pools. Resistant pools were maintained by splitting (1:3) and refeeding every 2 to 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 Supplementary Methods.

Sanger Sequencing of BTK/PLCG2 Mutants

See Supplementary Methods.

Creation of Lyt2- and Gfp-Expressing Vectors for BTK/PLCG2 Alleles and Other Genes

See Supplementary Methods.

Virus Production and Transduction

See Supplementary Methods.

Culture of Cells in Outgrowth Experiments of IR Cells and Cells Expressing Mutant Alleles of Resistance Genes

See Supplementary Methods.

Flow Cytometry for Gfp+ and Lyt2+ Outgrowth Experiments

See Supplementary Methods.

Flow Cytometry for Live-Cell Counting (Calcein/EtBr)

See Supplementary Methods.

Exome Sequencing

See Supplementary Methods.

RNA-seq Gene-Expression Profiling

See Supplementary Methods.

Ibrutinib-Response Gene-Expression Signature Definition and Gene-Expression Signature Enrichment

See Supplementary Methods.

Evolution of ibrutinib Resistance: Barcode Method

One million HBL1 or TMD8 ABC DLBCL cells were transduced with a retroviral vector bearing a random 60 mer barcode and a puromycin resistance gene (pRSMSpg; ref. 67), such that each cell received one unique barcode (multiplicity of infection of 1:3). Cells were selected with puromycin (1 μg/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 (0.5 nmol/L week 1 to 10 nmol/L 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; ref. 67), using primerSTAR HS DNA polymerase (Takara, #R010B). See Supplementary Methods. Library DNA was purified and quantituated as in ref. 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 (Supplementary Table S2A). Barcodes were identified from the first 18 bp of unique sequence after the multiple cloning site from the vector. Relative depletion or enrichment of each barcode was calculated, compared with the starting population.

Evolution of ibrutinib Resistance: Expression of Barcode Method

To capture single-cell gene expression, 300 ABC DLBCL cells (HBL1 or TMD8) were transduced with the empty Perturb-Seq base vector library (19) 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 from the same cultures, ~6,000 cells were captured for single-cell analyses.

Single-cell suspensions were loaded on the 10x Genomics Chromium platform at a concentration to capture ~5,000 cells (10x Genomics; 1001027, 1000269, 1000215, and 1000190), and scRNA-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 adapter and partial TruSeq Read1 sequence (AATGATACGGCGACGCAGACGATCTGATCTCACACTTTTCTCCTACAGCAGGCTC-TAG). Gene-expression libraries and expressed barcode libraries were pooled and sequenced on a Next-Seq 500, with a Read1 of 26 cycles, Index1 of 8 cycles, and Read2 of 96 cycles. Fastq files were demultiplexed using the Cell Ranger v3.0.1 pipeline (10x Genomics) with –barcode-mismatch 0 setting to avoid potential index collision between expressed barcode library indices and gene expression indices. Demultiplexed reads from the expressed barcode libraries were processed using Bowtie. After expressed barcodes were corrected for 1-bp 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 data set. For each sample, the top observed expressed barcodes based on highest unique molecular identifier count was determined to be that individual cell’s expressed barcode. Samples representing part of the same culture, but at different time points, were combined, and only expressed barcodes observed in at least 10 cells in the entire data set were retained for further analysis. Cells were assigned resistance phenotypes (Supplementary Table S2B) as determined by both DNA and RNA analyses from the same populations. Expressed barcode gene expression was displayed as a t-SNE projection using the 10x Genomics Loupe Browser (V4.0). See Supplementary Methods for further details.

10x scRNA-seq Transcriptomics

Single-cell suspensions were loaded on the 10x Genomics Chromium platform at target capture number of ~5,000 cells, and scRNA-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. Fastq files were processed through the 10x Cell Ranger Pipeline and visualized via the 10x Loupe program (V4.0).
STRING Analyses of Gene Sets
See Supplementary Methods.

Fast ATAC-seq to Identify Regions of Open Chromatin
See Supplementary 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 100 bp, and for each experiment, the number of bins that overlapped were counted. Peak regions were defined by bins that 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 samples’ 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:

$$\text{Peak count value} = \frac{\# \text{ of reads in the sample peak} \times (\text{parental total counts/sample total})}{\text{# of reads in the sample peak}}$$

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 (https://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 refs. 36 and 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 ±100 kb) were identified and associated with that peak. Transcription factor motif enrichment was performed by inputting chromosomal peak coordinates 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 50 bp of overlap were identified by overlap analysis in GALAXY.

Short Hairpin RNA and sgRNA Sequences
See Supplementary Methods.

CRISPR Screening to Determine Essentiality of Genes in Parental Cells and IR Pools
See Supplementary Methods.

Enumeration of Cells with Expression of RAC2 in scRNA-seq Samples above the Parental Average
See Supplementary Methods.

Flow Cytometry for Intracellular Proteins
See Supplementary Methods.

Creation and Expression of Human RAC2–BioID2
See Supplementary Methods.

Identification of the RAC2 Interactome by Mass Spectrometry
See Supplementary Methods.

CLL Patient Samples
All patients provided written informed consent under an NIH, National Heart, Lung, and Blood Institute (NHLBI) Institutional Review Board–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 onibrutinib (NCT01500733) were collected at the time of clinical progression. Clinical data and outcome of therapy have been described previously (refs. 69 and 70, respectively). Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll (Millipore Sigma, F4375). Peripheral blood mononuclear cells were then washed twice, resuspended in FBS containing 10% DMSO, and stored in a liquid nitrogen freezer.

PLA for RAC2 Interactions
See Supplementary Methods.

High-Throughput Drug Screening
See Supplementary Methods.

PLCG Activity Assay
The day prior to the assay, 5 million cells in 8 mL of media in T25 flasks (Thermo Fisher, 169900) were prepared with vehicle or the drugs as indicated. The next morning, aliquots of 300 μL 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 calcine/EBBr method. The second plate was used to make cell lysates to assess PLC activity by competitive ELISA (Cisbio721P1PEA). 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/): NIH Protocol #METB054. See Supplementary Methods for details.

Authors’ Disclosures
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Authors’ Contributions
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