SUPPLEMENTARY APPENDIX

miR-497 suppresses cycle progression through an axis involving CDK6 in ALK-positive cells

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Supplemental legends:

Supplemental Figure 1: Validation of si-ALK transfection and crizotinib treatment in NPM-ALK-positive lymphoma cells. Protein levels of NPM-ALK and p-NPM-ALK were assessed by western blotting in NPM-ALK(+) KARPAS-299 (KARPAS), COST and SU-DHL1 cells transfected with a siRNA targeting ALK mRNA (si-ALK) or with negative control siRNA (si-CTL) **(A)** or treated or not (PBS) with crizotinib **(B).** The GAPDH protein served as an internal control to ensure equal loading. Results from one representative experiment are shown.

Supplemental Figure 2: The MIR497 promoter is methylated in NPM-ALK-positive lymphoma cells. (A) Schematic representation of the position of 6 CpG dinucleotides in the promoter region of *MIR497HG*. Percentage of DNA methylation, assessed by bisulfite conversion and pyrosequencing in **(B)** untreated NPM-ALK(+) KARPAS-299 (KARPAS), SU-DHL1, COST cells and in CD4 lymphocytes activated using CD3/CD28 antibodies or **(C)** treated with decitabine. Data represent means ± SEM (bars) from 3 independent experiments; * $p<0.05$, ***$P<0.0001$, unpaired two-tailed Student's t-test with Welch's correction.

Supplemental Figure 3: Efficiency of miR-497 mimic transfection in NPM-ALK-positive lymphoma cells. NPM-ALK(+) COST, SUDHL1 and KARPAS-299 cells were transfected with negative control miRNA (miR-CTL), mimic miR-497 (miR-497, **A, B, C**) or mimic miR-195 (miR-195, **B**). MiRNA expression was analyzed 72h after transfection by qRT-PCR. SNORD44 served as a relative control. The relative expression levels of miR-195 and miR-497 were expressed as the $2^{-\Delta\Delta Ct}$ relative to miR-CTL conditions.

Supplemental Figure 4: NPM-ALK-positive lymphoma cells express CDK4, CDK6 and Rb proteins. Protein levels of NPM-ALK, p-NPM-ALK, CDK4, CDK6, Rb and GAPDH were assessed by western blotting in NPM-ALK(+) KARPAS-299 (KARPAS), COST and SU-DHL1 cells**.** The GAPDH protein served as an internal control to ensure equal loading. Results from one representative experiment are shown.

Supplemental Figure 5: Silencing efficiency of CDK6, CCNE1, CDC25A and E2F3 after siRNA transfection in NPM-ALK-positive lymphoma cells. NPM-ALK(+) COST, SUDHL1 and KARPAS-299 (KARPAS) cells were transfected with a siRNA targeting CDK6, CCNE1, CDC25A and E2F3 mRNAs (si-CDK6, si-CCNE1, si-CDC25A and si-E2F3, respectively) or with negative control siRNA (si-CTL). **(A)** Quantitative RT-PCR analysis of CDK6, CDC25A, CCNE1 and E2F3 mRNA expression. *GAPDH* was used as an internal control and relative mRNAs expression was expressed as the $2^{-\Delta\Delta Ct}$ relative to si-CTL conditions. **(B)** Protein levels of CDK6, CCNE1, CDC25A and E2F3 were assessed by western blotting in KARPAS-299, COST and SU-DHL1 cells. The GAPDH protein served as an internal control to ensure equal loading. Results from one representative experiment are shown. (**C)** Densitometric analysis was performed using ImageJ software from Wayne Rasband (NIH). Relative protein expression was expressed relative to si-CTL conditions. Data represent means ± SEM (bars) from 3 independent experiments. *$P<0.05$, **$P<0.001$, ***$P<0.0001$ and *ns*: not significant; unpaired 2-tailed Student's t test with Welch's correction.

Supplemental Figure 6: CDK6, CCNE1 and E2F3 transfection in NPM-ALK-positive lymphoma cells induce cell cycle alterations. Cell cycle analysis of NPM-ALK(+) COST and KARPAS-299 (KARPAS) cells transfected with a siRNA targeting E2F3, CDK6 or CCNE1 mRNAs (si-E2F3, si-CDK6 and si-CCNE1 respectively) or with negative control siRNA (si-

CTL). Data represent means ± SEM (bars) from 3 independent experiments. *P<0.05, ***P<0.0001 and ns: not significant; unpaired 2-tailed Student's t test with Welch's correction.

Supplemental Figure 7: CDK6, CCNE1 and E2F3 transfection induces apoptosis in NPM-ALK-positive cells. Assessment of caspase 3/7 activity in NPM-ALK(+) KARPAS-299 (KARPAS) and COST cells transfected with a siRNA targeting CDK6, CCNE1 and or E2F3 mRNAs (si-CDK6, si-CCNE1 and si-E2F3 respectively) or with negative control siRNA (si-CTL). Data represent means ± SEM (bars) from 3 independent experiments. **P<0.001; unpaired 2-tailed Student's t test with Welch's correction.

Supplemental Figure 8: Silencing efficiency of CDK6, CCNE1 and E2F3 after transfection of pool of siRNAs. Quantitative RT-PCR analysis of CDK6, CCNE1 and E2F3 mRNA expression in NPM-ALK(+) KARPAS-299 (KARPAS) and COST cells transfected with pooled siRNAs targeting CDK6, CCNE1 and E2F3 mRNAs or with negative control siRNA (si-CTL). GAPDH was used as an internal control and relative mRNAs expression was expressed as the $2^{-\Delta\Delta Ct}$ relative to si-CTL conditions. Data represent means ± SEM (bars) from 3 independent experiments. *P<0.05, **P<0.001, ***P<0.0001; unpaired 2-tailed Student's t test with Welch's correction.

Supplemental Figure 9: Ectopic expression of miR-497 induces cell cycle arrest and reduces in vitro growth of NPM-ALK-positive COST and SU-DHL-1 cells. Cells were transfected with negative control miRNA (miR-CTL) or mimic miR-497 (miR-497). Cells in sub-G1, S and G2M phases of the cell cycle were sorted based on DNA content. Cell cycle distribution was measured 24h post-transfection and showed as a mean of the number of cells in each phase of the cell cycle (sub-G1, S and G2M) of 3 independent experiment **(A)** or as a representative cell cycle distribution for each condition **(B)**. *P < 0.05, **P < 0.001, ***P < 0.0001 and *ns*: not significant; using unpaired 2-tailed Student's t test with Welch's correction.

Supplemental Figure 10: Palbociclib decreased Cdk4/6-specific phosphorylation of Rb protein. (A) Protein levels of Rb and pRb were assessed by Western-blotting in NPM-ALK(+) SU-DHL1, COST and KARPAS-299 (KARPAS) cells treated (1µM) or not (0) with Palbociclib. The GAPDH protein served as an internal control to ensure equal loading. Results from one representative experiment are shown. (**B**) Densitometric analysis was performed using ImageJ software from Wayne Rasband (NIH). Relative protein expression was expressed relative to Rb expression.

Supplemental Figure 11: Silencing of CDK4 does not reduce cell proliferation in ALK-positive lymphoma cells. NPM-ALK(+) COST (**A, B and C**), KARPAS-299 (KARPAS) (**D, E and F**) and SU-DHL1 (**G, H and I)** cells were transfected with either an irrelevant siRNA (si-CTL) or a siRNA targeting CDK4**. (C, F and I)** Quantitative RT-PCR analysis of CDK4 mRNA expression in NPM-ALK(+) COST, KARPAS and SU-DHL1 cells transfected with siRNAs targeting CDK4 mRNAs or with negative control siRNA (si-CTL). GAPDH was used as an internal control and relative mRNA expression was expressed as the 2–ΔΔCt relative to si-CTL conditions. Cell growth **(A, D and G)** and apoptosis **(B, E and H)** were evaluated after si-CDK4 and si-CTL transfection in COST, KARPAS and SU-DHL1 cells. Data represent means ± SEM (bars) from 3 independent experiments.

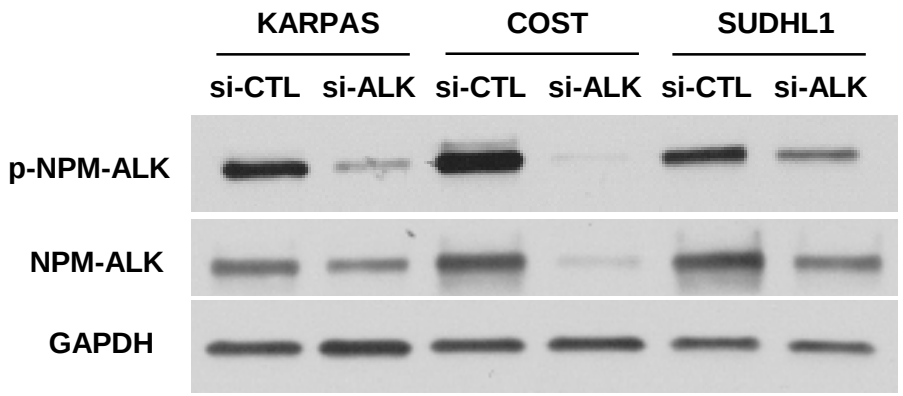
Supplemental Figure 12: Expression profile of trio genes CCNE1, CDK6 and E2F3 could predict chemotherapy treatment outcome of NPM-ALK(+) pediatric lymphomas (A) Quantitative RT-PCR analysis of CDK6, CCNE1 and E2F3 mRNA expression in NPM-ALK(+)

lymphoma patients (n=55). Data were normalized against equivalent mRNA levels from reactive lymph node tissue samples (RLN; n=12). Data represent means ± SEM (bars), ***P<0.0001; unpaired 2-tailed Student's t test with Welch's correction. (B) ROC (Receiver Operating Characteristics) curve obtained from CCNE1, CDK6, E2F3 genes-based score from NPM-ALK(+) ALCL samples (n=44) for whose event free survival is known; Cut-off value (-0.038) and AUC (Air under curve; 0.65) were calculated. Kaplan-Meier survival curves stratified by event free survival (cut-off obtained with ROC curve) (C) the patients (D) including the NPM-ALK(+) pediatric ALCL patients. (E) Box-plot distributions of the gene score in "relapsing" after chemotherapy cure (grey, n=23) and "non-relapsing" (white, n=19) groups in NPM-ALK(+) pediatric lymphomas. *P<0.05; unpaired 2-tailed Student's t test".

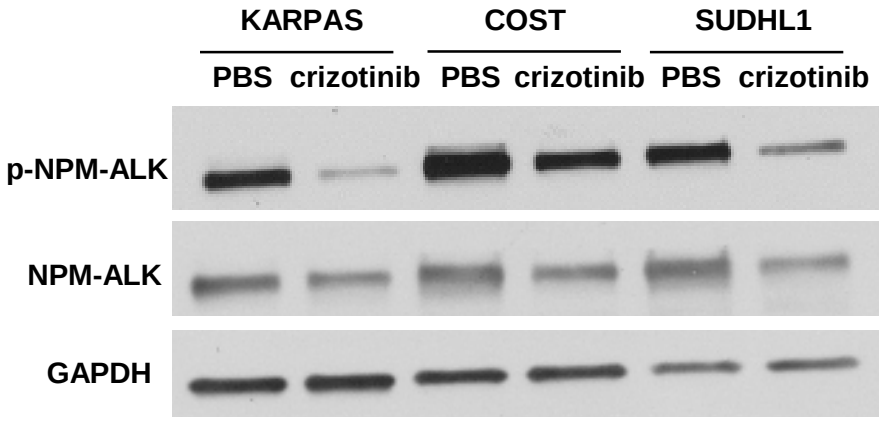
Supplemental Table 1: Sequences of siRNAs and primers used for quantitative real-time PCR and pyrosequencing analysis.

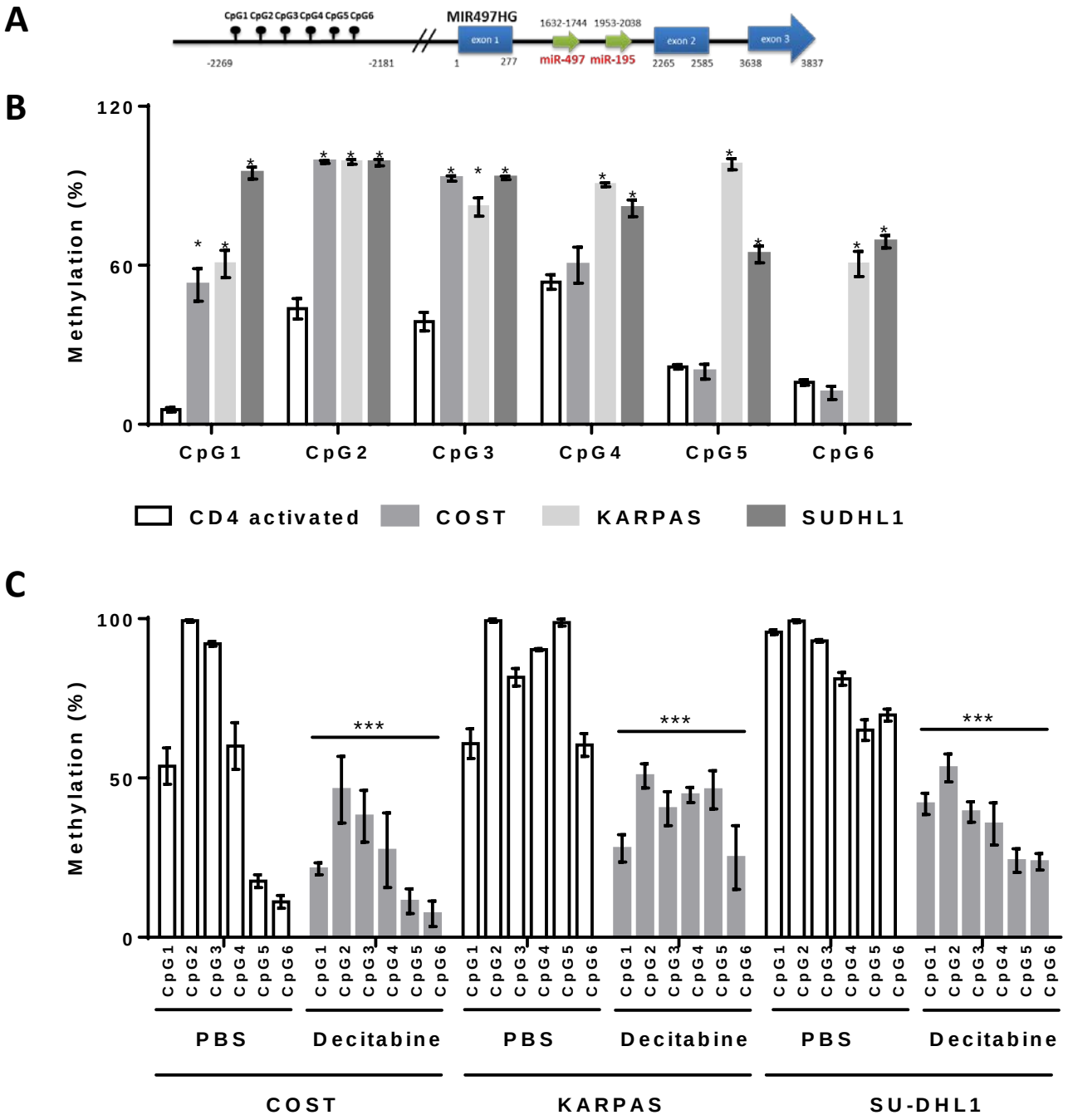
Supplemental Table 2: MiRNAs with differential expression in NPM-ALK(+) ALCL lymph node primary tissues sorted according to fold change in expression *(adapted from Congras et al, [22]).*

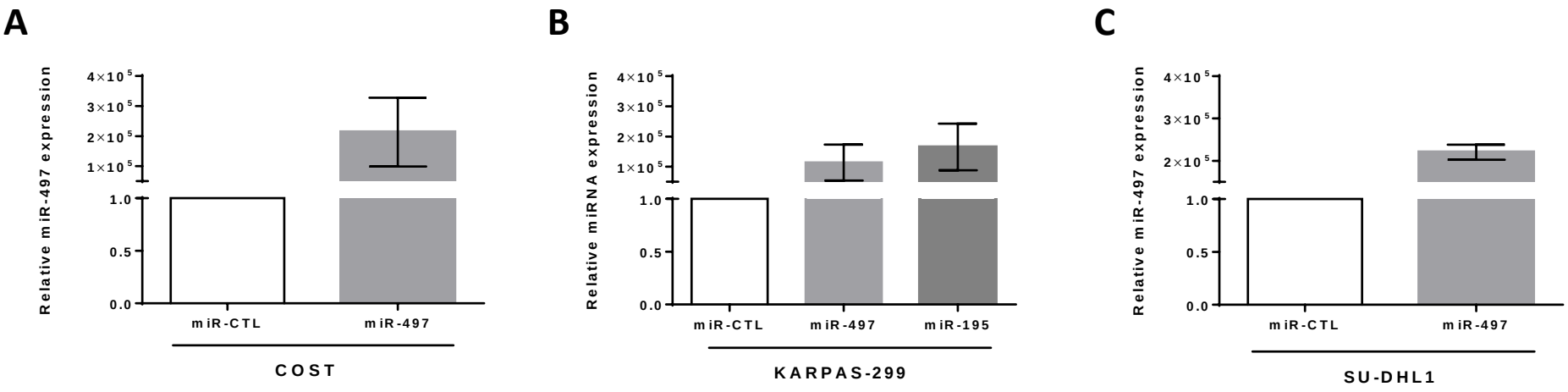
A

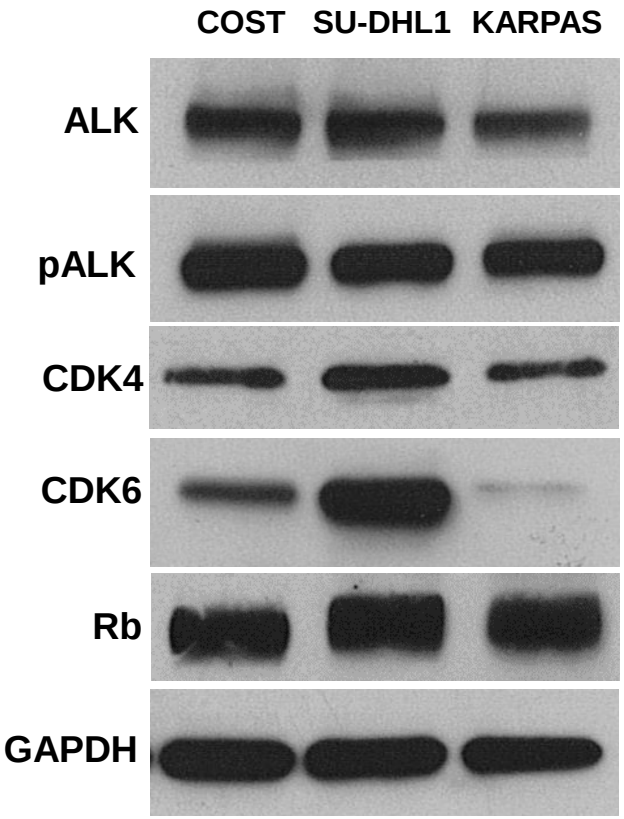

KARPAS
COST
SUDHL1
si-CTL
si-ALK
si-CTL
si-ALK
si-CTL
si-ALK
p-NPM-ALK
NPM-ALK
GAPDH

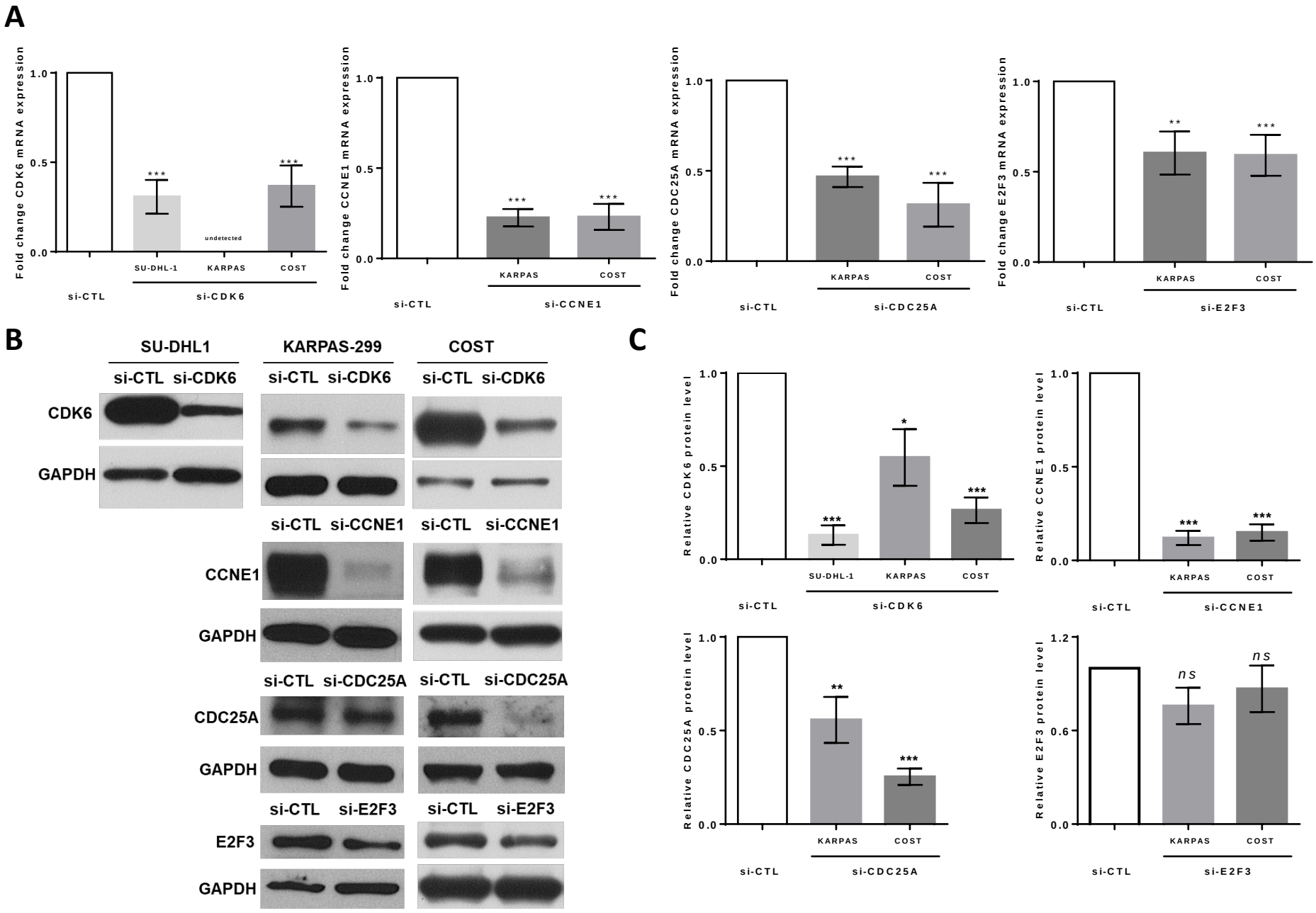
B

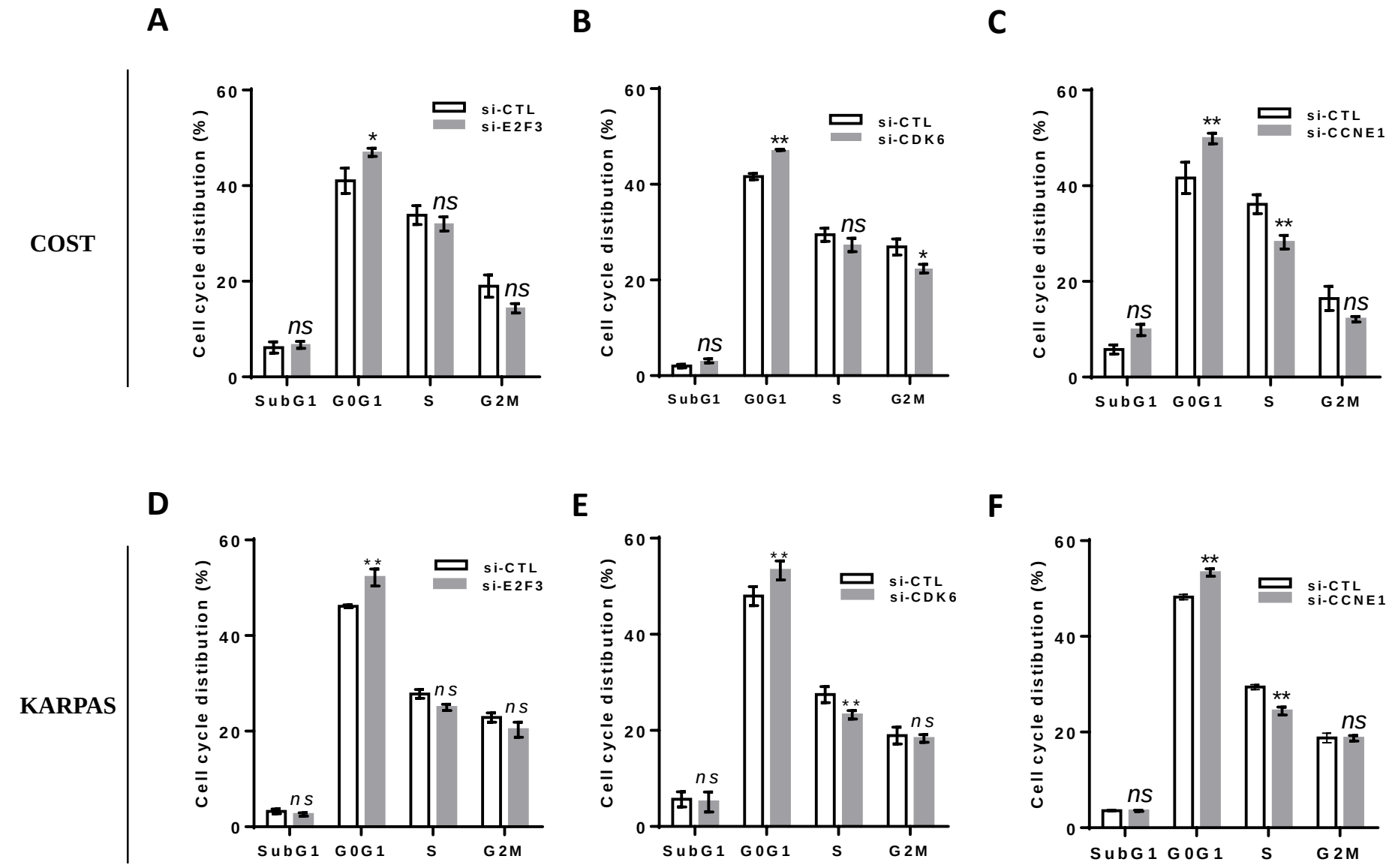

KARPAS
COST
SUDHL1
PBS
crizotinib
PBS
crizotinib
PBS
crizotinib
p-NPM-ALK
NPM-ALK
GAPDH

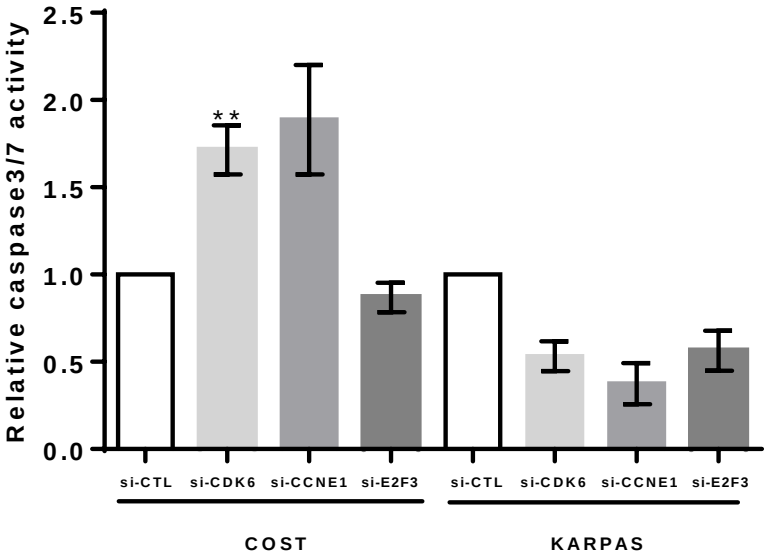

A
CpG1 CpG2 CpG3 CpG4 CpG5 CpG6
MIR497HG
1632-1744 1953-2038
exon 1
exon 2
exon 3
-2269
-2181
1
277
miR-497 miR-195
2265 2585
3638
3837
B
Methylation (%)
120
60
0
CpG1 CpG2 CpG3 CpG4 CpG5 CpG6
CD4 activated
COST
KARPAS
SUDHL1
C
Methylation (%)
100
50
0
***
CpG1 CpG2 CpG3 CpG4 CpG5 CpG6
PBS
Decitabine
COST
KARPAS
SU-DHL1

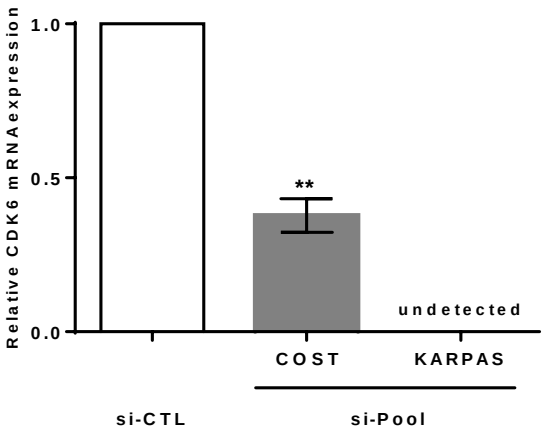

A
Relative miR-497 expression
4×10^5
3×10^5
2×10^5
1×10^5
1.0
0.5
0.0
miR-CTL
miR-497
COST
B
Relative miRNA expression
4×10^5
3×10^5
2×10^5
1×10^5
1.0
0.5
0.0
miR-CTL
miR-497
miR-195
KARPAS-299
C
Relative miR-497 expression
4×10^5
3×10^5
2×10^5
1.0
0.5
0.0
miR-CTL
miR-497
SU-DHL1

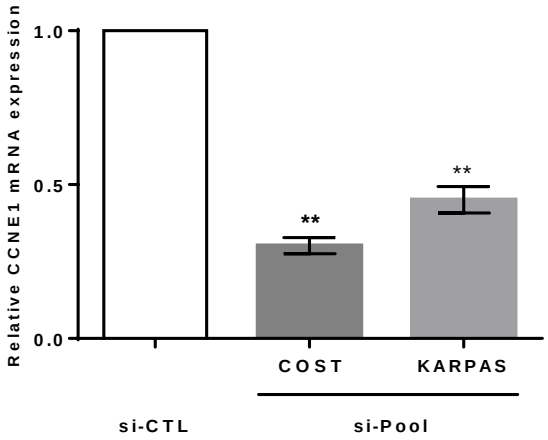

COST
SU-DHL1
KARPAS
ALK
pALK
CDK4
CDK6
Rb
GAPDH

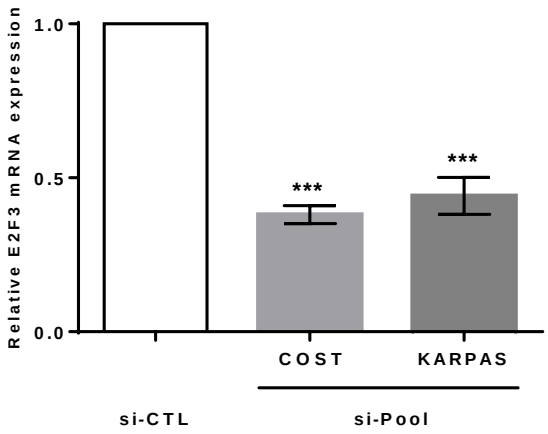

A
Fold change CDK6 mRNA expression
1.0
0.5
0.0
***
undetected
***
SU-DHL-1
KARPAS
COST
si-CTL
si-CDK6
Fold change CCNE1 mRNA expression
1.0
0.5
0.0
***
***
KARPAS
COST
si-CTL
si-CCNE1
Fold change CDC25A mRNA expression
1.0
0.5
0.0
***
***
KARPAS
COST
si-CTL
si-CDC25A
Fold change E2F3 mRNA expression
1.0
0.5
0.0
**
***
KARPAS
COST
si-CTL
si-E2F3
B
SU-DHL1
KARPAS-299
COST
si-CTL si-CDK6
si-CTL si-CDK6
si-CTL si-CDK6
CDK6
GAPDH
si-CTL si-CCNE1
si-CTL si-CCNE1
CCNE1
GAPDH
si-CTL si-CDC25A
si-CTL si-CDC25A
CDC25A
GAPDH
si-CTL si-E2F3
si-CTL si-E2F3
E2F3
GAPDH
C
Relative CDK6 protein level
1.0
0.5
0.0
***
*
***
SU-DHL-1
KARPAS
COST
si-CTL
si-CDK6
Relative CCNE1 protein level
1.0
0.5
0.0
***
***
KARPAS
COST
si-CTL
si-CCNE1
Relative CDC25A protein level
1.0
0.5
0.0
**
***
KARPAS
COST
si-CTL
si-CDC25A
Relative E2F3 protein level
1.2
0.6
0.0
ns
ns
KARPAS
COST
si-CTL
si-E2F3

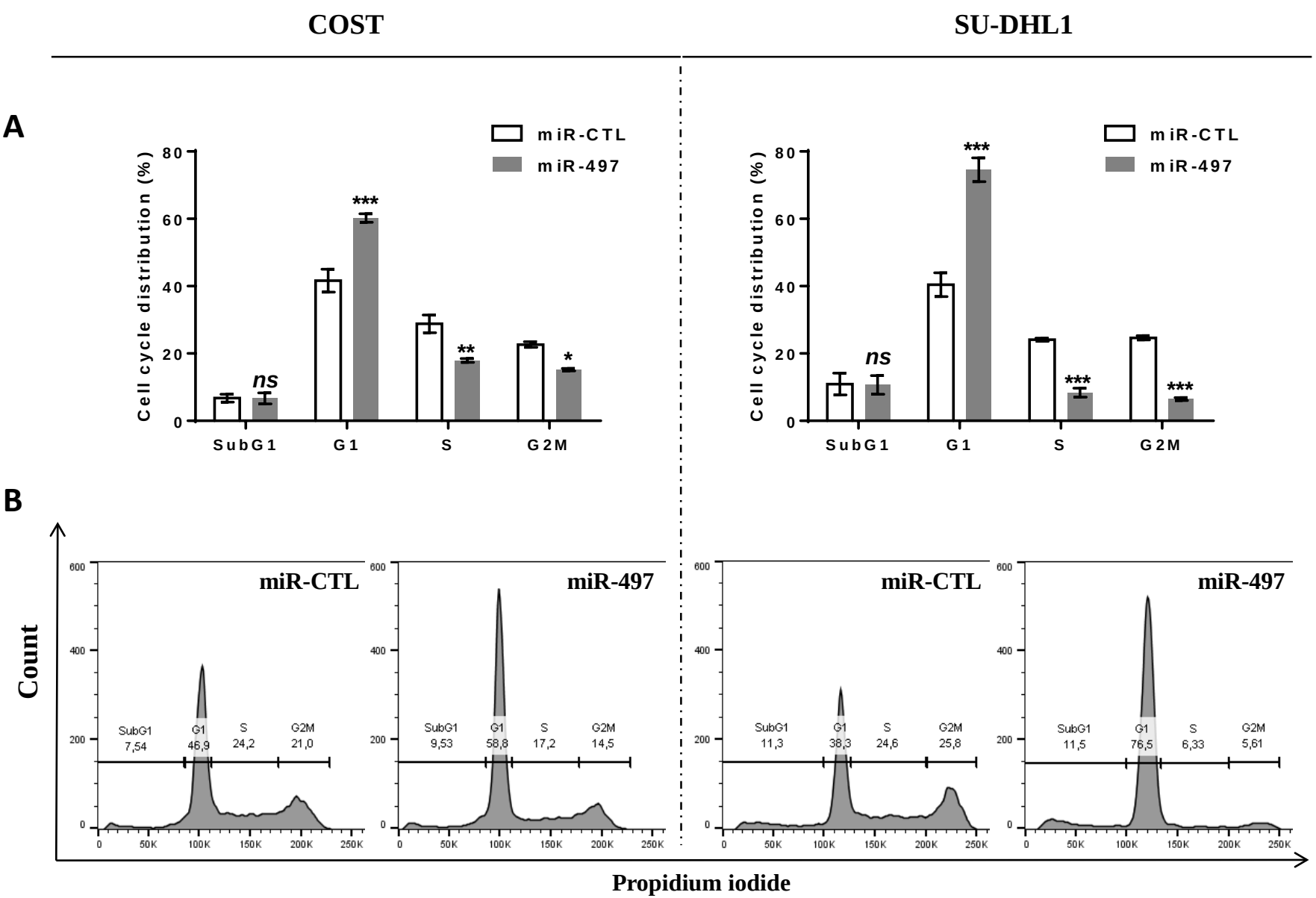

A
B
C
D
E
F
COST
KARPAS
Cell cycle distibution (%)
si-CTL
si-E2F3
si-CDK6
si-CCNE1
SubG1
G0G1
S
G2M
ns
*
**

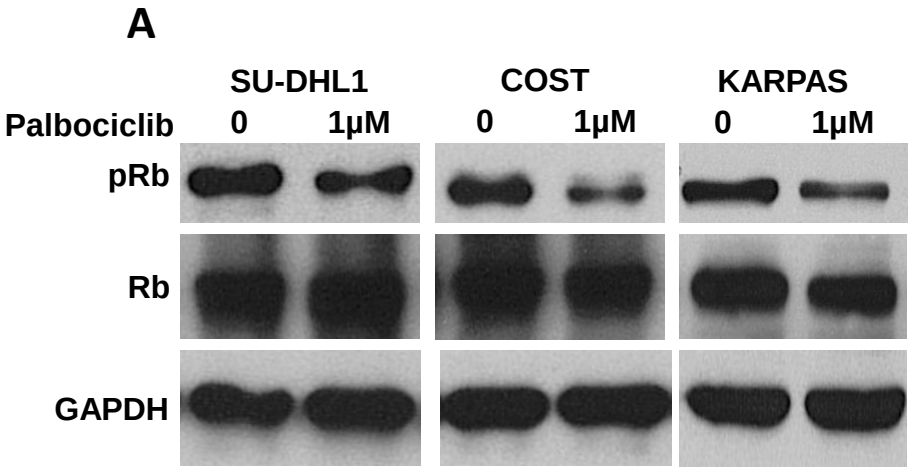

Relative caspase3/7 activity
2.5
2.0
1.5
1.0
0.5
0.0
**
si-CTL
si-CDK6
si-CCNE1
si-E2F3
si-CTL
si-CDK6
si-CCNE1
si-E2F3
COST
KARPAS

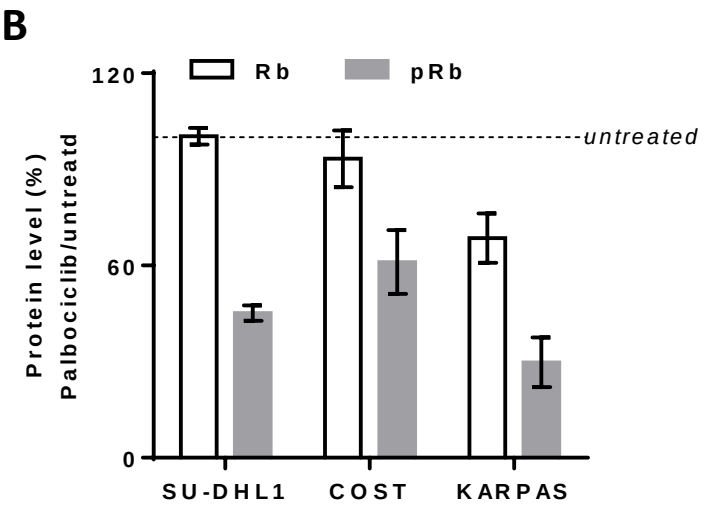

Relative CDK6 mRNA expression
1.0
0.5
0.0
**
undetected
COST
KARPAS
si-CTL
si-Pool

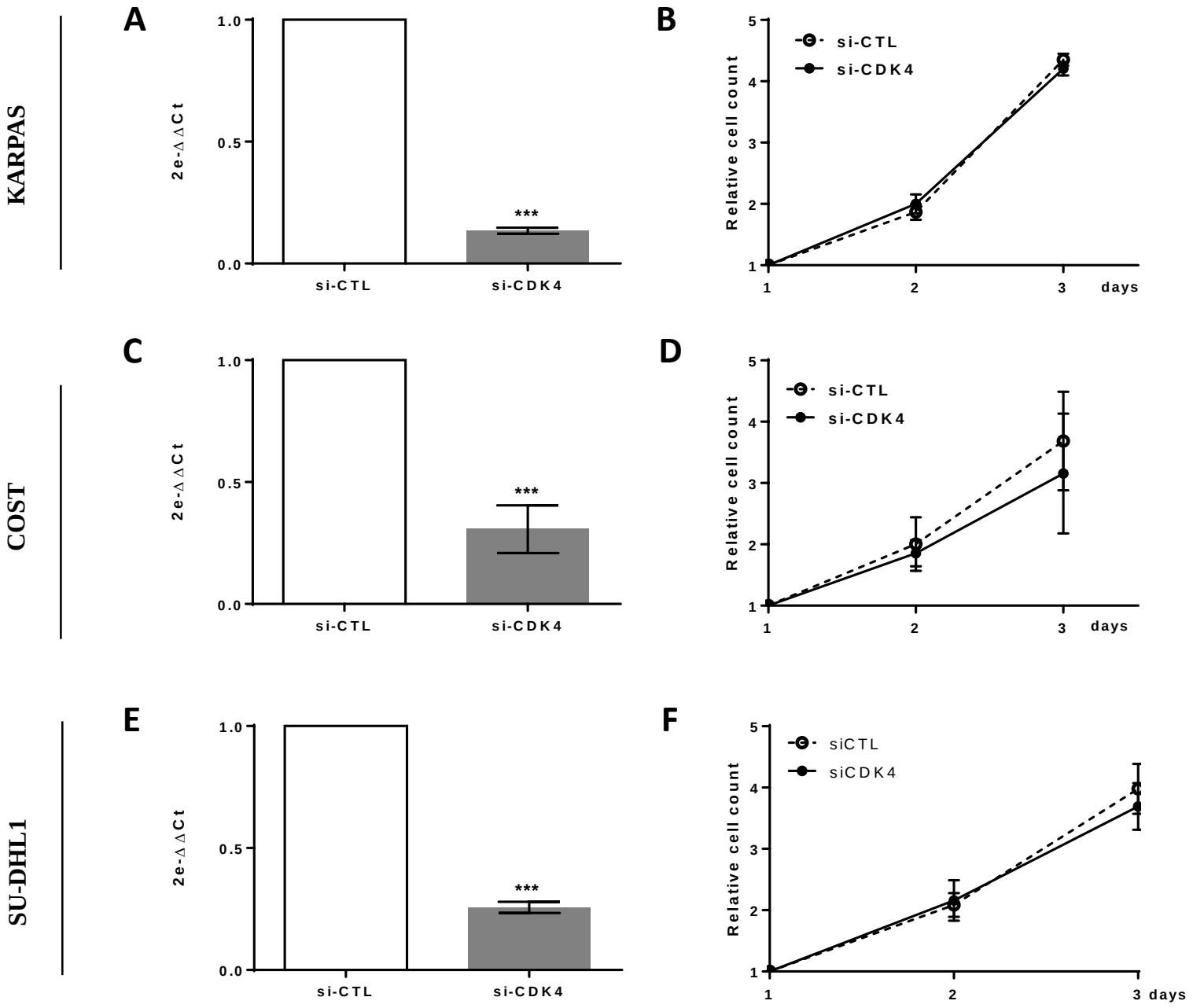

Relative CCNE1 mRNA expression
1.0
0.5
0.0
**
**
COST
KARPAS
si-CTL
si-Pool


Relative E2F3 mRNA expression
1.0
0.5
0.0
***
***
COST
KARPAS
si-CTL
si-Pool

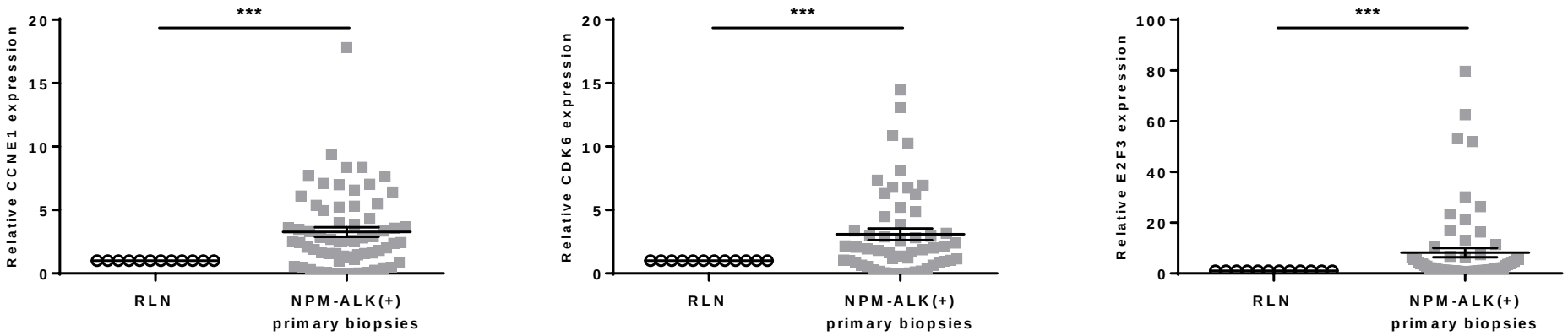

COST
SU-DHL1
A
miR-CTL
miR-497
Cell cycle distribution (%)
ns
***
**
*
SubG1
G1
S
G2M
B
Count
Propidium iodide
miR-CTL
miR-497
SubG1 7,54
G1 46,9
S 24,2
G2M 21,0
SubG1 9,53
G1 58,8
S 17,2
G2M 14,5
SubG1 11,3
G1 38,3
S 24,6
G2M 25,8
SubG1 11,5
G1 76,5
S 6,33
G2M 5,61


A
SU-DHL1
COST
KARPAS
Palbociclib
0
1µM
0
1µM
0
1µM
pRb
Rb
GAPDH

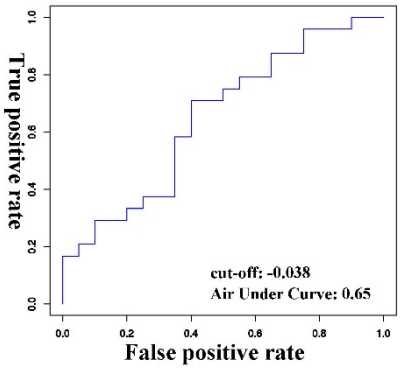

B
Rb
pRb
120
60
0
untreated
Protein level (%)
Palbociclib/untreatd
SU-DHL1
COST
KARPAS


KARPAS
COST
SU-DHL1
A
B
C
D
E
F
2e-ΔΔCt
1.0
0.5
0.0
***
si-CTL
si-CDK4
Relative cell count
5
4
3
2
1
days
siCTL
siCDK4

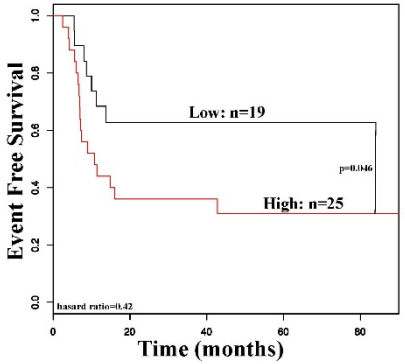
A


***
Relative CCNE1 expression
20
15
10
5
0
RLN
NPM-ALK(+)
primary biopsies
***
Relative CDK6 expression
20
15
10
5
0
RLN
NPM-ALK(+)
primary biopsies
***
Relative E2F3 expression
100
80
60
40
20
0
RLN
NPM-ALK(+)
primary biopsies

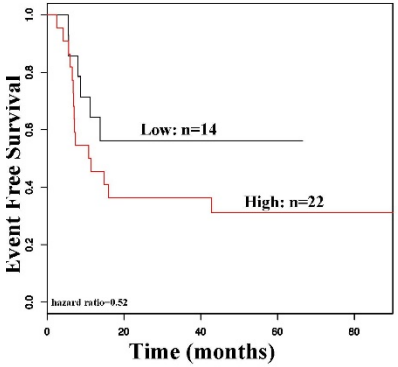
B


True positive rate
cut-off: -0.038
Air Under Curve: 0.65
False positive rate

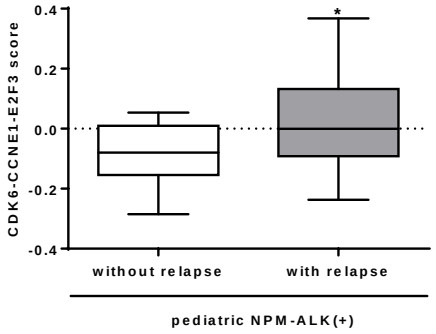
C


Event Free Survival
Low: n=19
p=0.046
High: n=25
hazard ratio=0.42
Time (months)

D


Event Free Survival
Low: n=14
High: n=22
hazard ratio=0.52
Time (months)

E


CDK6-CCNE1-E2F3 score
0.4
0.2
0.0
-0.2
-0.4
*
without relapse
with relapse
pediatric NPM-ALK(+)

qRT-PCR primers	
CDC25A-foward	CTGTCGCCTGTCACCAACCTG
CDC25A-reverse	TCGGAGGAGCCCATTCTCTGC
CDK6-foward	CCCCAGAGTCTGATTACCTGC
CDK6-reverse	ACATAGCCTCTGCCCAAGC
CCNE1-foward	GGTTTCAGGGTATCAGTGGTGCG
CCNE1-reverse	TCTGTGGGTCTGTATGTTGTGTGC
E2F3-foward	GCACTACGAAGTCCAGATAGTCC
E2F3-reverse	GGCTCAGGAGCTGAATGAA
CCND3-foward	CTGGATCGCTACCTGTCTTG
CCND3-reverse	TCCCACTTGAGCTTCCCTAG
GAPDH-foward	CGGGAAGCTTGTGATCAATGG
GAPDH-reverse	GGCAGTGATGGCATGGACTG
MLN51-foward	TAATCCCAGTTACCCTTATGCTCCA
MLN51-reverse	GTTATAGTAGGTCACTCCTCCATATACCTGT
siRNA	
CDC25A siRNA	CUGUGAUAUUGAUAGCUUU
CDK6 siRNA	UUCUACGAAACAUUUCUGC
CCNE1 siRNA	GAGCUGUUUCUGAGGAGCC
E2F3 siRNA	AAACGCGGUAUGAUACGUCUC
STAT3 siRNA	AACAUCUGCCUAGAACGGCUA
si-CTL	Smart Pool 4609 (Dharmacon)
bisulfite sequencing primers	
MiR-497-F	TGTTTTTTAGGAGGGGATTAGG
MiR-497-Rb	[Btn]TCACCTCCAAAAACATCTAAATAACAATAT
MiR-497-sequence	TTTAGGTTGGGGTTTTTA

Downregulated miRNAs in human	**Total**	**miRNA names**
NPM-ALK(+) and NPM-ALK(-) ALCL lymph node primary tissues ††	24	let-7a, let-7c, let-7g, miR-1201, miR-1256, miR-126, miR-140-3p, miR-150, miR-151-5p, miR-204, miR-26a, miR-29a, miR-29c, miR-30b, miR-342-3p, miR-342-5p, miR-361-3p, miR-361-p, miR-374a*, miR-384, miR-548, miR-655, miR-99a
NPM-ALK(+) ALCL lymph node primary tissues ††	39	let-7b, let-7d, miR-100, miR-10b, miR-1179, miR-122, miR-1259, miR-125b, miR-1277, miR-1279, miR-1324, miR-139-3p, miR-139-5p, miR-145, miR-155, miR-15b, miR-190b, miR-195, miR-19a*, miR-26b, miR-30a, miR-30a*, miR-30d, miR-31, miR-449a, miR-497, miR-508-5p, miR-509-3p, miR-514, miR-545* , miR-548g, miR-561, miR-590-3p, miR-599, miR-603, miR-606, miR-609, miR-769-5p, miR-802
NPM-ALK(-) ALCL lymph node primary tissue ††	8	let-7e, miR-194, miR-199a-3p, miR-26a-1*, miR-26a-2*, miR-335, miR-499-5p, miR-633

Normalized against equivalent miRNA levels from normal reactive lymph node (n=3)

adaptated from Congras et al, [22]