PNAS

Supporting Information for

ALK fusion NSCLC oncogenes promote survival and inhibit NK cell responses via *SERPINB4* expression

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This PDF file includes:

SI Appendix

Materials and Methods

Cell culture

Non-tumorigenic human bronchial epithelial cell line (NL20) was purchased from ATCC and maintained in Ham's F12 medium supplemented with 5% of fetal bovine serum (FBS), 1% of penicillin/streptomycin, 1X Insulin-Transferrin-Selenium-Ethanolamine (ITS-X) (Thermo Fisher Scientific), 10 ng/ml of epidermal growth factor (Thermo Fisher Scientific), and 500 ng/ml of hydrocortisone (Sigma-Aldrich) at 37℃ and 5% CO_2. CUTO8, CUTO9, CUTO29, YU1077, H3122 and H2228 were maintained in RPMI1640 supplemented with 5% FBS, 1% of penicillin/streptomycin at 37 °C and 5% CO_2. The CUTO 8, CUTO 9 and CUTO 29 EML4-ALK cell lines were derived from ALK-positive patients at the University of Colorado-Anschutz Medical Center. Molecular characterization of these CUTO lines as well as the original source of tumor tissue and the patient informed consent have been described in prior publications (1).

Plasmid construction and stable cell line generation

ALK fusion gene cDNA corresponding to EML4(exon1-exon13)-ALK(exon20-exon29)-V1, EML4(exon1-exon6)-ALK(exon20-exon29)-V3, KIF5B(exon1-exon24)-ALK(exon20-exon29) and TFG(exon1-exon3)-ALK(exon20-exon29) were synthesized by Genscript. Synthesized genes were cloned into pcDNA3 followed by PCR amplification of inserted genes using Phusion High-Fidelity DNA Polymerase system (Thermo Fisher Scientific) and ALK fusion specific primer pairs as listed in *SI Appendix*, Table S3. Fusion genes were sub-cloned

into pLVX-TetOne vector using In-Fusion HD cloning system (Takara bio). All plasmids were confirmed by Sanger sequencing. NL20 cells were transfected with ALK fusion constructs (pLVX-TetOne-EML4-ALK-V1, pLVX-TetOne-EML4-ALK-V3, pLVX-TetOne-KIF5B-ALK and pLVX-TetOne-TFG-ALK) or pLVX-TetOne (empty vector) respectively using X-tremeGENE™ HP DNA transfection reagents (Roche) according to the manufacturer's instruction. After transfection, cells were selected with 1µg/ml puromycin (Thermo Fisher Scientific) to generate stable cell lines. After western blot analysis of ALK expression and activated downstream signaling, three independent clones per ALK fusion variant were isolated by limited dilution. Resistant single colonies were maintained in complete medium containing 0.5 µg/ml of puromycin (Thermo Fisher Scientific).

Cell growth curves

NL20-ALK cells were plated in 48-wells plates (8000 cells/per well), and serum starved for 18 h, followed by doxycycline induction (300 ng/ml for EML4-ALK-V1 and KIF5B-ALK, 100 ng/ml for EML4-ALK-V3 and TFG-ALK) in the presence or absence of 30nM lorlatinib (Selleckchem). Cell confluence was monitored for at least 4 days and analyzed by IncuCyte® Live-Cell Analysis System (Essen BioScience).

Mouse xenograft assay

Female BALB/cAnNRj-Foxn1nu mice (Janvier Laboratory) 4-6 weeks old were housed with access to food and water ad libitum in a 12:12 light-dark cycle. Animals were allowed to acclimatize for 1 week before any procedures. Four

days before injection, diet were changed for half of the mice to a diet with 625 mg/kg Doxycycline (#TD.09628, Envigo), the other mice continued on normal chow diet (#2016, Envigo). Doxycycline was renewed every 2-3 days. 2 x 10^6 cells were induced with doxycycline at 300 ng/ml for 24 h before harvesting. 1.5 x 10^6 cells in serum-free medium mixed with Matrigel Matrix at a ratio of 1:1 were injected into the flank of the animal. The total injection volume was 100 µL. For each fusion-protein-construct, 5 animals per group, doxycycline and chow, were injected. The animals were followed and considered to have an active tumour once a diameter of 10 mm had been observed. Animals with an active tumour were sacrificed and samples taken for western blot and immunohistochemistry. All experimental procedures and protocols were performed in accordance with the Regional Animal Ethics Committee approval, Jordbruksverket (1890-201).

Immunoblotting analyses

Cells were lysed with RIPA buffer (Thermo Fisher Scientific) containing cOmplete™ protease inhibitor cocktail (Roche) for 30 mins on ice. Resulting lysates were centrifuged at 13000 rpm/ 4°C for 15 mins and supernatants were used for immunoblotting. Lysates were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with either 3% bovine serum albumin or 5% non-fat milk and probed with primary antibodies overnight at 4°C. After incubation with secondary antibodies at RT for 1 h, signal was detected by using Amersham ECL Prime reagent (GE Healthcare Life Sciences) and LI-COR Odyssey Fc imaging system (LI-COR).

Antibodies and reagents

Antibodies against p-Y1278-ALK (1:2000; #6941), ALK (1:3000; #3633), beta-actin (1:10000; #4970), p-S473-AKT (1:2000; #4060), AKT (1:2000; #4691), p-T202/Y204-ERK (1:3000; #4370), p-Y705-STAT3 (1:1000; #9145), STAT3 (1:1000; #4904), Tubulin (1:5000; #2144), GAPDH (1:5000; #5174), p-S265-FRA1 (1:1000; #5841), FRA1 (1:1000; #5281), p-S63-c-Jun (1:1000; #91952), c-Jun (1:1000; #9165), p-Tyr-1000 (1:2000; #8954), NF-κB1 p105/p50 (1:1000; #12540), NF-κB2 p100/p52 (1:1000; #4882), PARP (1:1000; #9532), p-T71/T53-ATF2 (1:1000; #24329), p-T334-MAPKAPK-2 (1:1000; #3007), p-T180/Y182-p38 (1:1000; #4511), Rabbit (DA1E) mAb IgG (#3900) and Granzyme M (1:1000; #37765) were purchased from Cell Signaling Technology. p38 (1:1000; cat. sc-271120) was purchased from Santa Cruz Biotechnology. pan-ERK1/2 (1:5000; cat.610123) was purchased from BD, SERPINB4 was purchased from Sigma (1:1000; cat. SAB2105452). Horseradish peroxidase (HRP)-conjugated secondary antibody, goat anti-mouse immunoglobulin G (IgG), goat anti-rabbit IgG (1:5000), and TNF-α were purchased from Thermo Fisher Scientific. Lorlatinib, JSH-23, CC-930, T-5224 and Brigatinib were from Selleckchem. Doxycycline and cycloheximide were purchased from Sigma-Aldrich or Merck Group.

Generation of polyclonally activated NK cells

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers by density gradient centrifugation using Lymphoprep (Stemcell technologies). NK cells were isolated by negative selection using the human

NK cell isolation kit (Miltenyi Biotec). Pure NK cells (>95%) were cultured together with irradiated allogenic PBMCs and 221G feeder cells in IMDM supplemented with 10% HI FCS, 2 mM L-glutamine, 1% PEST, 600 IU/ml IL-2 (Chiron) and 5 µg/ml phytohemagglutinin (PHA-M, Sigma cat. L8902). After 5 to 7 days, the medium was gradually changed to medium without PHA-M. The concentration of NK cells was maintained at 1-2 x 10^6 cells/ml and cells were frozen down in rounds. Before use in functional assays, thawed NK cells were cultured for at least 2 weeks.

NK cell co-culture and Caspase-3/7 activity measurements

NL20-ALK cells were treated with doxycycline (300 ng/ml) for 2 days, prior to plating on 96-well plates (4000 cells/ per well). After 16 h of incubation, polyclonally activated NK cells were re-suspended in complete F12 medium containing caspase 3/7 green reagent (1:2000, Essen BioScience) and 16000 cells were added to each well. The caspase3/7 signal was measured and analyzed by IncuCyte® Live-Cell Analysis System (Essen BioScience).

Overexpression of Serpin B4 and siRNA knockdown of STAT3

NL20 cells were transiently transfected with pcDNA3.1-SERPINB4-C-(k)DYK (OHu23419, Genscript) and vector control respectively using X-tremeGENE™ HP DNA transfection reagent (Roche) according to manufacturer's instruction. After 48 h incubation, transfected cells were subjected to TNF-α (100 ng/ml) or H_2O_2 (final concentration 0.05 M) treatments. For siRNA knockdown, Silencer® Select siRNAs for STAT3 and negative controls were purchased from Thermo Fisher Scientific and transfected to NL20-ALK cells using Lipofectamine 2000

(Thermo Fisher Scientific) according to the manufacturer's protocol. The final concentration of siRNA for each transfection reaction was 40 nM. Sequences of siRNAs were listed in *SI Appendix* Table S3. At 48 h post transfection, cell lysates were collected for immunoblotting analysis.

Generation of CRISPR/Cas9 Engineered *SERPINB4* KO NL20-ALK cells

Single guide RNA (5'-AUUGGCAAUGAUACGACAC-3') (Sigma) targeting the DNA sequence of *SERPINB4* and TrueCut™ Cas9 Protein v2 (Thermo Fisher Scientific) were co-transfected in NL20-TFG-ALK or NL20-EML4-ALK-V3 cells using Lipofectamine CRISPRMAX Cas9 transfection reagents (Thermo Fisher Scientific) following the manufacturer's protocol. After 48 h, KO efficiency was evaluated by immunoblot analysis and *SERPINB4* KO cells were subjected to single clone selection by limited dilution method. A total of 4 colonies for NL20-TFG-ALK and 3 colonies for NL20-EML4-ALK-V3 were selected and depletion of *SERPINB4* was validated by immunoblot analysis.

Immunoprecipitation

Cells were washed once with ice-cold PBS and lysed in immunoprecipitation lysis buffer containing 0.025 M Tris, 0.15 M NaCl, 1% NP-40, 5% glycerol and 1X cOmplete™ protease inhibitor cocktail (Roche) at 4°C for 20 min. Cell lysates were centrifuged at 14000 rpm for 15 min. Supernatants were collected and protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Anti-NF-κB1 or NF-κB2 antibodies were incubated with total protein (500 µg/per reaction) at 1:100 ratio overnight at 4°C. Protein G Sepharose beads (Merck) were added and incubated at 4°C for 1 h.

Immunocomplexes were subsequently washed 3 times with lysis buffer and boiled in 60 µl of 1X SDS sample buffer for 10 min. Supernatants were further used for immunoblotting analysis.

Sample preparation for MS-based phosphoprotein analysis

Pooled ALK fusion clones were serum-starved for 18 h and treated with doxycycline (EML4-ALK-V1, KIF5B-ALK at 300 ng/ml and EML4-ALK-V3, TFG-ALK at 100 ng/ml) for 6 h followed by 1 h of lorlatinib treatment (30 nM). For phosphor protein analysis, treated cells were washed once with ice-cold PBS and lysed in urea lysis buffer containing 20 mM Hepes (pH 8.0), 9 M urea, 1 mM activated sodium orthovanadate, 2.5 mM sodium pyrophosphate, and 1 mM β-glycerol phosphate. Total protein concentration was determined using BCA kit as described above. For each sample, 30 mg of protein was subjected to custom phosphoprofiling by Cell Signaling Technology as described previously (2). In brief, samples were trypsinized and immunoprecipitated using P-Tyr-1000 (CST #8803) or P-Ser/Thr (CST #25081) motif antibodies. LC-MS/MS analysis was performed as described previously (2). Samples from EML4-ALK-V1, V3 (+/-dox, +/-lorlatinib) were performed in 2 biological replicates (n=2). KIF5B-ALK and TFG-ALK were performed once (n=1), and technical duplicates were performed for all conditions. MS/MS spectra were evaluated using a Comet and the Core platform from Harvard University (3).

RNA-seq analysis

RNA-seq analysis was performed on three independent stable clones for each NL20-ALK variant. Each NL20 ALK clone variant was sequenced in duplicate. RNA-seq paired-end reads (read length 150 base pairs) were aligned to the GRCh38 (human cell line data) reference genome using hisat2 (4). The average alignment efficiency was 92.5%. Genes were annotated using GENCODE 29 and quantified using HTSeq (5, 6). Only protein coding genes were used for further analysis. Differential gene expression was determined using DESeq2 (7). Genes were considered differentially expressed if their absolute log2 fold change values were above 1.5 at FDR-adjusted *P*-values (*P*adj) below 0.01, considering a hyperbolic threshold as described previously (8).

Phosphoproteomics analysis

Differential phosphorylation was determined using the R Differential Enhancement of Proteomics data (DEP) package (default settings) (9). Phosphosites were considered differentially phosphorylated when the absolute log2 fold change values were above 1.5 at FDR-adjusted p values below 0.05, considering a hyperbolic threshold as described earlier (8). SH2 and PTB domain containing protein IDs were downloaded from InterPro (ref: https://doi.org/10.1093/nar/gkac993).

Gene set enrichment analysis

Preranked gene set enrichment analyses (GSEA) were performed using the R *fgsea* package (*fgseaMultilevel* function, default parameters) with ranking

based on the DEseq2 statistic (RNA-seq) or log2 FC value (proteomics). Unranked GSEA was performed using Fisher's exact test followed by false discovery rate (FDR) correction using the Benjamini-Hochberg method (10). Hallmark and Canonical pathway gene sets were downloaded from the Molecular Signatures Database v7.2 (11).

Statistical analysis

Two-sample t-tests were performed to test the significance of the biological effects of manipulated gene expression. Analyses were carried out using GraphPad Prism. Two-tailed p-values < 0.05 were considered statistically significant.

Data and statistical analysis.

Statistical analyses were performed with either GraphPad Prism 7/8 software or R statistical package (v4.0). Statistical tests are indicated in the respective sections and figure captions. Multiple testing corrections were performed using the Benjamini-Hochberg method (10).

Supplementary Table legend

Supplementary Table S1. RNA-seq differential expression results.

Differential expression determined between cell lines containing the ALK fusion and control cell lines without the fusion. Results for 4 different fusions as indicated by tab names.

Supplementary Table S2. Phosphoproteomics differential phosphorylation results.

Differential phosphorylation determined between cell lines containing the ALK fusion and control cell lines without the fusion as well as results for differential phosphorylation after treatment with lorlatinib. Results for 4 different fusions and 4 lorlatinib treated cells as indicated by tab names.

Supplementary Table S3. Sequence of oligonucleotides for PCR and siRNA knockdown.

SI References

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Fig. S1

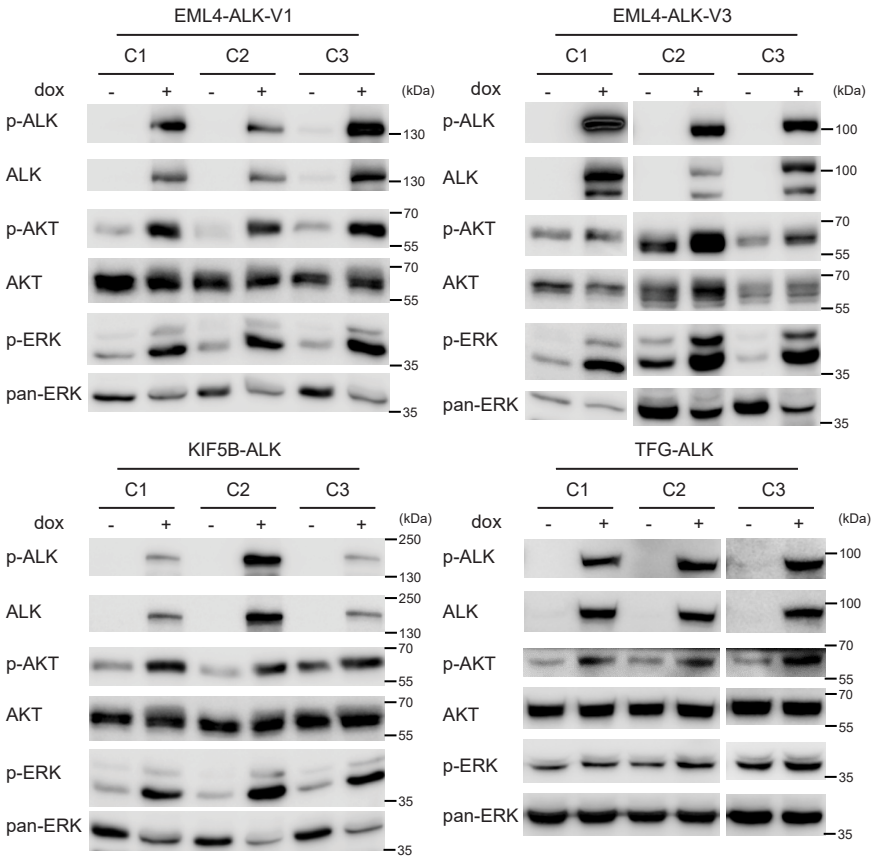


Fig. S1. ALK expression and activated downstream targets in NL20-ALK individual clones.

ALK fusions were induced in NL20-ALK individual clones with doxycycline. Lysates collected from three individual clones of each variant were immunoblotted with pY1278-ALK, pan-ALK, pS473-AKT, AKT, p-T202/Y204-ERK and pan-ERK as indicated.

Fig. S2

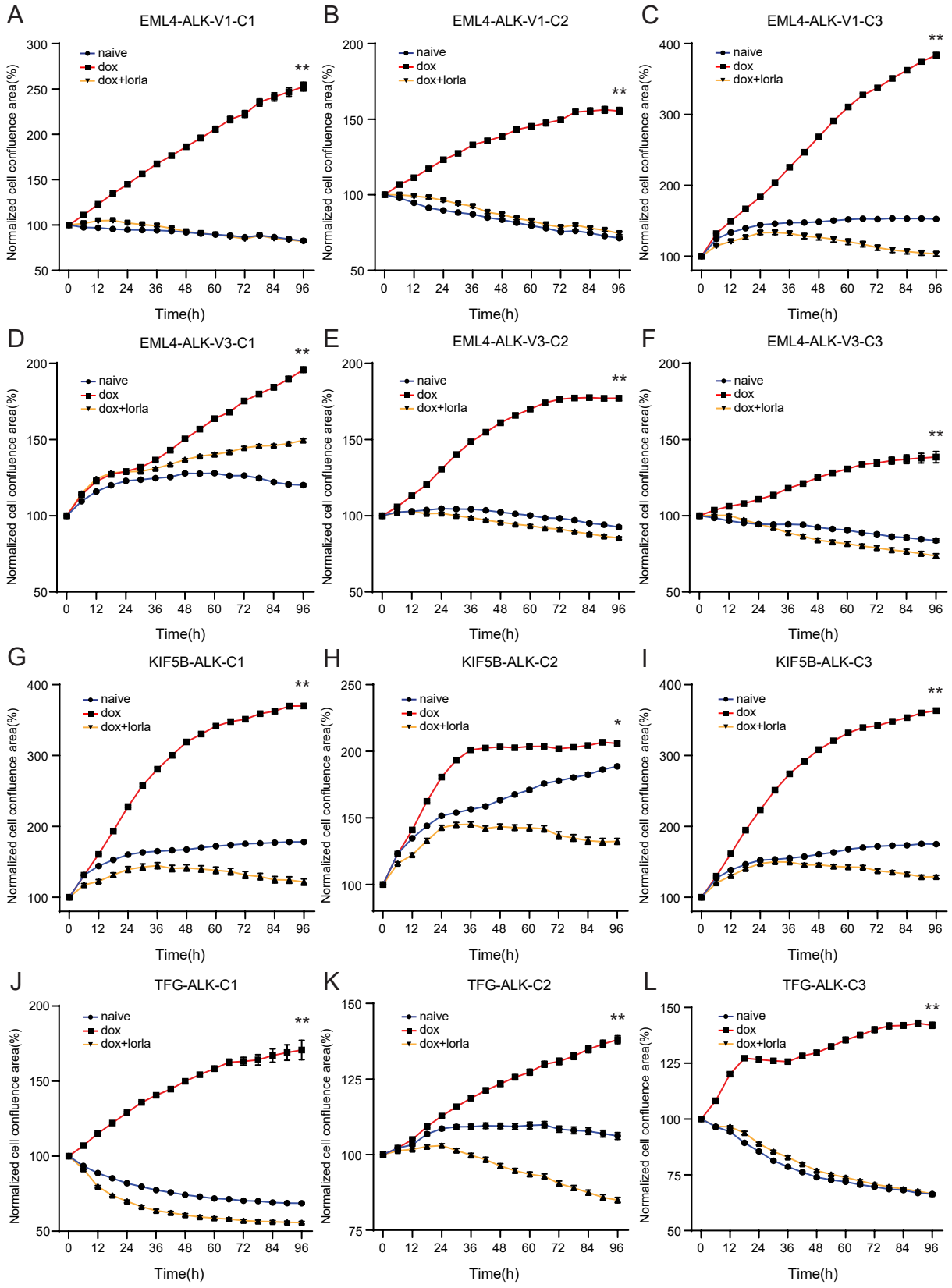


Fig. S2. Growth curve of dox-induced NL20-ALK individual clones.

A-L. NL20-ALK individual clones were treated with DMSO (blue), doxycycline (red) or doxycycline plus lorlatinib 30 nM (orange). Cell confluence was monitored using an IncuCyte® Live Cell Analysis system. Data points represent mean ± SEM of normalized cell confluence conducted in triplicate (*, P<0.05; **, P<0.01, two-tailed paired t-test). One typical experiment of three independent experiments is shown.

Fig. S3

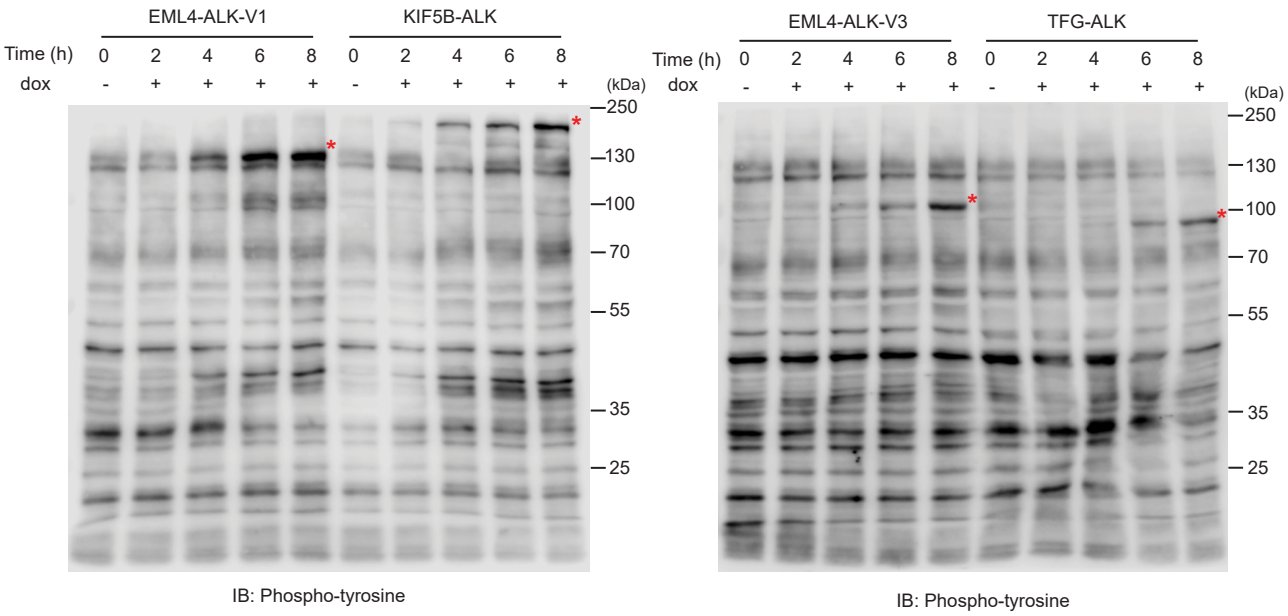


Fig. S3. Total phosphotyrosine phosphorylation in dox-induced NL20-ALK cells. ALK fusions were induced in NL20-ALK fusion expressing cells with doxycycline over an 8 h time-course. Lysates collected from each time point were immunoblotted with p-Y1000. Induced expression of each ALK fusion variant is indicated by asterisk.

Fig. S4

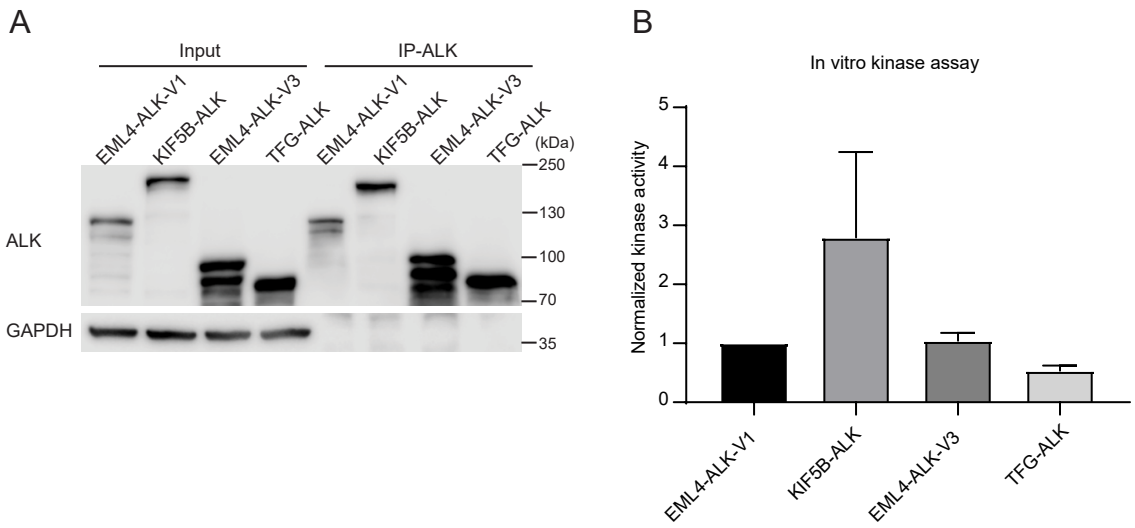
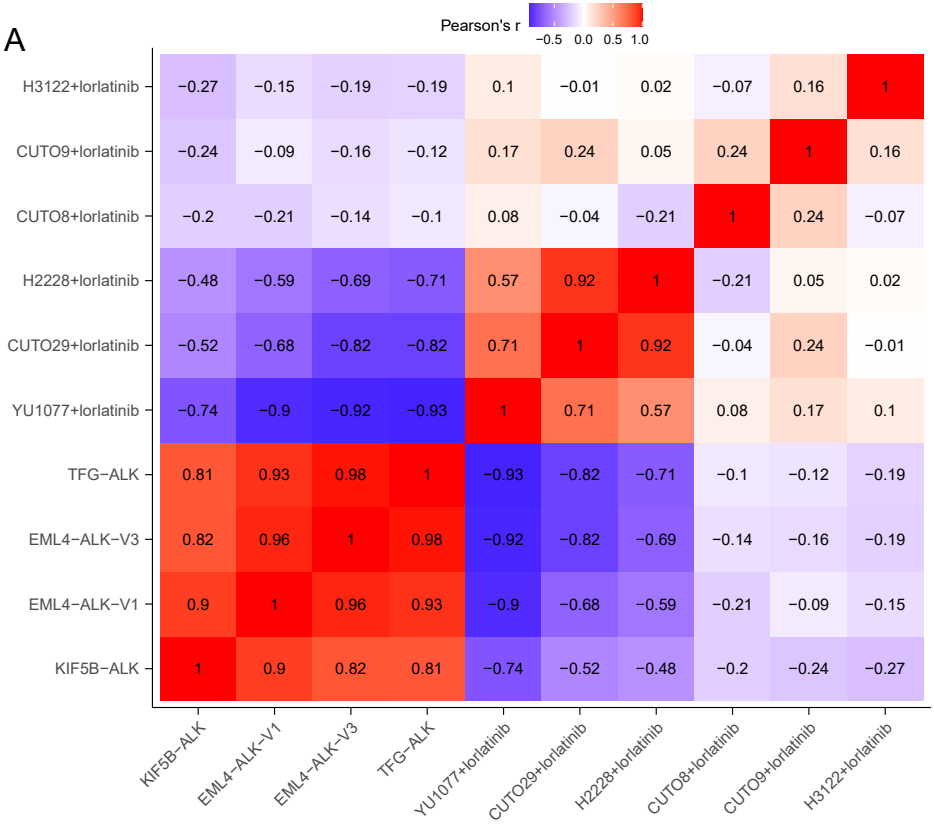


Fig. S4. Determination of ALK kinase activities by *in vitro* kinase assay.

A. ALK proteins were immunoprecipitated and immunoblotted with anti-ALK antibodies. The intensity of ALK expression of each variant was quantified by Image J and used for normalization of *in vitro* kinase assays. **B.** Kinase activity of each fusion protein was measured using a standard curve generated by the control sample provided in the assay kit and activity was normalized to all bands by immunoprecipitated ALK intensity.

Fig. S5


A
Pearson's r
−0.5 0.0 0.5 1.0
H3122+lorlatinib −0.27 −0.15 −0.19 −0.19 0.1 −0.01 0.02 −0.07 0.16 1
CUTO9+lorlatinib −0.24 −0.09 −0.16 −0.12 0.17 0.24 0.05 0.24 1 0.16
CUTO8+lorlatinib −0.2 −0.21 −0.14 −0.1 0.08 −0.04 −0.21 1 0.24 −0.07
H2228+lorlatinib −0.48 −0.59 −0.69 −0.71 0.57 0.92 1 −0.21 0.05 0.02
CUTO29+lorlatinib −0.52 −0.68 −0.82 −0.82 0.71 1 0.92 −0.04 0.24 −0.01
YU1077+lorlatinib −0.74 −0.9 −0.92 −0.93 1 0.71 0.57 0.08 0.17 0.1
TFG−ALK 0.81 0.93 0.98 1 −0.93 −0.82 −0.71 −0.1 −0.12 −0.19
EML4−ALK−V3 0.82 0.96 1 0.98 −0.92 −0.82 −0.69 −0.14 −0.16 −0.19
EML4−ALK−V1 0.9 1 0.96 0.93 −0.9 −0.68 −0.59 −0.21 −0.09 −0.15
KIF5B−ALK 1 0.9 0.82 0.81 −0.74 −0.52 −0.48 −0.2 −0.24 −0.27
KIF5B−ALK
EML4−ALK−V1
EML4−ALK−V3
TFG−ALK
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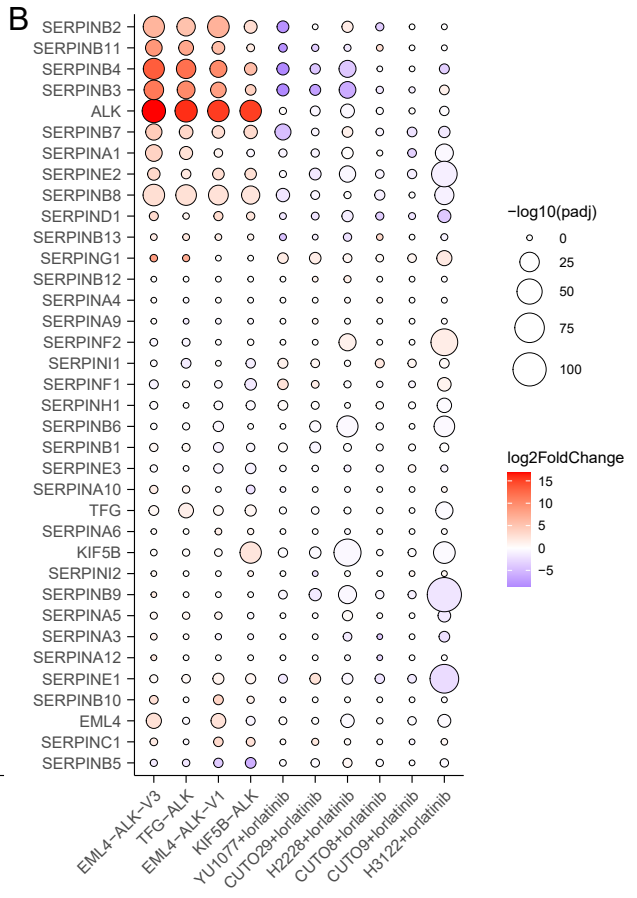
H3122+lorlatinib


B
SERPINB2
SERPINB11
SERPINB4
SERPINB3
ALK
SERPINB7
SERPINA1
SERPINE2
SERPINB8
SERPIND1
SERPINB13
SERPING1
SERPINB12
SERPINA4
SERPINA9
SERPINF2
SERPINI1
SERPINF1
SERPINH1
SERPINB6
SERPINB1
SERPINE3
SERPINA10
TFG
SERPINA6
KIF5B
SERPINI2
SERPINB9
SERPINA5
SERPINA3
SERPINA12
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SERPINB10
EML4
SERPINC1
SERPINB5
EML4−ALK−V3
TFG−ALK
EML4−ALK−V1
KIF5B−ALK
YU1077+lorlatinib
CUTO29+lorlatinib
H2228+lorlatinib
CUTO8+lorlatinib
CUTO9+lorlatinib
H3122+lorlatinib
−log10(padj)
0
25
50
75
100
log2FoldChange
15
10
5
0
−5

Fig. S5. *SERPINB* transcriptomic response to x-ALK fusions.

A. Correlation plot showing Pearson correlation coefficients of 13 *SERPINB* genes (logFC values) between different samples as indicated. **B.** Heatmap showing logFC values of all *SERPIN* genes, ALK and fusion partners in different samples as indicated. Hierarchical clustering performed on rows and columns of both plots (dendrograms not shown for visualisation purposes).

Fig. S6

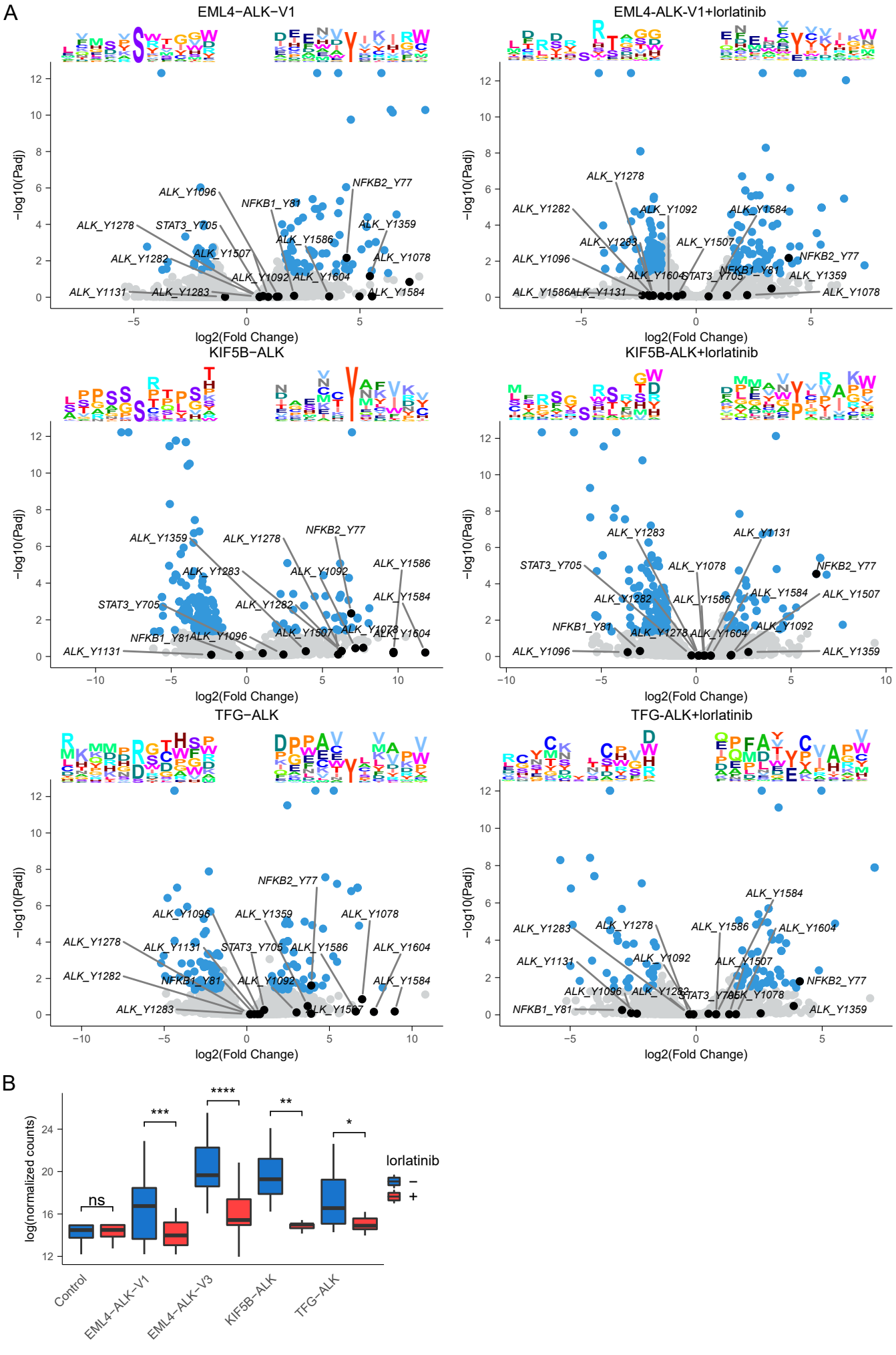
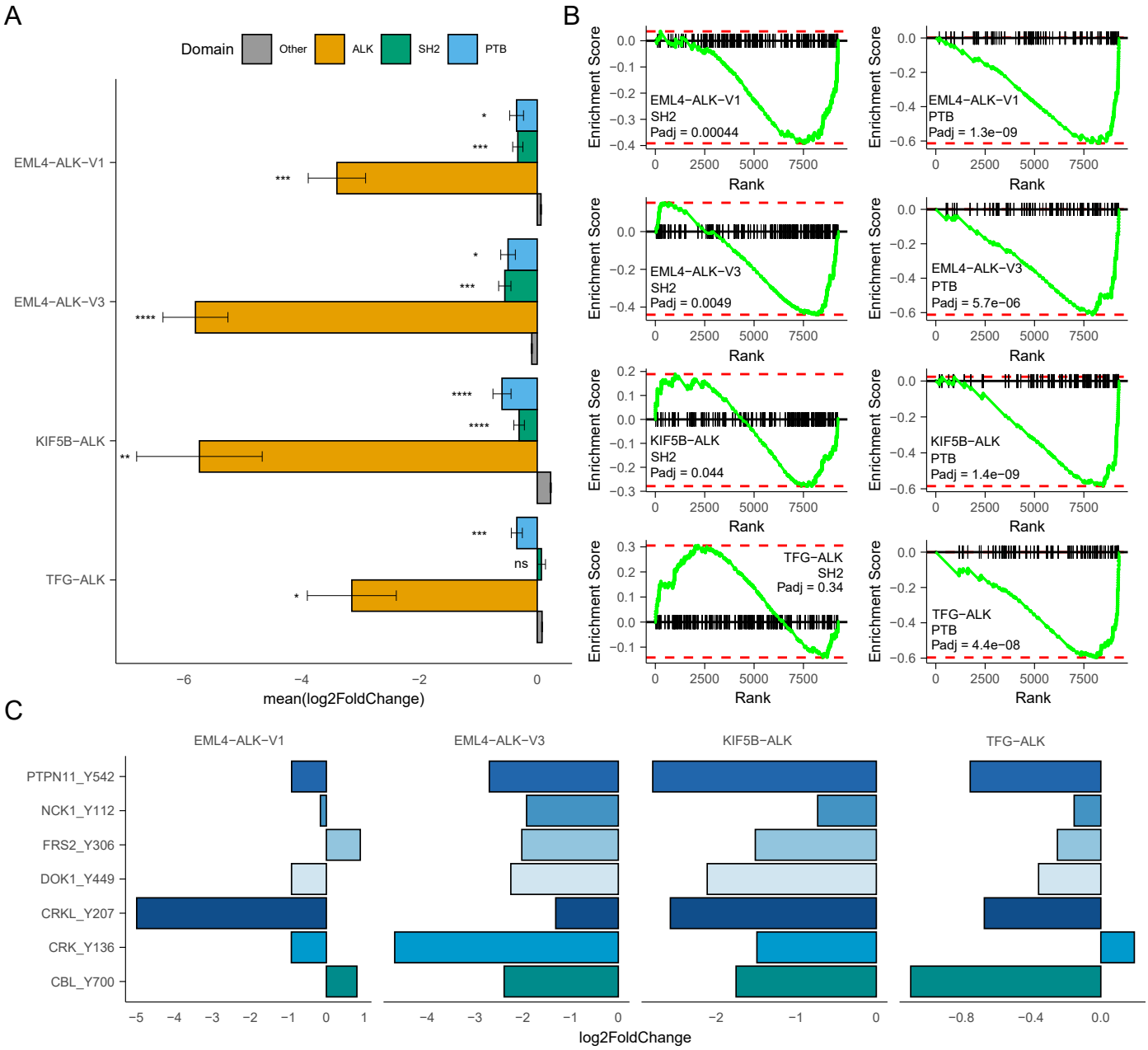

A
EML4−ALK−V1
EML4-ALK-V1+lorlatinib
KIF5B−ALK
KIF5B-ALK+lorlatinib
TFG−ALK
TFG-ALK+lorlatinib
−log10(Padj)
log2(Fold Change)
B
log(normalized counts)
lorlatinib
Control
EML4−ALK−V1
EML4−ALK−V3
KIF5B−ALK
TFG−ALK
ns

Fig. S6. Phosphoproteomic response to EML4-ALK-V1, KIF5B-ALK and TFG-ALK in the absence and presence of lorlatinib treatment.

A. Volcano plots showing phosphoproteomic response to induction of EML4-ALK-V1, KIF5B-ALK and TFG-ALK fusions (left panel) as well as treatment with lorlatinib of respective fusion (right panel). On top, sequence logo plots displaying position-specific enrichment ±5 amino acids centred around the phosphorylation site for hypo- and hyperphosphorylated sites as indicated. **B.** Box plot of phosphorylation of all ALK sites in non-treated cells vs lorlatinib-treated cells as indicated. Asterisks symbolizing significance for Student's t-test ns, non-significant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

Fig. S7


A
Domain
Other
ALK
SH2
PTB
EML4−ALK−V1
EML4−ALK−V3
KIF5B−ALK
TFG−ALK
mean(log2FoldChange)
B
Enrichment Score
Rank
EML4−ALK−V1
SH2
Padj = 0.00044
EML4−ALK−V1
PTB
Padj = 1.3e−09
EML4−ALK−V3
SH2
Padj = 0.0049
EML4−ALK−V3
PTB
Padj = 5.7e−06
KIF5B−ALK
SH2
Padj = 0.044
KIF5B−ALK
PTB
Padj = 1.4e−09
TFG−ALK
SH2
Padj = 0.34
TFG−ALK
PTB
Padj = 4.4e−08
C
EML4−ALK−V1
EML4−ALK−V3
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TFG−ALK
PTPN11_Y542
NCK1_Y112
FRS2_Y306
DOK1_Y449
CRKL_Y207
CRK_Y136
CBL_Y700
log2FoldChange

Fig. S7. Lorlatinib-dependent phosphoproteomic response to ALK fusion induction in proteins containing SH2 and PTB domains.

Differential phosphoprotein expression between lorlatinib-treated x-ALK fusion cells and untreated cells. **A.** Bar plot showing mean log2FC in sites located in proteins harboring an SH2 domain (green), PTB domain (blue), ALK site (orange) or none of the above (grey), with error bars indicated. T-test performed between the latter and each of the three previously mentioned groups. Asterisks indicating significance (Student's t-test). NS, nonsignificant; *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. **B.** Running score plots of ranked enrichment analysis on sites found in phosphotyrosine binding domain containing proteins with either SH2 or PTB domains in each of the different fusion variants, as indicated. **C.** Bar plots of log2FC of selected sites in several phosphotyrosine binding domain containing proteins in response to lorlatinib treatment in each of the four fusion variants, as indicated.

Fig. S8

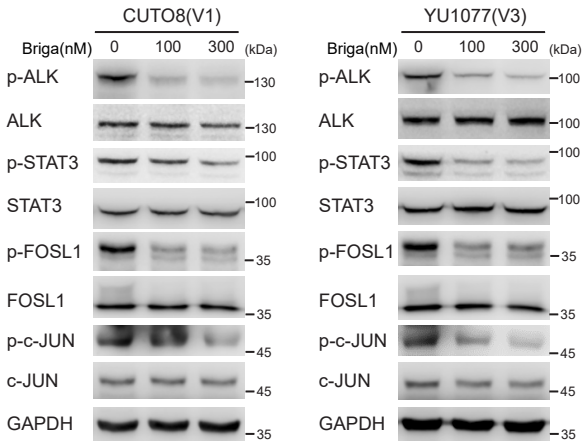

Fig. S8. ALK inhibition decrease phosphorylation of STAT3 and AP1 in ALK positive patient derived cell lines.

CUTO8(V1) and YU1077(V3) cell lines were treated with the ALK TKI brigatinib (100 nM or 300 nM) and lysates were immunoblotted with pY1278-ALK (p-ALK), pan-ALK, pY705-STAT3 (p-STAT3), pan-STAT3, pS265-FOSL1 (pFOSL1), FOSL1, pS63-c-JUN (p-c-JUN), pan-c-JUN and GAPDH as indicated. Phospho-ALK (pY1278-ALK) was employed as readout of ALK inhibition.