BTK kinase activity is dispensable for the survival of diffuse large B-cell lymphoma

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Title: BTK kinase activity is dispensable for the survival of diffuse large B-cell lymphoma

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Abstract
Inhibitors targeting Bruton's tyrosine kinase (BTK) have revolutionized the treatment for various B cell malignancies but are limited by acquired resistance after prolonged treatment as a result of mutations in BTK. Here, by a combination of structural modeling, in vitro assays, and deep phospho-tyrosine proteomics, we demonstrated that four clinically observed BTK mutations—C481F, C481Y, C481R, and L528W—inactivated BTK kinase activity both in vitro and in diffuse large B-cell lymphoma (DLBCL) cells. Paradoxically, we found that DLBCL cells harboring kinase-inactive BTK exhibited intact B cell receptor (BCR) signaling, unperturbed transcription, and optimal cellular growth. Moreover, we determined that DLBCL cells with kinase-inactive BTK remained addicted to BCR signaling and were thus sensitive to targeted BTK degradation by the proteolysis targeting chimera (PROTAC). By performing parallel genome-wide CRISPR-Cas9 screening in DLBCL cells with wild-type or kinase-inactive BTK, we discovered that DLBCL cells with kinase-inactive BTK displayed increased dependence on Toll-like receptor 9 (TLR9) for their growth and/or survival. Our study demonstrates that the kinase activity of BTK is not essential for oncogenic BCR signaling and suggests that BTK’s non-catalytic function is sufficient to sustain the survival of diffuse large B-cell lymphoma.

Introduction
During B cell development, antigen-mediated cross-linking of BCR activates the non-receptor tyrosine kinases BTK (1). BTK then promotes the activation of phospholipase Cγ2 (PLCγ2), which hydrolyzes phosphatidylinositol 4,5-bisphosphate to elicit an increase of intracellular Ca2+ (2,3). The resulting Ca2+ flux activates diverse transcriptional programs to promote B cell proliferation and differentiation (4,5). Oncogenic mutations, microbial antigens, or autoantigens can co-opt BCR signaling to support the growth and/or survival of
malignant B cells, resulting in B-cell leukemias and lymphomas (6-11). Inhibitors that target BTK (BTKi) have emerged as breakthrough therapies for treating a variety of B-cell malignancies, such as chronic lymphocytic leukemia/small lymphocytic lymphoma, mantle cell lymphoma, and Waldenström’s macroglobulinemia (12-14). However, durable response to BTKi is hampered by acquired resistance after prolonged treatment (15,16).

First- and second-generation BTKi, such as ibrutinib, acalabrutinib, and zanubrutinib, inhibit the kinase activity of BTK by binding to its ATP-binding pocket and then covalently modifying a cysteine residue at the position 481 (C481) of BTK (17,18). Correspondingly, the most common mechanism of BTKi resistance occurs through mutations changing this reactive cysteine into serine (C481S) and less frequently into phenylalanine, tyrosine or arginine (C481F, C481Y and C481R) (19,20). In addition, several non-C481 mutations have been observed in patients resistant to irreversible BTKi (19,21) and more recently, in relapsed CLL patients treated with the noncovalent BTK inhibitor pirtobrutinib (22). Whereas previous studies have clarified their mechanism of resistance to BTKi, whether these BTK mutations affect the biochemical activity and/or function of BTK in malignant B cells are not well understood.

Results

C481F/Y/R and L528W impair BTK kinase activity in vitro

In an attempt to explore the biochemical impact of clinically observed BTK mutations, we first docked ATP into the active site of BTK kinase domain. In the resulting structural model, the adenine ring of ATP formed hydrogen bonds with the side chain of T474 and the backbone of M477 in BTK. In addition, the phosphate group of ATP was locked in the active site of BTK by forming two hydrogen bonds with the side chain of K430. Residues C481 and L528 were located below the binding pocket and showed no direct interaction with ATP (Fig.
Next, we evaluated whether clinically observed BTK C481 and non-C481 mutations might affect ATP binding. Whereas C481S was not expected to affect the mode of ATP binding, substitutions of C481 by bulky side chains of phenylalanine (C481F), tyrosine (C481Y), or arginine (C481R) were all predicted to generate steric clashes to the sugar ring or the phosphate group of ATP (Fig. 1B). Similarly, leucine at position 528 mutated to tryptophan (L528W) caused a steric clash to the adenine ring of ATP (Fig. 1B). To verify these predictions, we purified recombinant BTK kinase domains and performed the thermal shift assay. We observed that ATP stabilized the kinase domains of BTK wild-type (WT), C481S, and C481R but did not stabilize the kinase domains of BTK C481F, C481Y, and L528W (Fig. 1, C-D). Compared to phenylalanine and tyrosine, arginine may be more flexible at the active site due to the lack of an aromatic ring in its side chain. Thus, ATP may gain access to the active site and stabilize the kinase domain of BTK C481R.

These observations prompted us to examine the kinase activity of BTK mutants using an in vitro kinase assay. By incubating BTK kinase domain with either a peptide substrate derived from PLCγ2 or a protein substrate (the SH3 domain of BTK containing an auto-phosphorylation site), we observed near complete lack of kinase activity of BTK C481F, C481Y and L528W in vitro (Fig. 1E). Notably, although C481R did not show a defect in ATP binding in the thermal shift assay, its kinase activity was significantly reduced comparing with WT BTK (Fig. 1E). Taken together, these results revealed that four clinically observed BTK mutations, C481F, C481Y, C481R, and L528W, impaired BTK kinase activity in vitro.

C481F/Y/R and L528W impair BTK kinase activity in DLBCL cells

To examine whether C481F/Y/R and L528W impaired BTK kinase activity in malignant B cells, we selected TMD8 and OCI-LY10, two DLBCL cell lines of the activated B-cell
subtype (9), as our experimental models because of their sensitivity to ibrutinib (Fig. S1A). We used a lentiviral vector to stably express BTK WT, C481S/F/Y/R, or L528W in TMD8 and OCI-LY10 cells at levels near their endogenous BTK (Fig. S1B) and then examined their sensitivity to ibrutinib. Whereas expression of WT BTK did not alter the sensitivity of TMD8 or OCI-LY10 to ibrutinib, expression of BTK C481S/F/Y/R and L528W conferred resistance to ibrutinib in both cell lines (Fig. S1C). These isogenic cell lines, in which endogenous BTK was inactivated by ibrutinib, allowed us to examine the biochemical and functional sequelae of BTK mutations.

Because BTK is a tyrosine kinase, we used deep phospho-tyrosine (pY) proteomics to profile changes of global pY patterns in DLBCL cells following BTK inactivation by ibrutinib. After proteolytic digestion, pY-modified peptides were enriched by a Src homology 2 (SH2)-domain-derived pTyr superbinder (23) followed by identification by mass spectrometry (Fig. 2A). We identified 176 distinct pY-modified peptides from TMD8 and OCI-LY10 cells (Table S2). Two of these peptides, BTK pY223 and pY361, showed greater than 75% reduction in both cell lines following ibrutinib treatment (Fig. S2A). Moreover, we used anti-IgM stimulation to enhance BCR signaling, resulting in the identification of 218 distinct pY-modified peptides (Table S2). Five of these pY-modified peptides, BTK pY223, BTK pY361, CCDC50 pY146, ESYT1 pY822, and OCIAD1 pY199, showed greater than 75% reduction in both cell lines following ibrutinib treatment (Fig. S2A).

We next used deep pY proteomics to compare the global pY patterns of TMD8 cells expressing BTK C481S/F/Y/R (treated with ibrutinib to inactivate their endogenous BTK) with that of parental TMD8 cells. Among the pY-modified peptides that showed greater than 75% reduction relative to parental cells, four were common in C481F/Y/R, including BTK pY223, BTK pY361, DNAJA1 pY381, and DOK1 pY409 (Fig. 2B). BTK pY223 is a well-known auto-phosphorylation site of BTK (24). Under basal conditions, BTK Y223
phosphorylation could be detected in parental TMD8 and BTK C481S-expressing cells, but was missing in TMD8 cells expressing BTK C481F/Y/R or L528W (Fig. 2C and Fig. S2B). Cross-linking of BCR by anti-IgM increased BTK Y223 phosphorylation in parental and BTK C481S-expressing TMD8 cells, but not in BTK C481F/Y/R- or L528W-expressing TMD8 cells (Fig. 2C and Fig. S2B). Similar observations were made in OCI-LY10 cells (Fig. S2, B-C).

To further validate findings from deep pY proteomics, we employed CRISPR genome editing in TMD8 to obtain a BTK L528W knock-in clone, which was 264-fold less sensitive to ibrutinib than parental TMD8 (Fig. S3, A-B). In contrast, the proliferation rate of BTK L528W knock-in clone was indistinguishable from parental TMD8 cells (Fig. S3C). Consistent with deep pY proteomics results, BTK Y223 phosphorylation was missing in BTK L528W knock-in TMD8 cells (Fig. 2D).

Activated BTK is known to phosphorylate PLCγ2 (Y753, Y759, and Y1217) (25). However, deep phospho-tyrosine (pY) proteomics showed that PLCγ2 phosphorylation was not affected by the loss of BTK kinase activity (Table S2). Moreover, western blotting confirmed the lack of correlation between PLCγ2 phosphorylation (pY759 and pY1217) and BTK kinase activity in both TMD8 and OCI-LY10 cells (Fig. 2C and Fig. S2, B-C). Similar observations were made in L528W knock-in TMD8 cells (Fig. 2D). Therefore, the identified pY sites of PLCγ2 are likely phosphorylated by a different kinase in DLBCL cells.

**Kinase-inactive BTK mutants support oncogenic BCR signaling in malignant B cells**

We next examined whether BTK kinase activity was required for oncogenic BCR signaling by measuring Ca\(^{2+}\) flux following BCR crosslinking. By testing a panel of B-cell lymphoma cell lines, we found that half of them displayed Ca\(^{2+}\) flux following anti-IgM stimulation (Fig. S4, A-B). Among the responding cell lines, TMD8 cells displayed Ca\(^{2+}\) flux peaked around 1
minute and subsided by 4 minutes post anti-IgM stimulation. Pretreatment with ibrutinib reduced the magnitude of the Ca\(^{2+}\) flux in parental TMD8 cells by 3.2-fold (Fig. 3, A-B). Comparable anti-IgM induced Ca\(^{2+}\) flux was observed in BTK L528W knock-in cells relative to parental cells, and the Ca\(^{2+}\) flux in BTK L528W knock-in cells could no longer be suppressed by ibrutinib (Fig. 3, A-B). These results suggest that TMD8 cells without BTK kinase activity maintain oncogenic BCR signaling.

Extending from findings in TMD8, we examined BCR signaling in a Burkitt’s lymphoma cell line Ramos, in which anti-IgM induced robust Ca\(^{2+}\) flux (Fig. S4A-B). Ramos does not rely on BCR signaling for survival; we thus isolated a BTK knockout clone of Ramos (Fig. S4C). The resulting BTK knockout cells showed reduced Ca\(^{2+}\) flux following anti-IgM stimulation (Fig. S4D). We were initially surprised that knocking out BTK did not completely abrogate anti-IgM induced Ca\(^{2+}\) flux; however, similar phenomena were observed in multiple previous studies (26,27), suggesting that BTK-independent mechanisms could contribute to residual Ca\(^{2+}\) flux in BTK knockout cells. We then expressed various BTK mutants and found all of them rescued the defective Ca\(^{2+}\) flux in BTK knockout Ramos cells to the same degree as WT BTK (Figure 3, C-D). These results altogether suggest that the kinase activity of BTK is dispensable for the induction of Ca\(^{2+}\) flux to transmit oncogenic BCR signaling.

**Kinase-inactive BTK does not alter gene expression in malignant B cells**

BCR signaling activates multiple transcription factors to sustain the growth and survival of malignant B cells (4,5). We therefore used RNA sequencing (RNA-seq) to examine whether loss of BTK kinase activity might affect gene expression downstream of BCR signaling (Table S3). By comparing parental TMD8 cells treated with vehicle or ibrutinib, we defined a list of 179 significantly upregulated genes and 192 significantly downregulated genes in
ibrutinib-treated cells (Fig. 3E and Fig. S5A). Gene ontology analyses revealed that ibrutinib treatment down-regulated genes involved in cytokine-mediated signaling pathways and regulation of cell adhesion and chemotaxis, consistent with previous studies (Fig. 3F) (28,29). Genes upregulated by ibrutinib did not enrich gene sets with statistical significance (Fig. S5F). By displaying these differentially expressed genes as a heatmap, we found that they were expressed at comparable levels in TMD8 cells expressing kinase-active BTK C481S or kinase-inactive BTK C481F/Y/R (Fig. 3E and Fig. S5, B-E). These results demonstrate that loss of BTK kinase activity does not affect gene expression downstream of BCR signaling.

**BTK kinase-inactivating mutations do not bypass BCR signaling**

We used a competitive cell growth assay to examine whether DLBCL cells deficient in BTK kinase activity remained dependent on BCR signaling for growth and survival (Fig. 4A). To ensure comparable CRISPR efficiencies, we isolated Cas9-transduced clones of parental TMD8 and BTK L528W knock-in cells and validated their dependencies on the pan-essential gene **POLD3** (Fig. S6A). We then used an sgRNAs targeting **BTK** and found that sgRNA-transduced cells from both parental and BTK L528W knock-in cells were depleted at comparable rates (Fig. 4B). The reduction of cell fitness due to loss of **BTK** could be fully rescued by cDNAs encoding either kinase-active BTK (WT and C481S) or kinase-inactive BTK (L528W and C481F/Y/R) (Fig. S6B).

We next transduced cells with sgRNAs that target several key genes of the BCR signaling pathway, including **CD79A/CD79B** (encoding components of the BCR complex), **SYK, BLNK** (encoding B cell linker protein), **PLCG2, PRKCB** (encoding protein kinase C β), **CARD11/BCL10/MALT1** (encoding components of the CBM signalosome complex), and **NFKB1** (encoding a nuclear factor kappa B subunit). We observed that both TMD8 parental cells and BTK L528W knock-in cells were equally dependent on these genes for survival (Fig.
 Altogether, these results demonstrate that DLBCL harboring BTK kinase-inactivating mutations do not bypass BCR signaling for growth and survival.

**Targeted degradation of BTK kinase-inactive mutants overcomes BTKi resistance**

Because DLBCL cells with kinase-inactive BTK remained addicted to BTK, we hypothesized that targeted BTK degradation by the proteolysis-targeting chimera (PROTAC) might be an effective strategy to overcome BTKi resistance (30). Thus, we employed a BTK PROTAC (BGB-15741) to hijack the E3 ubiquitin ligase CRL4<sub>CRBN</sub> to degrade BTK (Fig. 4C). BGB-15741 treatment induced the degradation of both wild-type BTK and BTK L528W in TMD8 cells, which could be blocked by the proteasome inhibitor MG132 and the CRBN binder lenalidomide (Fig. S7, A-B). TMD8 and OCI-LY10 cells harboring kinase-inactive BTK mutations were sensitive to the anti-proliferative effect of BGB-15741 (Fig. 4D). Using quantitative mass spectrometry, we examined the proteome-wide selectivity of BGB-15741. In TMD8 cells treated with BGB-15741 for six hours, BTK and JUN were the only two proteins significantly depleted (Fig. S7C and Table S4). By querying previous genome-wide CRISPR/Cas9 screening in TMD8 (31), we found that JUN was not essential for the survival of TMD8 (Fig. S7D). Thus, we conclude that the anti-proliferative activity of BGB-15741 is a result of BTK degradation. Taken together, the BTK PROTAC BGB-15741 promotes the degradation of kinase-inactive BTK mutants to overcome resistance to irreversible BTKi.

**BTK kinase-inactivating mutations increase TLR9 dependency in DLBCL cells**

To unbiasedly explore whether BTK kinase-inactivating mutations might result in alterations of DLBCL genetic dependencies, we performed parallel genome-wide CRISPR-Cas9 screening in parental TMD8 and L528W knock-in cells. After lentiviral transduction of the
sgRNA library, we propagated cells for three weeks and then performed next generation sequencing to quantify the abundance of each sgRNA in surviving cells (Fig. 5A).

MAGeCK (Model-based Analysis of Genome-wide CRISPR/Cas9 Knockout) algorithm ranked 12 significantly depleted genes in L528W knock-in cells relative to parental cells (Fig. 5B and Table S5). Among these 12 candidates, STRING analysis revealed that Toll-like receptor 9 (TLR9), unc-93 homolog B1 (UNC93B1), and canopy homolog 3 (CNPY3) formed a protein-protein interaction network (Fig. 5B). TLR9 is a member of the Toll-like receptor, which localizes to the endosomes and senses microbial DNA to trigger pro-inflammatory signaling (32). CNPY3 and UNC93B1 are required for proper TLR9 folding and localization to endosomes, respectively (33-35). In addition, sgRNAs targeting the non-receptor tyrosine kinase HCK was also depleted in L528W knock-in cells relative to parental cells (Fig. 5B).

We next used the competitive cell growth assay (Fig. 4A) to validate findings from the CRISPR-Cas9 screening. When transduced with sgRNAs targeting TLR9, BTK L528W knock-in cells were depleted with significantly faster kinetics relative to parental cells (Fig. S8A). To exclude clonal effect in the competitive cell growth assay, we used the suite of isogenic TMD8-Cas9 cells expressing BTK C481S/F/Y/R and L528W and inactivated their endogenous BTK by ibrutinib. When transduced with the sgRNA targeting POLD3, TMD8-Cas9 cells expressing different forms of BTK were depleted with similar kinetics, suggesting their comparable CRISPR efficiencies (Fig. 5C). In contrast, when transduced with sgRNAs targeting TLR9, UNC93B1, CNYP3, or HCK, BTK kinase-inactive cells (C481F/Y/R and L528W) were depleted with significantly faster kinetics than BTK kinase-active cells (C481S). (Fig. 5C and Fig. S8B). Similar observations were made in OCI-LY10-Cas9 cells (Fig. 5C and Fig. S8B). Taken together, we conclude that DLBCL cells with kinase-inactive BTK are more dependent on TLR9 signaling for their growth and/or survival.
**Discussion**

Our study unveiled a collection of clinically observed BTK mutations that not only cause resistance to irreversible BTKi, but also inactivate the kinase activity of BTK. These mutations affect two residues in BTK, C481 and L528, substituting them with residues containing bulky side chains, resulting in steric hindrance to ATP binding. By studying the biochemical and functional properties of these BTK mutants in DLBCL cell lines, we made the unexpected finding that kinase-inactive BTK mutants were as efficient at transducing BCR signaling as their wild-type counterpart. Similar observations of kinase-inactive BTK mutants have been made in other experimental systems. Tomlinson et al. observed that a kinase-inactive BTK (K430E) could restore BCR-induced calcium flux and ERK-MAPK activation in *BTK*-deficient DT40 cells (36). In addition, BTK C481F and C481Y mutants have been reported to lack auto-phosphorylation activity in HEK 293T and DT40 cells (20,27). Together with our results, BTK’s non-catalytic activity instead of kinase activity is required for oncogenic BCR signaling to support the growth and survival of malignant B cells.

Although protein kinases are known primarily as enzymes catalyzing phosphorylation, accumulating evidence has revealed their non-catalytic functions, such as allosteric regulation of other enzymes, scaffolding the assembly of signaling complexes, and regulation of transcription (37). Our parallel CRISPR-Cas9 screening in DLBCL cells with kinase-active versus kinase-inactive BTK revealed increased genetic dependency of BTK kinase-inactive cells on *TLR9, UNC93B1, CNPY3*, and *HCK*. Thus, inactivation of BTK kinase activity resulted in a loss of cellular fitness only when these genes were inactivated.

HCK is a member of the SRC family of cytoplasmic tyrosine kinases (SFKs), and is expressed in myeloid cells and B lymphocytes (38). High levels of HCK have been reported
in various types of leukemia, such as multiple myeloma and acute lymphoblastic leukemia (39,40). In our study, we demonstrated that DLBCL cells with kinase-inactive BTK displayed increased genetic dependence on HCK, compared to DLBCL cells with kinase-active BTK. Consistent with our finding, a recent study implicated the activation of HCK in malignant B cells with kinase-inactive BTK (27). Functions and mechanisms of HCK activation in DLBCL cells with kinase-inactive BTK require further investigation.

TLR9, a member of the toll-like receptor family expressed in mammalian immune cells, is a pattern recognition receptor for unmethylated CpG-DNA from bacteria and viruses (41). Ligand-bound TLR9 initiates the production of type I interferons and proinflammatory cytokines to activate host antibacterial or antiviral immune response (42-44). Previous studies have reported that BTK was required for TLR9 signaling in monocytic THP1 cells and mouse B cells (45,46). In addition, proximity labeling experiments revealed the physical association of BCR with TLR9 and MYD88 into a super complex in DLBCL cells (31). These observations suggest that BTK mediates the crosstalk between BCR and TLR9 signaling pathways. Together with our results, the inactivation of BTK kinase activity in DLBCL cells, although not impacting BCR signaling, may weaken the crosstalk between BCR and TLR9 signaling, resulting in increased dependency on TLR9. The biochemical and functional interactions between TLR9 and kinase-inactive BTK warrant future studies.

BTKi has transformed the treatment for various B cell malignancies. Thus far, it has been generally believed that BTKi acts by inhibiting the BTK’s catalytic function. Our study raises the intriguing question regarding the exact mechanism of action of BTKi. BTKi such as ibrutinib not only inactivates BTK kinase activity but also shuts down oncogenic BCR signaling. Given our finding that BTK kinase activity is dispensable for oncogenic BCR signaling, we propose that BTKi in clinical use may target BCR signaling by impairing
BTK’s non-catalytic function. Collectively, our findings set the stage for studying BTK’s non-catalytic functions in various forms of B cell malignancies.

**Experimental Procedures**

Recombinant protein purification, cell line engineering, western blotting, proteomic methods and chemical synthesis are described in Supplementary Methods. Sources of cell lines, antibodies, plasmids, and compounds are described in Table S1. All human lymphoma cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum and 2 mM L-glutamine, and were confirmed to be mycoplasma free on a weekly basis using a PCR-based assay.

**Thermal shift assay**

Recombinant BTK kinase domain was diluted to 2 μM with the assay buffer (50 mM Tris pH 7.4, 10 mM MgCl$_2$ and 2 mM MnCl$_2$). ATP (1 mM) was added to the diluted protein. Twenty microliter of protein-ATP mix was combined with 5 μl of 1:200 diluted SYPRO Orange (Sigma-Aldrich, St. Louis, MO, USA). After incubation on ice for 20 minutes, fluorescence measurements were performed using a CFX96 Touch Real-Time PCR instrument (Bio-Rad, Hercules, CA, USA). The temperature was increased from 10°C to 95°C with an increment of 0.5°C and equilibration time of 10 seconds at each temperature prior to measurement. The melting temperature (Tm) was defined as the temperature corresponding to the maximum value of the first derivative of fluorescence transition.

**In vitro BTK kinase assay**

Recombinant BTK kinase domain (1 μM) and PLCγ2 peptide (40 μM) or recombinant BTK SH3 domain (40 μM) were diluted with the kinase assay buffer (25 mM Tris pH 7.5, 150 mM...
NaCl, 5% glycerol, 20 mM MgCl₂ and 1 mM DTT) and mixed with ATP (50 μM). Reactions (20 μl) were incubated at room temperature for 0, 2.5, 5, 10, 20, 40, and 60 minutes. ADP level was measured using ADP-Glo™ Kinase Assay kit (Promega, Madison, WI, USA). Luminescence was recorded by EnSpire multimode reader (PerkinElmer, Waltham, MA, USA). Relative ADP level was determined with GraphPad Prism using baseline correction (by normalizing to 0 minute).

**Phospho-tyrosine proteomics**

A total of 5x10⁷ cells were treated with 10 nM ibrutinib for 6 hours followed by a 5-minute stimulation with 10 µg/ml anti-IgM (Jackson ImmunoResearch, West Grove, PA, USA; 109-006-129). Procedures of deep phospho-tyrosine proteomics are described in Supplementary Methods.

**Ca²⁺ flux measurement by flow cytometry**

Cells were rinsed 3 times with DPBS and incubated in the dark with 1 μM Fluo-4-AM (Beyotime, Shanghai, China; S1060) diluted in DPBS at 37°C for 30 minutes. Dye-loaded cells were washed 3 times and resuspended in DPBS for an additional 20-minute incubation at room temperature. Fluorescence measurements were performed using BD Accuri™ C6 Plus flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using an air-cooled argon ion laser (488 nm excitation). Stimulation with 10 µg/ml Anti-IgM (Jackson ImmunoResearch) was performed by injection with a syringe. For data analysis, the relative fluorescence units (RFU) were normalized as (F-F₀)/F₀, in which F₀ was defined as the mean of fluorescence 5-10 second before anti-IgM stimulation. F was defined as the average fluorescence intensity per second. Areas under the calcium flux curves were determined with GraphPad Prism.
**RNA sequencing and gene ontology analysis**

Total cellular RNA was purified from cells treated with vehicle (DMSO) or 10 nM ibrutinib for 24 hours. Standard RNA-seq was performed by Berry Genomics, Beijing, China. Sequencing reads were aligned to the human GRCh38 reference transcriptome using Botwie2 (47) followed by gene-level quantification with RSEM (48). Differential gene expression analyses were performed with DESeq2 (49) with the following cutoff: absolute log2-transformed fold change greater than 2 and $P$ value less than 0.01. Gene ontology analysis of differentially expressed genes was performed with clusterProfiler (50).

**Competitive cell growth assay**

Cas9-transduced cell lines were infected with the indicated sgRNA lentivirus at a low multiplicity of infection (MOI=0.2-0.4). Percentages of transduced cells (sgRNA$^+$) marked by mNeonGreen-2A-ZsGreen1 were quantified every 2–3 days using BD Accuri™ C6 Plus flow cytometer (BD Biosciences) and normalized to day 4 or 5.

**Parallel genome-wide CRISPR-Cas9 screening**

The human CRISPR Brunello library (51) was transduced into TMD8-Cas9 and TMD8-Cas9 $BTK$ L528W knock-in cells at a low multiplicity of infection (MOI=0.2-0.3) and a coverage of ~400 cells per sgRNA. After puromycin (1 μg/ml) selection, transduced cells were cultured for 3 weeks. Library preparation for sequencing was carried out in PCR performed on genomic DNA isolated from cells. Sequencing reads were analyzed by MAGeCK (52) to determine relative sgRNAs abundance.

**Cell viability assay**
Eight thousand cells were plated per well in 96-well microplates (Corning, Corning, NY, USA). Cells were treated with serial dilutions of ibrutinib or BGB-15741 with a D300e digital dispenser (Tecan, Männedorf, Switzerland). Cell survival was measured 96 hours later using CellTiter-Glo luminescent cell viability assay kit (Promega). Luminescence was recorded by EnSpire multimode reader (PerkinElmer). Half maximal inhibitory concentration (IC₅₀) was determined with GraphPad Prism using baseline correction (by normalizing to DMSO control), the asymmetric (five parameter) equation, and least squares fit.

**Statistical analysis**

Statistical analyses were performed with GraphPad Prism 8.0. Student’s t-test was used to evaluate the statistically significant difference between two sample groups. When comparing more than two independent groups, ANOVA was used to evaluate statistical significance. Multiple comparison tests were performed when ANOVA was significant. All tests were two-tailed and $P < 0.05$ was considered statistically significant.

**Data availability**

Raw data of RNA-seq results have been deposited in Gene Expression Omnibus (accession number GSE207322). All of the datasets generated during the study are available from the corresponding author upon reasonable request.

**Supporting information**

This article contains supporting information (53-59).

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**Author contributions**

Hongwei Yuan, Yutong Zhu, and Yalong Cheng performed experiments and analyzed data; Junjie Hou, Fengjiao Jin, Menglin Li, and Wei Jia performed deep pY proteomics; Zhenzhen Cheng and Haimei Xing constructed TMD8 BTK L528W knock-in cell lines; Mike Liu and Ting Han designed and supervised the study; Ting Han and Hongwei Yuan prepared the manuscript with input from all authors.

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**Conflict of interest**

Yutong Zhu, Zhenzhen Cheng, Haimei Xing, and Mike Liu are employees and owning stocks of BeiGene (Beijing) Co., Ltd., Beijing, China. Junjie Hou, Fengjiao Jin, Menglin Li, and Wei Jia are employees and owning stocks of Deepkinase Co., Ltd, Beijing, China. Hongwei Yuan, Yalong Cheng, and Ting Han declare no competing interests.

**References**


11. Ngo, V. N., Young, R. M., Schmitz, R., Jhavar, S., Xiao, W., Lim, K.-H., Kohlhammer, H., Xu, W., Yang, Y., Zhao, H., Shaffer, A. L., Romesser, P., Wright,


kinase is sufficient to activate multiple downstream signaling pathways via PLC Gamma 2 in B cells. *BMC Immunology* 2, 4


Figure legend

Figure 1. C481F, C481Y, C481R, and L528W impair BTK kinase activity in vitro. (A) A modeled structure of wild-type BTK kinase domain (PDB 6J6M) in complex with ATP. (B) Details of the modeled interface between indicated BTK kinase domain and ATP. Key residues of BTK and ATP are shown as sticks. Carbon atoms of ATP are shown in champagne. Hydrogen bonds between BTK and ATP are shown as yellow dotted lines. (C) Thermal shift of recombinant BTK kinase domain in the presence of DMSO or 1 mM ATP from three technical replicates. (D) Melting temperature (Tm) quantification of data in (C). Data are the mean of three technical replicates. Significance was analyzed using Student’s t-test, two-tail, paired (ns: not significant; ** P < 0.01). (E) In vitro kinase assay of recombinant BTK kinase domain using a synthesized PLCγ2 peptide (left) and recombinant BTK SH3 domain (right) as substrates. Data are the mean ± SD of three technical replicates. Comparisons between each mutant with wild-type BTK were analyzed using one-way ANOVA with Dunnett’s multiple comparison tests (ns: not significant; * P < 0.05, ** P < 0.01, *** P < 0.001).

Figure 2. BTK C481F, C481Y, C481R, and L528W are kinase-inactive in DLBCL cells. (A) Schematic of phospho-tyrosine (pY) proteomics. (B) Venn diagram depicting the overlap of pY-modified peptides identified in parental TMD8 cells and TMD8 cells expressing BTK C481S/F/Y/R with indicated treatments. The 4 overlapping peptides in C481F/Y/R are listed. (C) Western blotting of total and phosphorylated BTK and PLCγ2 in TMD8 cells expressing BTK C481S/F/Y/R and L528W with indicated treatments. (D) Western blotting of total and phosphorylated BTK and PLCγ2 in parental and BTK L528W knock-in TMD8 cells with
indicated treatments. Cells were pretreated with 10 nM ibrutinib for 6 hours and then stimulated with 10 μg/ml goat anti-human IgM for 5 minutes.

**Figure 3. Kinase-inactive BTK mutants support oncogenic BCR signaling in malignant B cells.** (A) Anti-IgM induced Ca^{2+} flux measurements in parental (left) and BTK L528W knock-in (right) TMD8 cells. Cells were pretreated with 10 nM ibrutinib for 12 hours followed by 10 μg/ml anti-IgM stimulation. Data are the mean ± SD of three biological replicates. (B) Area under the curve (AUC) quantification of data in (A). Significance was analyzed using two-way ANOVA with Tukey’s multiple comparison tests (ns: not significant; *** P < 0.001). (C) Anti-IgM induced Ca^{2+} flux measurements in BTK knock-out (KO) Ramos cells expressing vector, BTK wild-type, C481F/Y/R and L528W. Cells were stimulated with 10 μg/ml anti-IgM. Data are the mean ± SD of three biological replicates. (D) Area under the curve (AUC) quantification of data in (C). Significance was analyzed using one-way ANOVA with Tukey’s multiple comparison tests (ns: not significant; **** P < 0.0001). (E) Heatmap of differentially expressed genes in indicated cell lines with or without 10 nM ibrutinib treatment for 24 hours. Row-wise Z score transformation was performed. (F) Gene ontology enrichment analysis of genes down regulated by ibrutinib in parental TMD8 cells.

**Figure 4. DLBCL cells with kinase-inactive BTK maintain dependence on BCR signaling for survival.** (A) Schematic of the competitive cell growth assay. Cas9-transduced cells were infected with lentivirus expressing an sgRNA and a fluorescent protein marker at a low multiplicity of infection (MOI=0.2-0.4). Flow cytometry was used to monitor the percentages of transduced GFP^+ cells every 2 or 3 days. (B) Viability effects (normalized to the control sgChr2-4) after CRISPR inactivation of BCR signaling-related genes in indicated
cells. Data are the mean of three technical replicates. (C) Chemical structure of BGB-15741. (D) BGB-15741 cytotoxicity measurements on TMD8 (left) and OCI-LY10 cells (right) expressing BTK wild-type, C481S/F/Y/R and L528W. Data are the mean ± SD of three biological replicates.

**Figure 5. Altered genetic dependencies of DLBCL cells with kinase-inactive BTK.** (A) Schematic of parallel CRISPR-Cas9 screening in parental and *BTK* L528W knock-in TMD8 cells. (B) Scatterplot depicting log₂ transformed average fold change of sgRNA abundance (*BTK* L528W knock-in divided by parental) and -log₁₀ transformed *P* value computed by MAGeCK. Functional association of genes identified in the CRISPR-Cas9 screen by STRING analysis is shown as an insert. (C) Viability effects (normalized to the control sgChr2-4) after CRISPR inactivation of indicated genes in indicated cell lines. Endogenous BTK of these cell lines were inactivated by ibrutinib. Data are the mean of three technical replicates from one representative experiment. Two independent experiments were performed. Significance was analyzed using one-way ANOVA with Dunnett's multiple comparison tests (*P* < 0.05, **P** < 0.01, ***P** < 0.001).
Figure 1

A  BTK kinase domain

B  WT  

C481S  

C481F  C481Y  

C481R  L528W  

C  

WT  

C481S  

C481F  C481Y  

C481R  L528W  

D  

E  PLCγ2 peptide  

BTK SH3 domain
Figure 2

A

B

C

D

>75% reduction relative to parental

Journal Pre-proof
Figure 3

A. TMD8 parental

B. DMSO Ibrutinib Parental

C. Control WT L528W BTK cDNA

D. BTK KO BTK KO + BTK cDNA

E. TMD8 + BTK cDNA

F. Down-regulated by ibrutinib in TMD8 parental

Gene Ratio

G. Journal Pre-proof
Figure 4

A

U6

SFFV

mNG-2A-ZsGreen1

sgRNA

Cas9+ cell

lentivirus

Flow cytometry every 2 or 3 d

B

sgRNA induction (days)

0 0.25 0.5 0.75 1.0

sgPOLD3

sgCD79A

sgCD79B

sgSYK

sgBLNK

sgBTK

sgPLCG2

sgPRKCS

sgBCL10

sgMALT1

sgCARD11

sgNFIX21

Relative viability (normalized to sgChr2-4)

TMD8 parental

TMD8 B7K L528W knock-in

C

BTK PROTAC

BGB-15741

D

Cell viability (% Vehicle)

125 100 75 50 25 0

10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6}

BGB-15741 (M)

BGB-15741 (M)

TMD8

OCI-LY10

IC_{50} (nM)

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**Figure 5**

**A**
- SFFV Cas9
- TMD8 parental_Cas9
- TMD8 L528W KI_Cas9
- sgRNA library
- sgRNA lentivirus
- 3-week growth
- Genome extraction, NGS

**B**
- log₁₀(P value)
- log₂(BTK L528W/WT)

**C**
- TLR9 sg-1
- UNC93B1 sg-1
- CNPY3 sg-1
- HCK sg-1
- POLD3 sg-1

Relative viability (normalized to sgChr2-4)

- BTK C481S
- BTK C481F
- BTK C481Y
- BTK C481R
- BTK L528W
Author contributions

Hongwei Yuan, Yutong Zhu, and Yalong Cheng performed experiments and analyzed data; Junjie Hou, Fengjiao Jin, Menglin Li, and Wei Jia performed deep pY proteomics; Zhenzhen Cheng and Haimei Xing constructed TMD8 BTK L528W knock-in cell lines; Mike Liu and Ting Han designed and supervised the study; Ting Han and Hongwei Yuan prepared the manuscript with input from all authors.

Each of the authors confirms that this manuscript has not been previously published and is not currently under consideration by any other journal. Additionally, all of the authors have approved the contents of this paper and have agreed to JBC’s submission policies.

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Conflict of interest

Yutong Zhu, Zhenzhen Cheng, Haimei Xing, and Mike Liu are employees and owning stocks of BeiGene (Beijing) Co., Ltd., Beijing, China. Junjie Hou, Fengjiao
Jin, Menglin Li, and Wei Jia are employees and owning stocks of Deepkinase Co., Ltd, Beijing, China. Hongwei Yuan, Yalong Cheng, and Ting Han declare no competing interests.