# A novel model of alternative NF-κB pathway activation in Anaplastic Large Cell Lymphoma

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## Supplementary Information

## Supplemental Experimental Procedures

## Materials and Methods

### Cell Culture.

ALK+ ALCL cell lines DEL, Karpas299, L-82, SUDHL1, SR786; ALK- ALCL cell lines MAC1, MAC2B, FEPD, TLBR1, TLBR2; HL cell line L540, T-ALL cell line Jurkat, DLBCL line U2932, and PMBL cell line MedB1 were grown in RPMI-1640 medium (Invitrogen) +10%FBS (Hyclone, Defined) +pen/strep (Invitrogen). All cell lines were grown in a 5% CO2 incubator at 37ºC, and wherein log phase when experiments were started.

### Generation of the inducible Cas9 expression cell lines.

All ALK+ and ALK- ALCL cell lines, except for FEPD, and the DLBCL line U2932, were transduced with an inducible Cas9 vector, single-cell cloned, and tested for Cas9 cutting efficiency and inducibility.

### Cell line Authentication.

All the cell lines used in this study, including all the parental and the engineered cell lines (Cas9 single cell clones) have been authenticated and verified by the STR analysis (DNA fingerprinting) method described in [1]. In brief, 16 pairs of primers are designed to recognize 16 different single-

and multiple-locus variable numbers of tandem repeats (VNTRs) in human genomics. Then the Polymerase chain reaction (PCR) was used to amplify the fragment length polymorphism (AmpFLP) of these 16 prominent and highly polymorphic minisatellite VNTR loci. The generated banding pattern (by electrophoretic system) was compared to the original banding pattern of this cell line in our databases of all original lymphoma cell lines.

**Lentiviral production and transduction.**

Lentivirus was produced by triple transfection of HEK-293FT cells with a lentiviral transfer vector, and the packaging plasmids psPAX2 and pMD2.G. Transfection was performed using TransIT-293 Transfection Reagent as recommended by the manufacturer. The viral supernatant was collected 48 hours following transfection, filtered through a 0.45 μm filter, and added to target cells.

**Depletion (loss-of-function) CRISPR library screen.**

The Focused lymphoma signaling CRISPR library was described in [2]. The Cas9 inducible ALCL cell line Del and DLBCL cell line U2932 L428 cell line were used for the depletion (loss-of-function) CRISPR library screen. In brief, 40 million cells were infected with the pooled lentiviral lymphoma signaling sgRNA library at a multiplicity of infection of 0.3 and selected with 2 μg/ml puromycin for 72-96 hours, commencing 48 hours after transduction. GFP percentage was examined to ensure the completion of selection. Doxycycline was then added to induce sgRNAs expression. After 21 days of in vitro culture, doxycycline induced cells and the un-induced (Day 0) were collected for genomic DNA extraction (Qiagen). sgRNA sequences were amplified by two rounds of PCR, with the second-round primers containing adaptors for Illumina sequencing. The resulting libraries were sequenced with single end read with dual-index 75 bp on a HiSeq2500. The sequence reads were trimmed to remove the constant portion of the sgRNA sequences with a

Fastx clipper, then mapped to the reference sgRNA library with bowtie2. After filtering to remove multi-aligning reads, read counts were computed for each sgRNA.

**CRISPR mediated gene disruption.**

sgRNA oligonucleotides (IDT) were phosphorylated and annealed and cloned into lentiviral expression vectors: pLKO.1-puro/GFP U6 sgRNA BfuAI stuffer for inducible sgRNA expression. For CRISPR mediated gene disruption, the inducible Cas9 expression cell lines were transduced with the lentiviral sgRNA expression vector. Knockout clones were identified by flow cytometry analysis or immunoblot. *The sgRNA sequences used in this study are listed as follows:*

| **NAME** | **Sequence** |
|---|---|
| CTRL | GTAGCGAACGTGTCCGGCGT |
| NFKB2 #1 | GGGAGGAGGGGAAGAAACCG |
| NFKB2 #2 | GTAGGGGCTGTAGGCCAGGG |
| IKKα #1 | GCAATGGAATACTGTTCTGG |
| IKKα #2 | TGCCCTCCCGCCCCATGGAG |
| NIK #1 | GAACACAGGGCTCTTCTCCA |
| NIK #2 | TCCCACTTTCCGCAGAACAC |
| STAT3 #1 | GTACCACAGGATGGACGCCC |
| STAT3 #2 | ACAGCTTCCCAATGGAGCTG |
| CD30 #1 | AAGTCACGCAGGCTGTACAG |
| CD30 #2 | TTGCAGATTCCAGACCCAGG |
| TRAF3 #1 | AAAGTTTGTGAAGACCGTGG |
| TRAF3 #2 | TCGGGCTGCACAGCACCAGG |
| JAK2 #1* | AAGAATGAAAGCCTTGGCCA |
| JAK2 #2 | GCACCTAAGAGACTTTGAAA |

* only targeting WT JAK2

**Patient samples.**

Tumor biopsy specimens were obtained from patients with ALK+ and ALK- ALCL. All human samples were anonymously coded as stipulated by the Declaration of Helsinki. Written informed consent was obtained from the patients. All samples were studied according to a protocol approved by the Institutional Review Board of Sun Yat−sen University Cancer Center.

**Nuclear and cytoplasmic extraction.**

Nuclear and cytoplasmic protein was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit from Thermo Scientific, according to manufacturer instructions.

**Cell viability MTS assay.**

Cells were seeded in triplicate 96-well tissue culture plates at 8000 cells/well in 100 μL of media under various treatment conditions. 50 μL of containing medicine medium were added to each well at day 2. After 4 days of treatment, 20 μL of CellTiter Aqueous one solution cell proliferation reagent (Promega) were added to each well and mixed for 5 minutes. The absorbance was read on a Spark multimode microplate reader (Tecan) after 2-4 h incubation. The background was subtracted using a media only control. All experiments were performed in triplicate.

**Retroviral construct used for ectopic expression.**

The retroviral vector for inducible cDNA expression was pMSCV-based with the cDNA expressed from a doxycycline-inducible cytomegalovirus (CMV) promoter in which a binding site for the bacterial tetracycline repressor was inserted at the transcription start site (derived from pCDNA4/TO (Invitrogen)). IKKα WT was obtained by RT PCR and sub-cloned into the pMSCV-puro retroviral expression vector. The IKKα S176/180E plasmid was generated by quick-change mutagenesis. p100 cDNA was obtained by RT PCR and fused with EGFP at the N terminal, then cloned into a the PBMN-IRES-NEO retroviral expression vector, to generate the p100-EGFP reporter. The STAT3 WT and mutant retroviral expression plasmids were as described by Chen et al [3].

**p100-EGFP reporter mini CRISPR library screen.**

ALCL cell line TLBR2 and DEL were transduced with a retroviral vector coding p100 cDNA which was fused with EGFP at the N terminal, selected and signal cell cloned for stable GFP expression. This p100-EGFP reporter line was then transduced with each individual sgRNAs in

the mini CRISPR library, and induced to express for 4 days. The relative GFP intensity in each sgRNA transduced samples was measured by FACS and normalized to Ctrl sgRNA transduced samples. The $log_2$ fold changes of GFP intensity in each sgRNA transduced sample vs Ctrl sgRNA were calculated and plotted. *The containing sgRNAs and sequences of the mini CRISPR library are presented in Supplementary Table S2.*

**Antibody and reagents.**

The antibodies used in this study were purchased as follows: anti-STAT3, anti-phosphor-STAT3, anti-JAK2, anti-p100/p52, anti-IKKα, anti-phosphor-p100, anti-NIK, anti-TRAF2, and anti-TRAF3 from Cell Signaling Technologies; anti-β-actin-HRP (C-11), and mouse monoclonal anti-CD30 from Santa Cruz Biotechnology; anti-ALK from Invitrogen. FASC antibodies anti-CD30 (BY88) from BioLegend. Isotype control antibodies were obtained from the same company as each experimental antibody. Secondary HRP-conjugated antibodies were obtained from GE Healthcare.

NIK inhibitor was described as [4], The ALK inhibitor crizotinib was obtained from Cell Signaling Technologies. The proteasome inhibitor bortezomib was obtained from Selleckchem. Tissue culture grade DMSO vehicle control was obtained from Sigma-Aldrich.

**Western blotting.**

Cell pellets were lysed in the modified RIPA buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% NP40, 0.25% deoxycholic acid, 1mM EDTA) supplemented with a protease inhibitor tablet and a phosphatase inhibitor tablet (Roche), 1mM DTT, 1mM $Na_3VaO_4$, 1mM PMSF. Protein concentrations were measured by BCA Protein Assay Kit (Thermo Scientific). Total proteins were separated on 4% to 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes.

**Co-immunoprecipitation.**

Cells were lysed in an endogenous lysis buffer (20mM Tris-HCl pH 7.6, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 30mM NaF, and 2mM sodium pyrophosphate) supplemented with complete protease inhibitor cocktail (Roche), phosphatase inhibitor tablet (Roche), 1mM DTT, 1mM $Na_3VaO_4$, 1mM PMSF. Cleared lysates were incubated overnight with anti-TRAF2, TRAF3, CD30 or control antibodies. Immunoprecipitates were washed 5 times with lysis buffer, separated by SDS-PAGE, transferred to nitrocellulose and analyzed by immunoblotting.

**Real-Time Quantitative PCR.**

Total RNA was extracted from ALCL cell lines with RNeasy mini kit (Qiagen). Total RNA was reverse transcribed with random primers and SuperScript III Reverse Transcriptase (Invitrogen). ABI7500 instrument (Applied Biosystems) was performed using the resultant cDNA. Gene expression was normalized to the expression of beta-2-microglobulin (B2M) or Actin for all samples. The following quantitative PCR primers were used:

| | FW | RV |
|---|---|---|
| NFKB2 | GGGCCGAAAGACCTATCCC | CAGCTCCGAGCATTGCTTG |
| CD30 | GGACACCTGTCATGGAAACC | GTGCCTGGGAACTTGACAAT |
| B2M | GTGGCCTTAGCTGTGCTCG | ACCTGAATGCTGGATAGCCTC |
| ACTIN | CGCGAGAAGATGACCCAGAT | GGGCATACCCCTCGTAGATG |

**RNA sequencing.**

For RNA sequencing analysis, total RNA was extracted using the RNeasy Kit (Qiagen) from DEL and TLBR2 cell lines transduced with Ctrl or STAT3 sgRNAs, selected and induced for 6 days. The bulk RNA-seq libraries were constructed using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB). The quality-ensured RNA-seq libraries were also pooled and sequenced on the Illumina HiSeq 2500 platform with 100 or 150 bp paired-end mode (sequenced by Novogene).

For RNA-seq data processing, raw sequence reads were aligned to the human genome (hg38) using the Tophat algorithm [5]; the Cufflinks algorithm [6] was implemented to assemble transcripts and

estimate their abundance. Cuffdiff [7] was used to statistically assess expression changes in quantified genes in different conditions.

**Data access.**

The high-throughput RNA sequencing data from this study have been submitted to the NCBI Sequence Read Archive (SRA) under accession number: SUB6534613.

**Gene set enrichment analysis.**

The expression dataset was analyzed with Gene Set Enrichment Analysis (GSEA) (http://software.broadinstitute.org/gsea/index.jsp) using the Molecular Signatures Database, MSigDB (http://www.broad.mit.edu/gsea/msigdb/index.jsp). GSEA identified significant sets of genes that were overrepresented at the top or bottom of the ranked set of genes that were differentially expressed between comparison groups. For our analyses, we used the MSigDB hallmark gene sets. Gene sets with FDR p-value < 0.05 were considered significant.

**shRNA expression in lymphoma cells.**

The retroviral vectors for shRNA expression were described before[8]. STAT3 shRNA were described in[3]. Sequences for control and target specific shRNAs are as following:

Control shRNA: CTCTCAACCCTTTAAATCTGA

ALK shRNA #1: CAGGGCGAGCTACTATAGAAA

ALK shRNA #2: GAGCATGGGTTCATCCTATTC

NIK shRNA: GGTGTGAAAGTCCAAATACAG

IKKα shRNA: GGTGGAAAGATAATACATAAA

**Chromatin Immunoprecipitation (ChIP) including ChIP-PCR.**

Chromatin immunoprecipitations were performed as described [2]. Briefly, for each chromatin preparation, $5x10^7$ cells were collected, resuspended in 50 ml of RPMI without FBS and cross-

linked in 1% formaldehyde for 5' at RT'. Cross-linking was quenched by 125mM glycine, 5' at RT. Cross-linked cells were first washed with ice-cold PBS and then resuspended in ice cold RIPA buffer (10mM Tris-HCl pH8, 140 mM NaCl, 1mM EDTA pH 8, 0.5mM EGTA, 1% Triton X-100, 0.3% SDS and 0.1% Sodium Deoxycholate) to a final concentration of $5x10^6$ cell/ml. DNA was sheared with a Diagenode Bioruptor® Plus sonicator. For each pull-down, $1x10^7$ chromatin cell equivalents were incubated overnight with antibodies of IgG (Rabbit, Bythel), STAT3 (Rabbit, CST), and then Protein G coupled magnetic beads (Dynal/Invitrogen) for 4h at 4°C. The beads were washed 4 times with Ripa Buffer, twice with LiCl Buffer (10mM Tris-HCl pH8, 250mM LiCl, 0.5% NP40, 0.5% Sodium Deoxycholate, 1mM EDTA), twice with TE pH8 and finally resuspended in 100 µl TE pH8 containing RNase A (0.2 µg/µl final). Unprocessed chromatin samples were processed in parallel as an input reference. Reverse cross-linking was performed over night at 65°C. The reverse cross-linked samples were then treated with Proteinase K for 2h at 50°C. DNA was purified with QIAquick PCR Purification columns (QIAGEN), according to manufacturer's instructions. ChIP samples and corresponding input DNA thus prepared were analyzed by real-time PCR after 30- to 250-fold dilution in triplicate using 2xSYBR green master mix (ABI) on an ABI7500 Taqman machine (40-45 cycles, annealing at 60°C) using self-designed primers, previously screened for lack of primer-dimer artifacts and for single species amplification. Signal values were calculated using ΔΔCt method (ABI).

ChIP-PCR primer sequences

| | |
|---|---|
| NFKB2_S+10_F | 5'-CGACCGCAAGAATAACTTCC-3' |
| NFKB2_S+10_R | 5'-ACGCTTGGCTTTCTCTGAAG-3' |
| NFKB2_S+1785_F | 5'-ATTCTGGGAAGCAGAACCTG-3' |
| NFKB2_S+1785_R | 5'-CAGACAGGTGGACAGACACG-3' |
| CD30_S-18_F | 5'-AGGGAGGCGTCTCCTAGTGT-3' |
| CD30_S-18_R | 5'-GTTGTTCCTCCGAGGTTTCA-3' |
| CD30_S+19241_F | 5'-TCTAGCCTGGGCAACAGAGT-3' |

| | |
|---|---|
| CD30_S+19241_R | 5'-TGTGAAGTAGGGGTCGGAGA-3' |
| CD30_S+19825_F | 5'-TGCTGACAAGGATGTGGAGC-3' |
| CD30_S+19825_R | 5'-GGGCATAAGGCATGTTCAGT-3' |

**Immunohistochemical (IHC) analysis.**

Immunohistochemical analysis of p-STAT3 and nuclear p52 expression in the human tumor specimens was performed as previously described [2]. Each slide was incubated for 30 minutes with rabbit antibodies to human p-STAT3 (Cell signaling), p100/p52 (Cell signaling) and RELB (Cell signaling). Intensity of staining was evaluated according to the following scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The proportion of all tumor cells found to express nuclear p52 or p-STAT3 was determined and then multiplied by the staining intensity score to obtain a final semi quantitative H score. All immunohistochemical images were evaluated by an experienced pathologist who was unaware of the identity of the specimens.

**Immunofluorescence confocal microscopy.**

The coverslips were coated with 1% poly-D-Lysine (Millipore) at 4C overnight before seeding cells in 24-well plates at a density of 2 $X10^5$ cells per well for 4 hours, and then were centrifuged at 2000 rpm for 5 mins. After removal of the cell supernatant, the cells were directly fixed with 4% paraformaldehyde in PBS for 20 mins, and then permeabilized with 0.1% Trtion X100 in PBS for 5 mins. After blocking with 5% BSA in PBS for 20 mins, cells were stained with an antibody against rabbit-anti-TRAF3 (CST, 1:100 dilution) and mouse-anti-CD30 (Santa Cruz, 1:100 dilution) for 4 C overnight, and then washed with PBST three times, followed by incubation with Alexa Fluor 594-labelled goat antibody to Rabbit IgG (1:2,000 dilution) and Alexa Fluor 633-labelled goat antibody to mouse IgG. After being washed with PBST three times, cells were mounted with ProLong Gold mounting medium (Invitrogen) containing 0.2 ug/ml DAPI, which stains nuclei. Confocal images were acquired using an Olympus confocal laser scanning

microscope. ImageJ software combined with plug-in programs was used to calculate the overlap coefficients of CD30 and TRAF3 for each confocal image. Costes' automatic threshold was applied. Rs = Pearson's Coefficient. 1 means perfect overlap, and 0 means random distribution of CD30 and TRAF3.

**STAT3 reporter assay.**

STAT3 expression constructs were co-transfected with a STAT3 reporter, and a Renilla luciferase control (for normalization) into HEK293 cells. 24 hours later, cells were lysed following the protocol of the Dual- Glo Luciferase Reporter Assay System (Promega). The ratio of STAT3 reporter to Renilla luminescence was measured and normalized to that in empty vector controls.

**Flow cytometry.**

To examine the surface CD30 expression, cells were pelleted and washed one time with ice cold FASC buffer (1% FBS in PBS) and incubated with antibodies at 4°C for at least 30 min. Stained cells were washed twice, resuspended in 200 μL FASC buffer and then analyzed using a flow cytometer.

**Statistical analyses.**

All experiments have been repeated and results reproduced. The number of repeats have been clearly described in Figure legends, and in Supplementary Figure legends (for immunoblots and IPs). Where possible, error bars and/or P values are shown to indicate statistical significance. In some figures, error bars are not visible due to their short heights relative to the size of the symbols. P values were calculated with paired student's t-test, or unpaired t-test (Fig. 1D). $P < 0.05$ was considered statistically significant.

**Supplementary Figure legends**

**Supplementary Figure 1: Related to Fig 1.**

**A.** Outline of the workflow of the depletion (loss-of-function) CRISPR library screens in lymphoma cell lines. **B.** Overview of the depletion CRISPR screen results. Shown are the ranking of all the genes (average of 10 sgRNAs for each gene) enriched in the sgRNA ON population of both DEL and U2932 lines. Y axis indicates the distribution of standardized enrichment scores (Z-scores) for each gene enrichment. The green lines indicate p=0.05. **C.** Conformational EXP for (**B)**. ALCL lines were transduced with NFKB2 or Ctrl sgRNAs along with GFP. The fraction of viable sgRNA-expressing cells relative to the total viable cell fraction at indicated times following induction of the indicated sgRNAs, normalized to day 0 values. *Error bars denote SD of triplicates.* **D.** Densitometric analysis of **Fig. 1B**. The relative NIK, p-p100, p52 and p100 signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to DEL cell line. Experiments repeated three times. *Error bars denote SEM of three independent experiments.*

**Supplementary Figure 2: Related to Fig 2.**

**A.** p value analysis of **Fig. 2A (left)**. P were calculated comparing the Day 0 to each time points of sgIKKα #1 and sgIKKα #2 induction, in all cell lines tested; purple color indicates $P < 0.05$; red color indicates $P < 0.01$. **B.** Densitometric analysis of **Fig. 2A (right)**. The relative p-p100, p52 and IKKα signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the Ctrl sgRNA conditions in MAC2B cell line. Experiments repeated three times. *Error bars denote SEM of three independent experiments.* **C. Left,** NIK positive ALK- ALCL line FEPD was transduced with IKKα or Ctrl shRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. **Right,** NIK negative ALK- ALCL line MAC1 and ALK+ line SUDHL1 were transduced with IKKα or Ctrl sgRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins.

**Supplementary Figure 3: Related to Fig 2.**

**A.** p value analysis of **Fig. 2B (left)**. P were calculated comparing the Day 0 to each time points of sgNIK #1 and sgNIK #2 induction, in all cell lines tested; purple color indicates $P < 0.05$; red color indicates $P < 0.01$. **B.** Densitometric analysis of **Fig. 2B (right)**. The relative p-p100, p52

and NIK signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the Ctrl sgRNA conditions in MAC2B cell line. Experiments repeated three times. *Error bars denote SEM of three independent experiments.* **C. Left,** NIK positive ALK- ALCL line FEPD was transduced with NIK or Ctrl shRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. **Right,** NIK negative ALK- ALCL line MAC1 and ALK+ line SUDHL1 were transduced with NIK or Ctrl sgRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins.

**Supplementary Figure 4: Related to Fig 3.**

**A.** Verification of the TLBR2 p100-EGFP reporter line. The TLBR2 p100-EGFP reporter line was transduced with indicated sgRNAs, the relative GFP intensity in each sgRNA transduced sample was measured by FACS. **B.** Mini CRISPR library screen using the p100-EGFP reporter in DEL line. The DEL p100-EGFP reporter line was transduced with each individual sgRNAs in the mini CRISPR library, and induced to express for 4 days. The relative GFP intensity in each sgRNA transduced sample was measured by FACS and normalized to Ctrl sgRNA transduced samples. The $\log_2$ fold changes of GFP intensity in each sgRNA transduced sample vs Ctrl sgRNA were calculated and plotted. **C.** Densitometric analysis of **Fig. 3B (right)**. The relative p100-EGFP, endogenous p100, p52 and STAT3 signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the Ctrl sgRNA conditions in the parental controls line. Experiments repeated three times. *Error bars denote SEM of three independent experiments.*

**Supplementary Figure 5: Related to Fig 3.**

**A.** Densitometric analysis of **Fig. 3C (left)**. The relative p52, NIK, and STAT3 signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the Ctrl sgRNA conditions in each cell lines. Experiments repeated three times. *Error bars denote SEM of three independent experiments.* **B. Left,** NIK positive ALK- ALCL line FEPD was transduced with STAT3 or Ctrl shRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. **Right,** NIK negative ALK- ALCL line MAC1 and ALK+ line SUDHL1 were transduced with STAT3 or Ctrl sgRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. *indicates that this sgRNA has limited effect to deplete STAT3 expression in this experiment. **C.** Expression of p-

STAT3 and nuclear p52 in ALCL primary cases by immunohistochemistry (IHC). Immunohistochemical p-STAT3 and p100/p52 staining are shown in two cases. Section of lymph nodes were examined microscopically using a 200X magnification. **D.** Expression of p-STAT3 and nuclear p52 in ALCL cell lines TLBR2 and MAC2B by immunohistochemistry (IHC). Immunohistochemical p-STAT3 and p100/p52 staining are shown. Cell block were examined microscopically using a 400X magnification.

**Supplementary Figure 6: Related to Fig 4.**

**A.** GSEA of NF-κB signatures. The analysis was based on STAT3 sgRNA vs. Control sgRNA transduced cells in TLBR2 and DEL lines. Positively correlated means "control sgRNA correlated" and negatively correlated means "STAT3 sgRNA correlated". Normalized enrichment score (NES) and normalized (NOM) P value are shown. **B. Top,** ALCL lines were transduced with STAT3 or Ctrl sgRNAs along with GFP. The fraction of viable sgRNA-expressing cells relative to the total viable cell fraction at indicated times following induction of the indicated sgRNAs, normalized to day 0 values. *Error bars denote SD of triplicates.* **Bottom,** p value analysis of the above chat. P were calculated comparing the Day 0 to each time points of sgSTAT3 #1 and sgSTAT3 #2 induction, in all cell lines tested; purple color indicates $P < 0.05$; red color indicates $P < 0.01$.

**Supplementary Figure 7: Related to Fig 5.**

**A.** Densitometric analysis of **Fig. 5A**. The relative CD30 and STAT3 signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the Ctrl sgRNA conditions in each cell lines. Experiments repeated three times. *Error bars denote SEM of three independent experiments.* **B. Left,** NIK positive ALK- ALCL line FEPD was transduced with STAT3 or Ctrl shRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. **Right,** NIK negative ALK- ALCL line MAC1 and ALK+ line SUDHL1 were transduced with STAT3 or Ctrl sgRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. **C.** Indicated ALCL lines were transduced with STAT3 or Ctrl sgRNAs along with GFP, induced to expression for 4 days. Surface CD30 expression in untransduced (GFP-) cells and sgRNA transduced (GFP+) cells was measured by flow cytometry.

**Supplementary Figure 8: Related to Fig 5.**

**A.** Densitometric analysis of **Fig. 5B**. The relative NIK, p52 and CD30 signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the Ctrl sgRNA conditions in each cell lines. Experiments repeated three times. *Error bars denote SEM of three independent experiments.* **B.** NIK negative ALK- ALCL line MAC1 and ALK+ line SUDHL1 were transduced with CD30 or Ctrl sgRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. **C.** Densitometric analysis of **Fig. 5C (left)**. The relative p52, CD30 and IKKα signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the Ctrl sgRNA conditions in each stable engineered cell lines. Experiments repeated three times. *Error bars denote SD of three independent experiments.* **D.** Densitometric analysis of **Fig. 5C (right)**. The relative p52, STAT3 and IKKα signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the Ctrl sgRNA conditions in each stable engineered cell lines. Experiments repeated three times. *Error bars denote SEM of three independent experiments.*

**Supplementary Figure 9: Related to Fig 6.**

**A.** Indicated ALCL lines were transduced with TRAF3 or Ctrl sgRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. The relative p52, NIK and TRAF3 signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the Ctrl sgRNA conditions in each cell lines (right). *Error bars denote SEM of three independent experiments.* **B.** Indicated ALCL lines were transduced with STAT3 or Ctrl sgRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. The relative TRAF3 and STAT3 signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the Ctrl sgRNA conditions in each cell lines (right). *Error bars denote SEM of four independent experiments.* **C. Left,** NIK positive ALK- ALCL line FEPD was transduced with STAT3 or Ctrl shRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. **Right,** NIK negative ALK- ALCL line MAC1 and ALK+ line SUDHL1 were transduced with STAT3 or Ctrl sgRNAs, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins. **D.** Densitometric analysis of **Fig. 6B (top)**. The relative immunoprecipitated CD30 and TRAF2 signal intensity were determined by densitometric analysis, and normalized to the Ctrl

IgG IP condition in MAC2B cell line. Experiments repeated three times. *Error bars denote SEM of three independent experiments.* **E.** TRAF2 IPs or total lysates from DEL and TLBR2 cells were immunoblotted for the indicated proteins. **F.** Densitometric analysis of **Fig. 6B (bottom)**. The relative immunoprecipitated CD30, TRAF2, and TRAF3 signal intensity (compared to input) were determined by densitometric analysis, and normalized to the Ctrl IgG IP condition in each cell lines. Experiments repeated three times. *Error bars denote SEM of three independent experiments.* **G.** Pearson's correlation coefficient for co-localization analysis of **Fig. 6C**. ImageJ software combined with plug-in programs was used to calculate the overlap coefficients of CD30 and TRAF3 for each confocal image. Costes' automatic threshold was applied. Rs = Pearson's Coefficient. 1 means perfect overlap, and 0 means random distribution of CD30 and TRAF3.

**Supplementary Figure 10: Related to Fig 7.**

**A.** Densitometric analysis of **Fig. 7A**. The relative p100, p52, p-STAT3 and STAT3 signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the untreated conditions in each cell lines. Experiments repeated three times. *Error bars denote SEM of three independent experiments.* **B.** Densitometric analysis of **Fig. 7B**. The relative p100, p52, p-STAT3, STAT3 and ALK signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the Ctrl shRNA conditions in each cell lines. Experiments repeated three times. *Error bars denote SEM of three independent experiments.* **C.** Lysates of indicated ALK- ALCL lines were analyzed by immunoblotting for the indicated proteins. **D.** Densitometric analysis of **Fig. 7C**. The relative PCMI-JAK2, JAK2, p-STAT3, STAT3, CD30 and p52 signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the Ctrl sgRNA conditions in each cell lines. Experiments repeated three times. *Error bars denote SEM of three independent experiments.* **E.** Densitometric analysis of **Fig. 7E**. The relative p52 signal intensity (compared to β-actin) were determined by densitometric analysis, and normalized to the control vector transduced samples. Experiments repeated three times. *Error bars denote SEM of three independent experiments.* **F.** NIK negative ALK- ALCL cell line MAC1 was transduced with a control vector, STAT3 WT or STAT3 D661Y mutant, selected and expression induced. Lysates were analyzed by immunoblotting for the indicated proteins.

**Supplementary Table legends**

**Supplementary Table 1: IHC score (H score) of nuclear p52 and p-STAT3 in primary ALCL cases.** Intensity of staining was evaluated according to the following scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The percentage score was determined according to the proportion of tumor cells found to express nuclear p52 (0%=0; <5%=1; >5, <10=2; >10,<25=3; >25=4) or p-STAT3 (0%=0; <10%=1; >10, <50=2; >50,<75=3; >75=4), and then multiplied by the staining intensity score to obtain a final semi quantitative H score.

**Supplementary Table 2: Histopathology and ALK- ALCL subtypes in primary ALCL cases.** The cases are ordered in the same sequence as in Supplementary Table 1.

**Supplementary Table 3:** The containing sgRNAs and sequences of the mini CRISPR library (**Fig. 3**).

**References:**


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5. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* 2009 May 1; **25**(9)**:** 1105-1111.

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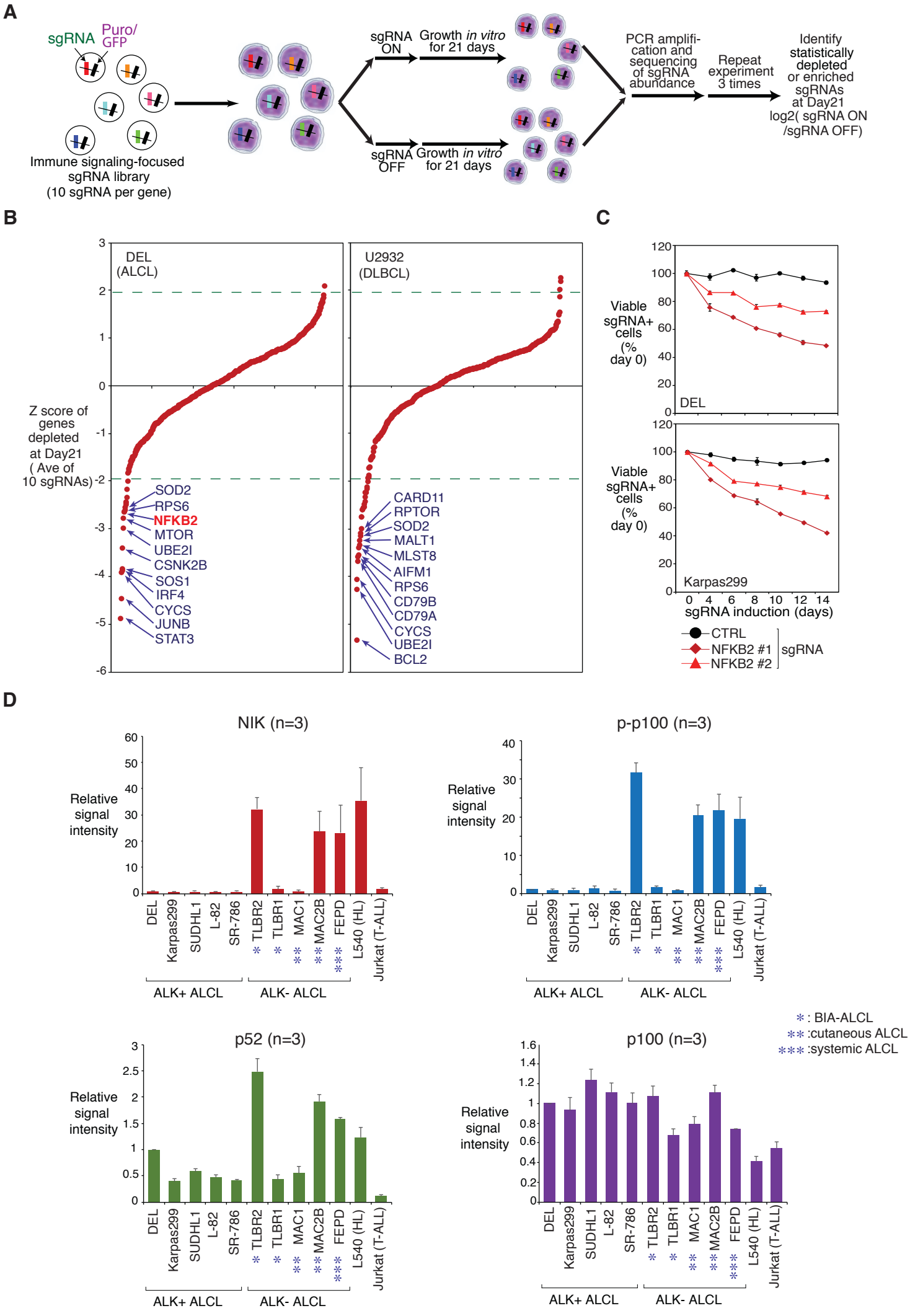
8. Ngo VN, Young RM, Schmitz R, Jhavar S, Xiao W, Lim KH*, et al.* Oncogenically active MYD88 mutations in human lymphoma. *Nature* 2011 Feb 3; **470**(7332)**:** 115-119.

# Supplemental Fig. 1



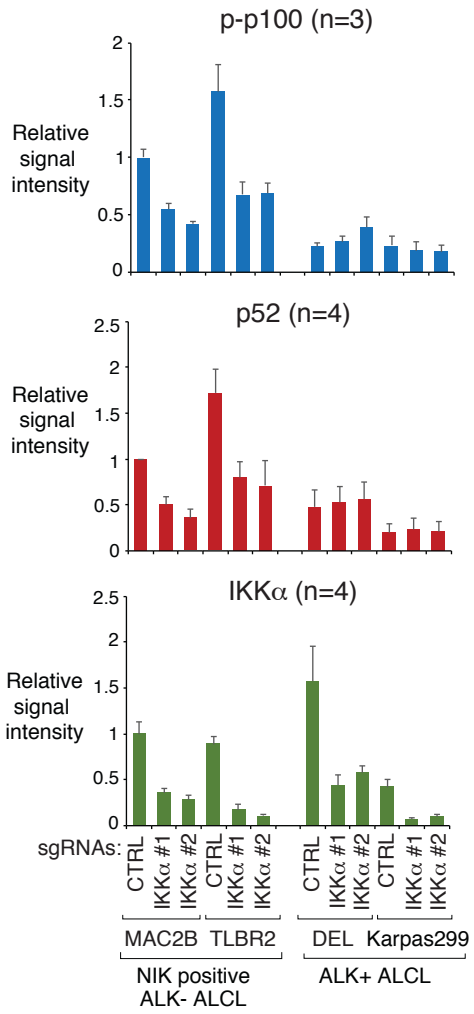
# Supplemental Fig. 2

## A

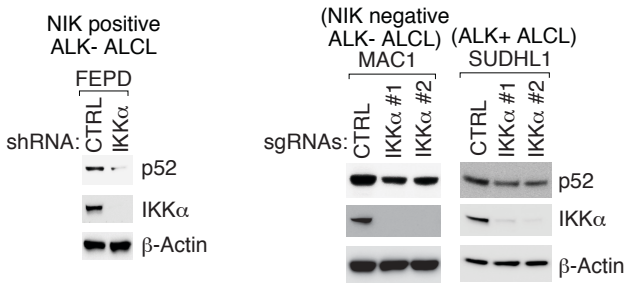
### p value for Fig. 2A (left)

| Cell Line | | Day 4 vs Day 0 | Day 6 vs Day 0 | Day 8 vs Day 0 | Day 10 vs Day 0 | Day 12 vs Day 0 | Day 14 vs Day 0 |
|---|---|---|---|---|---|---|---|
| DEL | SgIKKα#1 | 0.291138487 | 0.175162632 | 0.073463961 | 0.100890222 | 0.069285365 | 0.051987601 |
| | SgIKKα#2 | 0.074995727 | 0.061089101 | 0.020940881 | 0.019415608 | 0.028920557 | 0.025029132 |
| | | | | | | | |
| Karpas299 | SgIKKα#1 | 0.259852443 | 0.069865473 | 0.125897918 | 0.101266093 | 0.112243499 | 0.101213822 |
| | SgIKKα#2 | 0.841427508 | 0.651227209 | 0.615426065 | 0.243928077 | 0.530507057 | 0.587970702 |
| | | | | | | | |
| MAC2B | SgIKKα#1 | 0.002243122 | 0.000407239 | 0.000342583 | 5.04893E-05 | 0.000150864 | 2.11346E-05 |
| | SgIKKα#2 | 0.000198236 | 0.001689721 | 0.000686095 | 0.000175656 | 0.000107734 | 0.000138187 |
| | | | | | | | |
| TLBR2 | SgIKKα#1 | 0.053081338 | 0.00013701 | 0.00010345 | 9.12691E-05 | 1.52621E-05 | 1.65162E-05 |
| | SgIKKα#2 | 0.078540346 | 0.001208742 | 8.96399E-06 | 0.000241155 | 0.000202152 | 0.000357733 |

## B




## C



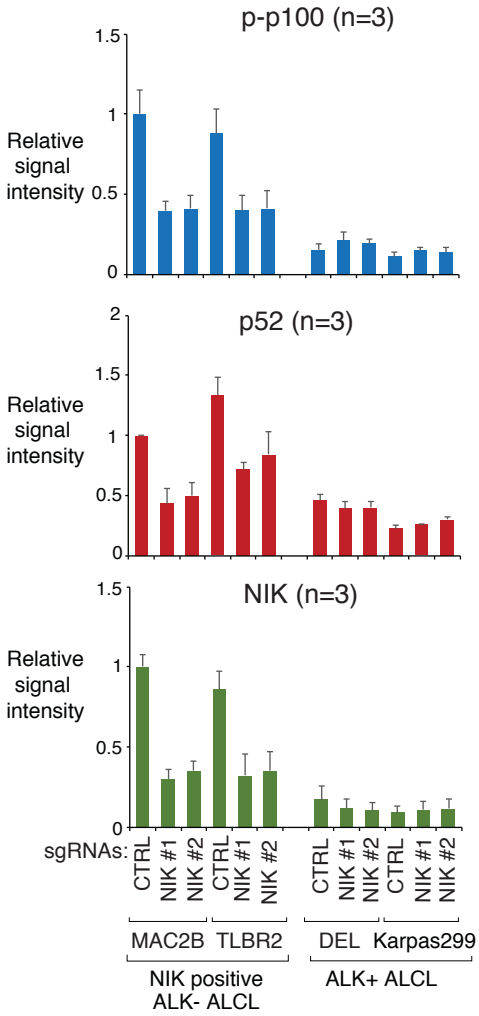
# Supplemental Fig. 3

## A

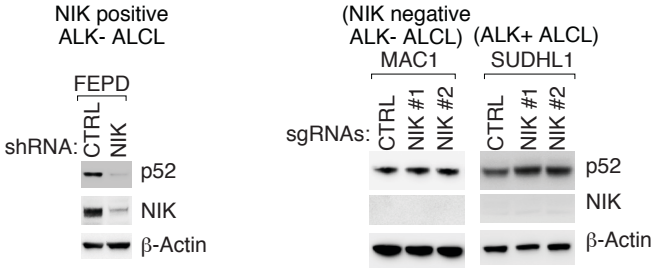
### p value for Fig. 2B (left)

| Cell Line | | Day 4 vs Day 0 | Day 6 vs Day 0 | Day 8 vs Day 0 | Day 10 vs Day 0 | Day 12 vs Day 0 | Day 14 vs Day 0 |
|---|---|---|---|---|---|---|---|
| DEL | sgNIK#1 | 0.887726508 | 0.990213536 | 0.428453263 | 0.256095921 | 0.134375017 | 0.10868538 |
| | sgNIK#2 | 0.370407026 | 0.179693671 | 0.055901334 | 0.041789682 | 0.029843349 | 0.026991015 |
| | | | | | | | |
| Karpas299 | sgNIK#1 | 0.269967441 | 0.126914461 | 0.101677467 | 0.072753203 | 0.0615781 | 0.030250697 |
| | sgNIK#2 | 0.01588263 | 0.006633657 | 0.001274925 | 0.00229331 | 0.005791245 | 0.018314594 |
| | | | | | | | |
| MAC2B | sgNIK#1 | 0.139590262 | 0.012526383 | 0.001912142 | 0.002420129 | 0.003498313 | 0.001505852 |
| | sgNIK#2 | 0.018738554 | 0.001584167 | 0.000444545 | 4.96833E-06 | 0.000352838 | 0.000378086 |
| | | | | | | | |
| TLBR2 | sgNIK#1 | 4.56506E-05 | 0.000380602 | 0.00025357 | 0.000366728 | 2.44632E-05 | 1.04232E-05 |
| | sgNIK#2 | 0.01269388 | 6.89122E-05 | 0.000124906 | 0.000548069 | 0.000318162 | 0.000198619 |

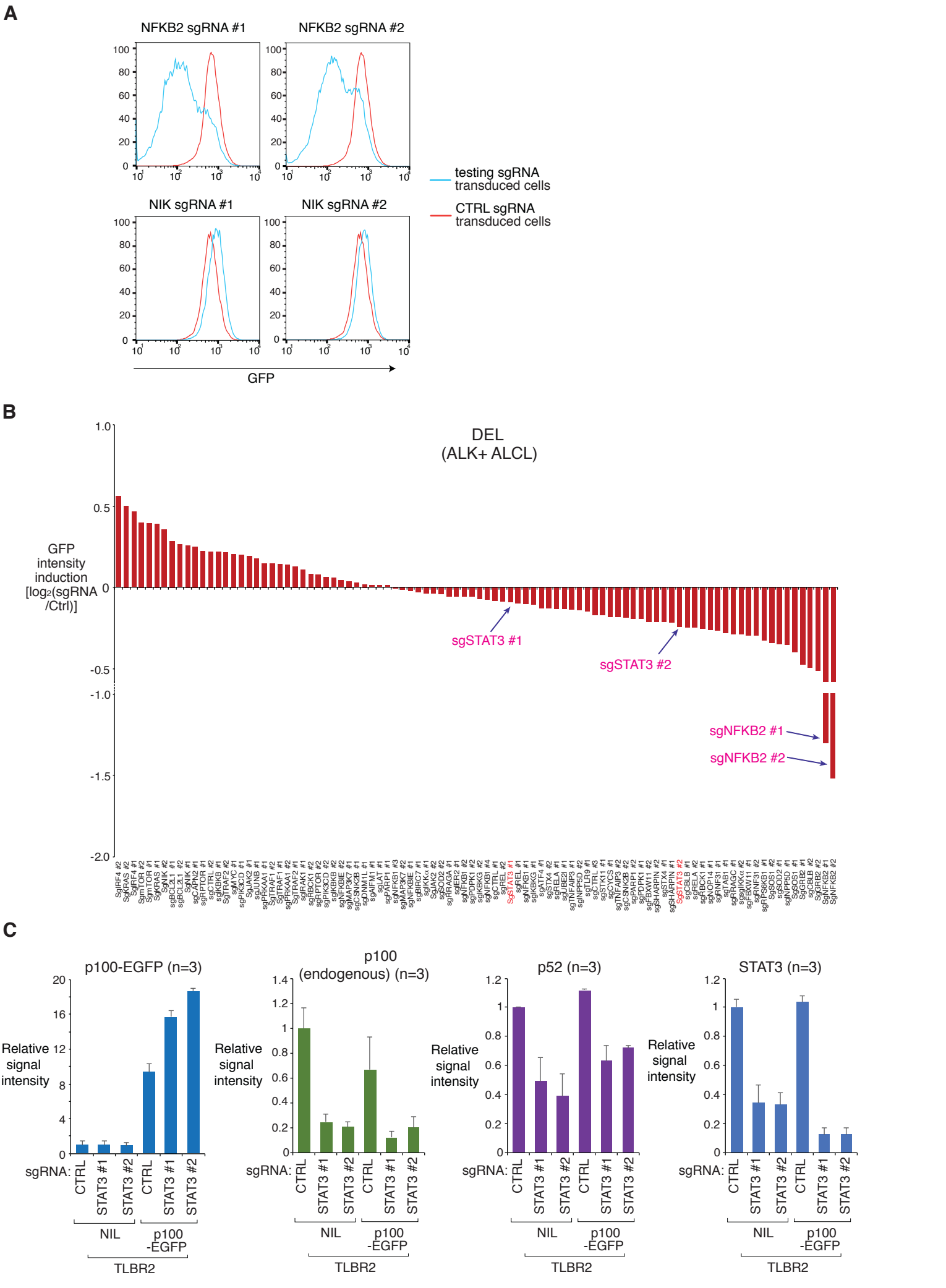
## B



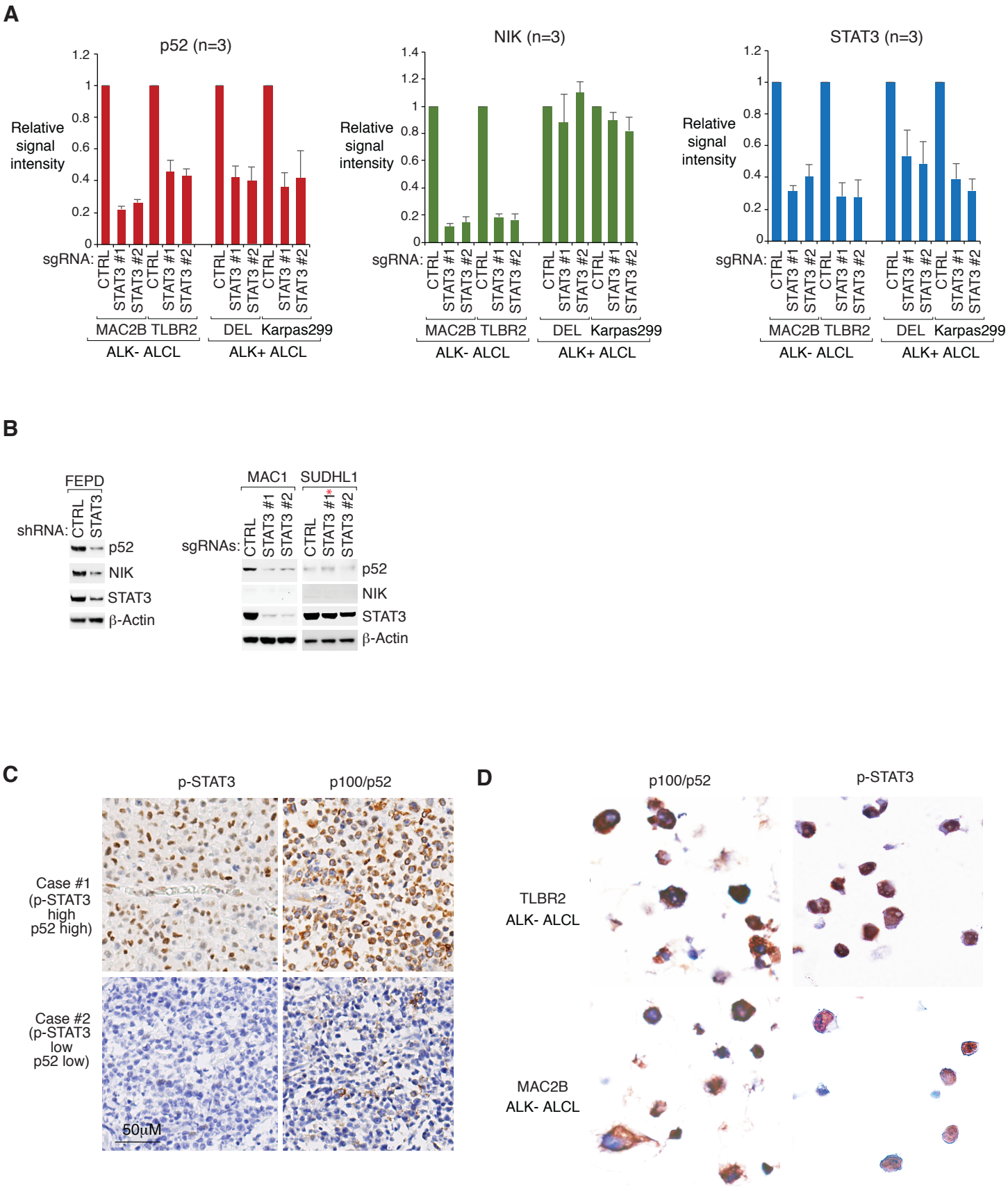

## C



# Supplemental Fig. 4

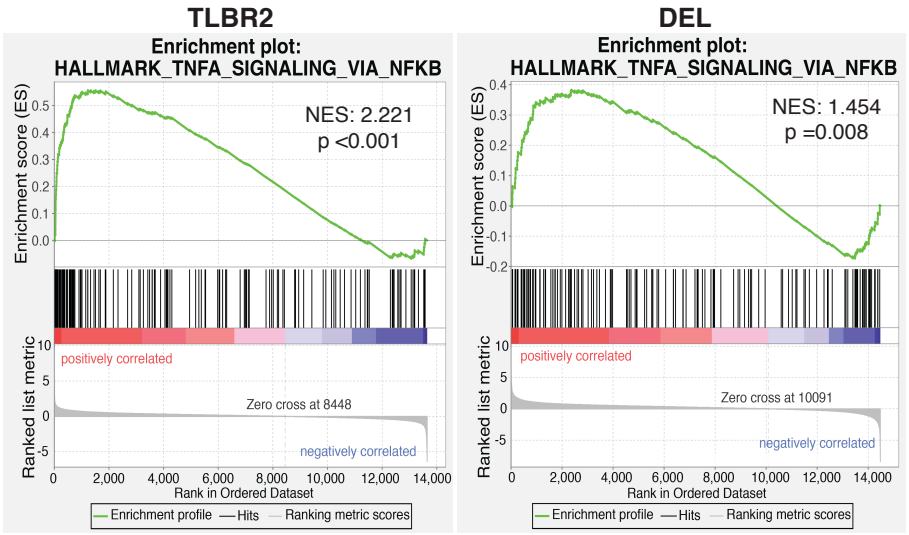


# Supplemental Fig. 5

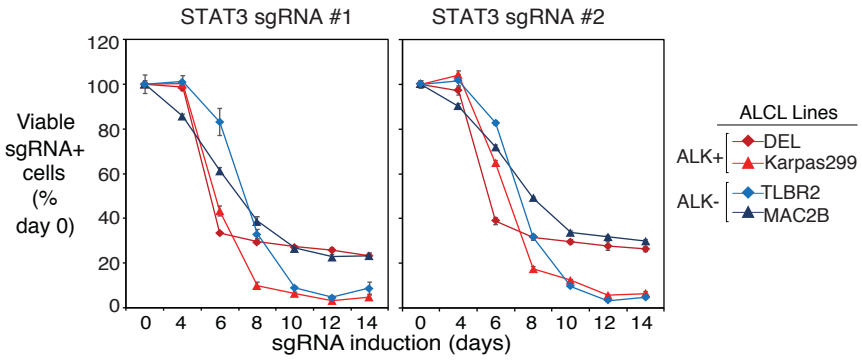


# Supplemental Fig. 6

**A**



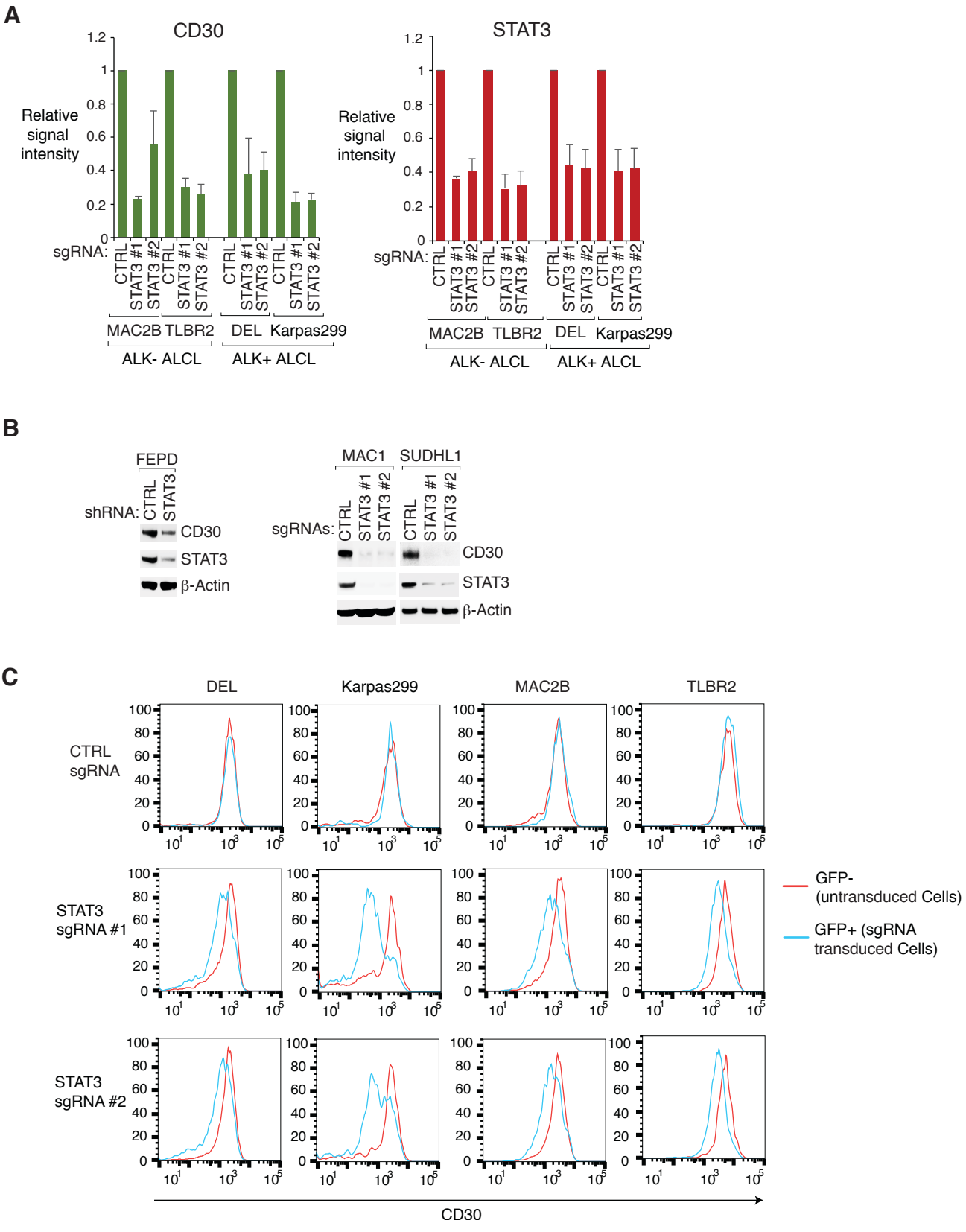

**B**



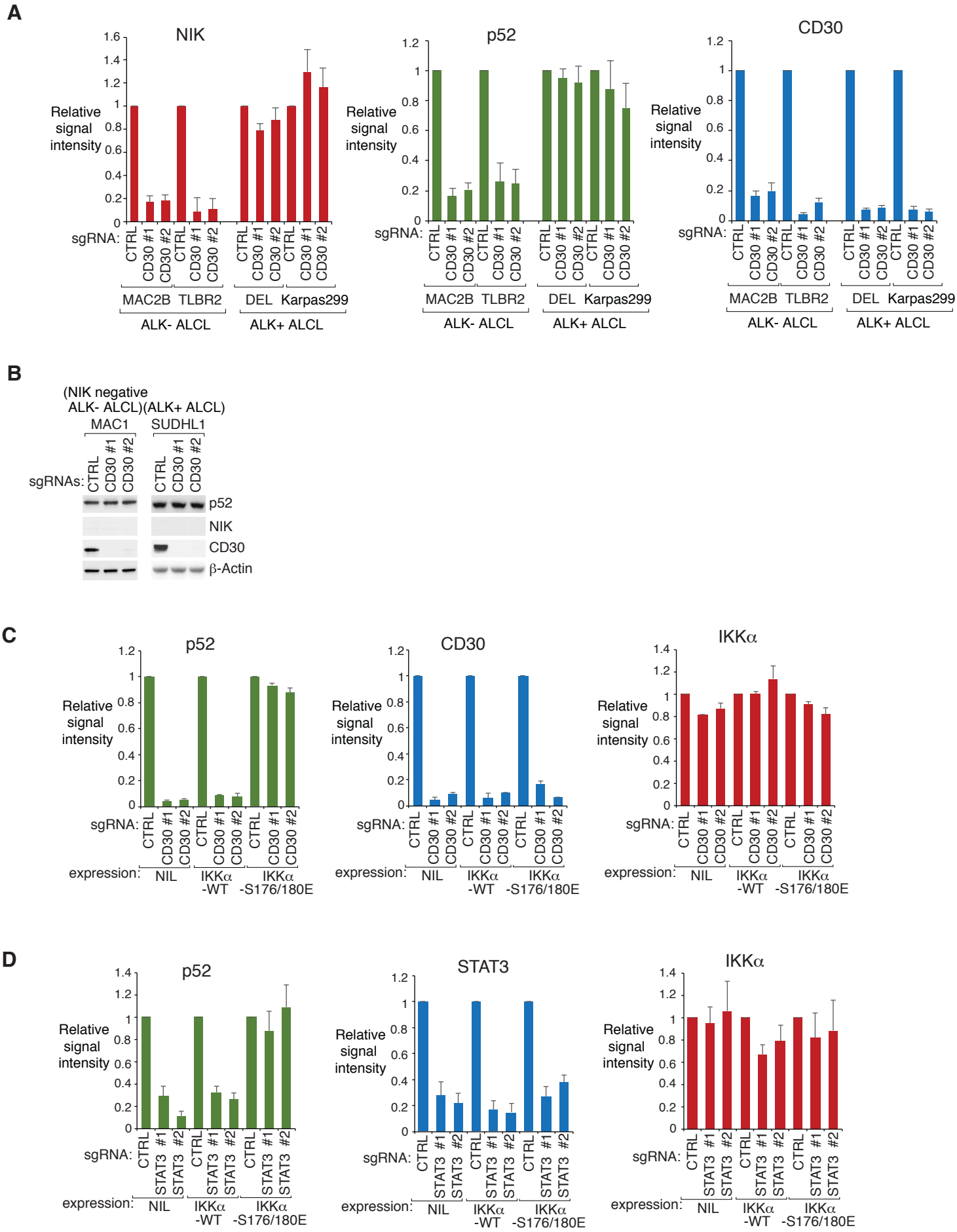

**P value of STAT3 sgRNAs toxicity assays**

| Cell Line | | Day 4 vs Day 0 | Day 6 vs Day 0 | Day 8 vs Day 0 | Day 10 vs Day 0 | Day 12 vs Day 0 | Day 14 vs Day 0 |
|---|---|---|---|---|---|---|---|
| DEL | SgSTAT3#1 | 0.391384774 | 0.000150323 | 4.4137E-06 | 3.04561E-05 | 0.000108802 | 0.000178886 |
| | SgSTAT3#2 | 0.309132031 | 0.000520717 | 0.000384282 | 0.000384678 | 0.000482088 | 0.000276612 |
| | | | | | | | |
| Karpas299 | SgSTAT3#1 | 0.88779058 | 7.81217E-05 | 0.000192632 | 0.000104873 | 0.000106219 | 0.000169123 |
| | SgSTAT3#2 | 0.092485868 | 0.000864993 | 6.61296E-05 | 4.38044E-05 | 2.30973E-05 | 2.24356E-06 |
| | | | | | | | |
| MAC2B | SgSTAT3#1 | 0.002244646 | 0.000176716 | 0.000199299 | 4.87431E-05 | 4.92928E-05 | 8.43334E-06 |
| | SgSTAT3#2 | 0.003540372 | 0.000940833 | 0.000426116 | 0.000178486 | 6.19371E-05 | 0.000197698 |
| | | | | | | | |
| TLBR2 | SgSTAT3#1 | 0.776611402 | 0.103971325 | 0.002492395 | 0.000901355 | 0.000674636 | 0.001898462 |
| | SgSTAT3#2 | 0.472338301 | 0.000733432 | 0.000510676 | 7.38049E-05 | 9.21402E-05 | 4.65429E-05 |

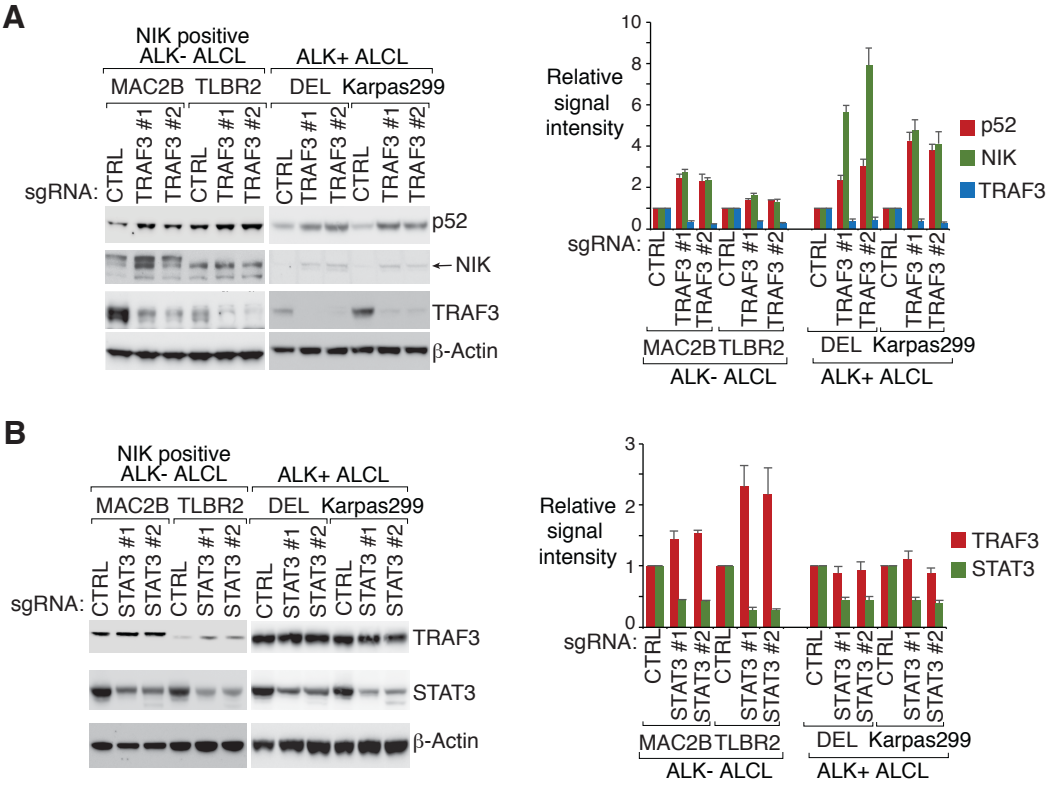
# Supplemental Fig. 7

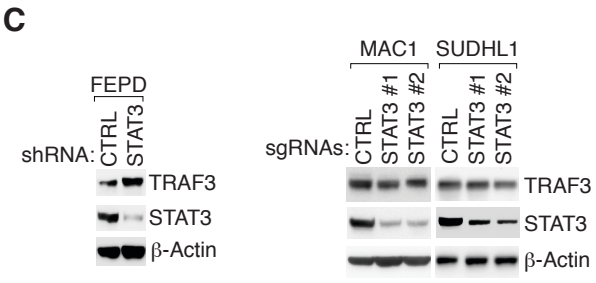


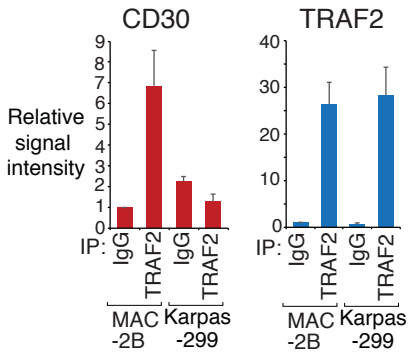
# Supplemental Fig. 8

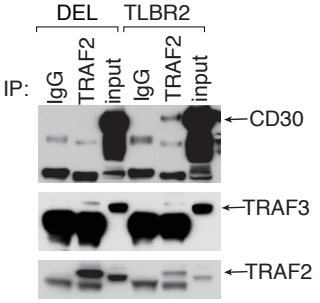


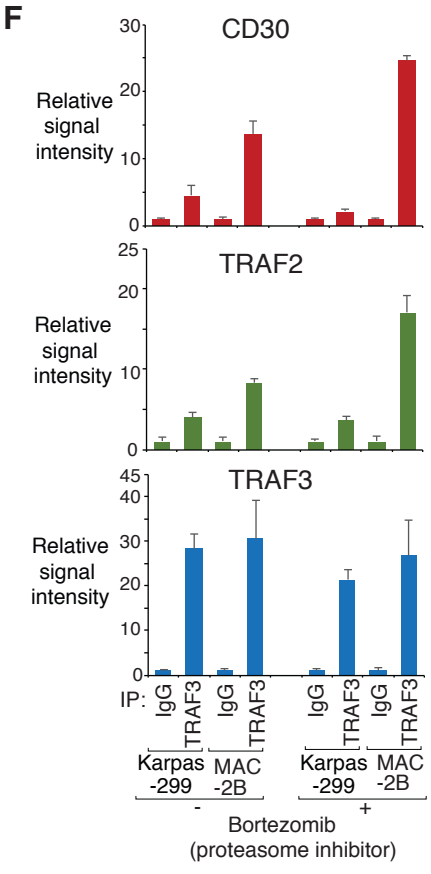
# Supplemental Fig. 9

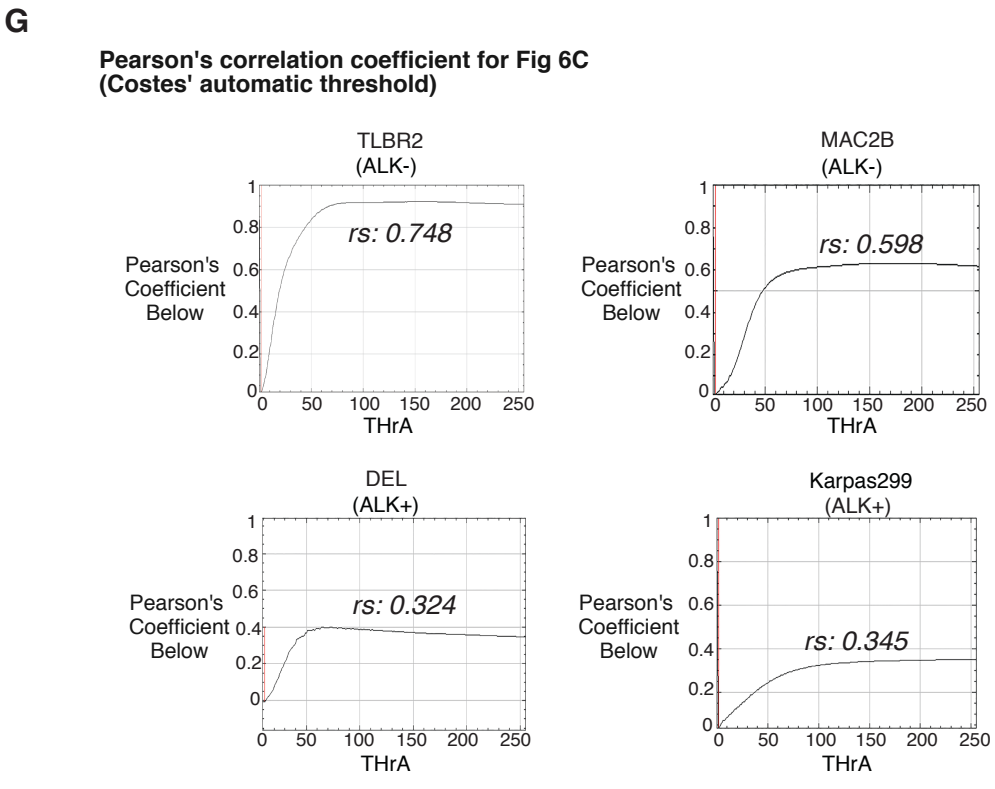


# Supplemental Fig. 10