SUPPLEMENTARY APPENDIX

STAT3-mediated activation of microRNA cluster 17~92 promotes proliferation and survival of ALK-positive anaplastic large cell lymphoma

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Supplementary Design and Methods

Immunoblotting

The following primary antibodies were used for western blotting: anti phospho STAT3, Tyr705, (1:1000; Cell Signaling Technology, Beverly, MA, USA); anti STAT3 (1:1000; Cell Signaling Technology Ltd, Beverly, MA, USA); anti Cyclin A (H432) (1:1000; Santa Cruz Biotechnology, inc.); anti Cyclin B1 (H433) (1:1000; Santa Cruz Biotechnology, inc.); anti Cyclin D3 (1:5000; Neomarkers Thermo Scientific); anti p21 (1:200; Neomarkers Thermo Scientific); anti caspase-3 cleaved, Asp 175, (1:1000; Cell Signaling Technology Ltd, Beverly, MA, USA); anti caspase-7 cleaved, Asp 198, (1:1000; Cell Signaling Technology Ltd, Beverly, MA, USA); anti BIM (1:1000; Cell Signaling Technology Ltd, Beverly, MA, USA); anti PARP (H 250) (1:500; Santa Cruz Biotechnology, inc.); anti cIAP1 (1:1000; Cell Signaling Technology Ltd, Beverly, MA, USA); anti cIAP2 (1:1000; Cell Signaling Technology Ltd, Beverly, MA, USA); anti XIAP (1:1000; Cell Signaling Technology Ltd, Beverly, MA, USA); anti α-actin (1:4000; Sigma-Aldrich Corp., St. Louis, MO, USA).

Cell cycle and apoptosis analysis by flow cytometry

Apoptosis was measured by flow cytometry after staining with the mitochondrion-permeable voltage-sensitive dye tetrametylrodamine methyl ester (TMRM; Molecular Probes, Eugene, OR). Cells ($5x10^5$) were washed once in PBS, incubated for 15 minutes at 37°C in HEPES buffer solution (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5mM CaCl) with 200 nM TMRM. Cells were analyzed by FACSCalibur using CellQuest software (BD Pharmingen Biosciences). For cell cycle analysis and DNA content determination, cells were fixed for 1 hour in 70% ethanol at 4°C. After washing, cells were treated with RNase (0.25 mg/ml) and stained with propidium iodide (50 μg/ml). Than cells were analyzed by FACSCalibur; the G1/G0-phase fraction was calculated using the CellQuest program (BD Pharmingen Biosciences).

RNA extraction and RT-qPCR

Total RNA was extracted by standard Trizol (Invitrogen, Carlsbad, CA) method and RNA concentration was quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). A total of 100 ng of RNA was reverse transcribed with the miScript Reverse Transcription Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Mature miRNA expression was assayed by miScript SYBR® Green PCR Kit (Qiagen, Valencia, CA), using the miScript Primer assay specific for the mature miRNA under study and normalized to RNU6B expression. Oligonucleotide pairs for gene expression analysis were designed with PrimerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences are available upon request. RT-qPCR assays were performed in triplicate in the Termal iCycler (BioRad) and calculated using the ΔCt method.

Gene expression profiling

Biological duplicate were used for each experimental condition. Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA) and purified using the RNeasy total RNA Isolation Kit (Qiagen, Santa Clarita, CA). RNA integrity was evaluated by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). cDNA and biotinylated cRNAs were generated by Illumina Total Prep RNA Amplification Kit (Ambion, Austin, TX). cRNAs quality and quantification was assessed by Bioanalyser. Hybridization was carried out on HumanHT-12 V6 bead chips (Illumina). Array washing, staining and scanning was performed using standard Illumina protocols. Detection data were processed with the XAS software21 using the following thresholds for significant detection: Differential Score >30, Detection >0.99, Fold Change >2.

Supplementary Tables

Name	ΔCt CTR	ΔCt shSTAT3	ΔΔCt
let-7d	8.9	10.5	-1.6
let-7i	n.d.	n.d.	n.d.
miR-100	8.2	6.2	2.0
miR-101	8.2	9.2	-1.0
miR-132	7.5	7.2	0.4
miR-133b	15.1	12.8	2.3
miR-16-2	n.d.	n.d.	n.d.
miR-22	7.5	7.1	0.5
miR-223	9.2	5.1	4.1
miR-34a	11.8	9.1	2.6
miR-500	9.6	11.1	-1.4
miR-505	12.7	12.0	0.7
miR-550	7.0	7.8	-0.8
miR-582	n.d.	n.d.	n.d.
miR-628	n.d.	n.d.	n.d.
miR-629	9.8	9.0	0.8
miR-7	5.2	6.6	-1.5
miR-886	n.d.	n.d.	n.d.
miR-93	1.2	1.7	-0.6
miR-98	10.6	11.8	-1.2
miR-106a	n.d.	n.d.	n.d.
miR-20b	3.2	3.8	-0.6
miR-18b	11.1	7.9	3.3
miR-92	0.4	0.7	-0.3
miR-363	8.9	8.3	0.6
miR-17-3p	8.4	8.9	-0.6
miR-17-5p	2.8	3.1	-0.2
miR-18a	5.0	5.2	-0.3
miR-19b	-0.9	-0.3	-0.6
miR-20a	0.2	1.0	-0.8
miR-181a	n.d.	n.d.	n.d.
miR-181b	6.4	5.5	0.9
miR-193b	11.1	11.7	-0.6
miR-365	5.9	6.5	-0.5
miR-143	12.2	9.3	3.0
miR-145	7.9	6.7	1.3
miR-221	4.9	3.2	1.7
miR-222	2.9	1.4	1.5
miR-23a	10.4	9.0	1.4
miR-24	1.7	1.0	0.6
miR-27a	3.5	2.6	0.9

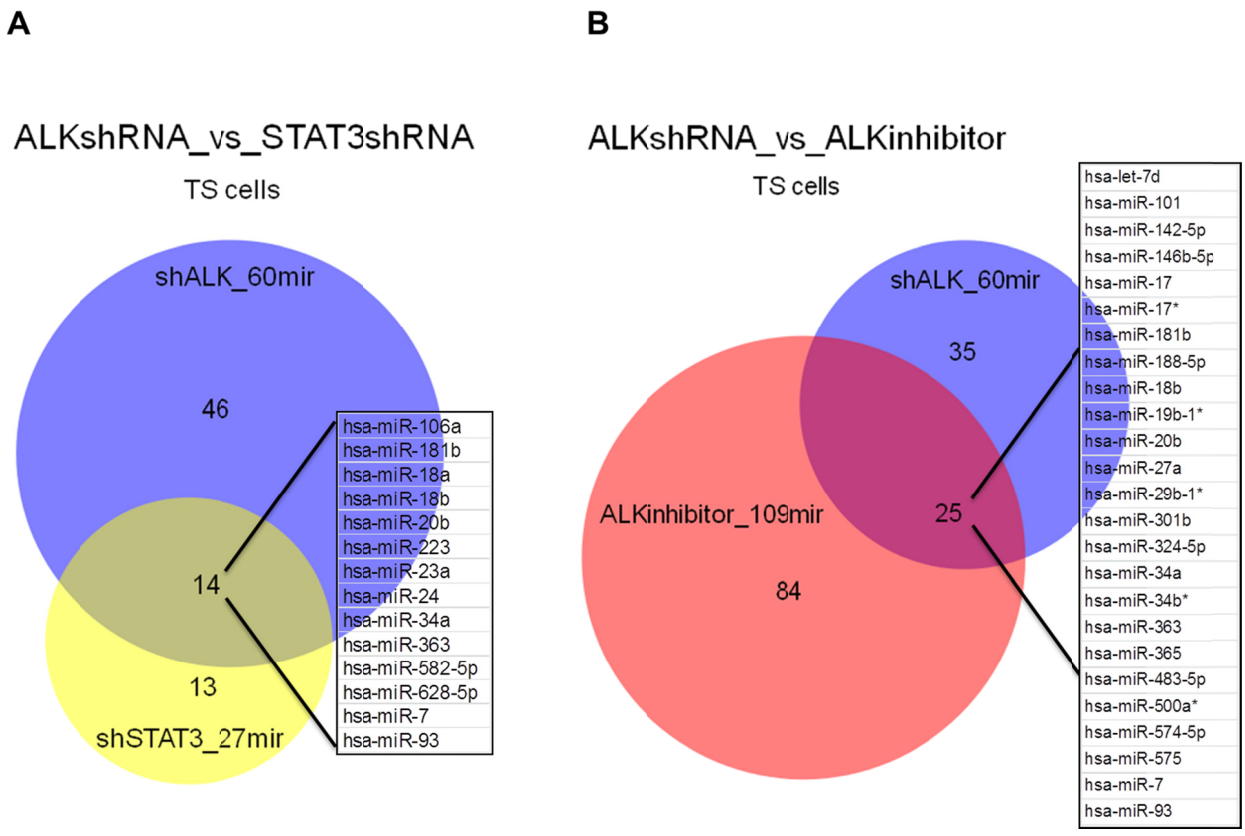
Supplementary Table S1. Validation of microRNAs modulated by STAT3 KD in JB-6 cells. JB-6 cells cotransduced with pLV-tTR-KRAB/DsRed and pLVTH-STAT3-S3S/GFP (S3S) lentiviral preparations were cultured in the absence (CTR) or presence (shSTAT3) of doxycycline (1 μg/ml) for 96 hours. Expression of indicated miRNA was detected by RT-qPCR and calculated using the ΔCt method. microRNAs accordingly modulated by STAT3 KD in JB-6 and SUP-M2 cells are shown in green, microRNAs inversely modulated are shown in red.

PROBE_ID	SYMBOL	CTRL_no Doxy	17-92_no Doxy	CTRL_+Doxy	17-92_+Doxy	FC
ILMN_2347145	DCN	111	111	805	136	5.91
ILMN_1732538	LBP	271	113	673	138	4.89
ILMN_1700888	ENPP1	192	163	2765	658	4.20
ILMN_1706505	COL5A1	840	358	2600	757	3.43
ILMN_1790529	LUM	144	143	453	142	3.19
ILMN_1758323	ACPP	2264	423	1364	481	2.83
ILMN_1760062	IFI44	401	224	1485	545	2.72
ILMN_1704353	IGSF3	223	140	641	235	2.72
ILMN_1812679	UPK1B	7139	5246	2324	865	2.69
ILMN_2147435	MAN2A1	1546	978	2083	789	2.64
ILMN_1772646	LOC388681	900	611	969	367	2.64
ILMN_2363489	BRE	6225	4489	17084	6497	2.63
ILMN_2094360	NR2F2	658	243	1226	477	2.57
ILMN_1811719	MYO7A	120	120	420	172	2.45
ILMN_1781285	DUSP1	3479	919	2035	861	2.37
ILMN_1736317	NALCN	204	184	636	270	2.35
ILMN_1697448	TXNIP	7135	4497	7772	3306	2.35
ILMN_1783956	ATP8B4	1057	569	2168	928	2.33
ILMN_3246538	LOC100133866	2871	1325	2725	1179	2.31
ILMN_1683194	DCN	118	105	274	119	2.30
ILMN_3221721	LOC728802	313	220	371	165	2.26
ILMN_1797236	TGM2	1831	877	1573	701	2.24
ILMN_1762529	SLC12A8	1886	1140	3610	1622	2.23
ILMN_1805665	FLRT3	1279	666	1216	547	2.22
ILMN_2095610	ANXA8	108	109	298	135	2.20
ILMN_1705066	BTBD11	1390	533	1622	737	2.20
ILMN_1739428	IFIT2	1390	733	658	301	2.19
ILMN_1778684	BRE	5901	3764	13277	6072	2.19
ILMN_1674063	OAS2	206	158	1293	592	2.19
ILMN_1691567	GNPDA2	1220	668	1866	861	2.17
ILMN_1776181	BIRC3	3426	1740	1947	902	2.16
ILMN_1679267	TGM2	6092	3241	5049	2350	2.15
ILMN_1705750	TGM2	8263	4005	11590	5406	2.14
ILMN_2388800	PPAP2B	877	465	1321	616	2.14
ILMN_2202915	FAR2	117	140	332	155	2.14
ILMN_2121816	GPR137B	927	523	1725	829	2.08
ILMN_2203149	TMPRSS12	253	129	379	182	2.08
ILMN_1771148	CNTN4	167	119	223	107	2.08
ILMN_1697220	NT5E	120	112	384	185	2.08
ILMN_1722622	CD163	14029	7993	19533	9455	2.07
ILMN_1673639	ABI3BP	140	117	248	121	2.05
ILMN_1691341	IL7R	401	271	4829	2356	2.05
ILMN_1726516	SCRIB	6664	4206	5983	2931	2.04
ILMN_1772910	GAS1	17837	9688	4298	2145	2.00

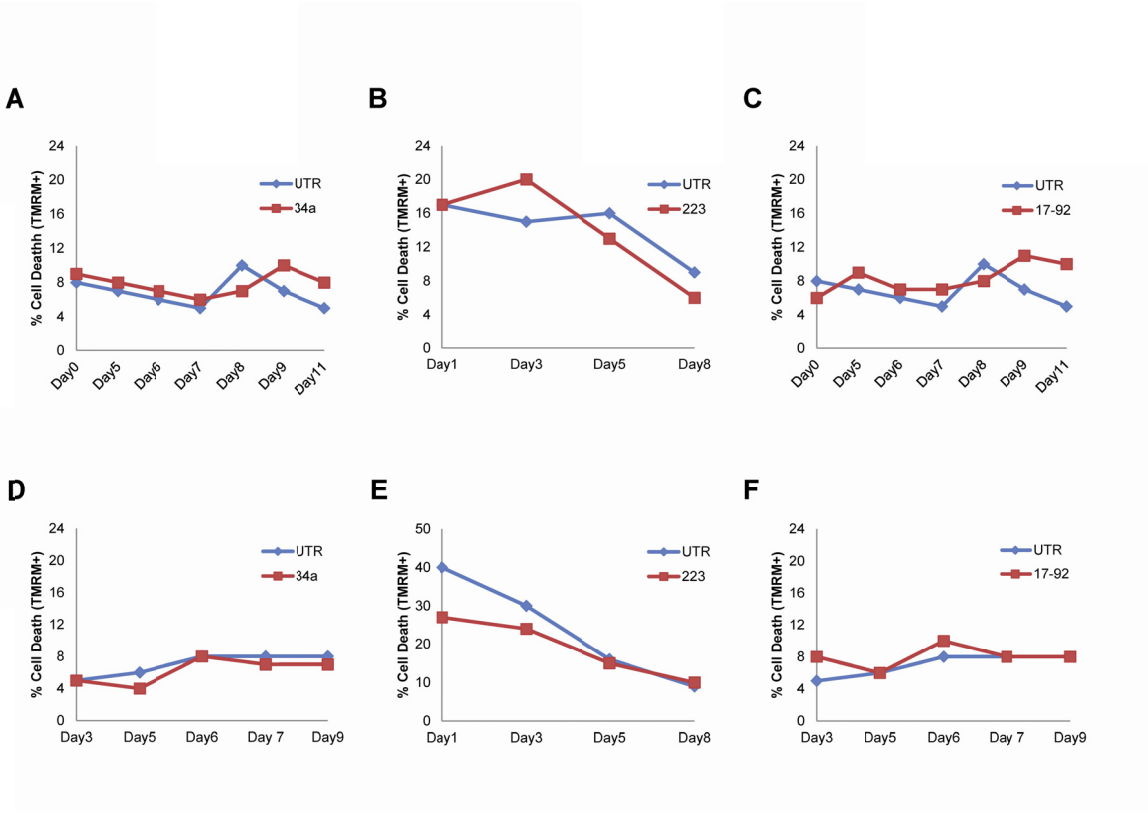
PROBE_ID	SYMBOL	CTRL_no Doxy	17-92_no Doxy	CTRL_+Doxy	17-92_+Doxy	FC
ILMN_2061116	MATN1	197	202	156	313	0.50
ILMN_1740875	FPR2	112	136	140	282	0.50
ILMN_1763537	LCTL	125	131	159	320	0.50
ILMN_3237926	CCNJL	170	226	150	314	0.48
ILMN_1671337	SLC2A5	3425	5618	1418	3043	0.47
ILMN_1780170	APOD	112	111	505	1094	0.46
ILMN_1796642	NCF2	139	156	254	555	0.46
ILMN_2339266	LAMA2	183	309	367	802	0.46
ILMN_2325837	CD3D	6476	8898	7183	15723	0.46
ILMN_2198878	INPP4B	148	204	140	310	0.45
ILMN_1815690	TIE1	130	213	164	366	0.45
ILMN_1717207	MMP25	244	565	516	1165	0.44
ILMN_1654396	ITGB2	5026	10669	12808	28946	0.44
ILMN_2342638	ASGR2	208	241	3246	7347	0.44
ILMN_1678095	SMPDL3B	114	124	165	375	0.44
ILMN_2319913	DGKA	182	263	321	730	0.44
ILMN_1670385	TUBAL3	712	781	583	1343	0.43
ILMN_1769546	RIN2	202	253	468	1094	0.43
ILMN_1715963	FBXO7	245	415	317	746	0.42
ILMN_1694966	ASGR2	129	141	1540	3641	0.42
ILMN_1651296	LOC143666	1768	2012	770	1825	0.42
ILMN_1696434	LAMA1	373	657	120	284	0.42
ILMN_1801040	SPN	314	329	484	1186	0.41
ILMN_1745807	TMEM62	2170	3433	1681	4206	0.40
ILMN_1671142	GPR68	658	1113	751	1994	0.38
ILMN_1721540	NWD1	97	114	216	585	0.37
ILMN_2109489	GZMB	28970	33596	7240	19816	0.37
ILMN_2043060	PLCH1	126	126	161	452	0.36
ILMN_1786847	TGM3	209	298	265	747	0.35
ILMN_1796423	CLIC3	116	120	341	979	0.35
ILMN_1698995	DES	118	133	222	640	0.35
ILMN_1754241	C16orf73	126	395	135	396	0.34
ILMN_1741917	OSCAR	183	298	244	733	0.33
ILMN_1759097	MLLT11	370	495	491	1473	0.33
ILMN_1766914	MFAP4	126	142	122	378	0.32
ILMN_1700268	QPRT	126	138	174	592	0.29
ILMN_1730628	RNASE2	134	141	222	756	0.29
ILMN_1794364	CTSW	109	107	213	726	0.29
ILMN_1806725	PDCD1	126	121	336	1315	0.26
ILMN_1777519	ITGB7	147	220	1447	5835	0.25
ILMN_2367418	OSCAR	218	282	250	1077	0.23
ILMN_1675190	RETN	121	125	122	540	0.22
ILMN_1711493	PRAM1	148	162	176	851	0.21
ILMN_1780255	KLK6	123	245	139	685	0.20
ILMN_2384181	DHRS9	143	133	162	851	0.19
ILMN_1796316	MMP9	144	929	7171	38696	0.19
ILMN_1710434	TBC1D10C	110	133	145	832	0.17
ILMN_1677108	CAPN13	152	434	138	929	0.15
ILMN_1733998	DHRS9	121	127	178	1392	0.13
ILMN_1784040	NAPSA	612	2899	475	5987	0.08

Supplementary Table S2. Genes modulated by miR-17~92 following inducible STAT3 KD in TS-SUP-M2 cells. Experiments were carried out 96 hours post doxycycline (+DOXY) or mock treatment (no DOXY). As controls (CTRL), we used TS-SUP-M2 cells not transduced or transduced with the antisense construct (17~92inv). Selection criteria: Fold Change (FC) >2, Differential Score >30, Detection >0.99.

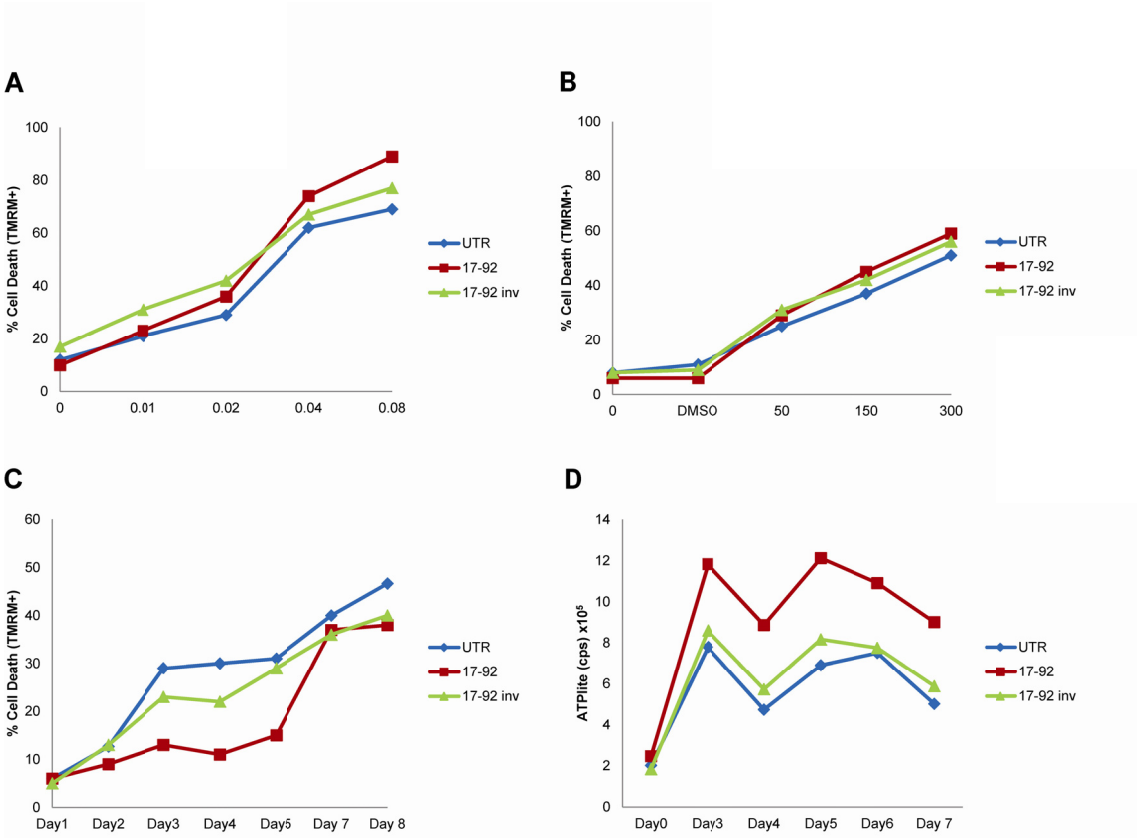
Supplementary Figures



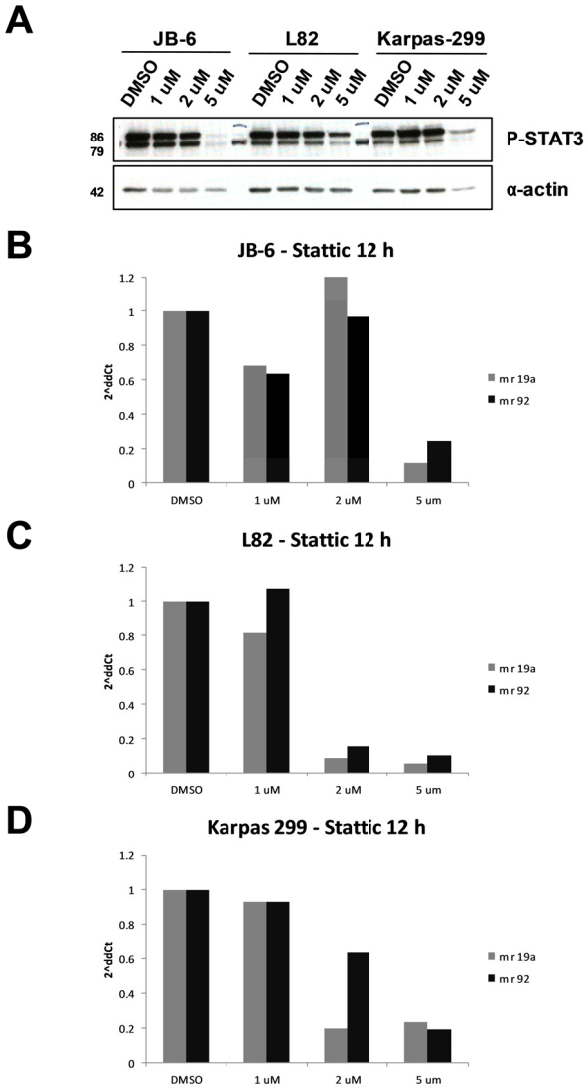
Supplementary Figure S1. (A) ALK/STAT3 miRNA signature obtained by overlapping miRNA expression profiling analyses in the ALK+ ALCL cell line TS-SUP-M2, following ALK (shALK) or STAT3 (shSTAT3) inducible KD. **(B)** ALK miRNA signature obtained by overlapping miRNA expression profiling analyses in the ALK+ ALCL cell line TS-SUP-M2, following ALK inhibition by CEP-28122 (ALKinhibitor) or ALK inducible KD (shALK).



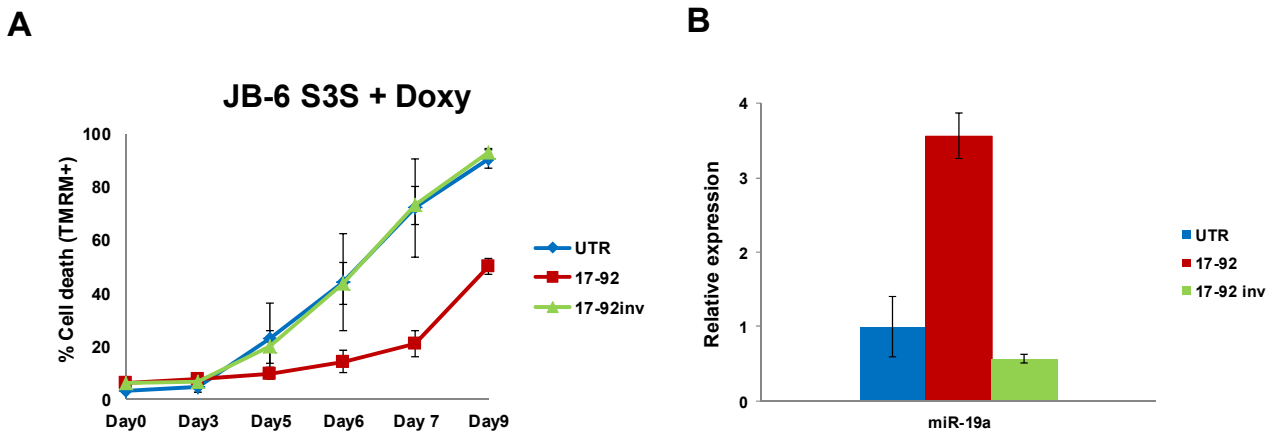
Supplementary Figure S2. Apoptosis analysis in TS-SUP-M2 (A-C) and JB-6 (D-F) cells expressing the indicated miRNAs at different time points in standard cell culture medium. Analysis was performed by TMRM staining-flow cytometry.



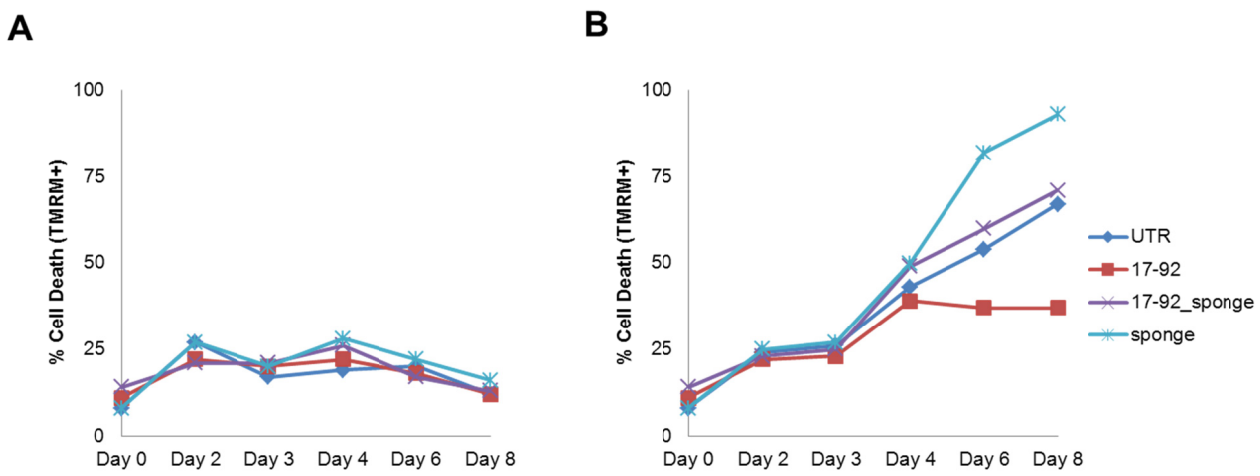
Supplementary Figure S3. Functional analysis of miR-17~92 cluster in TS-SUP-M2 S3S cells infected with lentivirus expressing the miR-17~92 cluster or the miR-17~92 inv as a control. (A) Apoptosis analysis of TS-SUP-M2 S3S cells expressing the indicated miRNAs after 48h of treatment with the indicated concentrations of Doxorubicin (B) and after 18h of treatment with the indicated concentrations of the ALK inhibitor CEP-14083. Analysis was performed by TMRM staining-flow cytometry. (C) Apoptosis analysis of TS-SUP-M2 S3S cells expressing the indicated miRNAs cultured in 2% FBS medium. Analysis was performed by TMRM staining-flow cytometry. (D) Metabolic activity analysis of TS-SUP-M2 S3S cells expressing the indicated miRNAs cultured in 2% FBS medium as determined by measuring the amount of ATP released by lysated cells.



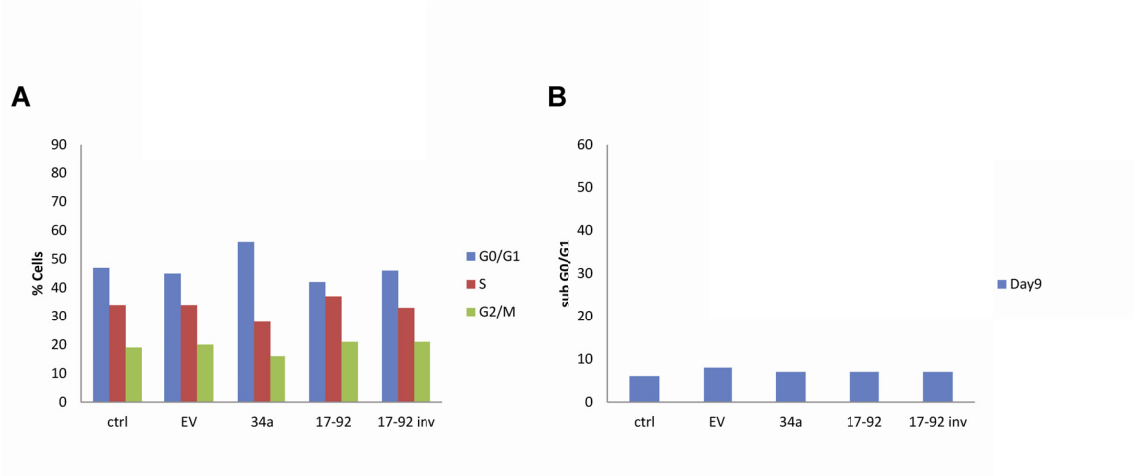
Supplementary Figure S4. Dose dependent inhibition of miR17~92 expression after treatment with increasing concentrations of the STAT3 inhibitor Stattic (Calbiochem Inc., Billerica, MA). ALK-positive ALCL cell lines JB-6, L82, and Karpas 299 were treated with the indicated concentration of Stattic for 12 hours. (**A**) STAT3 phosphorylation was assayed by immunoblotting using a specific phospho-STAT3 (Y705) antibody (upper panels). Anti-α-actin was used as a loading control. **(B-D)** Levels of two representative miR17~92 cluster members (miR-19a and miR-92) were analyzed by RT-qPCR.



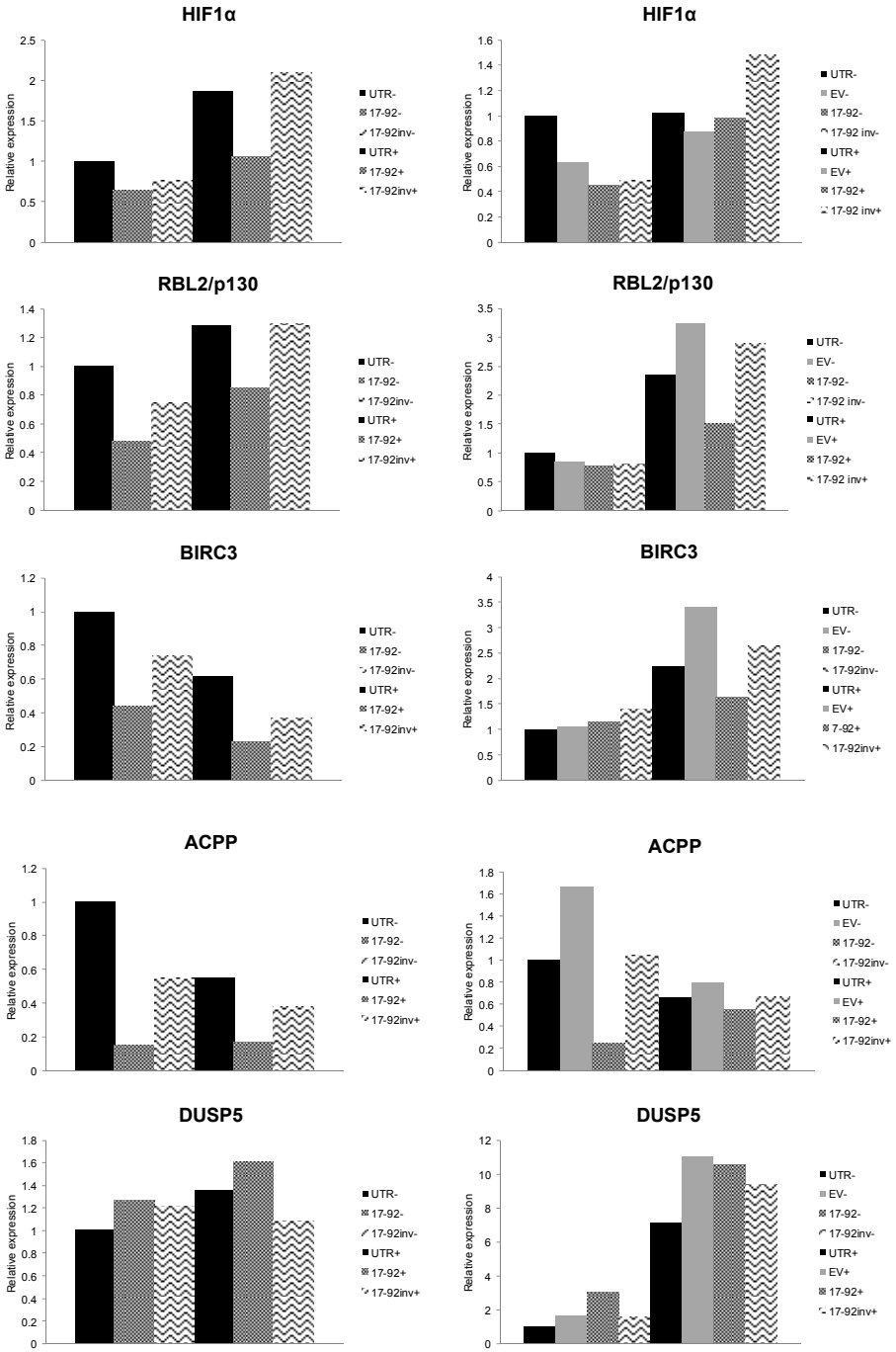
Supplementary Figure S5. **(A)** Apoptosis analysis in JB-6 S3S cells expressing the indicated miRNAs at different time points after induction of STAT3 KD by doxycycline. Analysis was performed by TMRM staining-flow cytometry. These findings are representative of 3 independent experiments. **(B)** miR-19a expression levels in JB-6 S3S cells transduced with lentiviral particles expressing the indicated miRNAs, as detected by RT-qPCR 4 days after infection.



Supplementary Figure S6. Apoptosis in TS-SUP-M2 S3S cells expressing miR 17~92 (17-92), a sponge targeting the entire miR-17-92 cluster (sponge), or both constructs (17-92_sponge) was analyzed at different time points in the absence **(A)** or in the presence **(B)** of doxycycline to induce STAT3 KD. Analysis was performed by TMRM staining-flow cytometry. The experiments were repeated three times with similar results.



Supplementary Figure S7. Cell cycle analysis and cell death of TS-SUP-M2 S3S cells expressing the indicated miRNAs in the absence of doxycycline treatment. (A) Cell cycle was analyzed by Propidium Iodide staining-flow cytometry. (B) Measurement of Sub-G0/G1 fraction as detected by Propidium Iodide staining-flow cytometry was used to quantify apoptotic cells. These findings are representative of 3 independent experiments.



Supplementary Figure S8. Comparison of Gene Expression Profiling (GEP) data to RT-qPCR analysis for potential miR-17~92 cluster targets. Expression of HIF1α, RBL2/p130, BIRC3, ACPP, and DUSP5 in TS-SUP-M2 S3S cells as detected by GEP (left panel), and by RT-qPCR (right panel), 96 hours and 8 days post doxycycline (+) or mock treatment (-).