Reversal of microRNA-150 silencing disadvantages crizotinib-resistant NPM-ALK-positive cell growth

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Supplemental Methods, Tables, and Figures

Supplemental Methods

miRNA and siRNA transfections. SiRNAs and miRNA mimics were transfected into ALCL cells maintained in exponential growth phase in IMDM and supplemented as follows: 5.106 of ALCL cells were rinsed with PBS, resuspended in 400 µl of serum-free IMDM medium containing 0.5 nmol of siRNAs (Table S1) or miRNA mimics (Ambion miR-CTL/4464058 and miR-150-5p/4464066-MC10070) then transferred into 4 mm cuvettes (Eurogentec). Cells were electroporated at 250 V and 950 µF using a Biorad Gene pulser and immediately mixed with 5 ml pre-warmed complete IMDM medium. RNA and protein extractions were performed for RT-qPCR or subsequent analysis by western blotting.

RNA extraction, reverse transcription and quantitative PCR. Total RNA from tumors, normal samples and cell lines were extracted using the TRIzol Reagent (Ambion) according to the manufacturer's instructions. The expression of miR-150 and RNU24 for human and snoRNA202 for mice (used as a reference gene) was measured using TaqMan Small RNA Assays provided by Applied Biosystems, according to the manufacturer's recommendations.

The expression of NPM-ALK, STAT3, MYB, CCNB1 and GAPDH (used as a reference gene) were measured as follows. 1 µg of total RNA mixed with 50 ng of random hexamers (Invitrogen) was reverse transcribed for 1h at 42°C by 200 units of SuperScript II Reverse Transcriptase (Invitrogen). The enzyme was then inactivated at 70°C for 15 min. Quantitative PCR (qPCR) amplifications were carried out using the Light Cycler 480 SYBR Green I Master Mix and gene-specific primers (listed in Table S1) on the Light Cycler 480 apparatus (Roche). Human GAPDH or mouse S14 was used as the reference or housekeeping gene (1). Data were presented as relative quantity.

Protein extraction and western blotting analyses. Total cell lysates were prepared by incubating KARPAS-299 cells in RIPA-buffer (20 mM Tris-HCl, 150 mM NaCl, 4 mM EDTA, 0.5 % Triton X100 and 0.2 % SDS) supplemented with proteases and phosphatases inhibitors: complete protease inhibitor cocktail tablets (Roche), 2 mM sodium orthovanadate, 20 mM sodium fluoride and 1 mM PMSF for 10 min on ice. The cells were then subjected to 2 pulses of sonication (10 s each separated by a 30 s incubation on ice) using a Bioblock Vibra-cell 72446 apparatus at 40 % of its power (Bioblock). Insoluble material was eliminated by centrifugation (10 min at 10 000 g). The protein concentration of the cell lysate was determined using a Bradford assay. Proteins were separated by electrophoresis on SDS polyacrylamide gels and electroblotted onto nitrocellulose membranes (Whatman). Proteins of interest were detected using primary antibodies and HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson Immuno Research). The antibodies used for western blotting are listed in Table S2.

Methylated DNA Immunoprecipitation (MeDIP) assays. Methylated DNA was immunoprecipitated as described previously (2) using 2 mg of sonicated genomic DNA. MeDIP samples were directly subjected to labeling and hybridization on a customized human promoter array (Agilent, Santa Clara, USA) (3), following the manufacturer's instructions. Finally, median-normalized log2 enrichment ratios (Medip/Input) were calculated using CoCAS software (4) and visualized using an IGB tool.

Array-CGH analysis. This was carried out as previously described by Dejean and coworkers (5). Data are available upon request from F. Meggetto and L. Lamant (lamant.l@chu-toulouse.fr).

Cell proliferation assays. The CellTiter 96AQueus One Solution provided by Promega was used to assess cell proliferation according to the manufacturer's instructions.

Cell cycle analysis using propidium iodide and flow cytometry. KARPAS-299 cells were washed twice with PBS and fixed overnight at -20°C 70 % ethanol. Cells were then washed with PBS and incubated for 30 min with 10 µg/ml of propodium iodide (Invitrogen) and 500 µg/ml of RNAse A (Sigma-Aldrich). Thereafter, cells were analyzed with a flow cytometer FACSCALIBUR (Becton Dickinson, San Jose, CA, USA).

Mitotic index. Cytospins of KARPAS-299 transfected either with miR-CTL or miR-150 mimic RNAs were stained with hematoxylin and eosin. For each condition, the number of mitotic cells was determined for a sample of 500 cells from two separate experiments.

Immunohistochemistry. Detection of the ALK protein was performed on formalin-fixed, paraffin-embedded tissue sections using a polyclonal rabbit antibody anti-ALK mAb (clone SP8; 1/100; Lab Vision Corporation), followed by antibody detection using the streptavidin-biotin-peroxidase complex (Vector Laboratories).

Soft agar assays for anchorage-independent cell growth. Two 2 ml layers of complete IMDM medium were laid in a 6 cm-dish. The bottom layer contained 0.5 % of agar (Sigma-Aldrich). The top layer 0.33% of agar and 2.105 of KARPAS-299 cells. The cells were fed twice a week with 500 µl complete IMDM medium. Colonies were stained with the MTT reagent (Thiazolyl Blue Tetrazolium Bromidefor) (Euromedex) for 2 h. Colony quantifications were carried out using the ImageJ quantification software (U.S, NIH Bethesda, MD, USA). An 8-bit-greyscale picture for each plate of soft-agar was used, and the threshold was adjusted to 130. The "Analyze particles" function was used to count the colonies (size (pixel^2) 10-10000, circularity 0.00 to 1.00). Experiments were done in triplicate.

Murine models and xenograft tumor assay. The generation of transgenic mice expressing NPM-ALK under the tetracycline regulatory system has been previously described (6). The tetracycline regulatory system was used to control transgene transcription; the addition of doxycycline (an analog of tetracycline) allowed the silencing of NPM-ALK expression (OFF condition). Expression of the transgene was obtained by removing doxycycline (100 µg/mL; Sigma-Aldrich) from the drinking water (ON condition). Lymph nodes from ON, OFF and control normal age-matched wild-type littermate mice (WT) were used for RNA and protein extractions. KARPAS-299 or Cost cells were transfected either with control or miR-150

microRNA mimics. 4.106 or 3.106 of KARPAS-299 or Cost-transfected cells respectively were then injected subcutaneously into both flanks of a 5 week-old female non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse (Janvier Labs, Le Genest, St Isle, France). Tumor volume was measured (5 for KARPAS-299 cells and 6 for Cost cells) three times a week (once a day at the end of the experiment) with a caliper, using the formula "length x width2xπ/6". At day 11 (for KARPAS-299) or day 15 (for Cost) mice were sacrificed. Subcutaneous tumors were then excised, divided into two pieces, one conserved at -80°C and the other fixed in 10 % neutral buffered formalin and embedded in paraffin for histochemical analysis.

Transcriptome analysis: Transcriptome analysis was used to compare lymph nodes from conditional NPM-ALK(+) mice (NPM-ALK(+) ON, *n*=11) with normal lymph nodes from age-matched wild-type littermate mice (WT, *n*=6) or healthy lymph nodes from doxycycline-treated mice (NPM-ALK(-) OFF, *n*=6). Data are available upon request from F. Meggetto.

References

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Table S1:

qRT-PCR primers	
S14-F	ATCAAACTCCGGGCCACAGGA
S14-R	CTGCTGTCAGAGGGGATGGGG
GAPDH-F	CGGGAAGCTTGTGATCAATGG
GAPDF-R	GGCAGTGATGGCATGGACTG
NPM-ALK-F	CAGTGCATATTAGTGGACAGCACTTAG
NPM-ALK-R	TGATGGTCGAGGTGCGGA
STAT3-F	TGCCTGTGGGAAGAATCACG
STAT3-R	TCTGCTGCTTCTCCGTCACC
c-MYB-F	ATCTCCCGAATCGAACAGATG
c-MYB-R	AAGCTCTATCACTCTCTGATC
CCNB1-F	CGGGAAGTCACTGGAAACAT
CCNB1-R	AAACATGGCAGTGACACCAA
ChIP primers	
miR150-A-F	AGGGAGCCAGGTGGAAACTC
miR150-A-R	CCTCTCACTCAGCCCCACAC
miR150-B-F	TGCCCCTTGCTGGTTCTCTA
miR150 B-R	GGGTTCCTGCCAGAGGAAGT
miR150-C-F	CCCCTCCCTGTACTCCCATC
miR150-C-R	CTTTGCGCATCACACAGAGG
miR150-D-F	CCCAACTAGGCGTGTGTGGT
miR150-D-R	ATGTGCTGAGGCCCTGTTGT
pSTAT3-F	TGATTCCCGCGTGGTAAGAG
pSTAT3-R	ACCGGAATGTCCTGCTGAAA
pc-MYC-F	GCCCTTTCCCCAGCCTTAGC
pc-MYC-R	AACCGCATCCTTGTCCTGTGAGTA
siRNAs	
NPM-ALK siRNAs	GGGCGAGCUACUAUAGAAA
STAT3 siRNAs	AACAUCUGCCUAGAACGGCUA
bisulfite sequencing primers	
forward bisulfite primer	GTTTTGTAGAGTTGGGGAGAGTATA
reverse bisulfite primer	CTACCCCCAACATAAAATAAAATAA

Table S2:

Antibody	**Company**	**Catalogue #**
ALK	Cell Signaling Tech	D5F3
P-ALK (Y1604)	Cell Signaling Tech	cs3341
ALK	Lab Vision Corporation	SP8
STAT3	Cell Signaling Tech	cs9132
P-STAT3 (Y705)	Cell Signaling Tech	cs9131
MYB	Abcam	ab45150
DNMT1	Imgenex	ING261A
β-actine	Sigma-Aldrich	A5441
GAPDH	Chemicon	MAB374

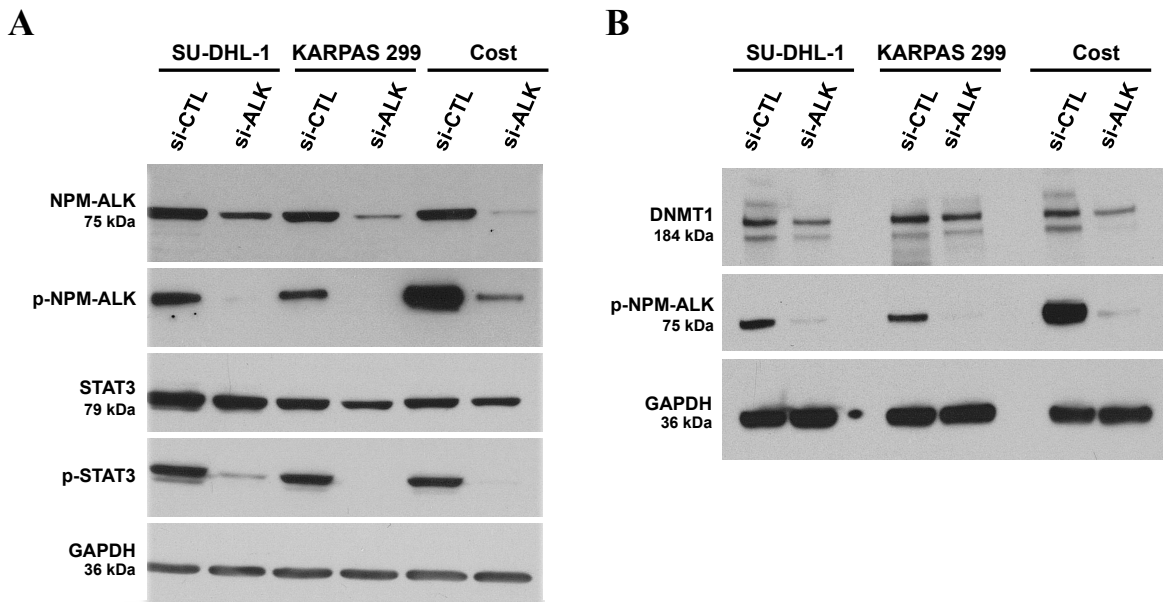
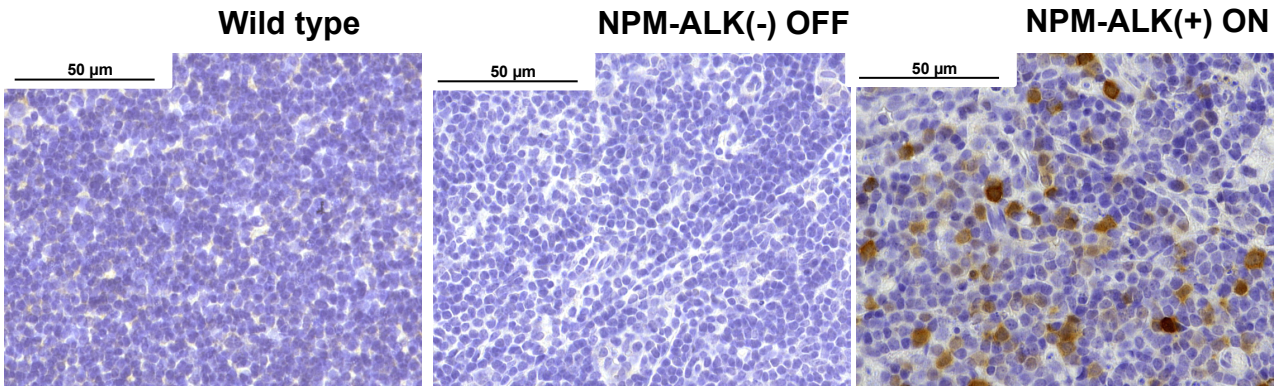


Figure S1: NPM-ALK depletion induces a reduction in p-STAT3 and DNMT1 levels.
Western-blotting analysis of NPM-ALK, p-NPM-ALK (the phosphorylated form of NPM-ALK on tyrosine 1604), STAT3, p-STAT3 (form of STAT3 phosphorylated on tyrosine 705) **(A)**, and DNMT1 **(B)** in SU-DHL-1, KARPAS-299 and Cost cells (NPM-ALK(+) ALCL cell lines) transfected with si-CTL or si-NPM-ALK (siRNA directed against NPM-ALK mRNA). The GAPDH protein served as an internal control to ensure equal loading.

A



B

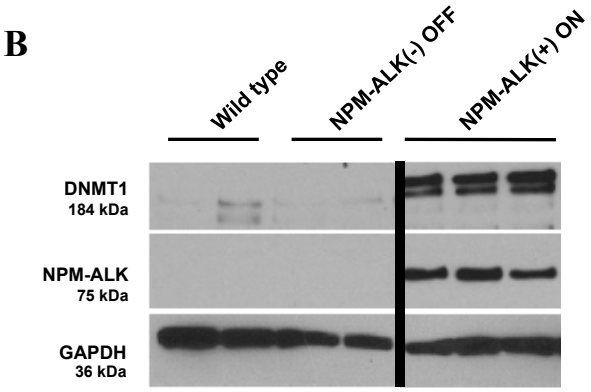


Figure S2: NPM-ALK(+) transgenic mice overexpress the DNMT1 protein.
(A) Representative **i**mmunohistochemical analysis of lymph nodes from wild type (n=2) or NPM-ALK transgenic mice containing a Tet-OFF system. Expression of the NPM-ALK transgene was both induced (NPM-ALK(+) ON, no doxycycline, n=3) or repressed (NPM-ALK(-) OFF, with doxycycline, n=2). Sections were stained with the rabbit anti-ALK antibody, and nuclei were counterstained with hematoxylin (scale bars: 50 µm, original magnification x40). **(B)** Western blot analysis of NPM-ALK, DNMT1 and GAPDH protein levels in lymph nodes from wild type, NPM-ALK(-) OFF and NPM-ALK(+) ON mice. The lanes were run on the same gel but were noncontiguous.

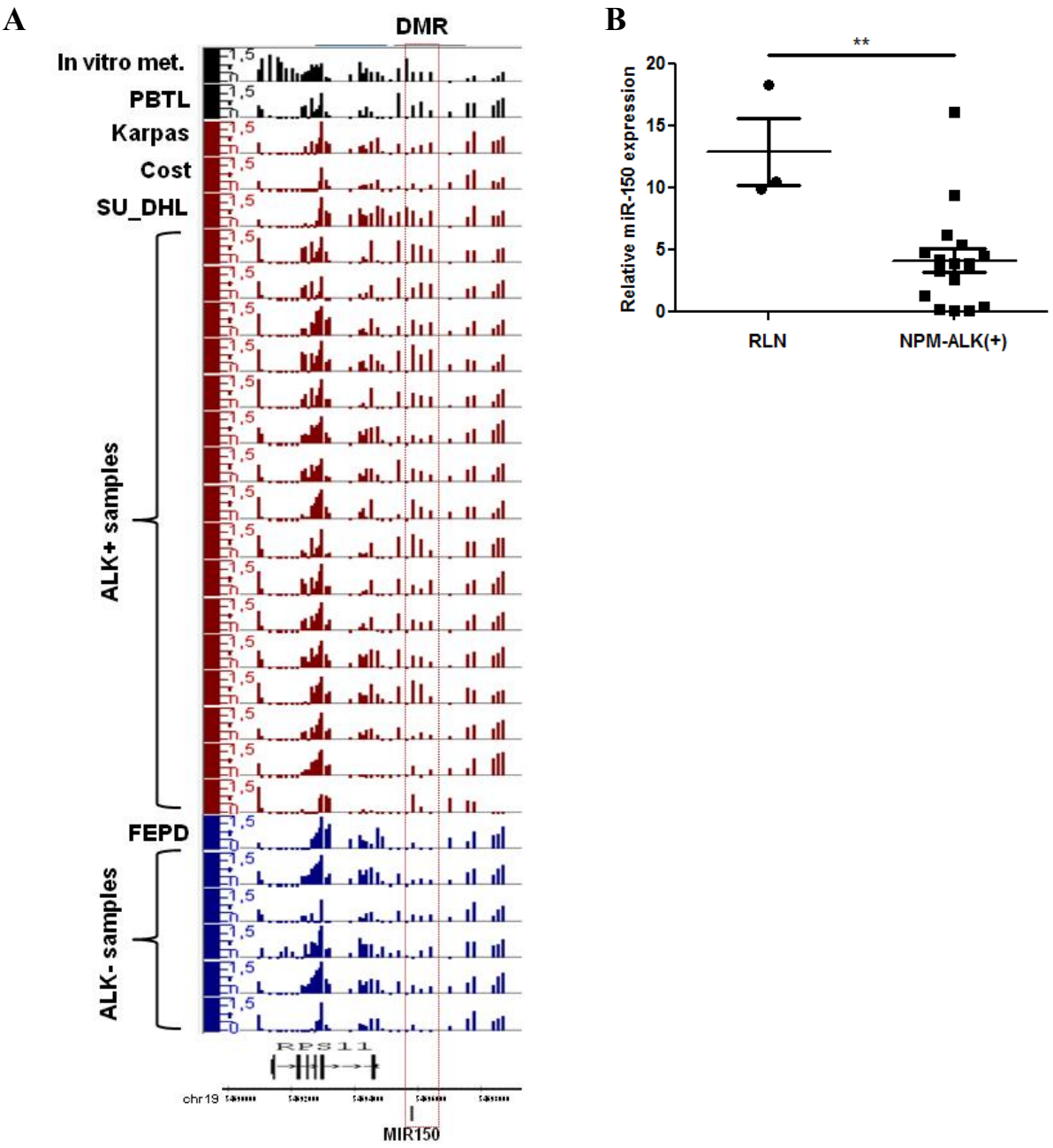


Figure S3: Quantification of DNA methylation levels at the miR-150 promoter and miR-150 expression levels in human ALCL cell lines and biopsies.

(A) Methylation profiles of the region around the *MIR150* gene. Each track shows the results of the Log2 enrichment ratio of the MeDIP/input signal from the indicated ALCL samples, the NPM-ALK(+) ALCL cell lines (KARPAS-299, SU-DHL-1, Cost), and the NPM-ALK(-) ALCL cell line, FE-PD. ALK(+) samples are shown in red and ALK(-) samples are in blue. Human peripheral blood T lymphocytes (PBTL) and in vitro methylated DNA were used as control samples. Gene annotation and chromosomal coordinates (HG18 assembly) are shown at the bottom of the panel. The differentially methylated region (DMR) close to the *MIR150* gene is indicated by a dotted rectangle. **(B)** miRNA-specific qRT-PCR analysis of miR-150 and RNU24 (used as the relative control) in human NPM-ALK(+) ALCL biopsies (n=17). miR-150 expression levels were expressed as the 2-ΔΔCt relative to reactive lymph nodes (RLN, n=3). Data represent mean ± SEM (bars) and asterisks depict statistically significant (**P < 0.001; unpaired 2-tailed Student's t test).

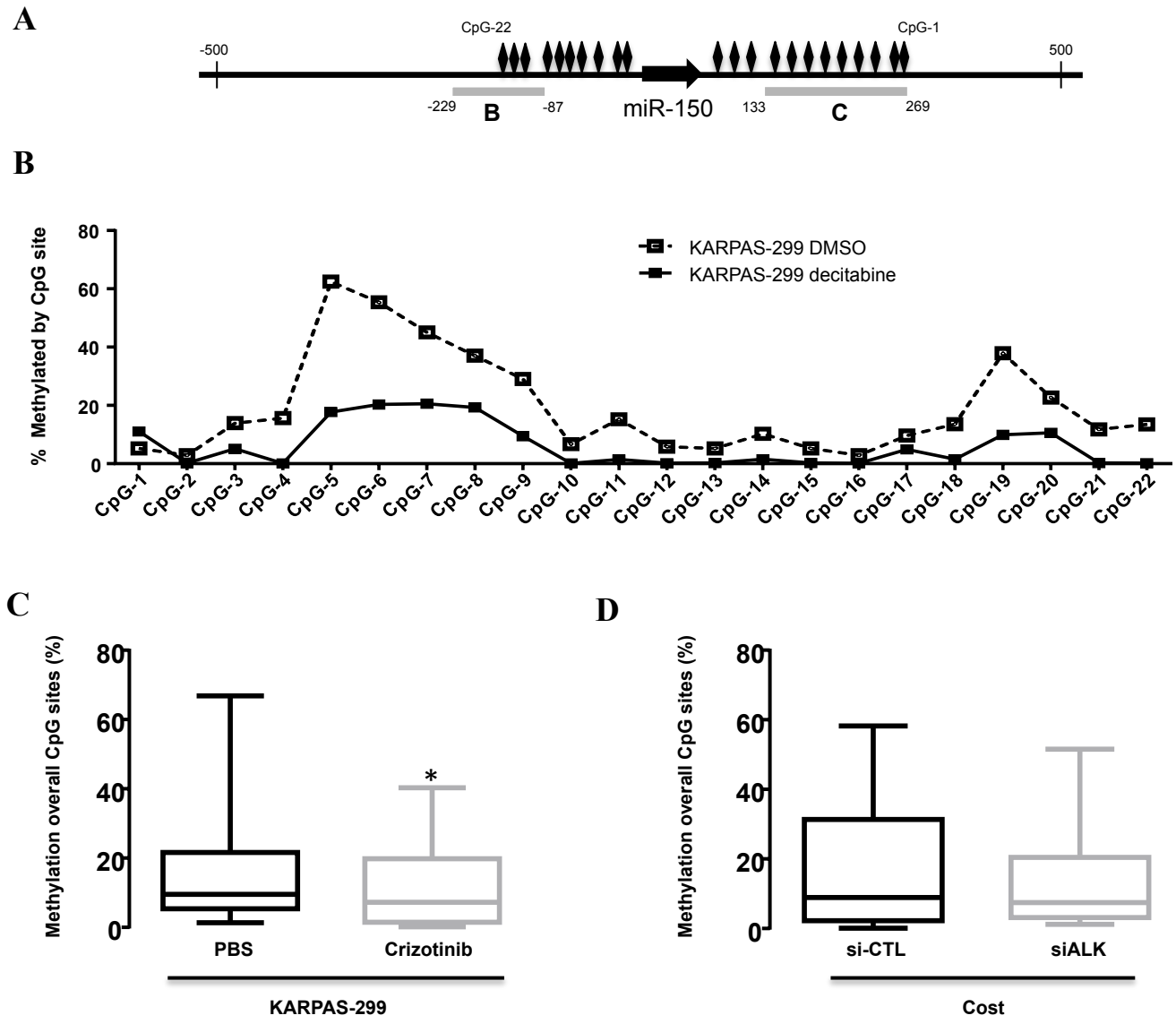


Figure S4: Methylation status of 22 CpGs surrounding of the *MIR150* gene.
CpG methylation was evaluated by bisulfite amplicon Illumina sequencing (GEO GSE69836). **(A)** Diagram of the CpG positions surrounding the *MIR150* gene. **(B)** Percentage of methylation of each of the 22 CpGs in KARPAS-299 cells treated or not (DMSO vehicle control) for 5 days with 5 µM decitabine. Methylation overall of 22 CpGs sites in **(C)** KARPAS-299 treated or not (PBS, vehicle control) with 500 nM crizotinib for 5 days, and **(D)** in Cost cells transfected with either siRNA targeting NPM-ALK (siALK) or siRNA negative control (si-CTL). . Data represent mean ± SEM (bars) and asterisks depict statistically significant results (*P < 0.05; unpaired 2-tailed Student's t test).

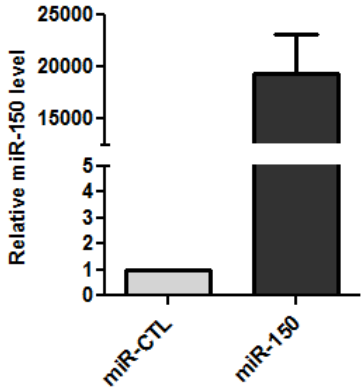


Figure S5: Efficiency of ectopic miR-150 mimic RNA transfection in KARPAS-299 cells. Cells were transfected with control microRNA (miR-CTL) or miR-150 microRNA (miR-150) and miR-150 expression was analyzed 72h after transfection by qRT-PCR. RNU24 served as a relative control. The relative expression levels of miR-150 were expressed as the $2^{-\Delta\Delta Ct}$ of miR-CTL-transfected KARPAS-299 cells. Data represent mean ± SEM (bars) (n=3).

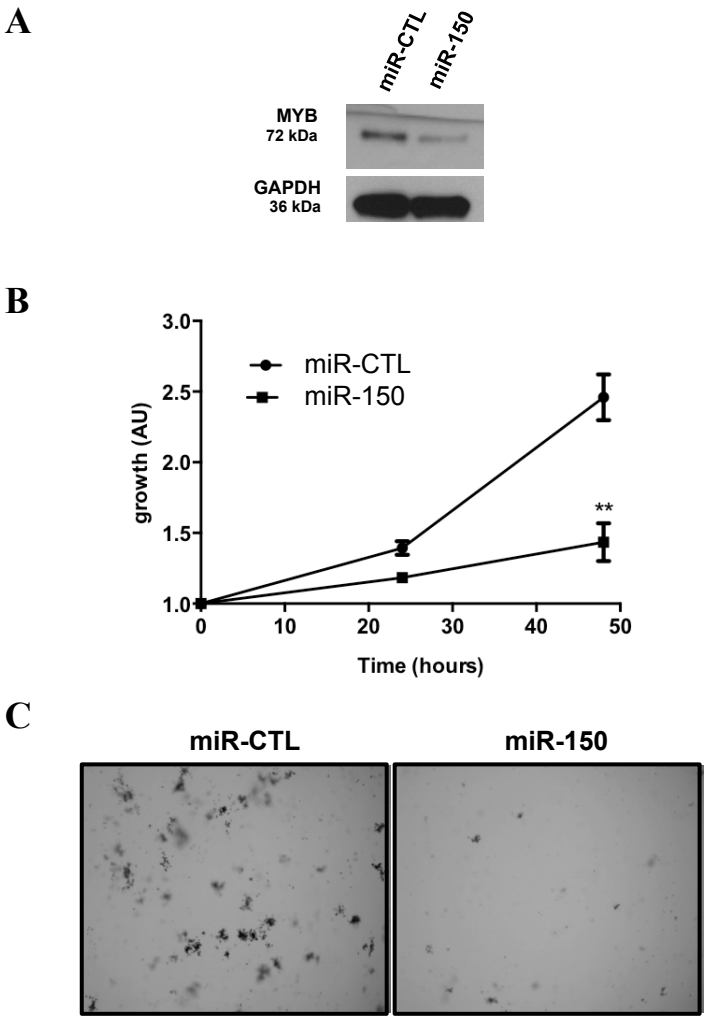


Figure S6: MiR-150 overexpression inhibits the proliferation of Cost cells *in vitro.*
(A) Western blotting analysis of MYB protein levels after transfection of Cost cells with miR-CTL or miR-150 microRNA mimics. GAPDH served as an internal control to ensure equal loading. **(B)** MTS colorimetric measurement of cell viability after control miR-CTL or miR-150 transfection in Cost cells. **(C)** Soft agar assay for anchorage independent cell growth of miR-CTL- and miR-150-transfected Cost cells. Representative images of the plate are shown (n=3, original magnification x10). Asterisks depict statistically significant results (*$P < 0.05$, **$P < 0.001$ and ***$P < 0.0001$; unpaired 2-tailed Student's t test).

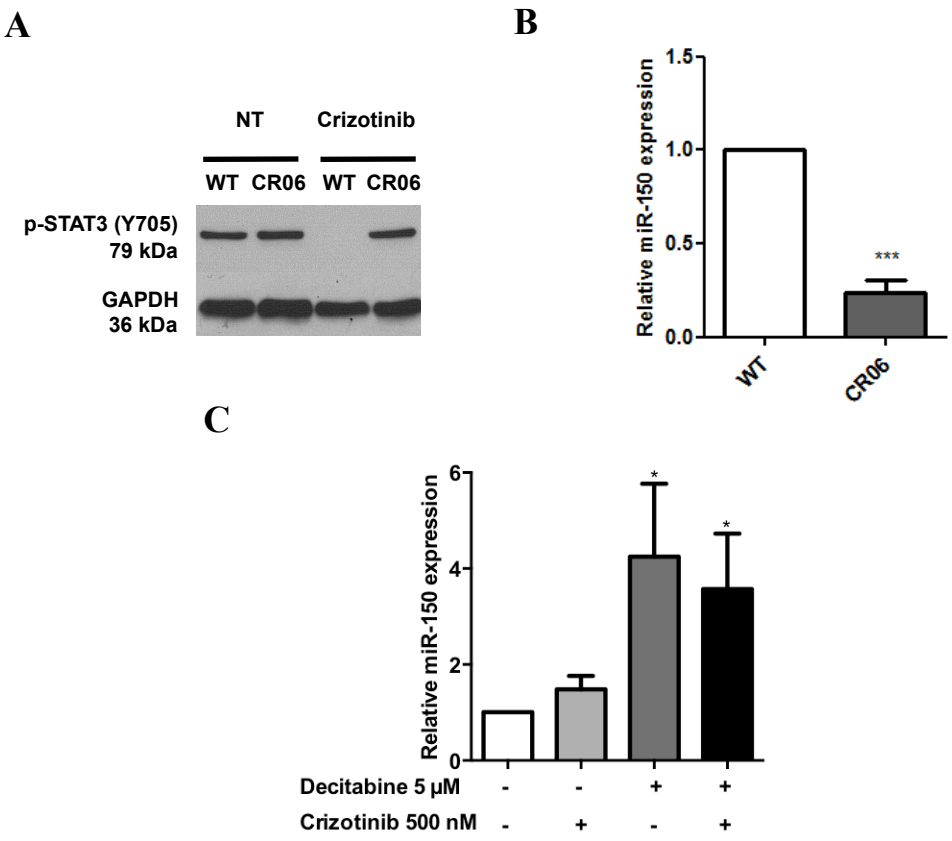


Figure S7: Decitabine treatment of crizotinib-resistant KARPAS-299-CR06 mutant cells induces miR-150 up-regulation.

(A) Western blotting was used to evaluate STAT activation (using antibodies directed against the activated form of STAT3 phosphorylated on tyrosine 705) in crizotinib-resistant KARPAS-299-CR06 cells (CR06) compared with KARPAS-299 parental cells (WT) treated with 500 nM crizotinib. The GAPDH protein served as an internal control to ensure equal loading. **(B)** miRNA-specific qRT-PCR analysis of miR-150 and RNU24 (relative control) in crizotinib-resistant KARPAS-299-CR06 and parental KARPAS-299 cells. **(C)** miR-150- and RNU24-specific qRT-PCR analysis in crizotinib-resistant KARPAS-299-CR06 cells after treatment with 500 nM crizotinib and/or 5 µM decitabine. The relative expression levels of miR-150 were expressed as the $2^{-\Delta\Delta Ct}$ to no treatment cells. Data represent mean ± SEM (bars) (n=3) and asterisks depict statistically significant results (*P < 0.05, **P < 0.001 and ***P < 0.0001; unpaired 2-tailed Student's t test).

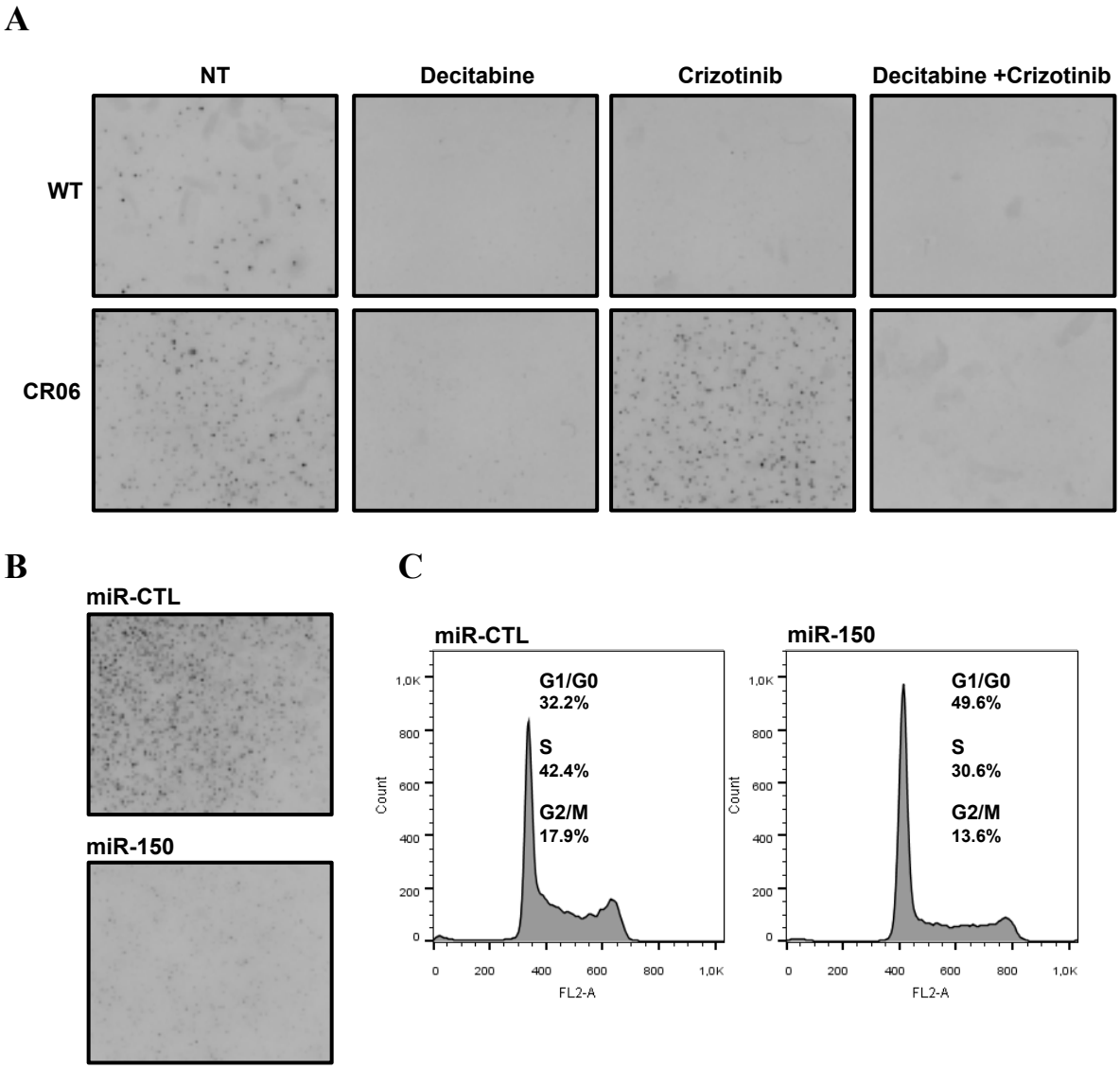


Figure S8: Effect of decitabine, crizotinib and miR-150 transfection on the growth of crizotinib-resistant KARPAS-299-CR06 cells.

Representative image of soft agar assays for parental crizotinib-sensitive-KARPAS-299 cells (WT) and crizotinib-resistant KARPAS-299-CR06 cells (CR06) (n=3, original magnification x1) **(A)** treated with 5 µM decitabine (5-Aza-2′-deoxycytidine), 500 nM crizotinib or a combination of both or **(B)** transfected with either control microRNA (miR-CTL) or miR-150 microRNA (miR-150) original magnification 1x. **(C)** Cell cycle analysis of miR-CTL- or miR-150-transfected KARPAS-299-CR06 cells (with propidium iodide incorporation measured by FACS, n=3).

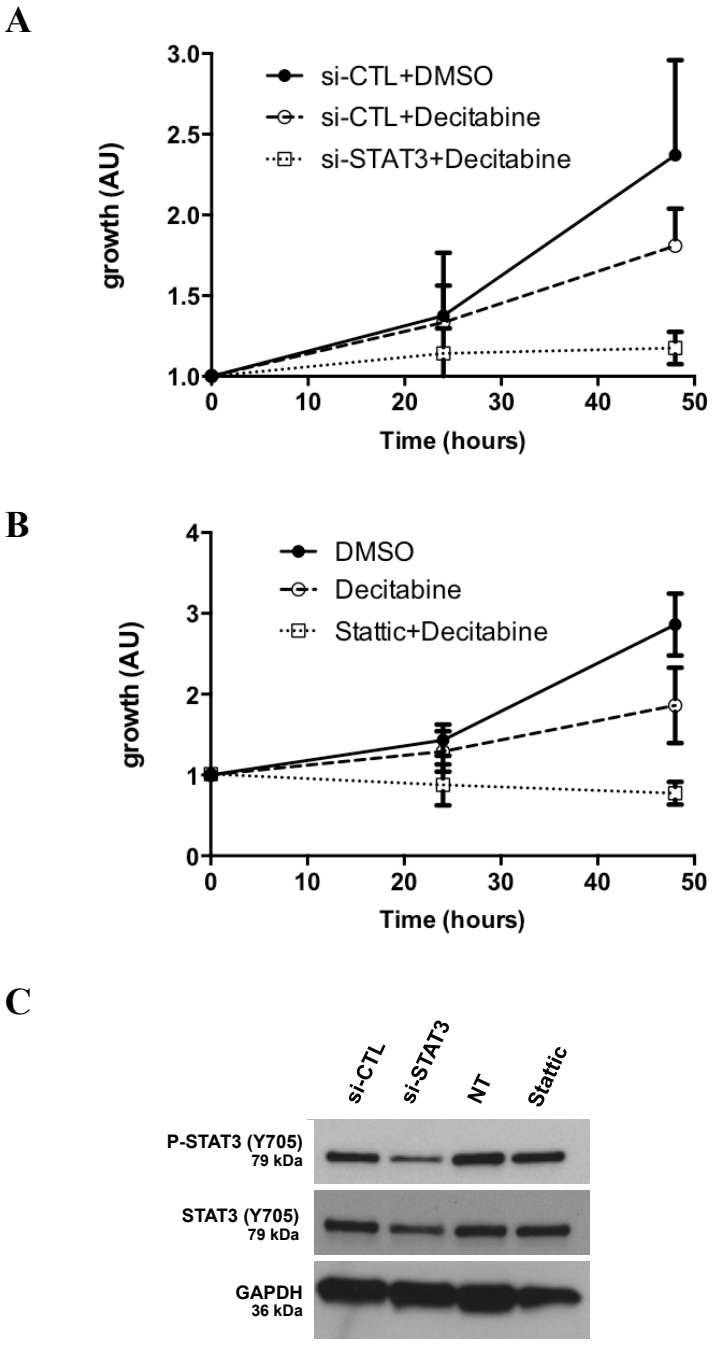


Figure S9: STAT3 pathway blockade in combination with decitabine treatment affects KARPAS-299-CR06 cell viability.

MTS colorimetric measurement (n=3) of the cell viability of KARPAS-299-CR06 cells **(A)** transfected with si-CTL or si-STAT3 and treated for 48h with DMSO vehicle control or 5 µM decitabine, or **(B)** treated for 48h with 5 µM Stattic and/or 5 µM decitabine or treated with DMSO (NT). **(C)** Western blotting was used to evaluate STAT3 inhibition in KARPAS-299-CR06 transfected with either si-CTL or si-STAT3 and either not treated or treated with 5 µM Stattic for 48h. The GAPDH protein served as an internal control to ensure equal loading.