SUPPLEMENTARY MATERIALS FOR

Targeting CCR7-PI3Kγ overcomes resistance to tyrosine kinase inhibitors in ALK-rearranged lymphoma

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Materials and methods

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MDAR Reproducibility Checklist

Data file S1

References (*81-86*)

Supplementary Materials and methods

Virus preparation and cell transduction

Lentiviruses were produced using the 3rd generation production system. Briefly, 293T cell lines were cultured in DMEM with 10% FBS. Cells at 70% confluency were co-transfected with pVSVG, pCMVR8.74, pRSV-Rev and a lentiviral vector expressing the construct of interest. Media was replenished 12/18h after transfection, and the supernatant was collected after 24 and 48h. Collected supernatants were passed through 0.22 μm filters, concentrated by ultracentrifugation (50,000 X g for 2hr) and pellets resuspended in 500 μl of sterile PBS. For infection, 5 x 10^4 cells were exposed to the prepared lentivirus along with polybrene (8μg/ml), cells with viral particles were spun down at 2500 RPM for 90 minutes and then incubated at 37° overnight.

PI3Kγ-specific and PI3Kα-specific shRNAs were purchased from SIGMA (for PI3Kγ shRNA#1, TRCN0000033280 and shRNA#2 TRCN0000195574).

Retroviruses were generated by transfection of retroviral vectors expressing wild-type PI3Kγ or the constitutively active form PI3Kγ^{CAAX}, wild-type PI3Kδ or the mutated form PI3Kδ^{E1021K} in 293 packaging cells. Transfected cells were incubated at 37^o C for 12/18h and supernatants containing viral particles were collected at 48 hours. 300 μl retroviral supernatants were used to transduce 5x10^4 ALK+ ALCL cells as previously described (*36*). For cell sorting enrichment, cells were induced with 1ug/ml doxycycline for 12h and sorted for GFP expression on a MoFlo High-Performance Cell Sorter (DAKO Cytomation).

Cell lysis and western blotting

Total cellular proteins were extracted using GST-FISH buffer (10nM $MgCl_2$, 150nM NaCl, 1% NP-40, 2% glycerol, 1mM EDTA, 25nM HEPES pH 7.5) mixed with 1mM

phenylmethylsulfonyl fluoride (PMSF), 10mM NAF, 1nM Na_3VO_4, and protease inhibitors (Roche, Germany). Total cell lysates were processed as previously described (*81*).

The following primary antibodies were used: anti-phospho-ALK (Y1604) (Cell Signaling Technology, Catalog#3341, RRID: AB_331047), anti-ALK (Cell Signaling Technology, CloneD5F3, Catalog#3633, RRID: AB_11127207), anti-PI3Kinase p110α (Cell Signaling Technology, CloneC73F8, Catalog #4249, RRID: AB_2165248), anti-PI3Kinase p110β (Cell Signaling Technology, CloneC33D4, Catalog #3011, RRID: AB_2165246)anti-PI3Kinase p110 δ (Cell Signaling Technology, CloneD1Q7R, Catalog #34050, RRID: AB_2799043), anti-PI3Kinase p110γ (*31*), anti-phospho-STAT3 (Y705) (Cell Signaling Technology, Catalog#9131, RRID: AB_331586), anti-STAT3 (Cell Signaling Technology, Clone79D7, Catalog#4904, RRID: AB_331269), anti-phospho-SHP2 (Y524) (Cell Signaling Technology, Catalog#3751, RRID: AB_330825), anti-SHP2 (Cell Signaling Technology, CloneD50F2, Catalog#3397, RRID: AB_2174959), anti-phospho-ERK (T202/Y204) (Cell Signaling Technology, Catalog#9101, RRID: AB_331646), anti-ERK (Cell Signaling Technology, Catalog#9102, RRID: AB_330744), anti-phospho-AKT (S473) (Cell Signaling Technology, Clone D9E, Catalog#4060, RRID: AB_2315049), anti-AKT (Cell Signaling Technology, Clone11E7, Catalog#4685, RRID: AB_2225340), anti-phospho S6 (Cell Signaling Technology, clone D57.2.2E, Catalog#4858, RRID: AB_916156) and anti-S6 (Cell Signaling Technology, clone 54D2, Catalog#2317, RRID: AB_2238583), anti-β-actin (Sigma-Aldrich, Catalog#A2066, RRID: AB_476693), anti-βTubulin (Cell Signaling Technology, Clone D3U1W, Catalog #86298, RRID:) AB_2715541.

Bioplex phosphoprotein detection assay (Bio-Rad, Hercules, CA) was used with antibodies provided by the manufacturer and conducted as previously described (*31*).

Fluorescence in situ hybridization

PIK3CG probe was purchased by Thermo Fisher Scientific. Quantification of amplified *PIK3CG* was performed using the Metafer Slide Scanning System (MetaSystems Hard & Software GmbH) as previously described (*54*). The image acquisition and the analysis of the cells were performed automatically by using the Metafer Slide Scanning System (MetaSystems Hard & Software GmbH), connected to the motorized microscope ZEISS Axio Imager.Z2 (Carl Zeiss). 3-channels images of a predefined area of the samples were acquired at a magnification of 40x. The DAPI channel was defined as the counterstain, and then used by the software for the automatic identification and selection of the suitable cells for the analysis. A "Z-stack" acquisition mode was used for the Red and Aqua channels, in order to provide a tridimensional evaluation of the corresponding FISH signals.

Histology and immunohistochemistry

For histology, tissue samples were formalin fixed and paraffin embedded, cut into 4 μm thick sections and stained with hematoxylin and eosin.

For immunohistochemistry, formalin-fixed sections were de-waxed in xylene, dehydrated by passage through graded alcohols to water; sections were microwaved in citrate buffer pH 6 for 15 minutes and then transferred to PBS. Endogenous peroxidase was blocked using 1.6% hydrogen peroxide in PBS for 10 minutes followed by washing in distilled water. Normal serum diluted to 10% in 1% BSA was used to block non-specific staining. The slides were then incubated for 1 hour with the following primary antibodies: anti-PI3Kγ (*31*); anti-ALK (clone 18-0266, Zymed); anti-CD30 (Dako); anti-Cleaved Caspase-3 (Asp175) (Cell Signaling Technology, Catalog#9661, RRID: AB_2341188), anti-murine Ki-67 (Abcam, Clone SP6, Catalog# ab16667, RRID: AB_302459). After washing, sections were incubated with biotinylated secondary goat antibody to rabbit IgG and visualized with the EnVision system (Dako).

Immunohistochemistry results for PI3Kγ have been evaluated by assigning an H-score to tumor samples as previously described (*82*).

Quantitative Real-Time (qRT) PCR analysis

Total RNA was extracted from cells using TRIZOL solution (Invitrogen), followed by cDNA preparation from 1 μg of total RNA. cDNA products were quantified by real-time PCR using SYBR Green Supermix (Bio-Rad) on CFX Opus Real-Time PCR System (Bio-Rad). Normalization was performed against the housekeeping human acidic ribosomal protein (HuPO) or actin according to the formula $2^{-\Delta\Delta Ct}$, where the $\Delta C_t = C_t$ (threshold cycle) gene of interest $-C_t$ internal control, as indicated by the manufacturer.

Gene	**Forward**	**Reverse**
PIK3CG	GGAGCGCTGGCAATTGAA	CGCAGATCATCACCATGTTT
PIK3CA	CAATCCCAGGTGGAATGAAT	TCCTCTTTAGCACCCTTTCG
PIK3CD	GCGGATGAAGCTGGTGGT	CAGGTCGCAGATGTTGATGT
CCR7	CTGGTGGTGGCTCTCCTTGTCA	GTTCCGCACGTCCTTCTTGG
HuPO	GCTTCCTGGAGGGTGTCC	GCTTCCTGGAGGGTGTCC

Cell proliferation and apoptosis assays

Cell proliferation analysis was performed using CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions, as previously described (*36*). Briefly, cells were plated in white walled 96-well plates (3 wells/sample) and analysed each day until day 4 using the GloMax-Multi Detection System (Promega).

For apoptosis assay cells were stained with 200nM tetrametylrodamine methyl-ester (TMRM) for 15 minutes in dark, washed twice in PBS and the percentage of apoptotic cells was measured by flow cytometry (BD FACSCALIBUR) using CellQuest Program.

Cell viability and drug sensitivity assays

Cell viability assay on human ALK+ ALCL cell lines was performed using MTS CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) Powder, Promega or CellTiter-Glo (Promega) according to manufacturer's instructions, as previously described (*36*). Briefly, cells were seeded into white walled 96-well plates (3 wells/sample). CellTiter-Glo reagent was added to each well and luminescence output data were taken at 0, 24, 48 and 72 hours by GloMax-Multi Detection System (Promega). All experimental points were a result of three to six replicates, and all experiments were repeated at least twice.

Drug sensitivity assays have been performed as previously described (*36*). Briefly, cells (2×10^3) were seeded in 96-well plates in RPMI complete medium. The following day, cells were treated with ALK TKIs (crizotinib, brigatinib, ceritinib, alectinib or lorlatinib using a ten-point dose titration scheme from 1nM to 1μM. After 48 or 72 hours, cell viability was assessed using CellTiter-Glo Luminescent Cell Viability Assay (Promega). Absolute inhibitory concentration (IC) values were calculated using four-parameter logistic curve fitting. All experimental points were a result of three to six replicates, and all experiments were repeated at least twice. The data were graphically displayed using GraphPad Prism 7 for Windows (GraphPad Software). Each point (mean ± standard deviation) represents growth of treated cells compared to untreated cells. The curves were fitted using a non-linear regression model with a sigmoidal dose response.

Chemokine assay

CCL19/21 in vitro stimulation was performed in ALK$^+$ ALCL cell lines. CCL19/21 chemokines (Human CCL19 (MIP-3β) and Human Exodus-2 (CCL21);) were diluted to 10ng/μL in 1%BSA/PBS. Cells were diluted to 3x10^6 cells/mL in complete RPMI 1640 and incubated with crizotinib (either 40nM or 300nM) or equal volume of vehicle (DMSO) for 3 hours at 37ºC and 5% CO_2. After incubation, cells were stimulated with 20ng/mL of CCL19 and CCL21, or equal volume of vehicle (1%BSA/PBS) for 1, 5 and 10 minutes. After cytokines stimulation, cells were harvested, and total proteins were extracted.

Flow cytometry analysis

CCR7 expression was evaluated with two different antibodies: PE-conjugated monoclonal rat anti-human CCR7 (CD197; clone 3D12) (BD Pharmingen) and PE-conjugated monoclonal mouse anti-human CCR7 (CD197; clone FR 11-11E8) (Miltenyi Biotec). Isotype control was performed with PE-conjugated mouse IgG1, k isotype Ctrl antibody (clone MOPC-21) (Biolegend). Briefly, from each cell line, 1x10^4 cells were washed in FACS buffer (PBS containing 1% of FBS and 2nM of EDTA) and then stained with CCR7 antibodies or isotype control for 20 minutes at 4ºC in the dark. Next, cells were washed twice with FACS buffer and events were acquired using the BD FACSCelesta flow cytometer (BD Biosciences). CCR7 expression profiling with anti-CCR7 CAP-100 was evaluated as previously described (*63*). To this end, indirect staining was performed by means of a PE-labeled goat anti-human IgG1 secondary antibody, acting as the detection antibody. Data analysis was performed with FlowJo software version 7 (FlowJo LLC, EUA).

RNA sequencing on crizotinib-sensitive and -resistant ALCL cell lines

Total RNA purification. RNA was purified from wild type and crizotinib resistant cell lines using RNeasy mini kit (Qiagen). Total RNA was estimated quantitatively and qualitatively with

Qubit RNA BR kit (ThermoFisher Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies) and then stored at -80°C until use. 1 μg of RNA was treated with DNAse (DNA-free kit, Ambion) and used for RNA-sequencing.

RNA-sequencing. Coding transcriptome was sequenced from libraries generated using TruSeq RNA Access library prep kit (Illumina) following manufacturer's instructions and starting from 40 ng of DNA-free RNA from wild type and crizotinib resistant cells. Libraries were quantified with Qubit DNA ds HS (LifeTechnologies) and run at the concentration of 1.6 pM on the NextSeq500 Illumina sequencer in 75 nts paired end sequencing mode following manufacturer instruction.

Bioinformatics analysis. Analyses were performed using the tools embedded in docker4seq package [https://pubmed.ncbi.nlm.nih.gov/29069297/], to guarantee bioinformatically reproducible results [https://pubmed.ncbi.nlm.nih.gov/30367595/]. Coding transcriptome RNAseq quantification was done using STAR/RSEM (*83, 84*). Reads were mapped against human genome (hg38) using ENSEMBL GTF (version 99) annotation. Differential expression analysis was done using DESeq2 Bioconductor package (*85*). RNAs with a $|Log_2FC| \geq 1$ and an adjusted p-value ≤ 0.1 were considered as differentially expressed. Principal Component Analysis (PCA), Venn diagrams and unsupervised hierarchical clustering were used to inspect and compare the expression data. Genes annotated. in the PI3K-AKT KEGG pathway (https://www.genome.jp/dbget-bin/www_bget?pathway+hsa04151).

Single cell RNA sequencing (scRNA-seq)

Single cell libraries generated with the Chromium Single Cell 3' Reagents were initially quality checked using the Tapestation instrument (Agilent) and then sequenced on a Novaseq 6000 (Illumina). Feature matrices were generated from raw fastq reads using cellranger v. 6.0.1; a total of 23255 and 17342 cells were successfully barcoded for primary lymph node and

PDX, respectively. Filtered count matrices were processed using Seurat package v4.0.4 (*86*). Individual experiments were loaded using the Read10X function. Per-cell gene counts, Unique Molecular Identifier (UMI) and fraction of mitochondrial reads were plotted to perform initial quality-check and filtering. UMI counts were subsequently normalized using a regularized negative binomial regression through the SCTransform function, while regressing for UMI and mitochondrial read fraction. Dimensionality reduction was initially performed by Principal Component Analysis (PCA) done on pearson residuals of the negative binomial regression, initially keeping the first 40 principal components. Uniform Manifold Approximation and Projection was then applied on the first 30 components of the PCA. Clusters were identified using the Louvain algorithm; the optimal cluster number was identified with Clustree v. 0.4.3. Cell identity was assessed both with SingleR, using the Human Primary Cell Atlas and the Encode databases, as well as by manual inspection of cell-specific markers.

Chromatin immunoprecipitation

ChIP-seq data were analyzed from previously published databases (*36*). Briefly, output BAM files were converted into BigWig track files using the 'callpeak' function of MACS2 v2.1.1 with the '-B –SPMR' option followed by the use of the BEDTools51 'sort' function and the UCSC utility 'bedgraphToBigWig'52, and the tracks were visualized in IGV v2.4.3. Narrow peaks were called with the MACS2 v2.1.1 software using input as controls and a q value cutoff of 0.00153. Metaplots and heatmaps were generated using ngsplot v2.6154.

Supplementary Figures

Fig. S1

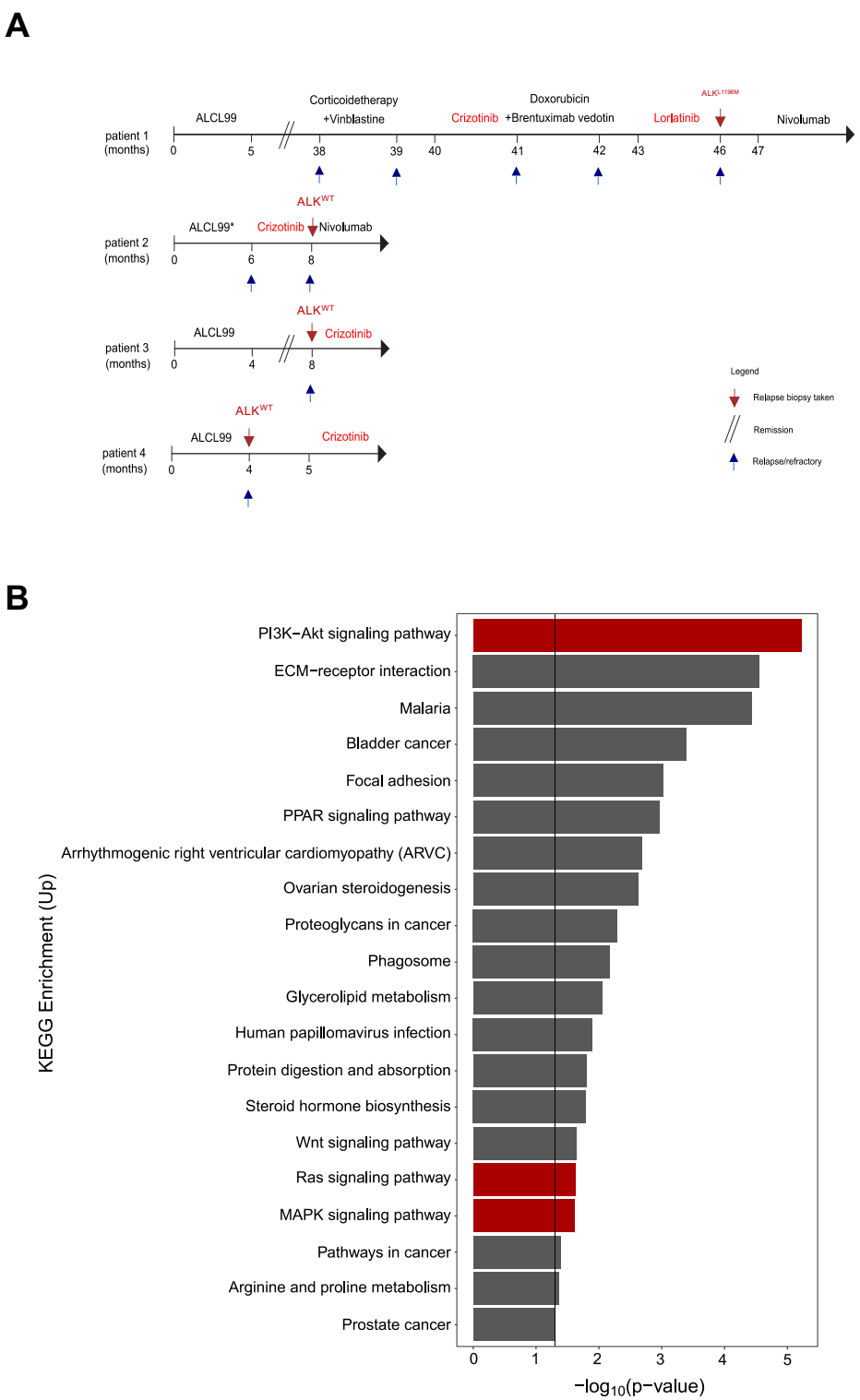


Fig. S1. PI3K-AKT signaling pathway is up-regulated in crizotinib-resistant ALCL patient tissue and cell lines. (**A**) Scheme of the treatment history of patients with ALCL(*17*). The red arrow indicates when the sample for RNA-seq was collected. (**B**) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for up-regulated genes identified by RNA-seq in samples from patients with ALK+ ALCL that relapsed on ALK inhibitor treatment (n = 2) versus sensitive to ALK inhibitor treatment (n = 2).

Fig. S2

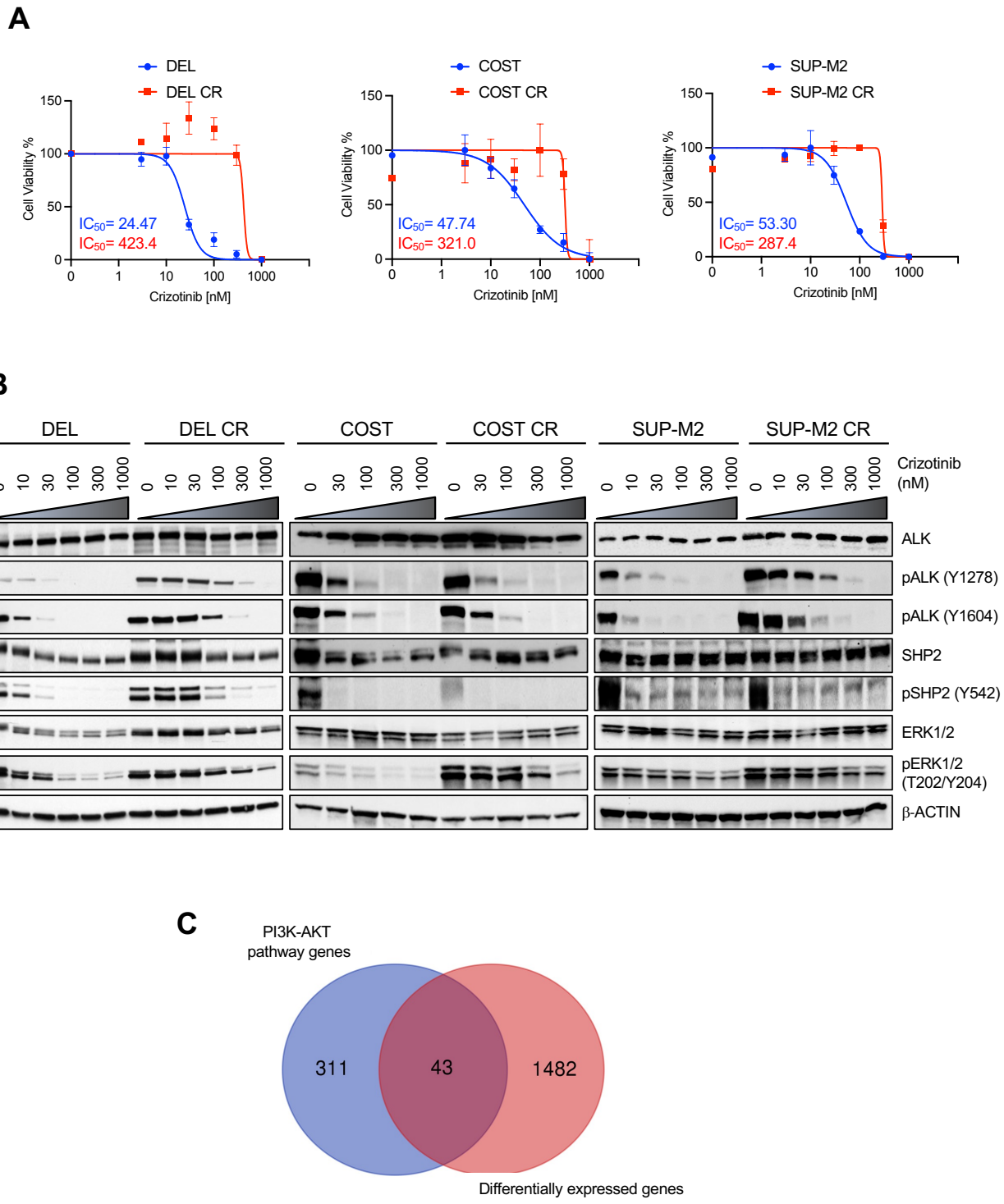
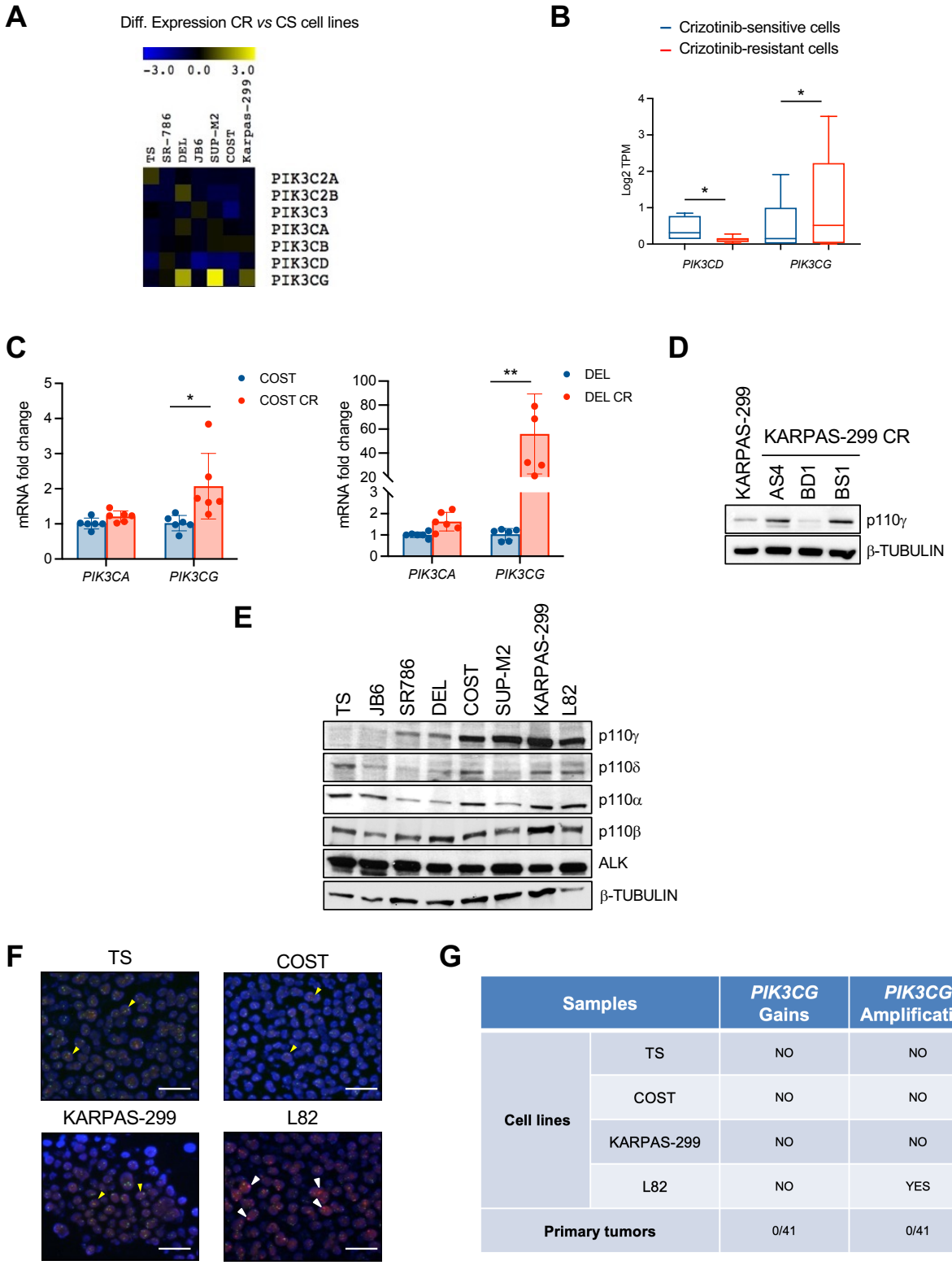

A
DEL
DEL CR
COST
COST CR
SUP-M2
SUP-M2 CR
Cell Viability %
Crizotinib [nM]
IC50= 24.47
IC50= 423.4
IC50= 47.74
IC50= 321.0
IC50= 53.30
IC50= 287.4
B
Crizotinib (nM)
ALK
pALK (Y1278)
pALK (Y1604)
SHP2
pSHP2 (Y542)
ERK1/2
pERK1/2 (T202/Y204)
β-ACTIN
C
PI3K-AKT pathway genes
311
43
1482
Differentially expressed genes

Fig. S2. Characterization of crizotinib-resistant ALCL cell lines. (**A**) Dose-response curves of 3 representative crizotinib-sensitive and crizotinib-resistant (CR) ALK+ ALCL cell lines (DEL versus DEL CR, COST versus COST CR, SUP-M2 versus SUP-M2 CR) treated with increasing concentrations of crizotinib for 72h. *n*=3 technical replicates. (**B**) Western blot analysis on crizotinib-sensitive and resistant (CR) ALK+ ALCL cell line treated with increasing concentrations of crizotinib for 3h. (**C**) Venn diagram indicating the number of genes differentially expressed in crizotinib-sensitive and resistant ALK+ ALCL cell lines and associated with the PI3K-AKT pathway genes. Fisher test p-value: 0.0012. Data are shown as means ± s.d. For western blots, β–actin was used as a loading control and two independent experiments with similar results were performed.

Fig. S3



Samples		*PIK3CG* Gains	*PIK3CG* Amplification
Cell lines	TS	NO	NO
	COST	NO	NO
	KARPAS-299	NO	NO
	L82	NO	YES
Primary tumors		0/41	0/41

Fig. S3. PI3Kγ is up-regulated in crizotinib-resistant ALCL cells. (**A**) Heat map showing the odds ratios for the detection of PI3K genes based on the RNA-seq data in paired crizotinib-sensitive (CS) and crizotinib-resistant (CR) ALK+ ALCL cells (only detected PIK3 genes are shown). Gene expression is shown as the log2 ratio between each gene and the average expression of the same gene in all cell lines. (**B**) mRNA expression for *PIK3CG* and *PIK3CD* in crizotinib-sensitive (CS) and crizotinib-resistant (CR) ALK+ ALCL cells. The boxes represent the first and third quartiles and the line represents the median. The whiskers represent the upper and lower limits of the range. TPM: Transcripts Per Million. (**C**) qRT-PCR expression analysis of *PIK3CA* and *PIK3CG* mRNA performed on paired crizotinib-sensitive and resistant ALK+ ALCL cells. *n*=3 technical replicates. (**D**) Immunoblot analysis of PI3Kγ expression performed on lorlatinib-sensitive and resistant ALCL cell line KARPAS-299 xenografts. (**E**) Immunoblot analysis performed on human ALK+ ALCL cells lines. (**F**) FISH analysis of ALK+ ALCL cell lines. Yellow arrows indicate normal content of DNA. White arrows indicate amplification of PIK3CG locus. (**G**) Gains or amplification of *PIK3CG* detected by FISH in 41 primary ALCL samples and cell lines. Data are shown as means ± s.d. **P*<0.05, ***P*<0.01. Significance was determined by unpaired, two-tailed Student's t-test.

Fig. S4

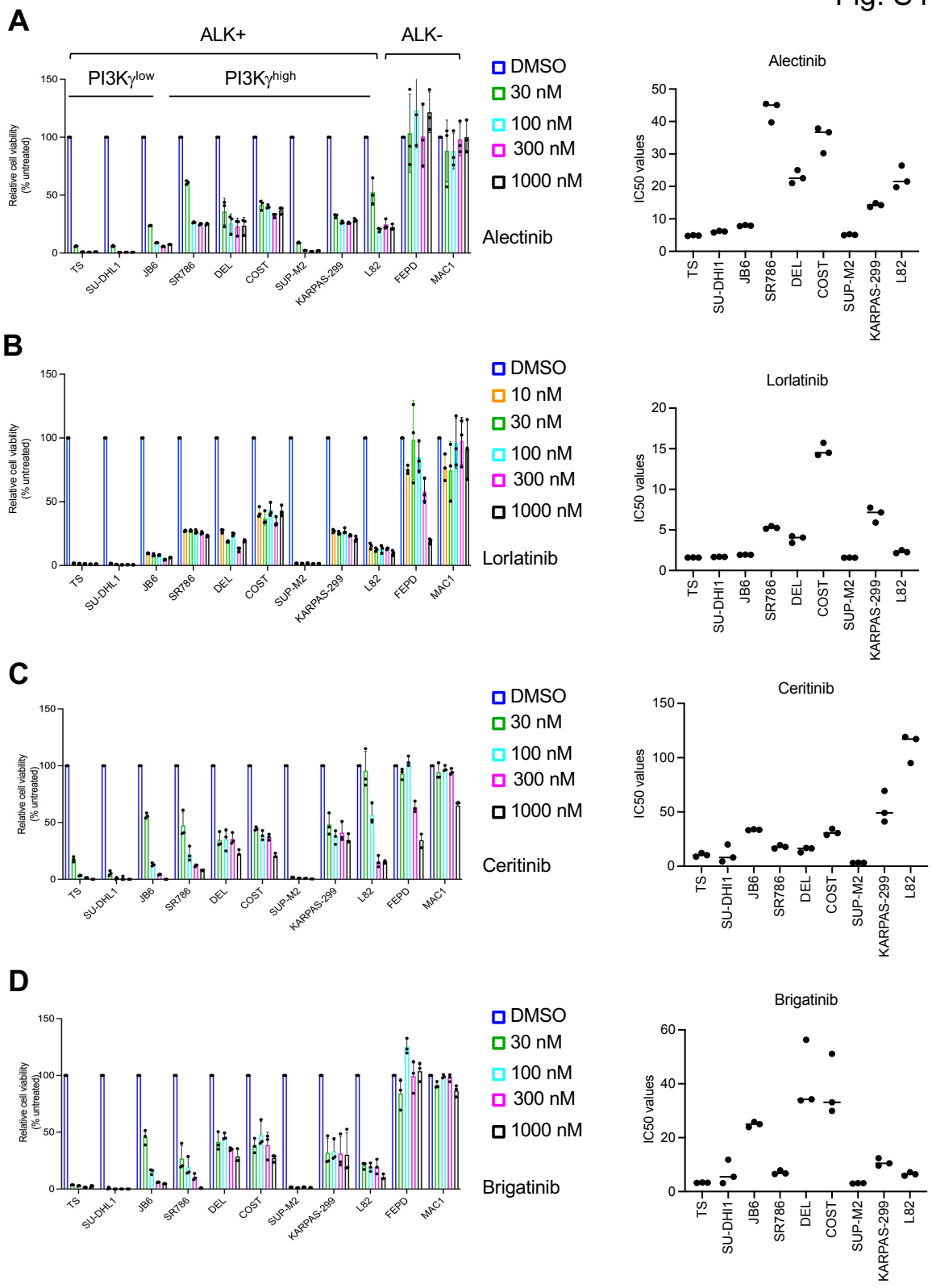
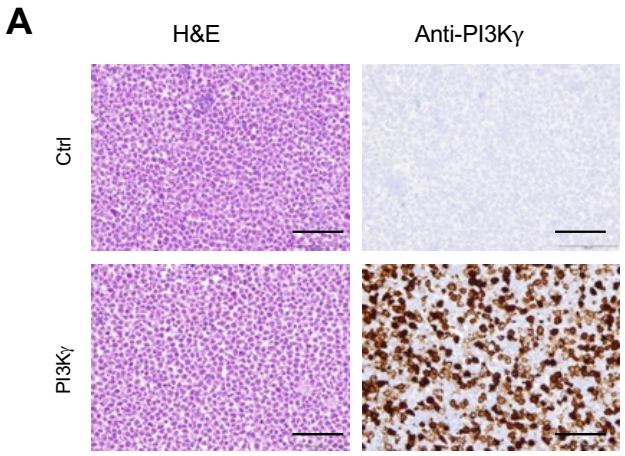

A
ALK+
ALK-
PI3Kγlow
PI3Kγhigh
Relative cell viability (% untreated)
DMSO
30 nM
100 nM
300 nM
1000 nM
Alectinib
Alectinib
IC50 values
B
DMSO
10 nM
30 nM
100 nM
300 nM
1000 nM
Lorlatinib
Lorlatinib
C
DMSO
30 nM
100 nM
300 nM
1000 nM
Ceritinib
Ceritinib
D
DMSO
30 nM
100 nM
300 nM
1000 nM
Brigatinib
Brigatinib
TS
SU-DHL1
JB6
SR786
DEL
COST
SUP-M2
KARPAS-299
L82
FEPD
MAC1

Fig. S4. High expression of PI3Kγ induce spontaneous resistance to ALK TKIs in ALK+ ALCL. (**A**-E**D**) Relative cell viability measured using CellTiter Glo. ALK+ and ALK- ALCL cells treated with increasing concentrations of ALK TKIs, alectinib (**A**), lorlatinib (**B**), ceritinib (**C**), brigatinib (**D**) (left panels). IC50 values are reported for each cell line (right panels). *n* = 3 technical replicates. Data are shown as means ± s.d.

Fig. S5


A
H&E
Anti-PI3Kγ
Ctrl
PI3Kγ

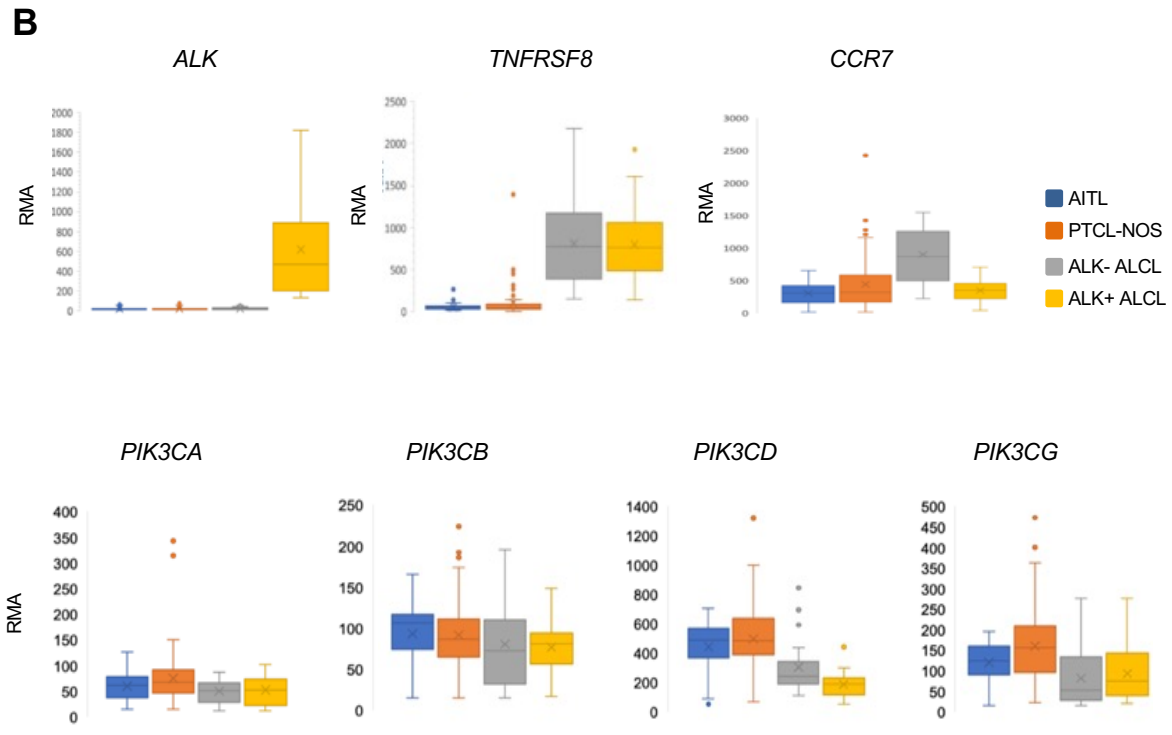

B
ALK
TNFRSF8
CCR7
RMA
AITL
PTCL-NOS
ALK- ALCL
ALK+ ALCL
PIK3CA
PIK3CB
PIK3CD
PIK3CG

Fig. S5. Expression of PI3Kγ in primary T cell lymphomas. (**A**) Validation of PI3Kγ antibody by IHC on formalin-fixed samples of cells transduced with a PI3Kγ encoding retrovirus. Scale bar = 100μm. (**B**) mRNA expression for *ALK, TNFSRF8 (CD30*)(*17*)*, CCR7, PIK3CA*, *PIK3CB*, *PIK3CD* and *PIK3CG* on different primary cases of human AITL (*n*=40), PTCL (*n*=74), ALK- ALCL (*n*=24) and ALK+ ALCL (*n*=30). The boxes represent the first and third quartiles and the line represents the median. The whiskers represent the upper and lower limits of the range. RMA: Robust Multi-Array Average.

Fig. S6

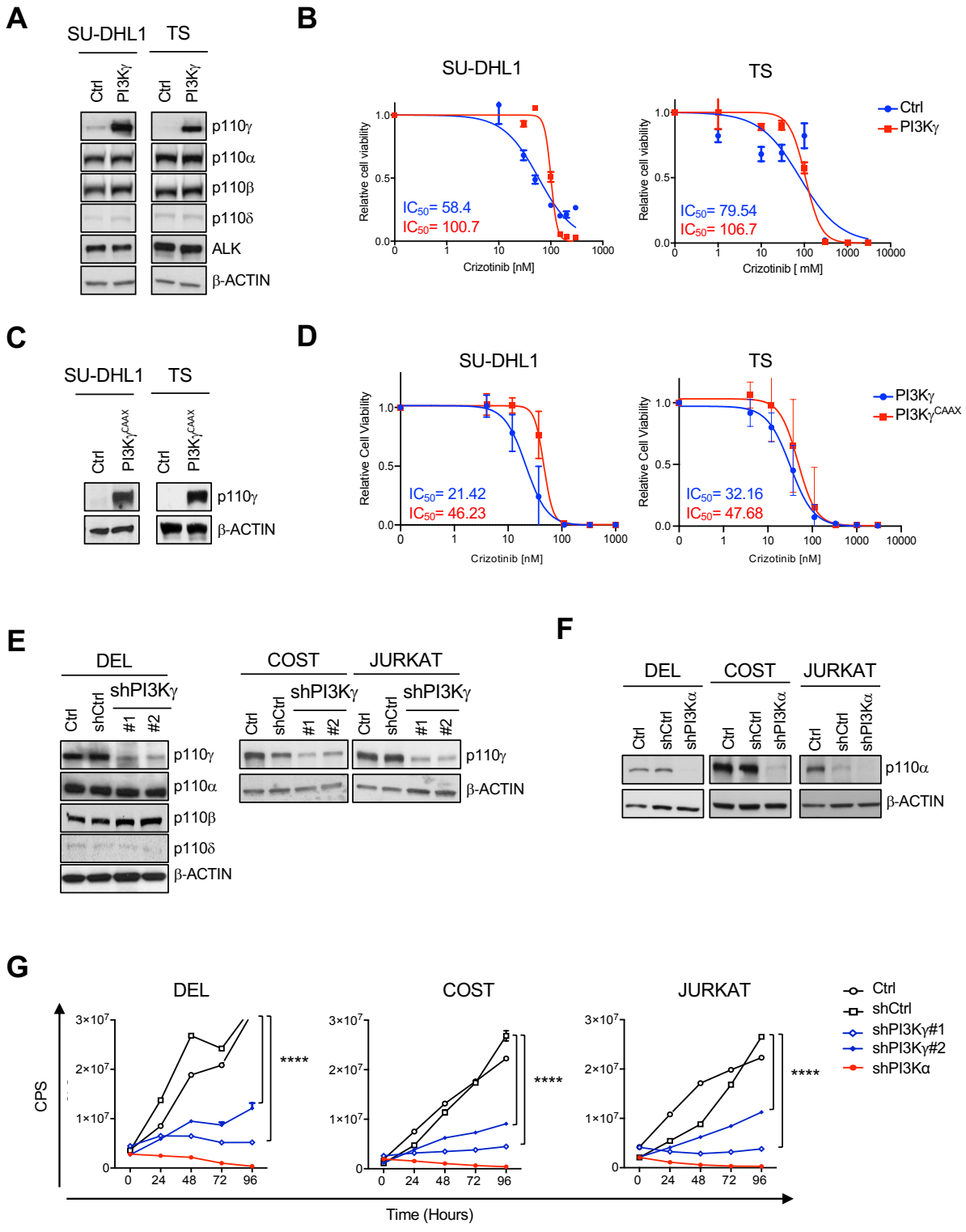

A
SU-DHL1
TS
Ctrl
PI3Kγ
p110γ
p110α
p110β
p110δ
ALK
β-ACTIN
B
SU-DHL1
TS
Relative cell viability
IC50= 58.4
IC50= 100.7
IC50= 79.54
IC50= 106.7
Crizotinib [nM]
Crizotinib [mM]
Ctrl
PI3Kγ
C
PI3Kγ^CAAX
D
Relative Cell Viability
IC50= 21.42
IC50= 46.23
IC50= 32.16
IC50= 47.68
E
DEL
COST
JURKAT
shCtrl
shPI3Kγ
#1
#2
F
shPI3Kα
G
CPS
Time (Hours)
shPI3Kγ#1
shPI3Kγ#2
shPI3Kα
****

Fig. S6. PI3Kγ mediates ALK TKI resistance in ALK+ ALCL. (**A**) Western blot analysis on low PI3Kγ ALK+ ALCL human cell lines (SU-DHL1 and TS) transduced with a PI3Kγ encoding retrovirus (PI3Kγ) or with an empty retrovirus (Ctrl). (**B**) Relative cell viability of ALK+ ALCL cells transduced as in (**A**) and treated with increasing concentration of crizotinib for 48h. *n*=3 technical replicates. (**C**) Western blot analysis on SU-DHL1 and TS cells transduced with an active form of PI3Kγ (PI3Kγ^{CAAX}). (**D**) Relative cell viability of SU-DHL1 and TS cells transduced with wild-type PI3Kγ or with PI3Kγ^{CAAX} and treated with increasing concentrations of crizotinib for 72h. *n*=4 biological replicates. (**E** and **F**) Western blot analysis on ALK+ ALCL human cells highly expressing PI3Kγ (DEL and COST) and, as a control cell line, a T cell lymphoma cell line (JURKAT) transduced with PI3Kγ shRNA (**E**), PI3Kα shRNA (**F**) or a scrambled shRNA (shCtrl). Two independent shRNA have been used to target PI3Kγ. (**G**) Cell growth of DEL, COST and JURKAT cell lines transduced as in (**E**) and (**F**). Cell proliferation was measured using CellTiter Glo at indicated time points. *n*=3 technical replicates. For western blots, β–actin was used as a loading control. Blots are representative of two independent experiments with similar results. CPS: Count Per Seconds. Data are shown as mean ± s.d. ****P<0.0001. Significance was determined by unpaired, two-tailed Student's t-test.

Fig. S7

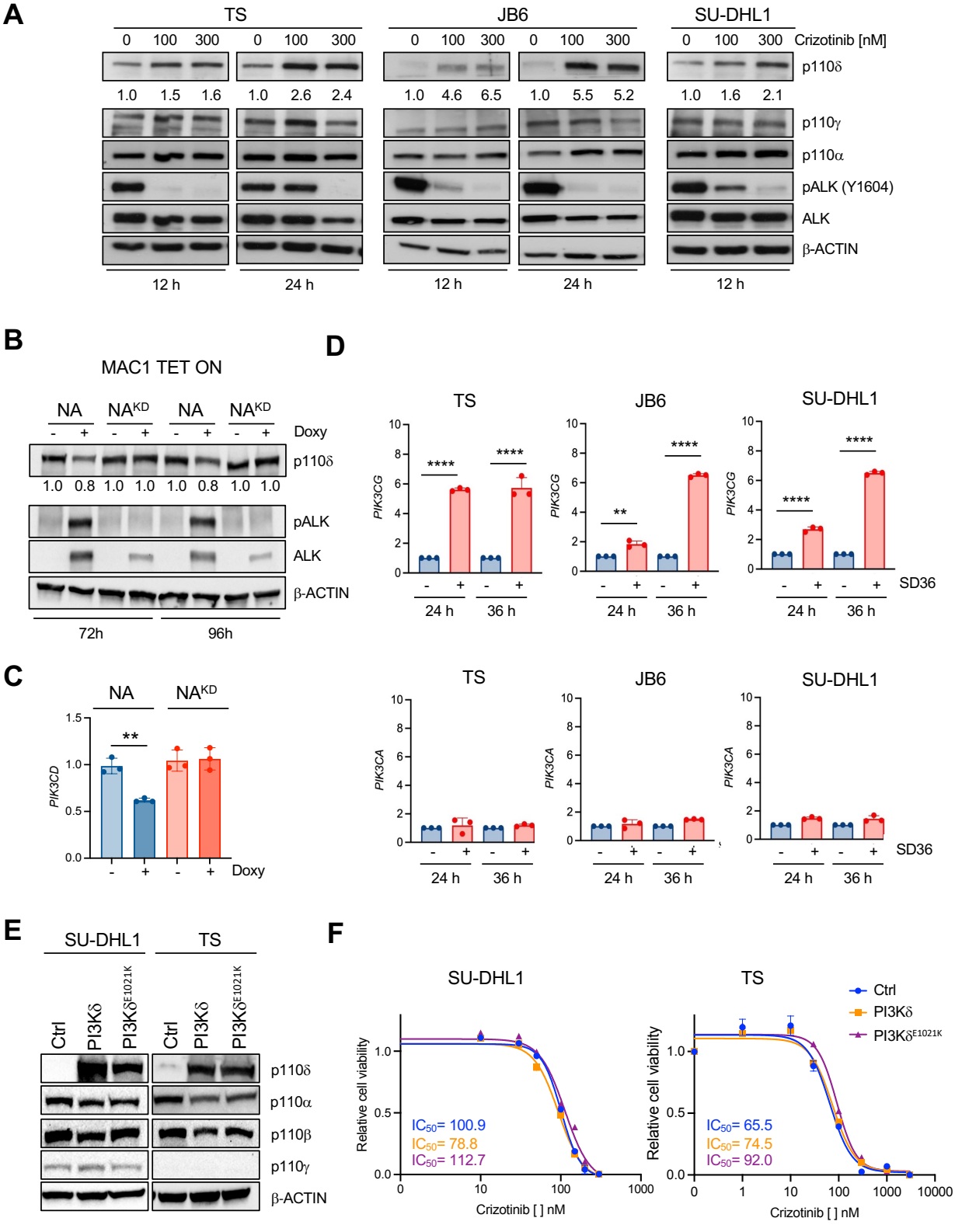

A
TS
JB6
SU-DHL1
0 100 300 0 100 300 0 100 300 0 100 300 0 100 300 Crizotinib [nM]
p110δ
1.0 1.5 1.6 1.0 2.6 2.4 1.0 4.6 6.5 1.0 5.5 5.2 1.0 1.6 2.1
p110γ
p110α
pALK (Y1604)
ALK
β-ACTIN
12 h 24 h 12 h 24 h 12 h
B
MAC1 TET ON
NA NA^KD NA NA^KD
Doxy
p110δ
1.0 0.8 1.0 1.0 1.0 0.8 1.0 1.0
pALK
ALK
β-ACTIN
72h 96h
C
NA NA^KD
PIK3CD
Doxy
D
TS JB6 SU-DHL1
PIK3CG
24 h 36 h
SD36
PIK3CA
E
SU-DHL1 TS
Ctrl PI3Kδ PI3Kδ^E1021K
p110δ
p110α
p110β
p110γ
β-ACTIN
F
SU-DHL1 TS
Ctrl
PI3Kδ
PI3Kδ^E1021K
Relative cell viability
IC50= 100.9
IC50= 78.8
IC50= 112.7
IC50= 65.5
IC50= 74.5
IC50= 92.0
Crizotinib [ ] nM

Fig. S7. PI3Kδ is repressed by ALK in ALK+ ALCL. (**A**) Western blot analysis on TS, JB6 and SU-DHL1 ALK+ ALCL human cell lines treated with different concentrations of crizotinb (100nM and 300nM). (**B**) Western blot analysis of ALK- ALCL cell line (MAC1) transduced with a doxycycline-inducible lentivirus (TET ON) expressing NPM-ALK or a kinase-dead form of NPM-ALK (NPM-ALK^{K210R}). (**C**) qRT-PCR analysis of *PIK3CD* mRNA expression performed on MAC1 TET ON cells transduced as in (**B**). *n*=3 technical replicates. **(D**) qRT-PCR analysis of *PIK3CG* (top panels) and *PIK3CA* (bottom panels) mRNA expression performed on ALK+ ALCL cell lines treated with SD36 (1μM) *n*=3 technical replicates. (**E**) Western blot analysis on SU-DHL1 and TS cells transduced with a wild-type PI3Kδ or an active form of PI3Kδ (PI3Kδ^{E1021K}). Control cells were transduced with an empty retrovirus (Ctrl). (**F**) Relative cell viability of SU-DHL1 and TS cells transduced with wild-type PI3Kδ or with PI3Kδ^{E1021K} and treated with increasing concentrations of crizotinib for 72h. *n*=3 technical replicates. For western blots, β–actin was used as a loading control and two independent experiments with similar results were performed. Data are shown as mean ± s.d. $^{**}P<0.01$, $^{****}P<0.0001$. Significance was determined by unpaired, two-tailed Student's t-test.

Fig. S8

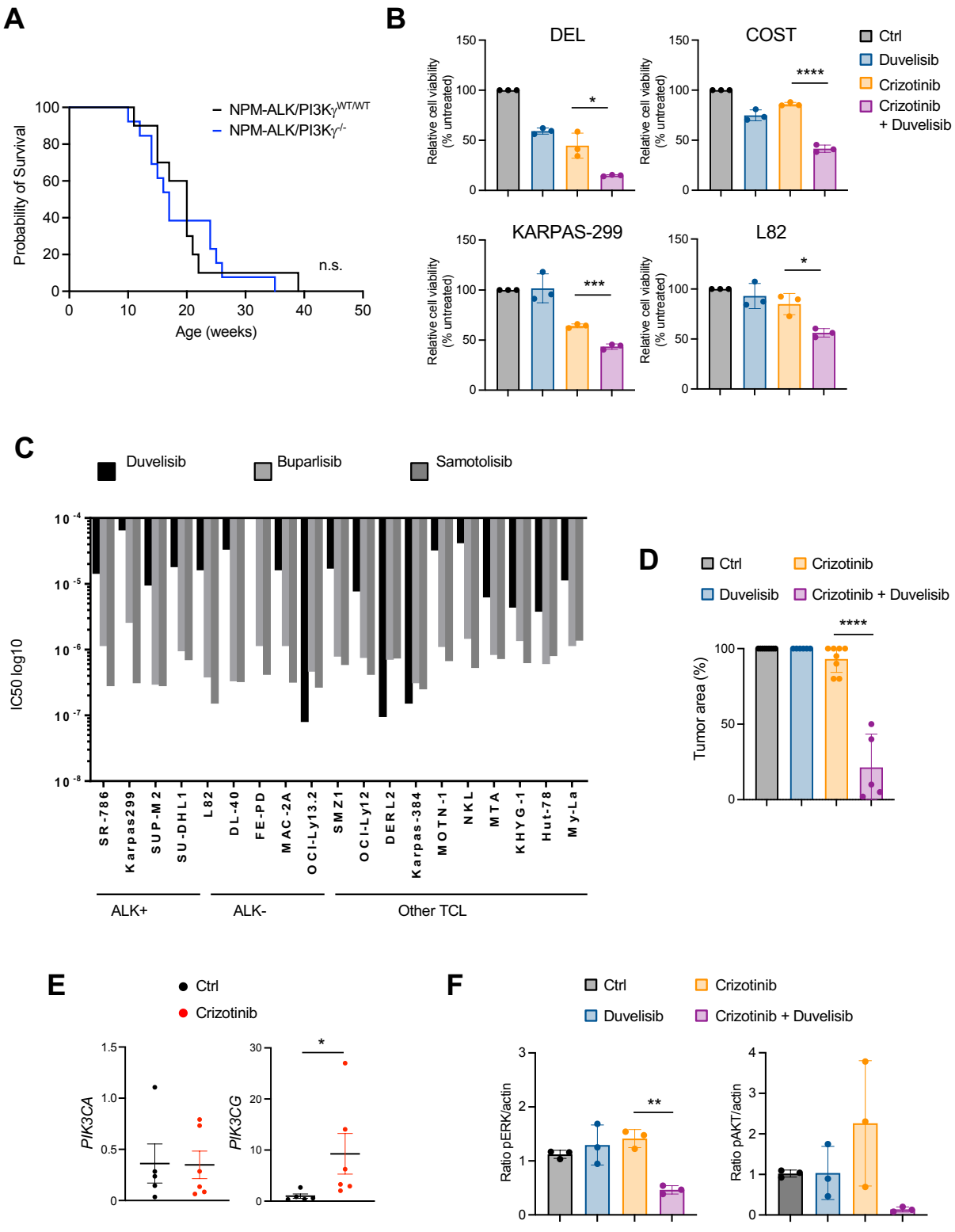

A
Probability of Survival
Age (weeks)
NPM-ALK/PI3Kγ WT/WT
NPM-ALK/PI3Kγ -/-
n.s.
B
DEL
COST
KARPAS-299
L82
Relative cell viability (% untreated)
Ctrl
Duvelisib
Crizotinib
Crizotinib + Duvelisib
C
Duvelisib
Buparlisib
Samotolisib
IC50 log10
SR-786
Karpas299
SUP-M2
SU-DHL1
L82
DL-40
FE-PD
MAC-2A
OCI-Ly13.2
SMZ1
OCI-Ly12
DERL2
Karpas-384
MOTN-1
NKL
MTA
KHYG-1
Hut-78
My-La
ALK+
ALK-
Other TCL
D
Ctrl
Crizotinib
Duvelisib
Crizotinib + Duvelisib
Tumor area (%)
E
Ctrl
Crizotinib
PIK3CA
PIK3CG
F
Ctrl
Crizotinib
Duvelisib
Crizotinib + Duvelisib
Ratio pERK/actin
Ratio pAKT/actin

Fig.S8. PI3Kγ accelerates ALK-dependent lymphomagenesis and duvelisib potentiates crizotinib treatment in ALK+ ALCL. (**A**) Kaplan-Meier survival curves of NPM-ALK transgenic mice crossed with PI3Kγ$^{-/-}$ mice (Black, NPM-ALK/ PI3Kγ$^{WT/WT}$, *n*=30 mice; Blue, NPM-ALK/ PI3Kγ$^{-/-}$; *n*=26 mice); n.s., not significant. (**B**) Relative cell viability in ALK+ ALCL cells treated with duvelisib (10μM), crizotinib (10nM) in single or in combination for 72h. *n*=3 technical replicates. (**C**) IC50 values of T cell lymphoma cell lines treated with the indicated inhibitors. TCL, T cell lymphoma (**D**) Histograms show the PDX tumor area in mice inoculated s.c. and treated as in Fig. 4D. Tumors were collected at day 16 for control mice, at day 20 for mice treated with duvelisib, at day 30 for mice treated with crizotinib and at day 40 for mice treated with crizotinib and duvelisib. (**E**) qRT-PCR analysis of *PIK3CA* and *PIK3CG* expression on ALK+ ALCL PDX samples collected from NSG mice treated as indicated in Fig. 4D and collected as indicated in (**D**). (**F**) Quantification of phosphorylated ERK (pERK) and AKT (pAKT) in PDX tumor samples blotted as shown in the western blot in Fig. 4. *n*=3 biological independent samples. Data are shown as mean ± s.d. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. Significance was determined by unpaired, two-tailed Student's t-test.

Fig. S9

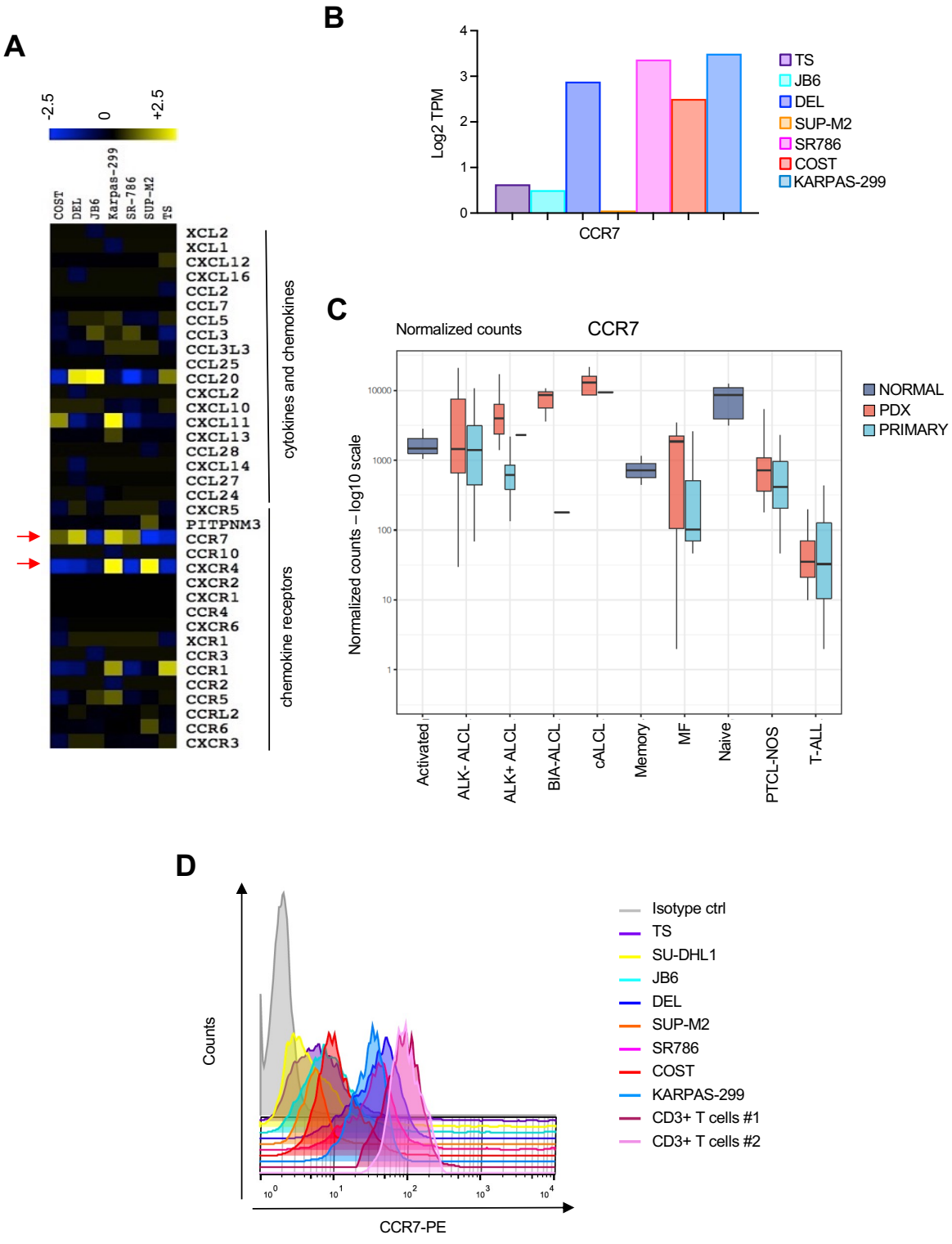

A
-2.5
0
+2.5
COST
DEL
JB6
Karpas-299
SR-786
SUP-M2
TS
XCL2
XCL1
CXCL12
CXCL16
CCL2
CCL7
CCL5
CCL3
CCL3L3
CCL25
CCL20
CXCL2
CXCL10
CXCL11
CXCL13
CCL28
CXCL14
CCL27
CCL24
CXCR5
PITPNM3
CCR7
CCR10
CXCR4
CXCR2
CXCR1
CCR4
CXCR6
XCR1
CCR3
CCR1
CCR2
CCR5
CCRL2
CCR6
CXCR3
cytokines and chemokines
chemokine receptors
B
Log2 TPM
CCR7
TS
JB6
DEL
SUP-M2
SR786
COST
KARPAS-299
C
Normalized counts
CCR7
Normalized counts – log10 scale
Activated
ALK- ALCL
ALK+ ALCL
BIA-ALCL
cALCL
Memory
MF
Naive
PTCL-NOS
T-ALL
NORMAL
PDX
PRIMARY
D
Counts
CCR7-PE
Isotype ctrl
TS
SU-DHL1
JB6
DEL
SUP-M2
SR786
COST
KARPAS-299
CD3+ T cells #1
CD3+ T cells #2

Fig.S9. CCR7 is specifically expressed in ALK+ cells in patients with ALCL. (**A**) Heat map showing the basal expression of chemokine ligands and receptors based on RNA-seq data in ALK+ ALCL cell lines (only detected genes are shown, CCL19/CCL21 were not detected). Gene expression is shown as the log2 ratio between each gene and the average expression of the same gene in all cell lines. Red arrows indicate CCR7 and CXCR4 mRNA expression. (**B**) CCR7 mRNA expression in ALK+ ALCL cells. TPM: Transcripts Per Million. (**C**) CCR7 mRNA expression by RNA sequencing analysis performed in PDX, primary tumors and T cells as indicated. (**D**) CCR7 basal cell surface expression in ALK+ALCL cells and CD3+ T cells measured by flow-cytometry.

Fig. S10

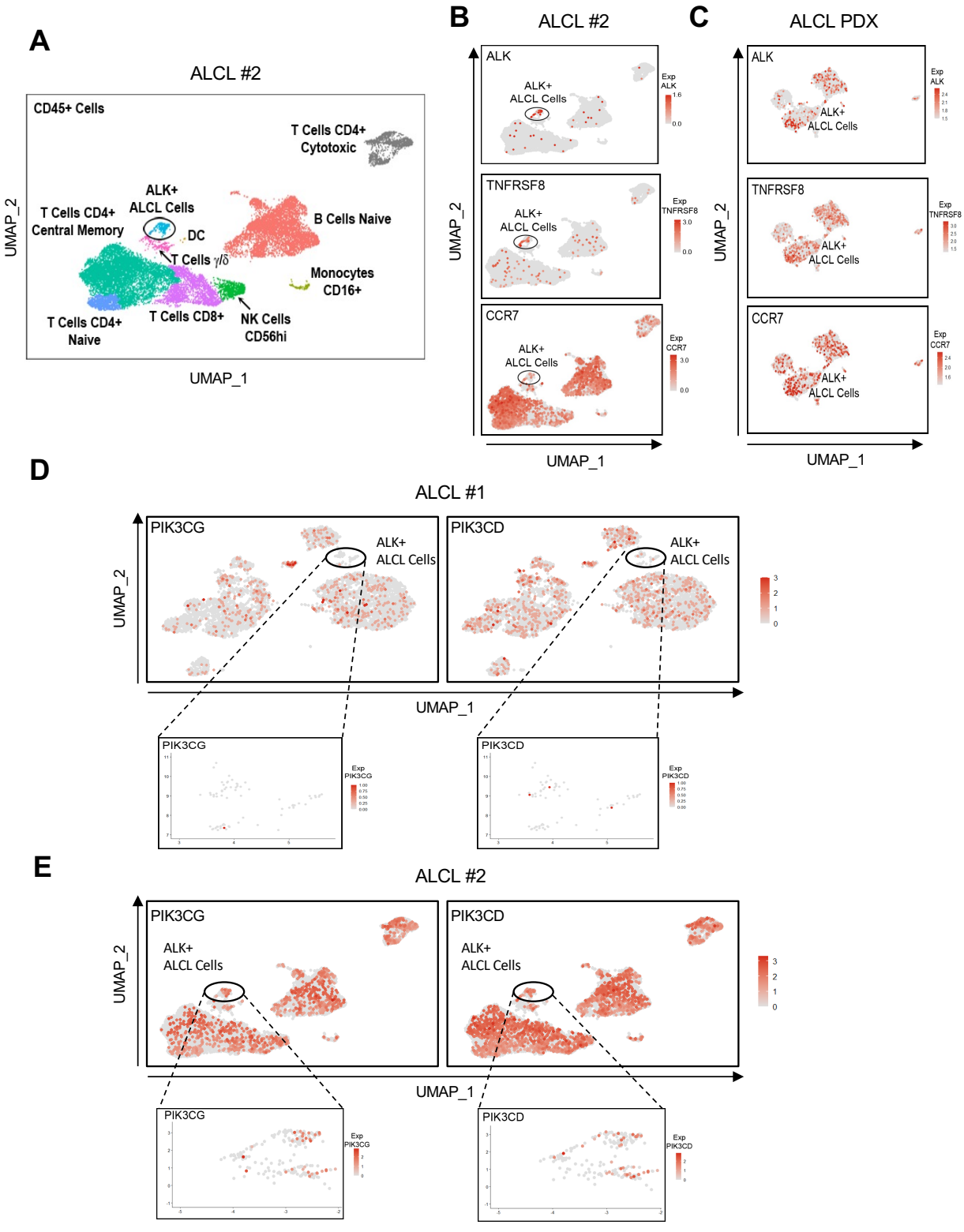

A
ALCL #2
CD45+ Cells
T Cells CD4+ Cytotoxic
ALK+ ALCL Cells
T Cells CD4+ Central Memory
DC
B Cells Naive
T Cells γ/δ
Monocytes CD16+
T Cells CD4+ Naive
T Cells CD8+
NK Cells CD56hi
UMAP_2
UMAP_1
B
ALCL #2
ALK
TNFRSF8
CCR7
ALK+ ALCL Cells
C
ALCL PDX
D
ALCL #1
PIK3CG
PIK3CD
E
ALCL #2

Fig.S10. ALK+ cells show a low to undetectable expression of PI3Kγ and PI3Kδ in primary ALK+ ALCLs. (**A**) UMAP plot of scRNA seq data for CD45+ cells from a primary lymph node of an ALK+ ALCL patient (#2), color-coded by the main group. (**B**) UMAP plots of scRNA seq data for CD45+ cells from the primary lymph node of ALK+ ALCL patient #2 for normalized expression of selected genes. (**C**) UMAP plots of scRNA seq data for CD45+ cells generated from an ALK+ ALCL PDX sample for normalized expression of selected genes. (**D** and **E**) UMAP plot of scRNA seq data for CD45+ cells from primary lymph nodes of patients with ALK+ ALCL (patient #1 in **D** and patient #2 in **E**) for normalized expression of *PIK3CG* and *PIK3CD.* In the boxes, UMAP plot of scRNA seq data for ALK+ ALCL cells.

Fig. S11

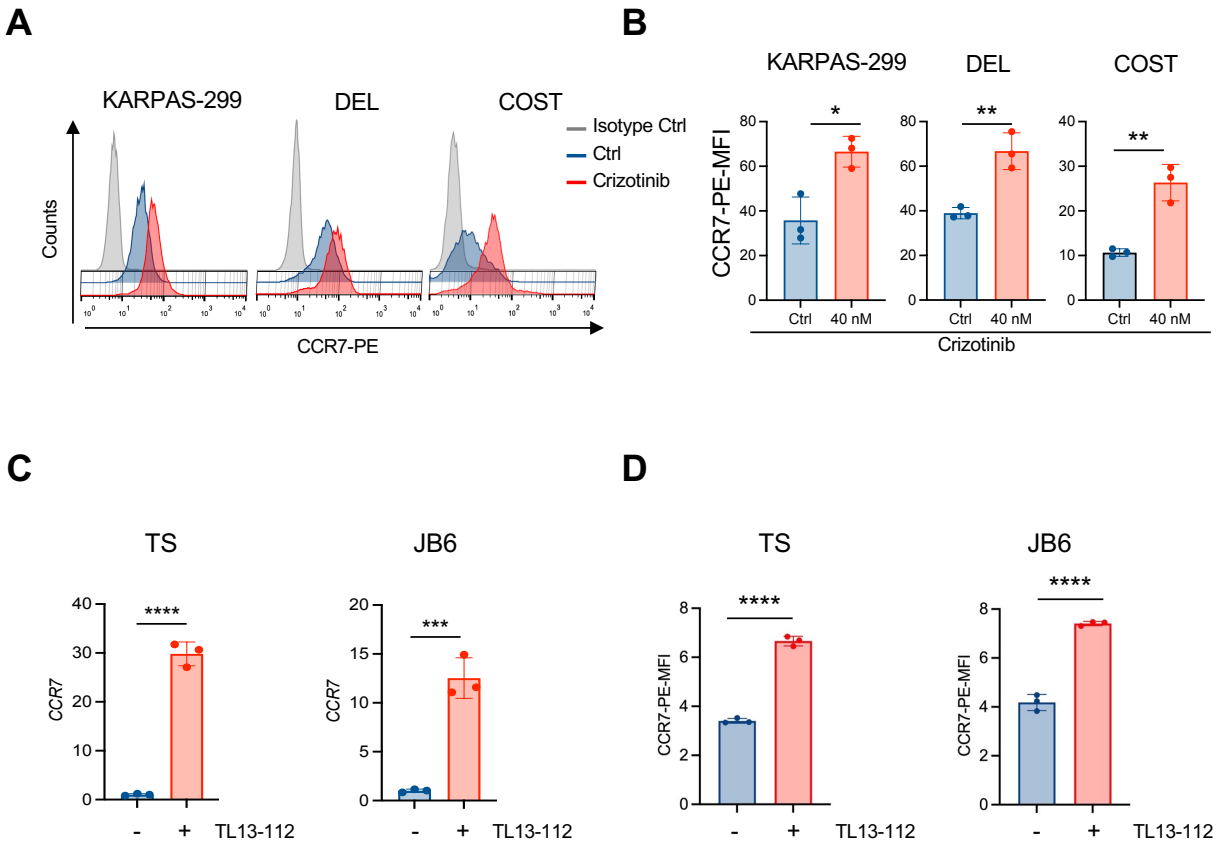

A
KARPAS-299
DEL
COST
Isotype Ctrl
Ctrl
Crizotinib
Counts
CCR7-PE
B
KARPAS-299
DEL
COST
CCR7-PE-MFI
Ctrl
40 nM
Crizotinib
C
TS
JB6
CCR7
TL13-112
D
TS
JB6
CCR7-PE-MFI
TL13-112

Fig. S11. CCR7 expression is suppressed by ALK activity. (**A**) CCR7 cell surface expression intensity in high PI3Kγ expressing ALK+ ALCL cell lines (KARPAS-299, DEL and COST) treated with crizotinib (40nM) for 24h. Ctrl, control. (**B**) Histograms show MFI of CCR7 cell surface expression in ALK+ ALCL cells treated with crizotinib as in (**A**). (**C**) *CCR7* mRNA expression by qRT-PCR in low PI3Kγ expressing ALK+ ALCL cell lines (TS and JB6) treated with TL13-112 (100nM) for 8h. (**D**) CCR7 cell surface expression intensity as measured by MFI in TS and JB6 treated with TL13-112 (50nM) for 72h. Data are shown as mean ± s.d. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. Significance was determined by unpaired, two-tailed Student's t-test.

Fig. S12

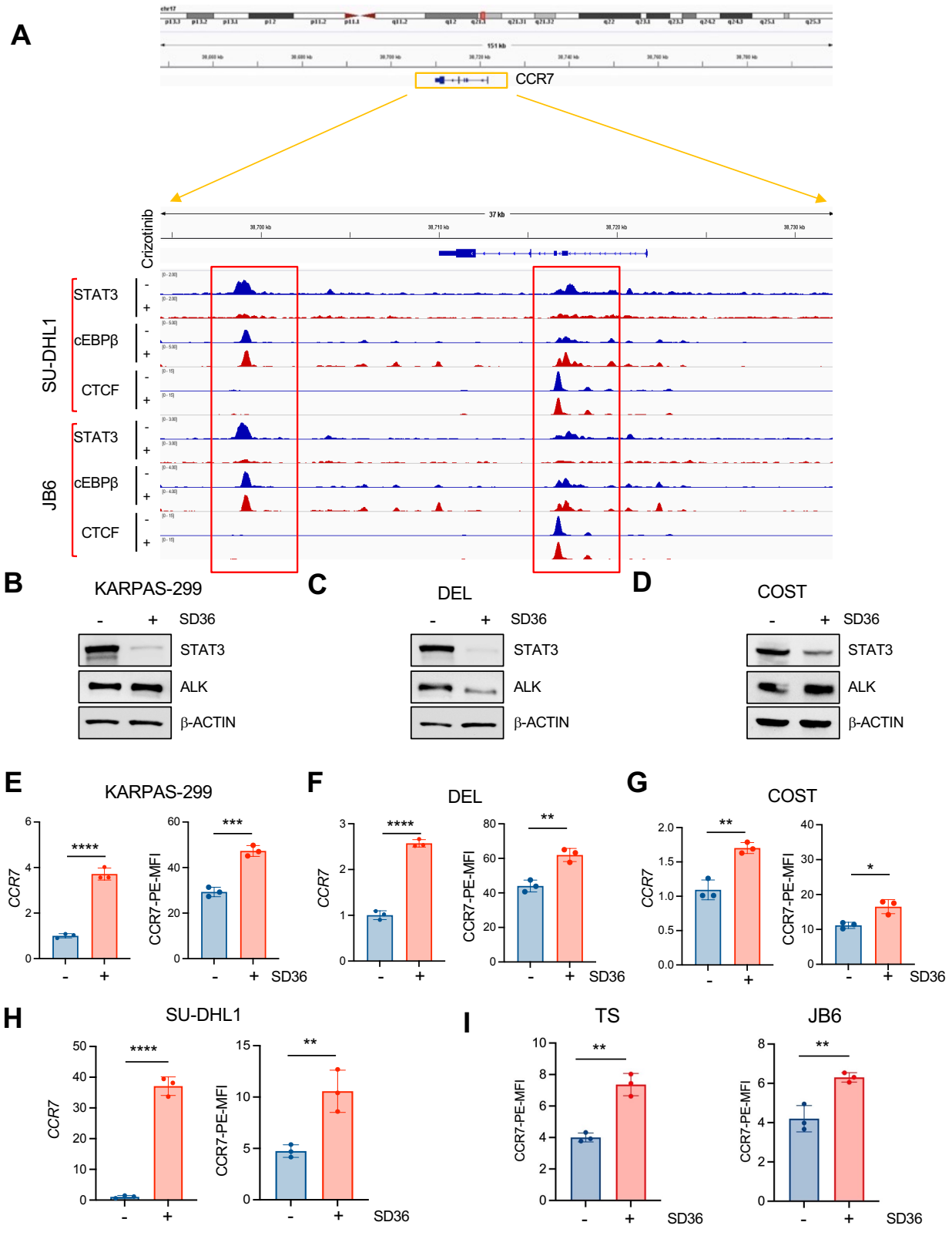

A
CCR7
Crizotinib
SU-DHL1
JB6
STAT3
cEBPβ
CTCF
B
KARPAS-299
C
DEL
D
COST
SD36
ALK
β-ACTIN
E
F
G
CCR7
CCR7-PE-MFI
H
SU-DHL1
I
TS
JB6

Fig. S12. CCR7 expression is suppressed by STAT3. (**A**) STAT3, C/EBP-β and CTCF ChIP-seq tracks at CCR7 gene on two representative ALK+ ALCL human cell lines (JB6 and SU-DHL1) treated with crizotinib (300 nM) for 3h. Experiment was performed one time on two independent cell lines with similar results. (**B**) Western blot of total cell lysates with the indicated antibodies of KARPAS-299 cell line treated with the STAT3 degrader SD36 (1μM).. (**C**) Western blot of total cell lysates with the indicated antibodies of DEL cell line treated with the STAT3 degrader SD36 (1μM). (**D**) Western blot of total cell lysates with the indicated antibodies of COST cell line treated with the STAT3 degrader SD36 (1μM). (**E**)*CCR7* mRNA expression by qRT-PCR (left panel) and CCR7 cell surface expression intensity as measured by MFI (right panel) in KARPAS-299 cell line treated with the SD36 degrader and collected at 36h. (**F**) *CCR7* mRNA expression by qRT-PCR (left panel) and CCR7 cell surface expression intensity as measured by MFI (right panel) in DEL cell line treated with the SD36 degrader and collected at 24h. (**G**) *CCR7* mRNA expression by qRT-PCR (left panel) and CCR7 cell surface expression intensity as measured by MFI (right panel) in COST cell line treated with the SD36 degrader and collected at 24h. (**H**) qRT-PCR analysis of *CCR7* mRNA expression performed on SU-DHL1 cell line treated with SD36 and collected at 24h. *n*=3 technical replicates. (**I**) CCR7 cell surface expression intensity as measured by MFI in TS and JB6 cell lines treated with SD36 (1μM) for 48h. For western blots, β–actin was used as a loading control and two independent experiments with similar results were performed. Data are shown as mean ± s.d. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. Significance was determined by unpaired, two-tailed Student's t-test.

Fig. S13

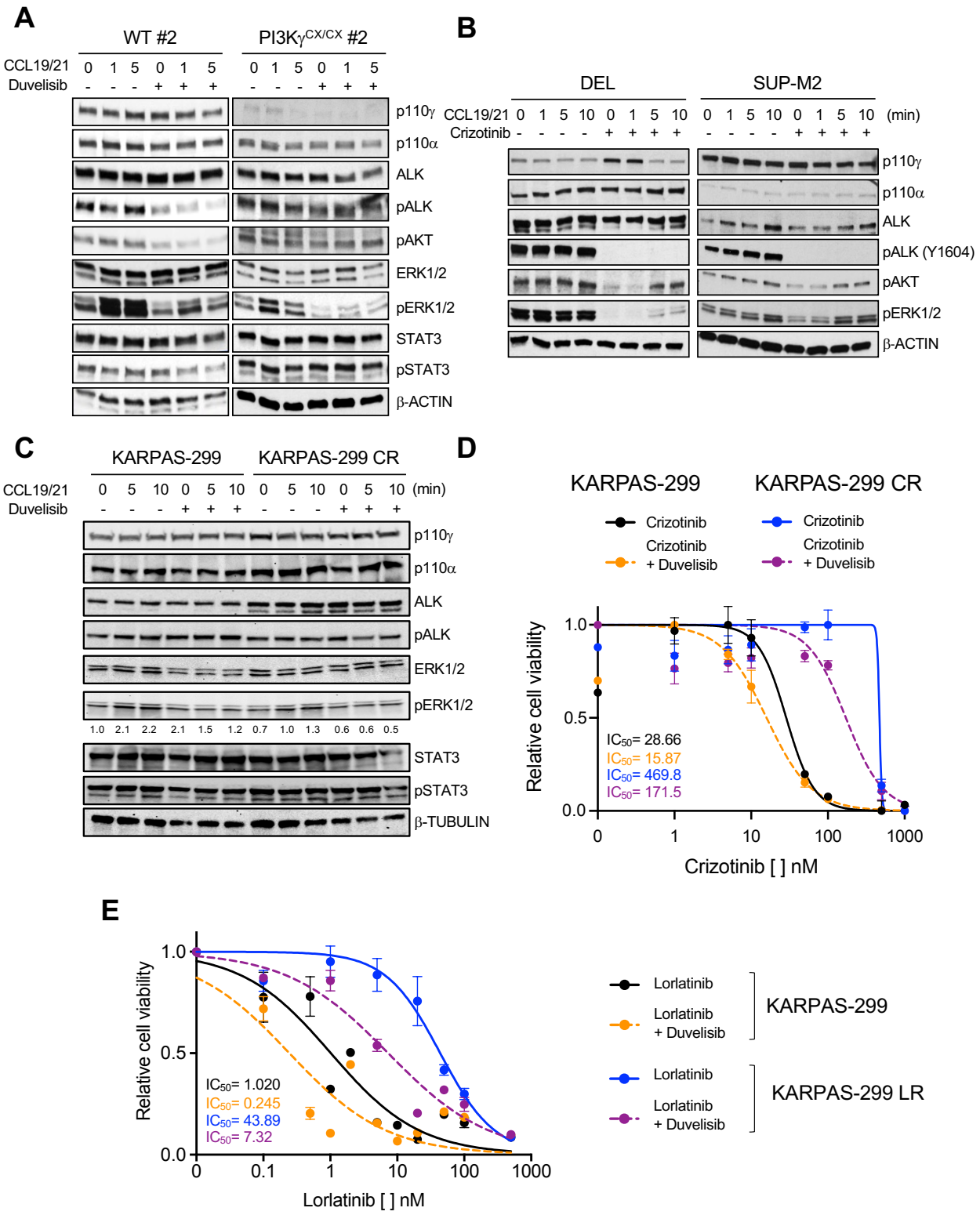

A
WT #2
PI3Kγ CX/CX #2
CCL19/21
Duvelisib
0 1 5 0 1 5
- - - + + +
p110γ
p110α
ALK
pALK
pAKT
ERK1/2
pERK1/2
STAT3
pSTAT3
β-ACTIN
B
DEL
SUP-M2
CCL19/21 0 1 5 10 0 1 5 10 0 1 5 10 0 1 5 10 (min)
Crizotinib - - - - + + + + - - - - + + + +
p110γ
p110α
ALK
pALK (Y1604)
pAKT
pERK1/2
β-ACTIN
C
KARPAS-299
KARPAS-299 CR
CCL19/21 0 5 10 0 5 10 0 5 10 0 5 10 (min)
Duvelisib - - - + + + - - - + + +
p110γ
p110α
ALK
pALK
ERK1/2
pERK1/2
1.0 2.1 2.2 2.1 1.5 1.2 0.7 1.0 1.3 0.6 0.6 0.5
STAT3
pSTAT3
β-TUBULIN
D
KARPAS-299
KARPAS-299 CR
Crizotinib
Crizotinib + Duvelisib
Relative cell viability
1.0
0.5
0.0
IC50= 28.66
IC50= 15.87
IC50= 469.8
IC50= 171.5
0 1 10 100 1000
Crizotinib [ ] nM
E
Relative cell viability
1.0
0.5
0.0
IC50= 1.020
IC50= 0.245
IC50= 43.89
IC50= 7.32
0 0.1 1 10 100 1000
Lorlatinib [ ] nM
Lorlatinib
Lorlatinib + Duvelisib
KARPAS-299
Lorlatinib
Lorlatinib + Duvelisib
KARPAS-299 LR

Fig. S13. CCR7 engagement by CCL19/21 activates the MAPK pathway through PI3Kγ. (**A**) Western blot analysis of NPM-ALK+ lymphoma cells derived from primary tumors in mice with the indicated genotypes stimulated with CCL19/21 (100ng/ml) and treated with duvelisib (1μM) for 3h. WT, wildtype. (**B**) Western blot analysis performed on human ALK+ ALCL cells lines stimulated with CCL19/21 (100ng/ml and left untreated or treated with crizotinib (300nM) for 3h. (**C**) Western blot analysis of crizotinib-sensitive (KARPAS-299) and crizotinib-resistant (KARPAS-299 CR) ALK+ ALCL cells stimulated with CCL19/21 (100ng/ml) in the presence or absence of duvelisib (1μM). (**D**) Dose-response curves of KARPAS-299 and KARPAS-299 CR cells treated with increasing concentrations of crizotinib in single or in combination with duvelisib (10μM). *n*=3 technical replicates. (**E**) Dose-response curves lorlatinib-resistant KARPAS-299 LR cells derived from xenografts and treated with increasing concentrations of lorlatinib alone or in combination with duvelisib (10μM). *n*=3 technical replicates. Data are shown as mean ± s.d. For western blots, β–actin or β–tubulin was used as a loading control and two independent experiments with similar results were performed.

Fig. S14

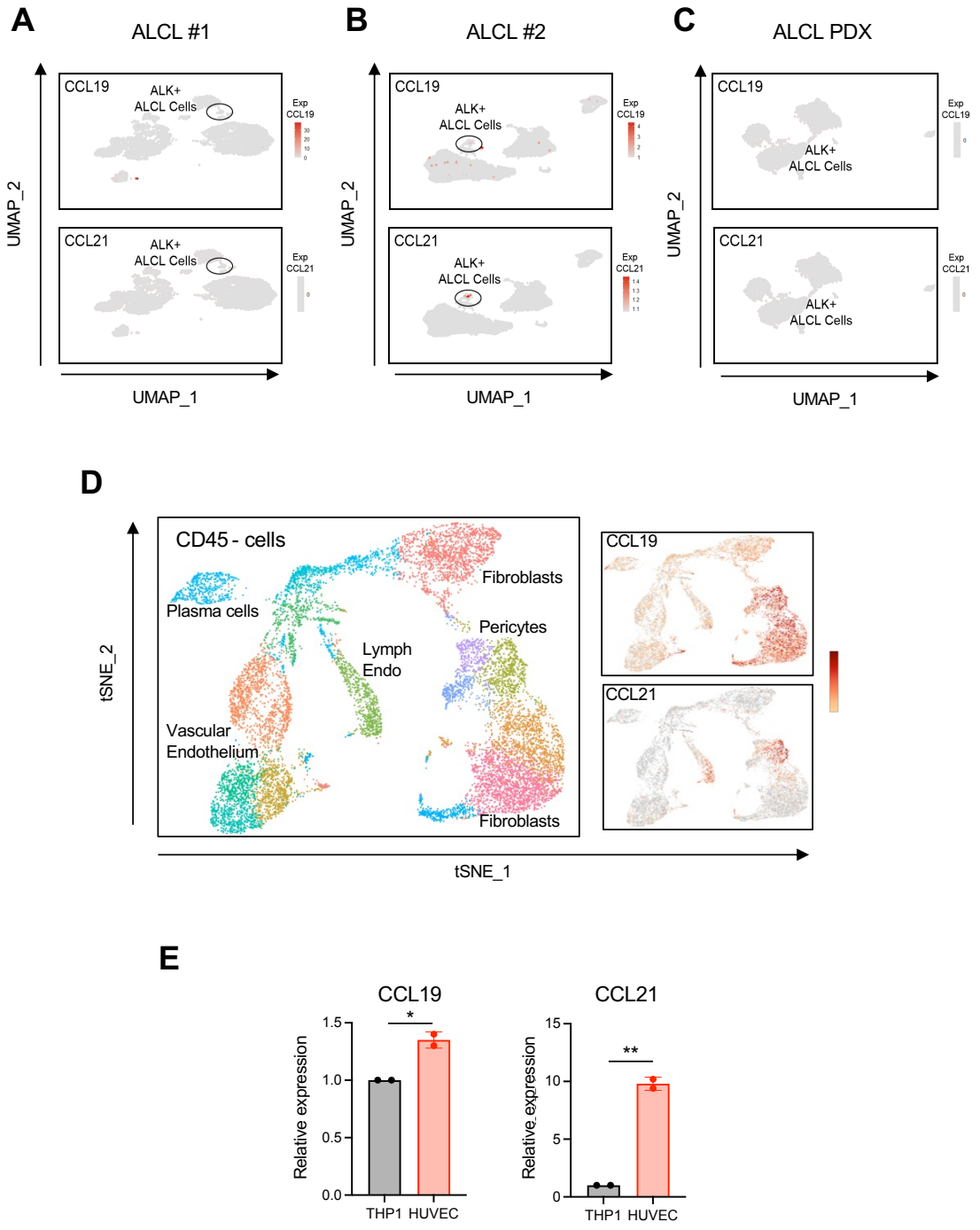

A
ALCL #1
CCL19
ALK+
ALCL Cells
Exp
CCL19
CCL21
ALK+
ALCL Cells
Exp
CCL21
UMAP_2
UMAP_1
B
ALCL #2
CCL19
ALK+
ALCL Cells
Exp
CCL19
CCL21
ALK+
ALCL Cells
Exp
CCL21
UMAP_2
UMAP_1
C
ALCL PDX
CCL19
ALK+
ALCL Cells
Exp
CCL19
CCL21
ALK+
ALCL Cells
Exp
CCL21
UMAP_2
UMAP_1
D
CD45 - cells
Plasma cells
Fibroblasts
Pericytes
Lymph
Endo
Vascular
Endothelium
Fibroblasts
CCL19
CCL21
tSNE_2
tSNE_1
E
CCL19
*
Relative expression
1.5
1.0
0.5
0.0
THP1
HUVEC
CCL21
**
Relative expression
15
10
5
0
THP1
HUVEC

Fig. S14. CCL19/21 are not expressed by ALK+ cells in primary ALCL and ALCL PDX. (**A** and**B**) UMAP plots of scRNA seq data for CD45+ cells generated from two different primary lymph nodes of patients with ALK+ ALCL (#1 and #2) for normalized expression of selected genes. (**C**) UMAP plots of scRNA seq data for CD45+ cells generated from an ALK+ ALCL PDX sample for normalized expression of selected genes. (**D**) t-SNE display of CD45- cells from a primary lymph node of an ALK+ ALCL patient, color coded by main group (left panel). t-SNE (CD45- cell positioning as shown in the left panel) display of normalized expression of selected genes (right panel). (**E**) qRT-PCR analysis of *CCL19* and *CCL21* mRNA expression in HUVEC cells compared to THP1 cells, used as positive control. *36B4* gene was used as housekeeping gene. *n*=3 technical replicates. Data are shown as means ± s.d. **P*<0.05, ***P*<0.01. Significance was determined by unpaired, two-tailed Student's t-test.

Fig. S15

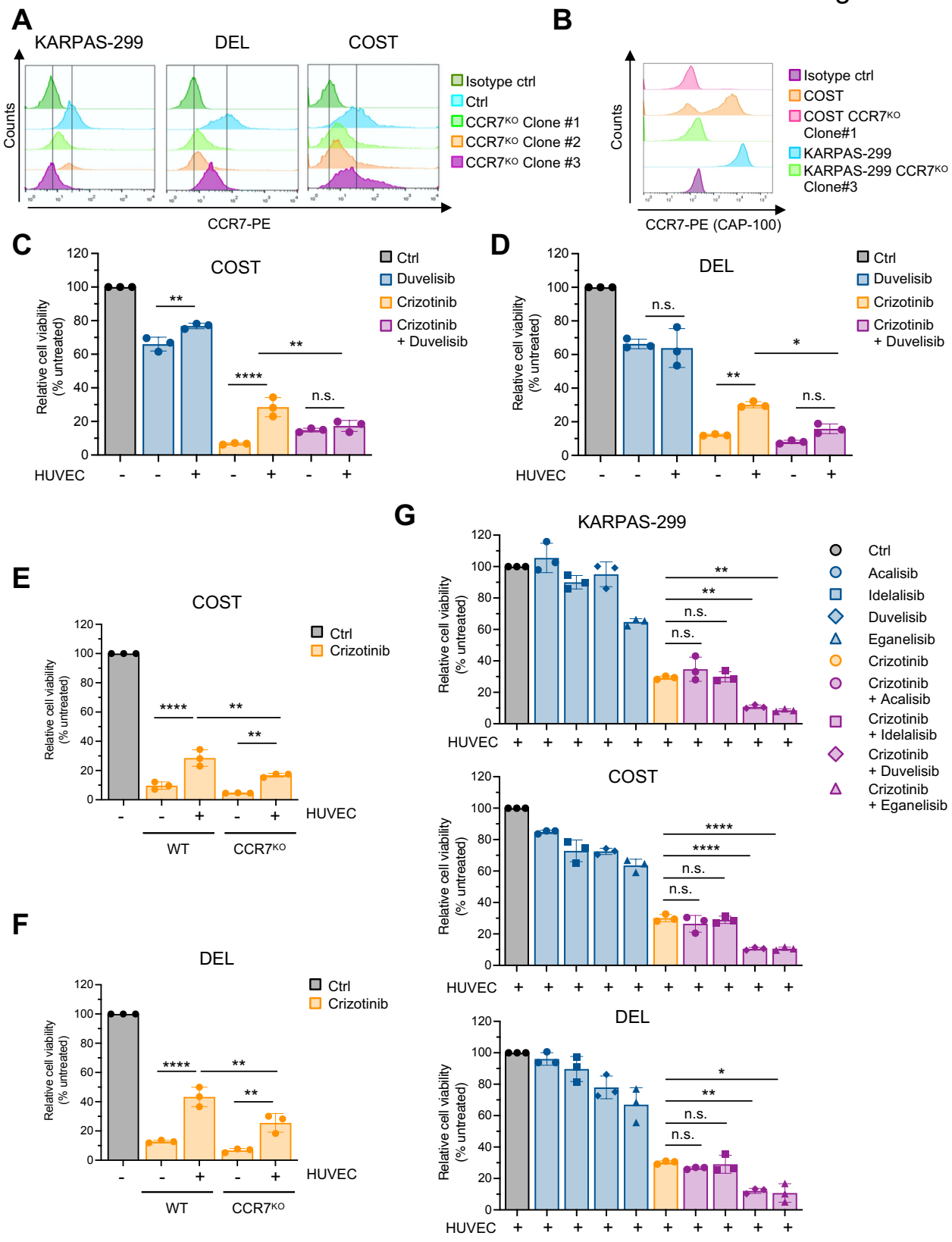

A
KARPAS-299
DEL
COST
Counts
CCR7-PE
Isotype ctrl
Ctrl
CCR7KO Clone #1
CCR7KO Clone #2
CCR7KO Clone #3
B
Counts
CCR7-PE (CAP-100)
Isotype ctrl
COST
COST CCR7KO Clone#1
KARPAS-299
KARPAS-299 CCR7KO Clone#3
C
COST
Relative cell viability (% untreated)
HUVEC
Ctrl
Duvelisib
Crizotinib
Crizotinib + Duvelisib
D
DEL
E
COST
WT
CCR7KO
F
DEL
G
KARPAS-299
COST
DEL
Acalisib
Idelalisib
Duvelisib
Eganelisib
Crizotinib
Crizotinib + Acalisib
Crizotinib + Idelalisib
Crizotinib + Duvelisib
Crizotinib + Eganelisib

Fig. S15. CCR7 protects ALCL cells from cell death induced by crizotinib. (**A**) CCR7 surface expression evaluated by flow-cytometry in ALK+ ALCL cell lines (KARPAS-299, DEL and COST) knocked out using CRISPR/Cas9 system. Clone # 3 was used for KARPAS-299 cell line; clone #1 for DEL and COST cell lines. (**B**) CCR7 deletion by CRISPR/Cas9 system was checked with the anti-CCR7 antibody CAP-100 in KARPAS-299 and COST clones. (**C**) Cell viability of $CCR7^{WT}$ and $CCR7^{KO}$ COST cell linesin co-culture with or without blood vessels (HUVEC) treated with crizotinib (300nM) or duvelisib (10μM) or in combination. (**D**) Cell viability of $CCR7^{WT}$ and $CCR7^{KO}$ DEL cell linse in co-culture with or without blood vessels (HUVEC) treated with crizotinib (300nM) or duvelisib (10μM) or in combination. (**E**) Cell viability of $CCR7^{WT}$ and $CCR7^{KO}$COST cell lines in co-culture with or without HUVEC treated with crizotinib (300nM). *n*= 3 biological replicates. (**F**) Cell viability of DEL, $CCR7^{WT}$ and $CCR7^{KO}$ DEL cell lines in co-culture with or without HUVEC treated with crizotinib (300nM). *n*= 3 biological replicates. (**G**) Cell viability of ALK+ ALCL cell lines (KARPAS-299, COST and DEL) in co-culture with HUVEC treated with crizotinib (300nM) or acalisib (10μM), or idelalisib (10μM), or duvelisib (10μM), or eganelisib (10μM), as single agents, or in combination. *n*= 3 biological replicates. Data are shown as means ± s.d. **P*<0.05, ***P*<0.01, *****P*<0.0001, n.s., not significant. Significance was determined by one-way ANOVA.

Supplementary tables

Table S1. ALK sequencing in crizotinib-resistant ALCL cell lines.

ALCL Cell Line	ALK Resistance Mutation	Prediction	Allele Frequency	Reference
DEL	D1160G	Damaging	0.159	not previously described
SR-786	L1152P	Damaging	0.063	Fribulet et al, Cancer Discov. 2014, 4: 662-673
TS	WT			
JB6	WT			
COST	WT			
SUP-M2	WT			
KARPAS-299	WT			

Table S3. Differentially expressed genes between crizotinib sensitive and resistant ALCL cell lines annotated in the KEGG PI3K-AKT Pathway.

Symbol	EnsemblID	baseMean	log2FoldChange	padj
RELN	ENSG00000189056	144	4,1	0,00
ITGB4	ENSG00000132470	3190	3,6	0,00
IL7R	ENSG00000168685	125	2,8	0,00
SPP1	ENSG00000118785	111	2,8	0,00
LAMA1	ENSG00000101680	46	2,6	0,00
ITGB5	ENSG00000082781	117	2,2	0,00
CDK6	ENSG00000105810	1890	1,7	0,00
BCL2L11	ENSG00000153094	19	1,6	0,08
PDGFA	ENSG00000197461	474	1,5	0,05
FGF9	ENSG00000102678	93	1,4	0,09
IRS1	ENSG00000169047	442	1,4	0,04
CHUK	ENSG00000213341	2254	1,3	0,01
CCNE1	ENSG00000105173	465	1,3	0,00
CCND3	ENSG00000112576	3414	1,2	0,00
MTOR	ENSG00000198793	2580	1,1	0,02
PRKCA	ENSG00000154229	179	1,0	0,03
MAP2K1	ENSG00000169032	6088	-1,0	0,00
PPP2R5C	ENSG00000078304	3635	-1,0	0,00
SOS2	ENSG00000100485	1654	-1,0	0,02
THBS3	ENSG00000169231	96	-1,0	0,09
ITGA4	ENSG00000115232	2302	-1,1	0,09
IL7	ENSG00000104432	97	-1,1	0,06
CREB3L4	ENSG00000143578	356	-1,1	0,00
MAPK3	ENSG00000102882	846	-1,1	0,00
ITGA6	ENSG00000091409	2379	-1,1	0,09
PCK2	ENSG00000100889	816	-1,2	0,00
ATF2	ENSG00000115966	1427	-1,2	0,00
EPOR	ENSG00000187266	407	-1,3	0,01
RBL2	ENSG00000103479	1441	-1,3	0,00
FOXO3	ENSG00000118689	158	-1,4	0,00
PIK3CD	ENSG00000171608	80	-1,4	0,10
GYS2	ENSG00000111713	99	-1,5	0,10
COL4A1	ENSG00000187498	31	-1,5	0,10
COL4A2	ENSG00000134871	691	-1,6	0,03
LAMB3	ENSG00000196878	17717	-1,7	0,02
CDKN1B	ENSG00000111276	771	-1,8	0,00
TNR	ENSG00000116147	80	-1,8	0,04

PPP2R5B	ENSG00000068971	355	-1,9	0,00
DDIT4	ENSG00000168209	794	-1,9	0,02
EFNA3	ENSG00000143590	54	-1,9	0,00
TNXB	ENSG00000168477	86	-2,0	0,02
F2R	ENSG00000181104	1565	-2,5	0,00
IL2RG	ENSG00000147168	404	-3,0	0,00

Legend: differentially expressed genes which are part of the PIK3 KEGG pathway

Table S4. ALK+ lymphoma cell infiltration in the leptomeninges or around blood vessels in the brain of mice inoculated i.v. with COSTCCR7WT and COSTCCR7KO.

	WT		**CCR7^{ko}**	
	Ctrl	Crizotinib	Ctrl	Crizotinib
N° of mice with meningeal lymphoma infiltration	3/3	5/5	4/4	0/5
N° of mice with perivascluar lymphoma infiltration	3/3	5/5	4/4	0/5