Vecabrutinib inhibits B-cell receptor signal transduction in chronic lymphocytic leukemia cell types with wild-type or mutant Bruton tyrosine kinase

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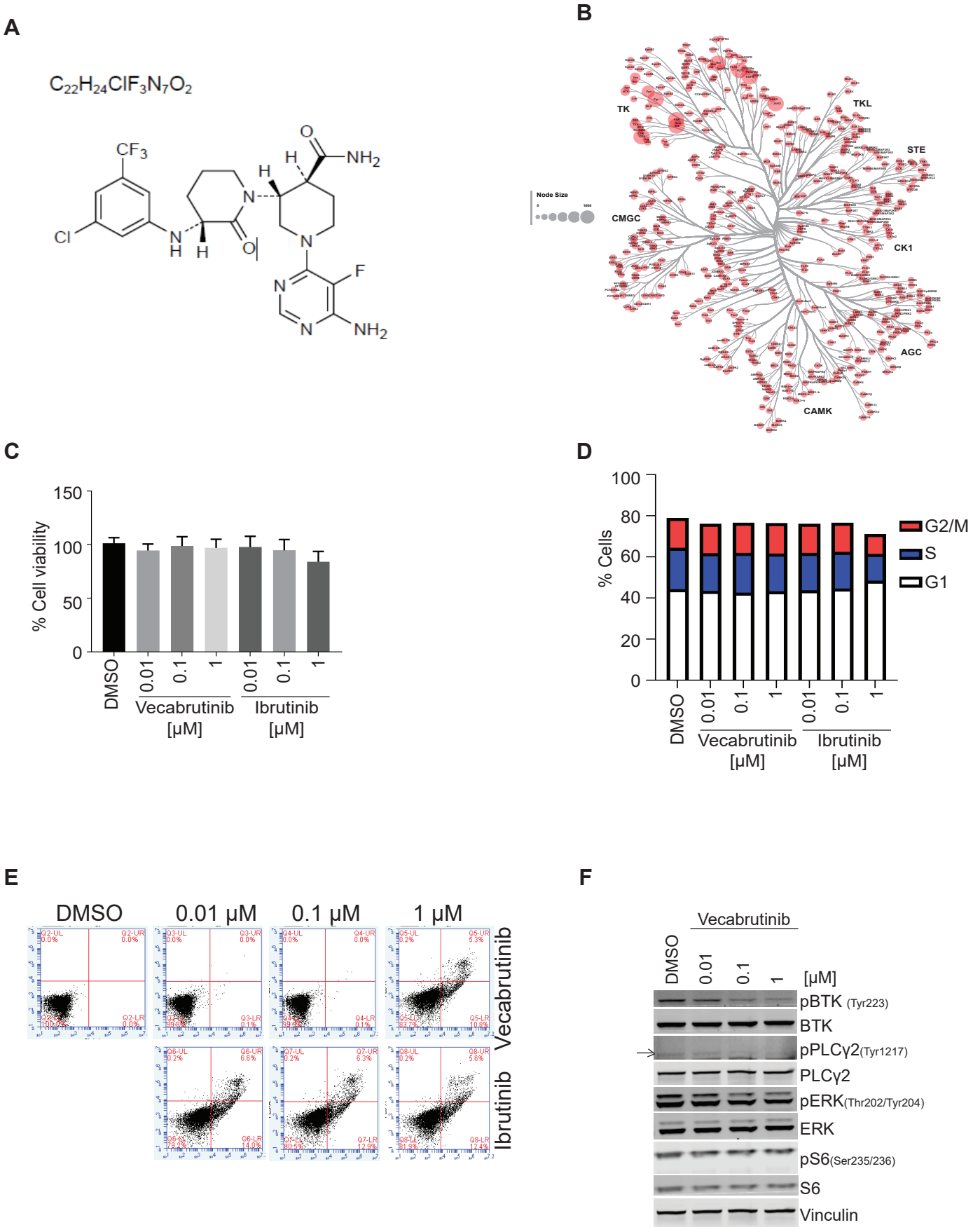
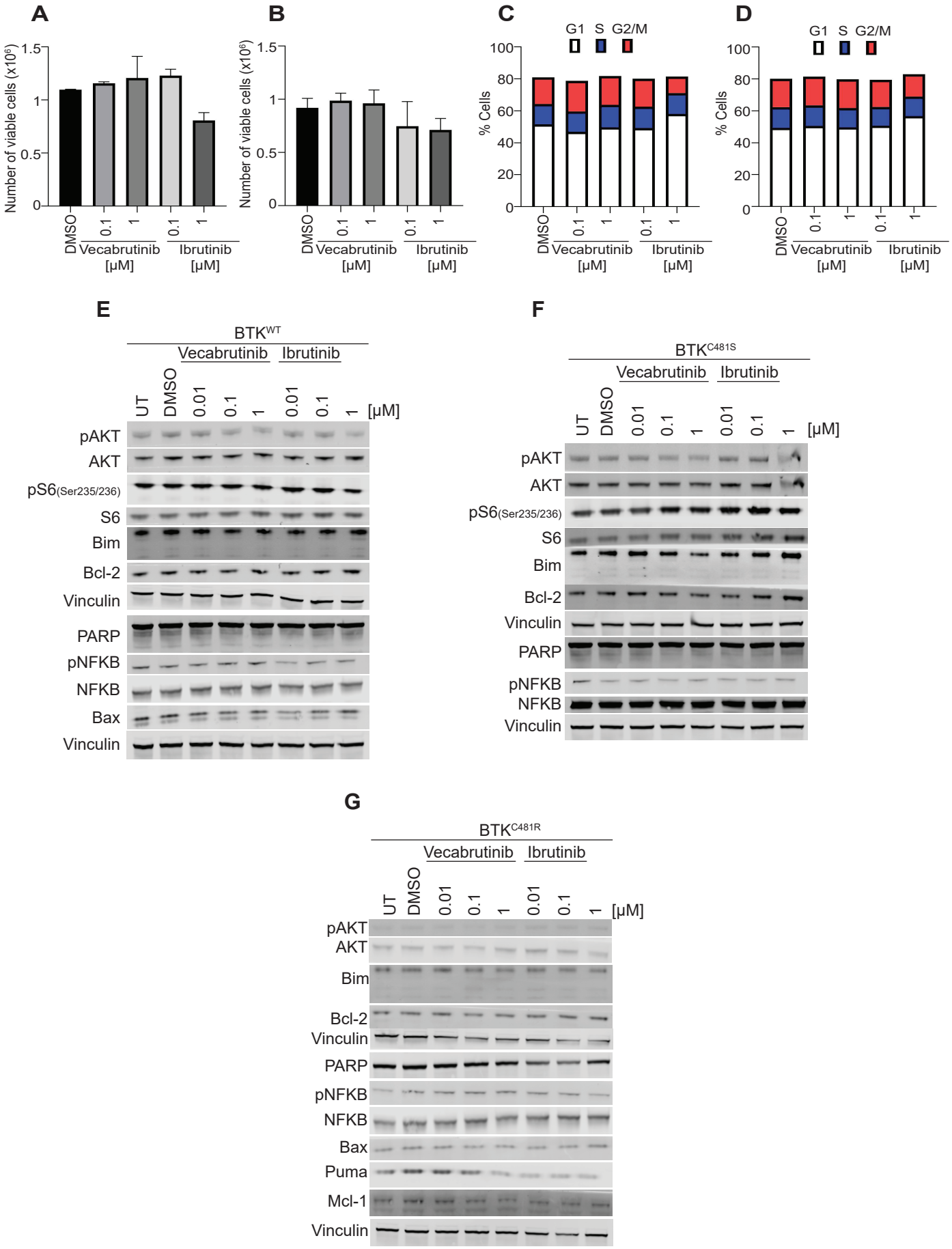
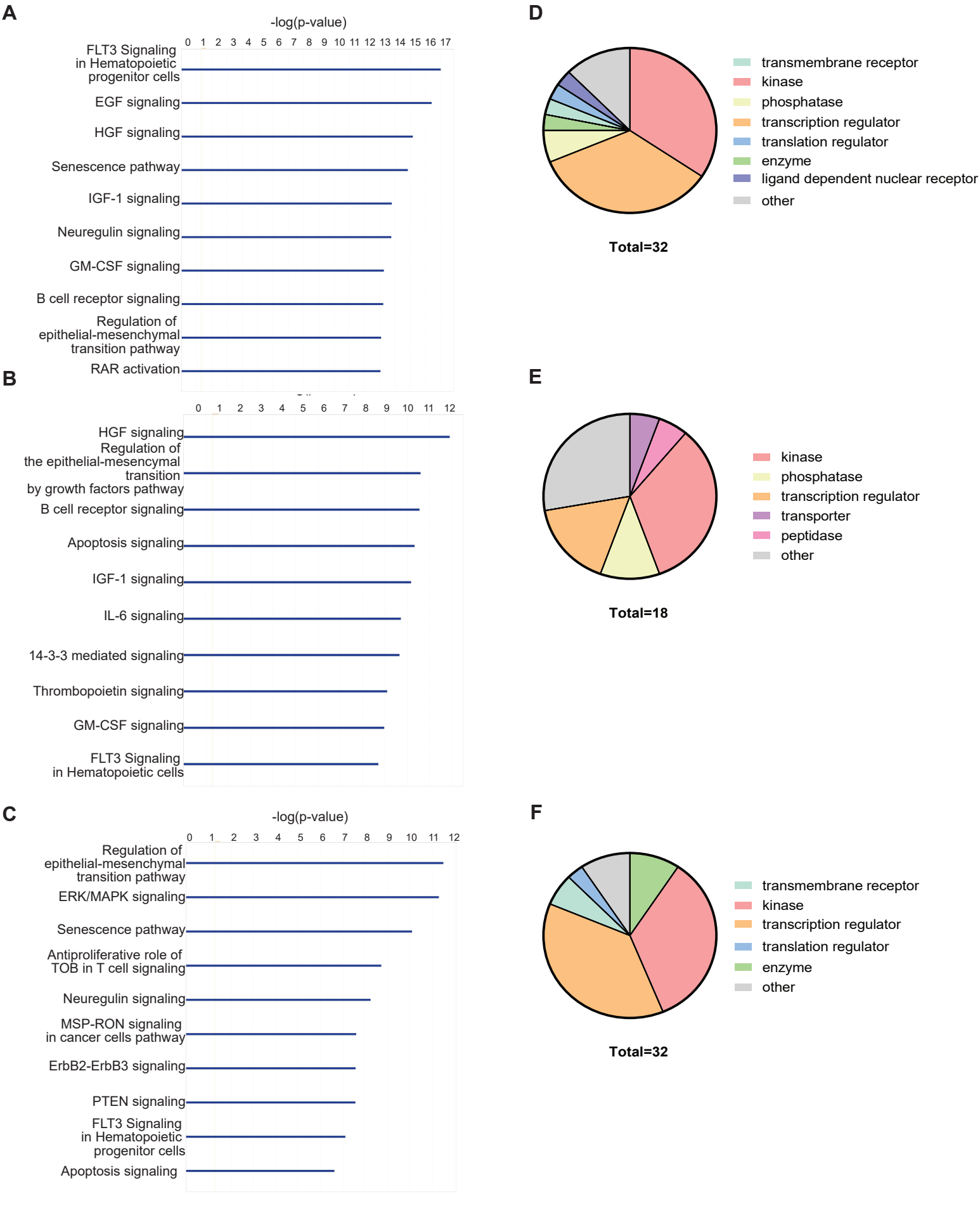
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure Legends

Supplementary Figure 1. Structure and kinome profiling of vecabrutinib and its effect on MEC-1 cell line.

(A) Chemical structure of vecabrutinib [(3R,3'R,4'S)-1'-(6-amino-5-fluoropyrimidin-4-yl)-3-[3-chloro(trifluoromethyl)anilino]-2-oxo[1,3'-bipiperidine]-4'-carboxamide]. Vecabrutinib was provided by Sunesis Pharmaceuticals. **(B)** IC_{50} values from Figure 1B were marked in an illustration of the human kinome using Coral (http://phanstiel-lab.med.unc.edu/CORAL/) to show selectivity. IC_{50} values higher than 500 nM were not plotted. **(C-D)**. Biological and molecular effects of vecabrutinib in the MEC-1 B-cell line. Exponentially growing non-transduced MEC-1 cells were incubated with vecabrutinib or ibrutinib at three concentrations (0.01 μM, 0.1 μM, and 1 μM) for 24 hours. **(C)** Effect of vecabrutinib or ibrutinib on cell viability. Cells were incubated in 96-well plates with 6 (n=6/concentration) and incubated for 24 hours. CCK8 was added to the wells, and the absorbance at 450 nm was measured using a microplate reader (n = 3). **(D)** Treated cells were fixed in 70% ethanol and stained with propidium iodide (PI), and cell cycle profiles were analyzed by flow cytometry (n = 3). **(E)** Cells treated with both drugs and stained with FITC-labeled annexin V and PI. Apoptotic cells were determined with flow cytometry. **(F)** Protein extracts were prepared from drug-treated cells and were subjected to immunoblot assays to determine levels of pBTK inhibition and downstream signaling of BTK in MEC-1 cells. Lamin B1 and vinculin were used as the loading control.

Supplementary Figure 2. The effect of vecabrutinib treatment on cell viability,cell cycle and non-BCR proteins in MEC-1 cells overexpressing WT or mutant BTK.

Green fluorescence protein (GFP)-labeled MEC-1 cell lines that stably overexpress wild-type BTK (BTK^{WT}) and mutant BTK (BTK^{C481S} or BTK^{C481R}) were generated by using standard lentiviral

transfection methods. For all experiments, cell populations that were more than 75% GFP+ were used. GFP-labeled MEC-1 cells were regularly sorted with use of a BD FACSAria Fusion Cell Sorter for the enrichment of transduced GFP+ cell populations in each cell line. **(A-B)** Cells (0.5×10^6/mL) were treated with drugs, and viable cells were counted at 24 hours using Vi-Cell XR Cell Viability Analyzer (n = 3). The graphs show the viability in **(A)** BTK^{C481S} and in **(B)** BTK^{C481R} cells. **(C-D)** Exponentially growing cells (0.5×10^6/mL) were incubated with drugs for 24 hours. At endpoint, cells were fixed in 70% ethanol, stained with PI, and analyzed by flow cytometry (n = 3). The graphs show the cell cycle profiles in **(C)** BTK^{C481S} and in **(D)** BTK^{C481R} cells. **(E-G)**. Protein extracts were subjected to immunoblot assays to determine levels of phospho-AKT (Ser473), AKT, phospho-S6 (Ser235/236), S6, Bim, Bcl-2, PARP, phospho-NFKB(Ser547), NFKB, Bax, Puma, and Mcl-1 in MEC-1 cells overexpressing **(E)** BTK^{WT}, **(F)** BTK^{C481S}, and **(G)** BTK^{C481R}. Vinculin was used as the loading control.

Supplementary Figure 3 Effect of vecabrutinib treatment on functional protein profiles of wild-type and mutant BTK-overexpressing MEC-1 cells

Exponentially growing MEC-1 cells harboring either wild-type or mutant BTK were treated with vecabrutinib at three concentrations (0.01, 0.1, and 1 μM) and incubated for 24 hours. Experiments were performed in biological triplicates (n = 3/cell line). At the end point, cells were collected, and protein was extracted and was subjected to RPPA that included 258 antibodies. Change in expression ($RPPA_{treatment[1\mu M]}$-$RPPA_{DMSO}$) values were used to determine the top 10 canonical pathways were identified by using Ingenuity Pathway Analysis (IPA) and analyzed by using the log2 ratios and were associated with **(A)** BTK^{WT}, **(B)** BTK^{C481S}, and **(C)** BTK^{C481R} cells. **(A)** Following genes were in the BTK^{WT} cell line pathways: FLT3 signaling in hematopoietic progenitor cells (AKT1, AKT3, EIF4EBP1, MAP2K1, MAPK1, MAPK14, PTPN11, RPS6KA1,

RPS6KB1, STAT3), EGF signaling (AKT1, AKT3, JUN, MAP2K1, MAPK1, MAPK14, RPS6KB1, SRC, STAT3), and HGF signaling (AKT1, AK3, CCND1, ETS1, JUN, MAP2K1, MAPK1, PTK2, PTPN11, STAT3). **(B)** Following genes were in the BTK^{C481S} cell line pathways: HGF signaling (ETS1, MAP2K1, MAPK1, MAPK8, PTK2, PTPN11, STAT3), regulation of epithelial-mesenchymal transition by growth factors pathway (ETS1, MAP2K1, MAPK1, MAPK8, PTPN11, STAT3, YAP1), and B-cell receptor signaling (ETS1, GAB2, MAP2K1, MAPK1, MAPK8, PTK2, PTPN11). **(C)** Following genes were in the BTK^{C481RS} cell line pathways: epithelial-mesenchymal transition (ARAF, ETS1, MAP2K1, MAPK1, NOTCH1, NOTCH2, PDGFRB, SMAD3, STAT3), ERK/MAPK signaling (ARAF, BAD, ETS1, HSPB1, ITGB1, MAP2K1, MAPK1, RPS6KA1, STAT3), and senescence pathway (ARAF, CCNB1, CDKN1B, ETS1, MAP2K1, MAPK1, RB1, SMAD1, SMAD3). **(D-F)** Types of proteins that were altered after vecabrutinib treatment. Pie charts were generated to display the proportions between types of target proteins. Change in expression ($RPPA_{treatment[1\mu M]}$-$RPPA_{DMSO}$) values were used to determine the targets**.** Number of proteins are listed at the bottom of the pie and protein groups are listed on the right. Changes are for **(D)** BTK^{WT}, **(E)** BTK^{C481S}, and **(F)** BTK^{C481R} cells.