

Supplemental Data

The synergism of MCL1 and glycolysis on pediatric acute lymphoblastic leukemia cell survival and prednisolone resistance

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Additional information Methods:

Cell culture and primary cells

Medium was supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin (Gibco), 0.125 µg/ml fungizone (Gibco), and 10% Fetal Calf Serum (Integro). Reh, 697 and Sem are BCP-ALL cell lines with an *ETV6-RUNX1*⁺, *E2A-PBX1*⁺, *MLL-AF4*⁺ translocation respectively. Jurkat is a mature tetraploid T-ALL cell line and Loucy is an immature early T-cell precursor (ETP-ALL) cell line. All cell lines were tested for their resistance to prednisolone. HEK293T, a human embryonal kidney cell line, was used for the production of viral particles.

Lentiviral production and infection

Lentiviral helper vectors pRSV-Rev (Addgene plasmid 12253), pMDLg/pRRE (Addgene plasmid 12251), pMD2.G (Addgene plasmid 12259) and psPAX2 (Addgene plasmid 12260) were provided by Prof. D. Trono (Geneva, Switzerland). pLKO.1 Mission short hairpin RNA (shRNA) vectors were purchased from Sigma-Aldrich, i.e. SHC005 against eGFP, and TRCN000005518 and TRCN0000197024 against *MCL1*.

Infections were performed as follow; 70-80% confluent HEK293T cells were transfected with sh*MCL1*, pMD2.G and psPAX2 complemented with CaCl₂ and HEPES-buffered saline in the presence of 25 µM chloroquine (Sigma). Virus-containing supernatant was collected, filtered 0.45µm, and concentrated by ultracentrifugation at 32,000rpm, 1hr, 4°C. Viral titers were determined with a HIV-1 p24 Antigen ELISA kit according to the manufacturer's protocol (ZeptoMetrix). Infection occurred during 45' 1800 rpm spin-oculation of 0.5*10⁶ cells/ml with 2.5 TU/cell viral particles and 5 µg/ml polybrene (Sigma-Aldrich). After 24h, infected cells were selected in 0.5 µg/ml in the case of 697 and Loucy, 1 µg/ml for Reh and Sem, and 2 µg/ml puromycin for Jurkat.

Quantitative RT-PCR

RNA was extracted using a Rneasy minikit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized from 1 µg RNA by 8 IU/µl MMLV (Promega), 20nM oligodT primers, 1µM random hexamer primers (Invitrogen), 200 µM dNTPs and 1 IU/µl RNAsin in MMLV-buffer (Promega). Primers used for the reference gene *RPS20*, were 5'-AAGGGCTGAGGATTTTG-3' (forward) and 5'-CGTTGCGGCTTGTTAG-3' (reverse).

Apoptosis measurement

0.2*10⁶ cells were incubated for 15 minutes in 200µl AnnexinV binding buffer (Molecular probes) containing 2 µg/ml propidium iodide (Molecular probes) and 1:1000 AnnexinV Alexa Fluor® 633 (Molecular probes).

Additional information Methods:

Reverse Phase Protein Array

Slides were stained with specific antibodies and incubated with a biotinylated secondary antibody. Slides were scanned using the NovaRay scanner and protein levels were calculated relative to the total amount of protein per sample using MicroVigene Software.

Western Blot and Immunoblotting

Cell pellets were lysed in lysis buffer supplemented with protease inhibitors, and protein concentration was quantified according to the BCA assay (Pierce).

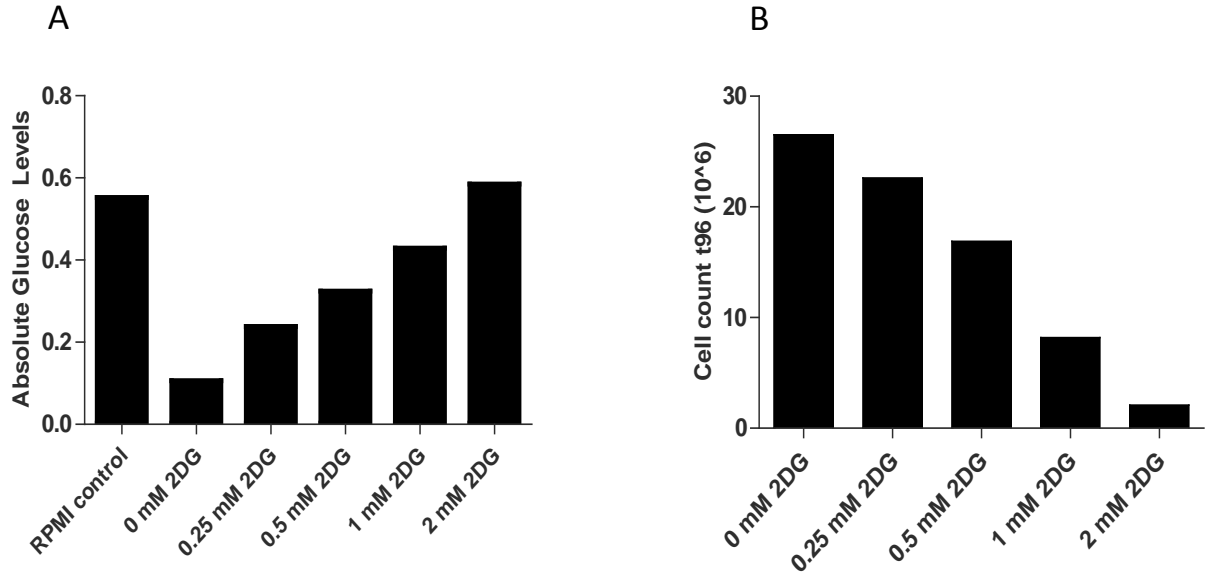
Glucose-consumption assay

Briefly, the supernatant of cultured cells was diluted 25 times in milliQ, supplemented with assay solvent containing Glucose Oxidase, Peroxidase and o-Dianisidine and incubated for 30 minutes at 37°C. Hereafter, 12N sulfuric acid was added and levels of the spectrophotometric end-product oxidized o-Dianisidine were measured at 540nm using the Versamax (Molecular Devices).

Synergistic effect

Synergistic effects were calculated from equi-effective drug concentrations by the following equation postulated by Berenbaum (15) $[\text{Drug A}_{\text{in combination with B}}] / [\text{Drug A}_{\text{alone}}] + [\text{Drug B}_{\text{in combination with A}}] / [\text{Drug B}_{\text{alone}}]$. A synergy factor (F_{syn}) <1 indicates synergy, whereas a F_{syn} of 1 indicates additivity and a $F_{\text{syn}} >1$ points to antagonism between two drugs.

Supplemental Figure 1.

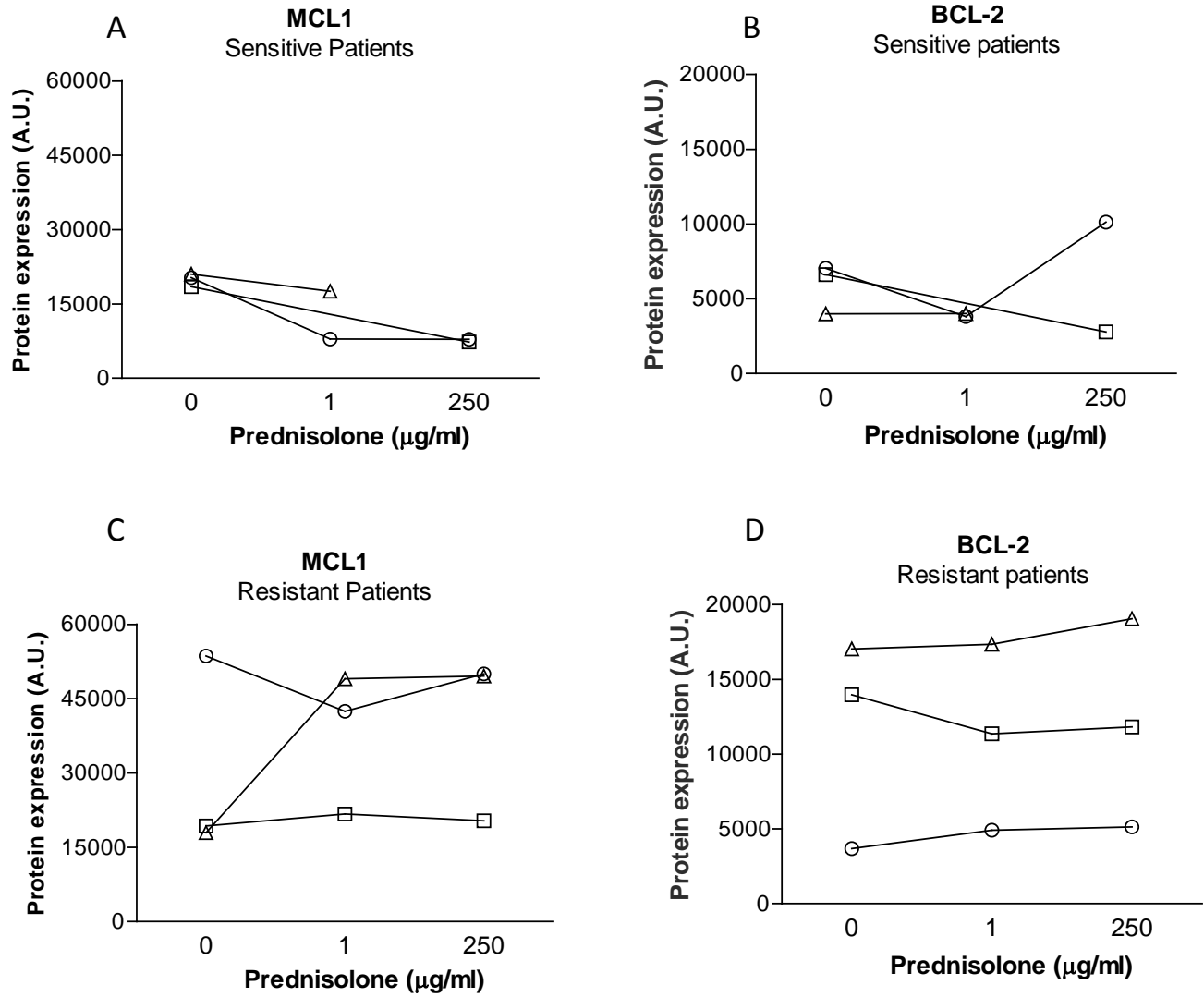


Supplemental Figure 1.

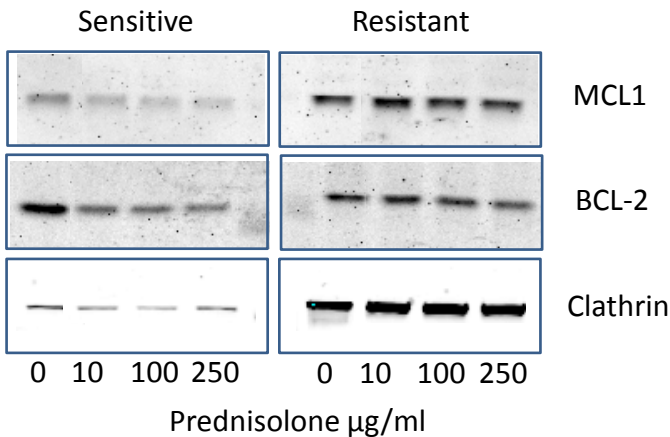
The effect of 2DG on glucose consumption and proliferation.

(A) Absolute glucose levels in the supernatant of *ETV6-RUNX1*⁺ BCP-ALL (REH) cells were measured with a glucose assay. RPMI control represents the amount of glucose present in culture medium incubated for 96 hours in the absence of 2DG. Cells cultured without 2DG have consumed >90% of the glucose present in RPMI culture medium after 96 hrs. Exposure to increasing concentrations of 2DG reduces the amount of glucose that is being consumed. In cells treated with 2mM 2DG the glucose levels are virtually unaffected compared to RPMI control medium, indicating that no glucose has been consumed. (B) *ETV6-RUNX1*⁺ BCP-ALL (REH) cells were counted by a trypan blue exclusion assay after exposure to 2DG for 96 hours. Glucose consumption (A) and cell viability (B) are correlated.

Supplemental Figure 2.



E

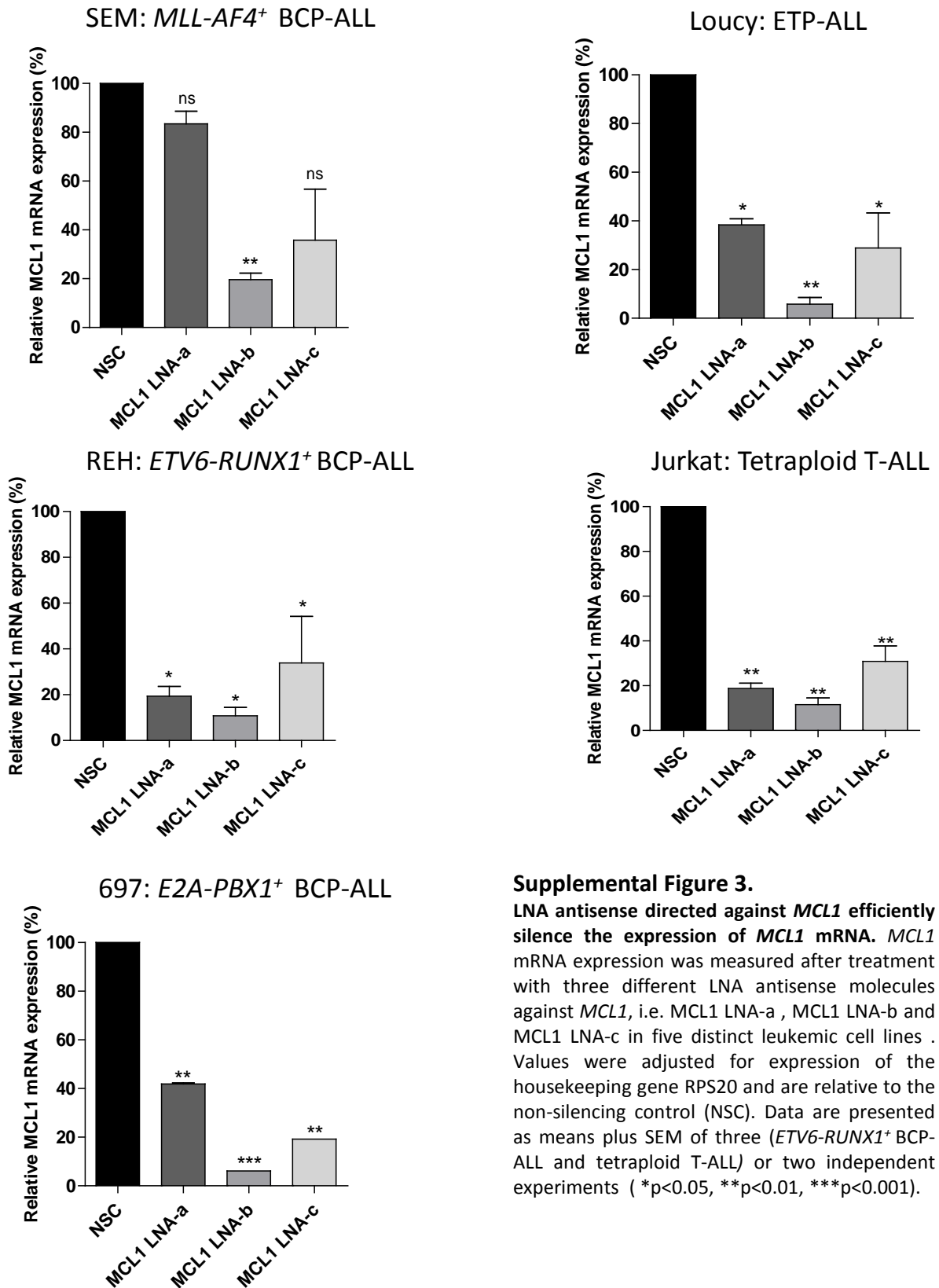


Supplemental Figure 2.

Downregulation of MCL1 by prednisolone is impaired in prednisolone resistant leukemic cells of patients.

(A-D) Individual MCL1 and BCL-2 expression levels after prednisolone exposure of prednisolone sensitive and resistant patients cells indicated in Figure 1. (E) MCL1 and BCL-2 western blot analysis of one sensitive patient and one resistant pediatric BCP-ALL patient's cells after *in vitro* exposure for 48 hours with 0, 10, 100 or 250 µg/ml prednisolone. Clathrin was used as loading control.

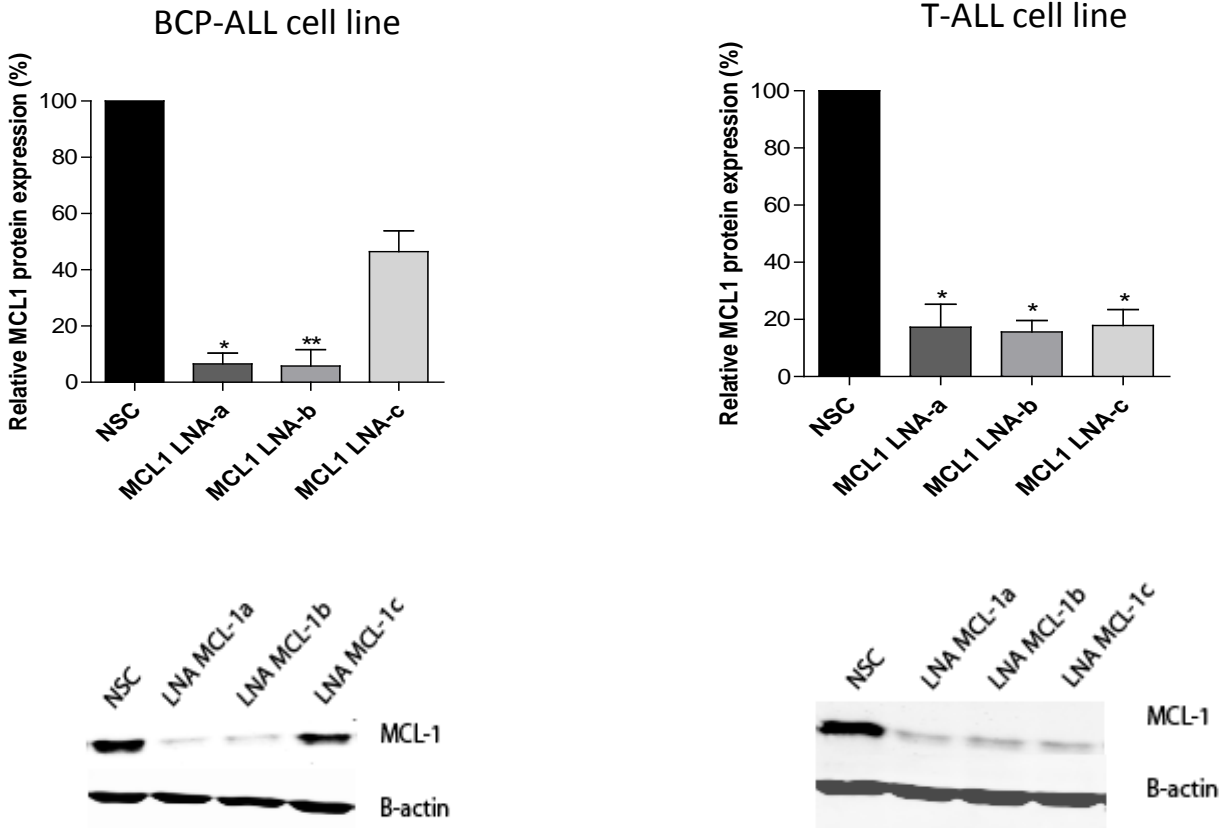
Supplemental Figure 3.



Supplemental Figure 3.

LNA antisense directed against *MCL1* efficiently silence the expression of *MCL1* mRNA. *MCL1* mRNA expression was measured after treatment with three different LNA antisense molecules against *MCL1*, i.e. MCL1 LNA-a, MCL1 LNA-b and MCL1 LNA-c in five distinct leukemic cell lines. Values were adjusted for expression of the housekeeping gene RPS20 and are relative to the non-silencing control (NSC). Data are presented as means plus SEM of three (*ETV6-RUNX1*⁺ BCP-ALL and tetraploid T-ALL) or two independent experiments (*p<0.05, **p<0.01, ***p<0.001).

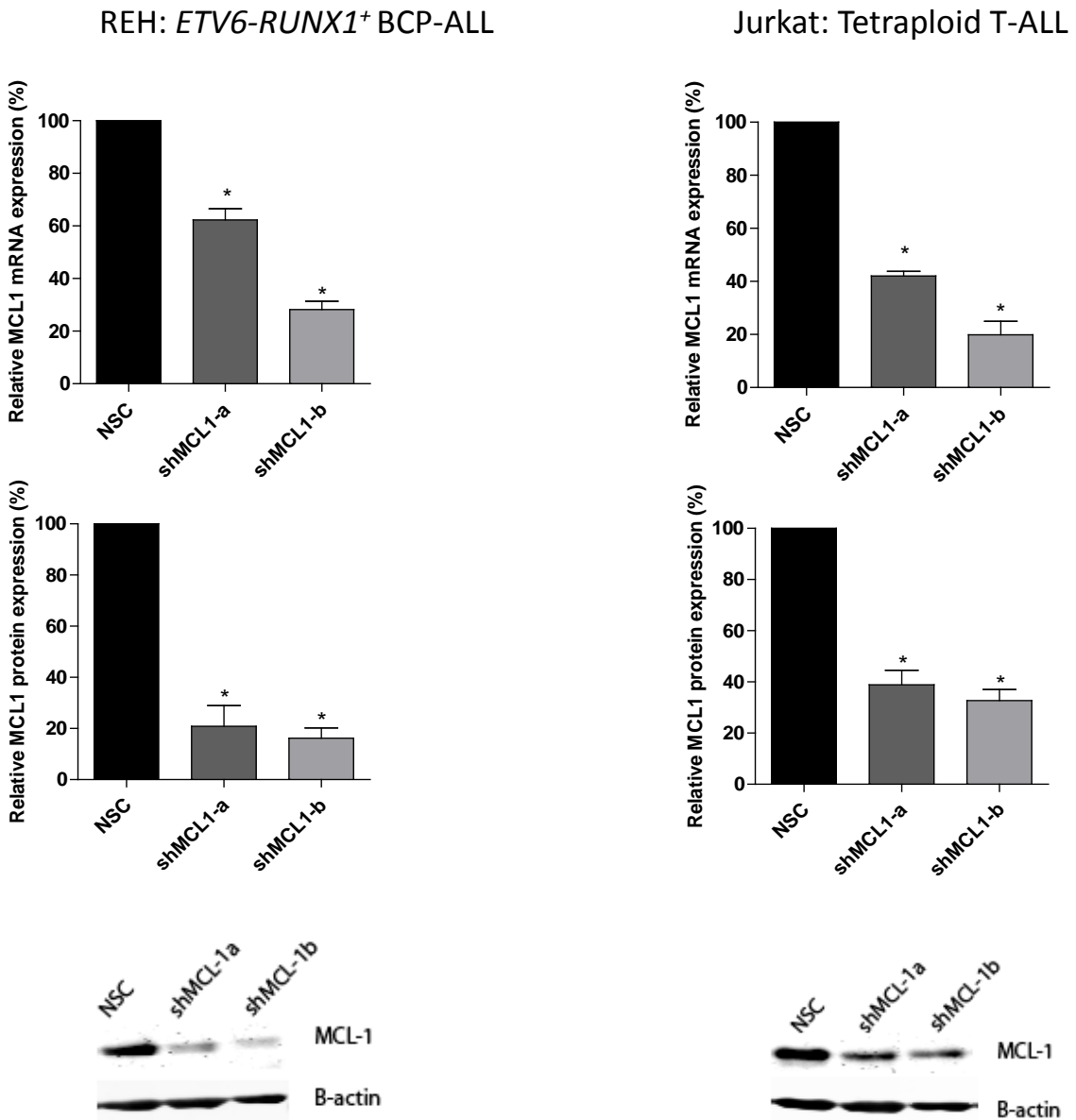
Supplemental Figure 4.



Supplemental Figure 4.

LNA antisense directed against MCL1 efficiently silence the expression of MCL1 protein. Protein expression of MCL1 was assessed at t168 with Western blot after LNA treatment. MCL1 protein expression was calculated with the Odyssey software, corrected for β -actin and is relative to the NSC. A representative Western blot for a BCP-ALL cell line, i.e. REH a *ETV6-RUNX1*⁺ BCP-ALL cell line and a T-ALL, i.e. Jurkat a Tetraploid T-ALL cell line is illustrated. Data are presented as mean plus SEM of three independent experiments (* $p < 0.05$, ** $p < 0.01$).

Supplemental Figure 5.

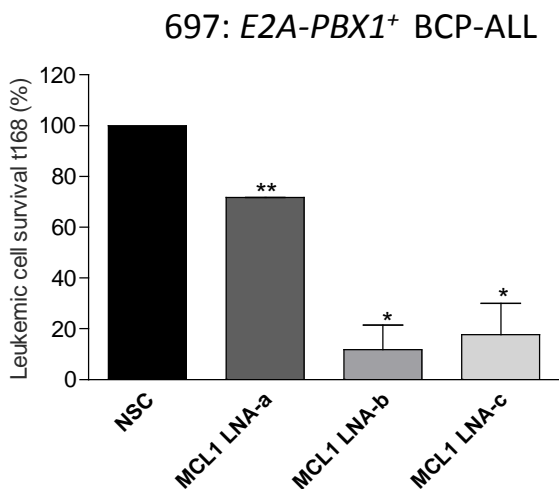
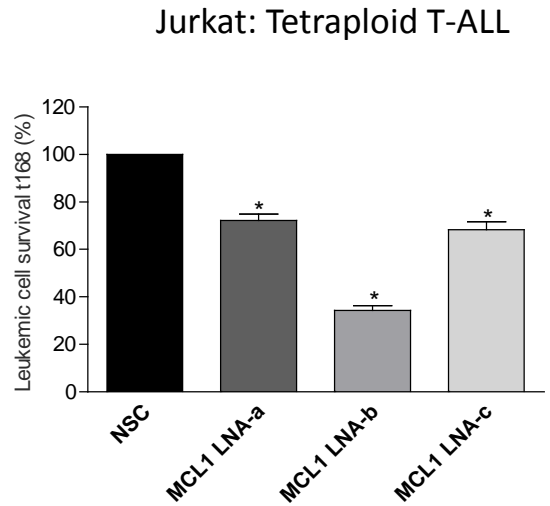
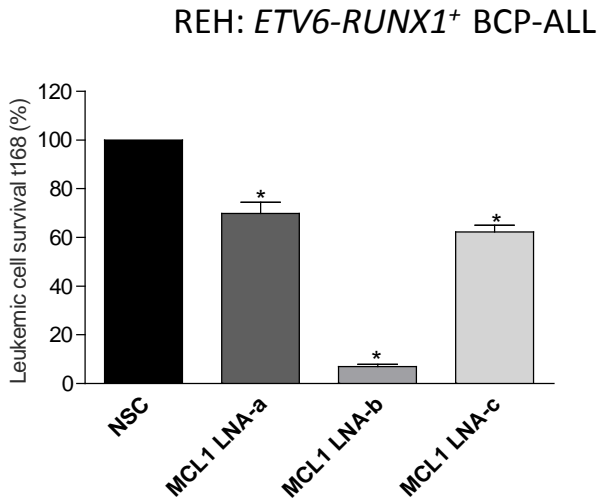
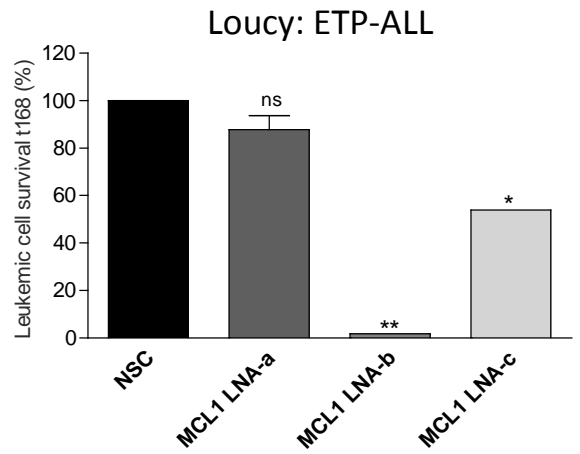
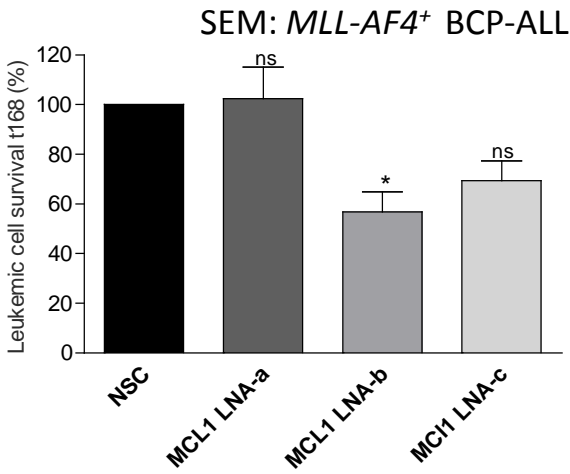


Supplemental Figure 5.

Short hairpins directed against *MCL1* efficiently silence the expression of *MCL1*.

MCL1 mRNA expression in *ETV6-RUNX1*⁺ BCP-ALL cell line and Tetraploid T-ALL cell line was measured after lentiviral knockdown of *MCL1* with two different constructs, i.e. shMCL1-a and shMCL1-b. Values were adjusted for expression of the housekeeping gene RPS20 and are relative to the non-silencing control (NSC). Protein expression of *MCL1* was assessed with Western blot after lentiviral knockdown. *MCL1* protein expression was calculated with the Odyssey software, corrected for β -actin and is relative to the NSC. A representative Western blot is illustrated. Data are presented as mean plus SEM of three independent experiments (* $p < 0.05$, ** $p < 0.01$).

Supplemental Figure 6.

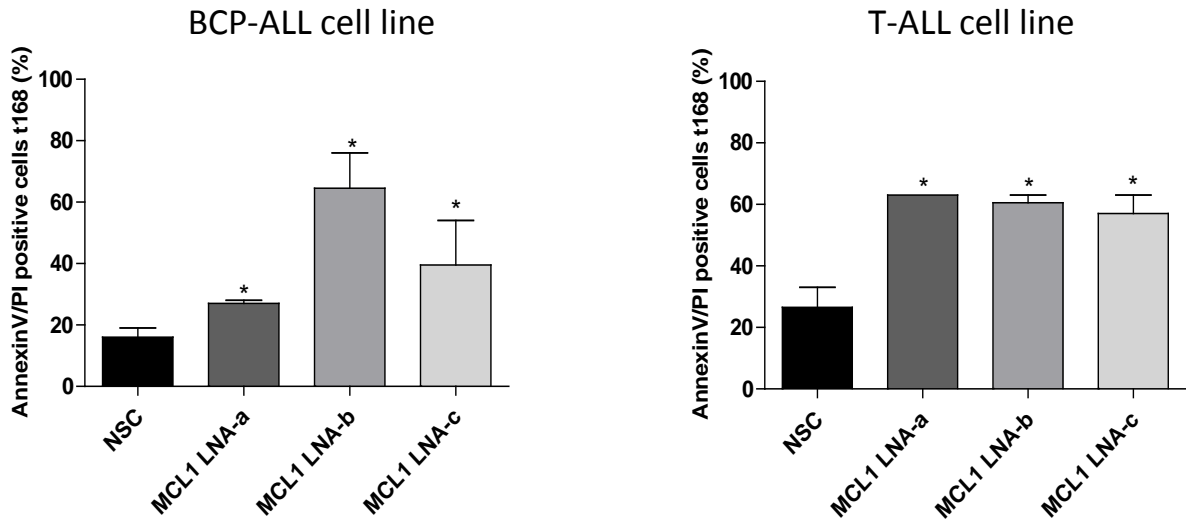


Supplemental Figure 6.

Knockdown of MCL1 by MCL1 LNA inhibits leukemic survival.

The effect of *MCL1* knockdown by MCL1 LNA on cell viability and cell count of five distinct leukemic cell lines was determined with a trypan blue exclusion assay and analyzed with the MACSQuant. Data are presented as mean plus SEM of three (*ETV6-RUNX1*⁺ BCP-ALL and Tetraploid T-ALL) or two independent experiments (* $p < 0.05$, ** $p < 0.01$).

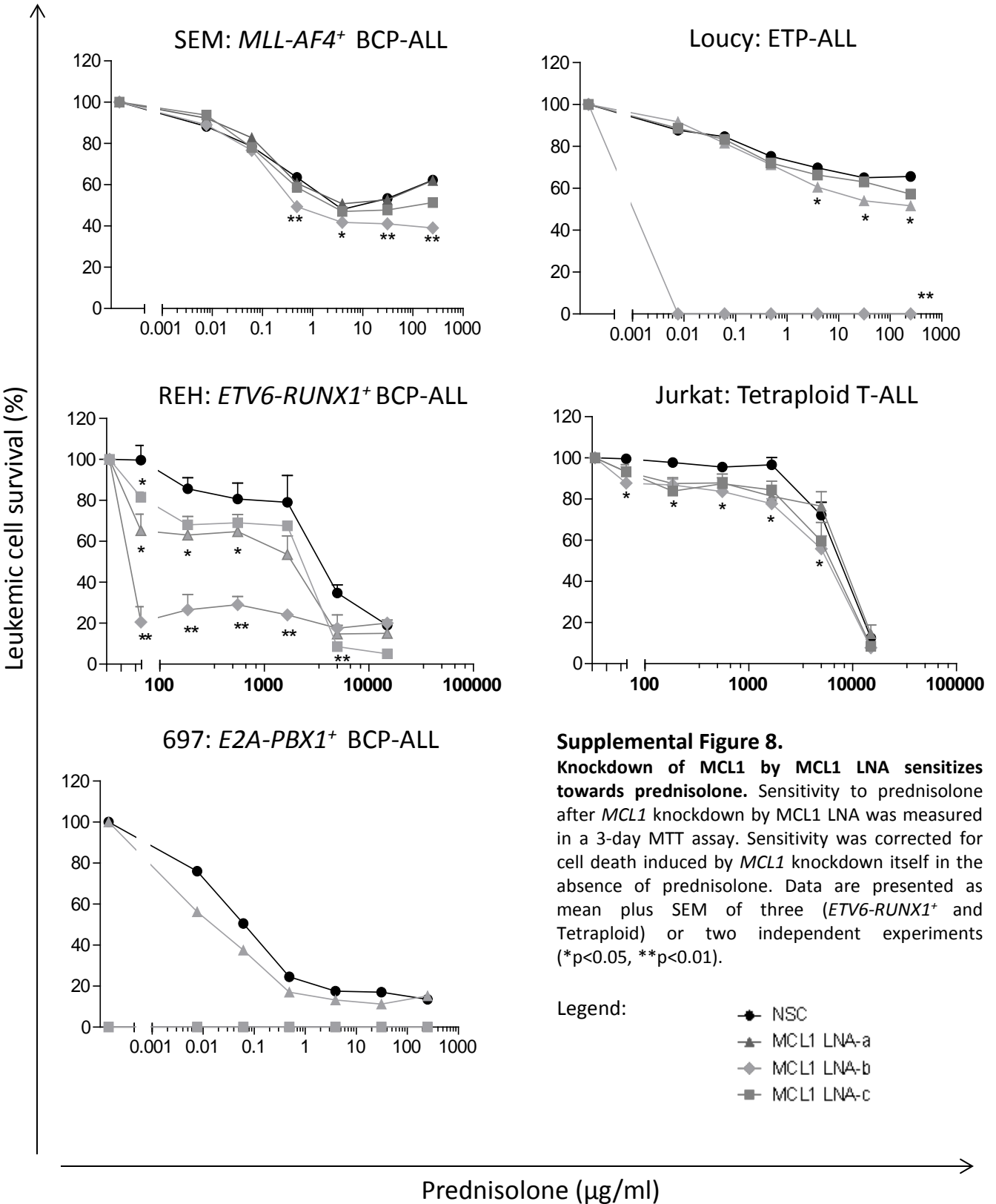
Supplemental Figure 7.



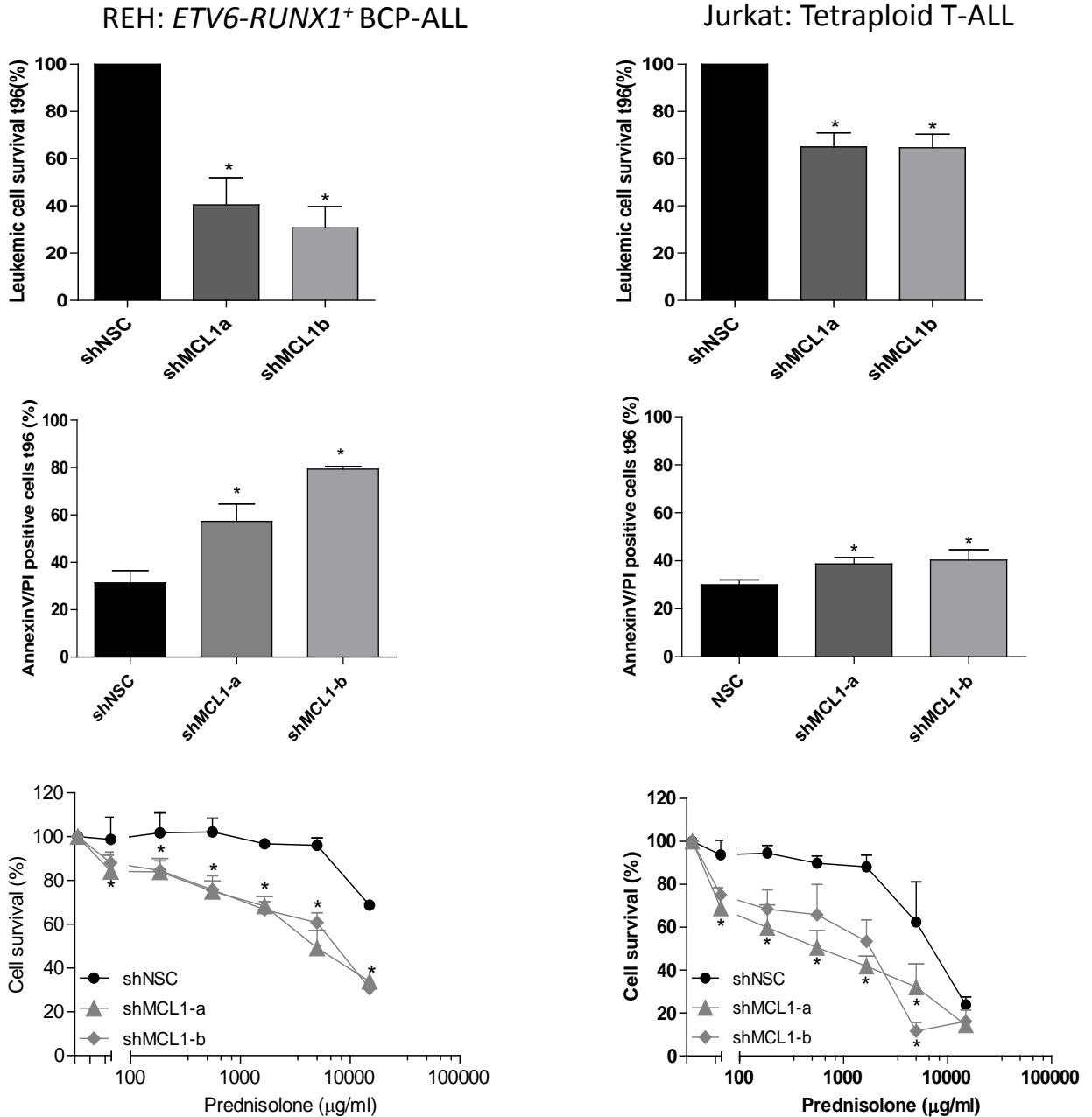
Supplemental Figure 7.

Knockdown of MCL1 by MCL1 LNA increases apoptosis. MCL1 knockdown-induced apoptosis in a representative BCP-ALL cell line, i.e. Reh an *ETV6-RUNX1*⁺ BCP-ALL cell line and a T-ALL cell line, i.e. Jurkat a Tetraploid T-ALL, was assessed on a flowcytometer using an AnnexinV/PI staining. The percentage of apoptotic cells was calculated using the quadrant method. Data are presented as means plus SEM of three independent experiments (* $p < 0.05$).

Supplemental Figure 8.



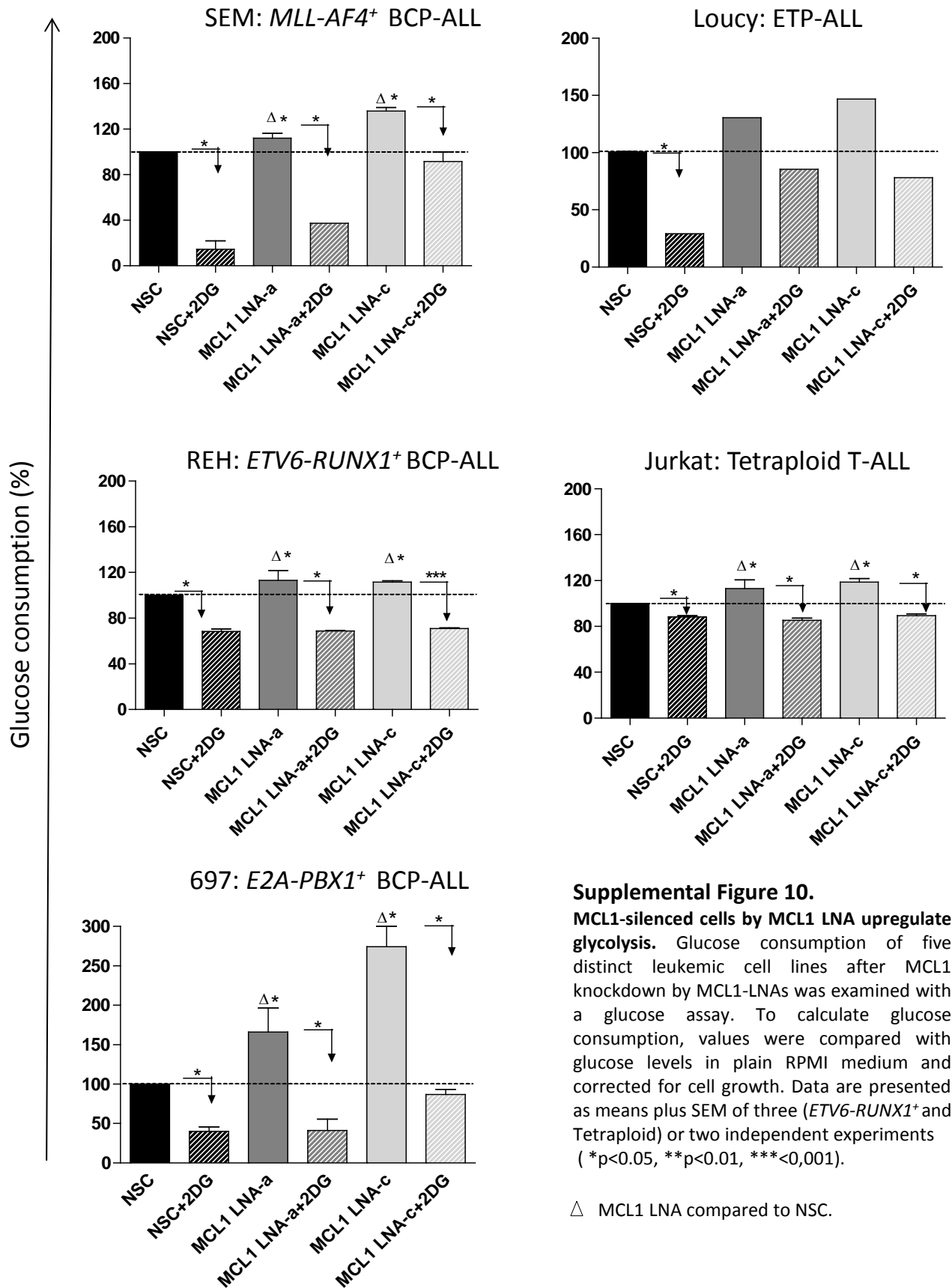
Supplemental Figure 9.

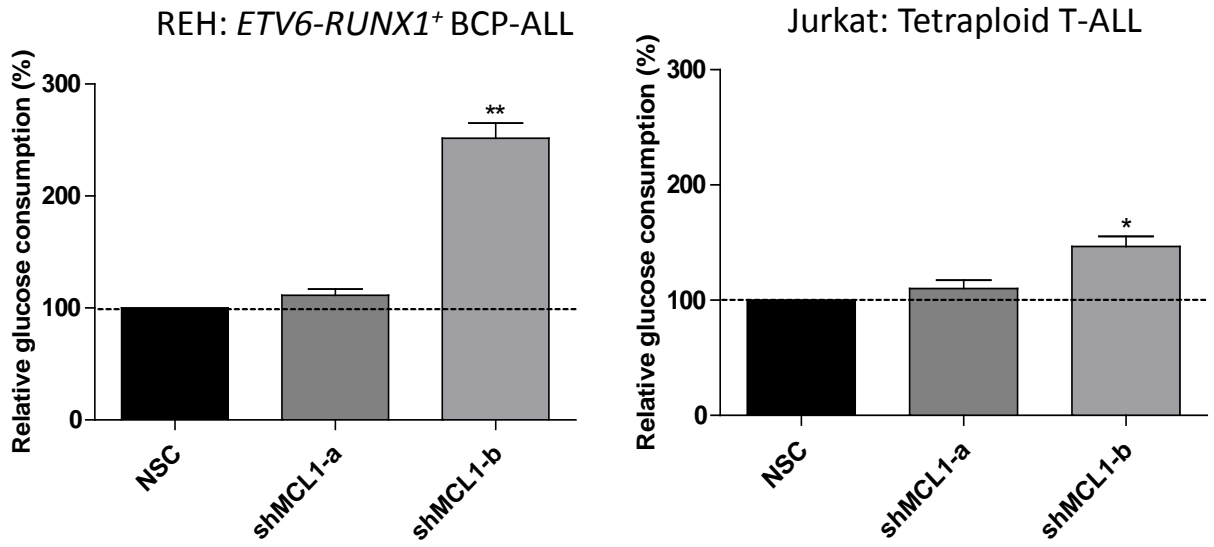


Supplemental Figure 9.

Knockdown of MCL1 by shMCL1 inhibits leukemic survival, increases apoptosis and sensitizes towards prednisolone. The effect of MCL1 knockdown by shMCL1 on cell viability and cell count of *ETV6-RUNX1*⁺ BCP-ALL cells and T-ALL cells was determined with a trypan blue exclusion assay and analyzed with the MACSQuant. *MCL1* knockdown-induced apoptosis in a BCP-ALL and T-ALL cell line was assessed on a flowcytometer using an AnnexinV/PI staining. The percentage of apoptotic cells was calculated using the quadrant method. Sensitivity to prednisolone after *MCL1* knockdown was measured in a 3-day MTT assay. Sensitivity was corrected for cell death induced by *MCL1* knockdown itself in the absence of prednisolone. Data are presented as mean plus SEM of three independent experiments (* $p < 0.05$, ** $p < 0.01$).

Supplemental Figure 10.



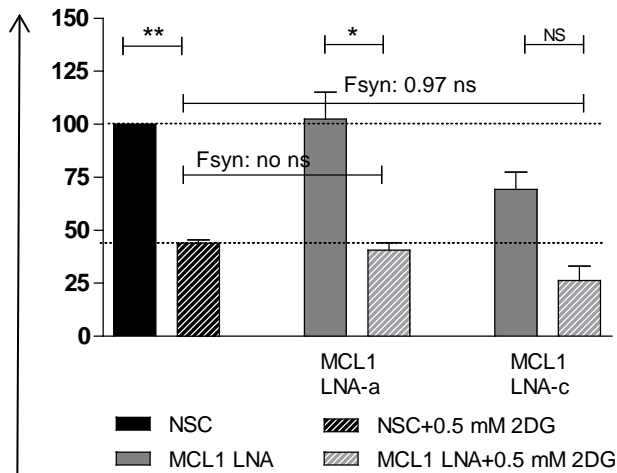


Supplemental Figure 11.

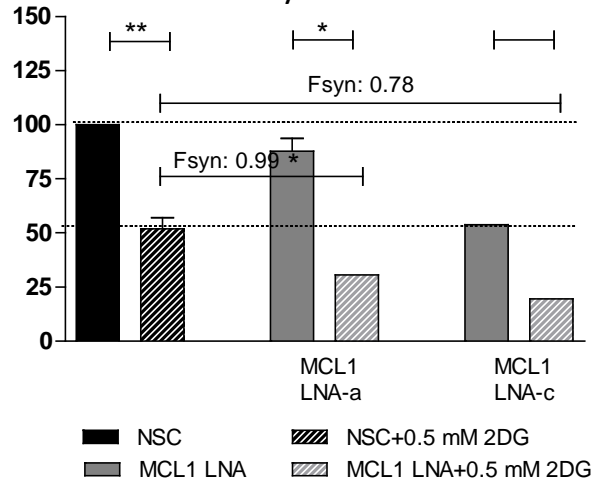
MCL1-silenced cells by shMCL1 upregulate glycolysis. Glucose consumption of a MCL1-silenced *ETV6-RUNX1+* BCP-ALL cell line and a tetraploid T-ALL cell line by shMCL1 was examined with a glucose assay. To calculate glucose consumption, values were compared with glucose levels in plain RPMI medium and corrected for cell growth. Data are presented as means plus SEM of three independent experiments (* $p < 0.05$, ** $p < 0.01$). Off note, shMCL1-a is the only construct that targets exon 2 of MCL1, in contrast to shMCL1-b, LNA-a, LNA-b and LNA-c which are directed against exon 3. It may be that targeting exon 2 containing MCL1 transcripts does not affect glycolysis and/or that interference with exon 3 is more important for a functional effect on glycolysis.

Supplemental Figure 12.

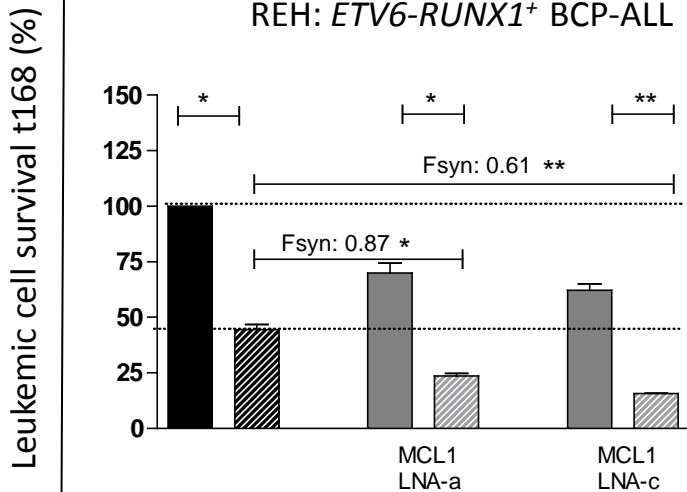
SEM: *MLL-AF4*⁺ BCP-ALL



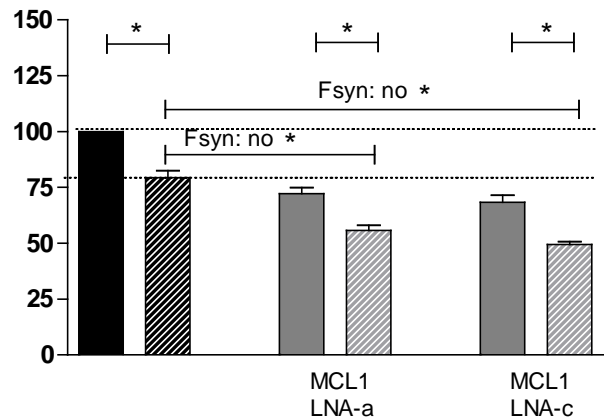
Loucy: ETP-ALL



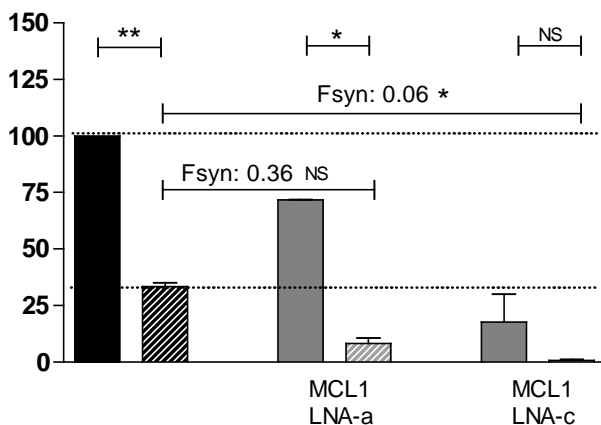
REH: *ETV6-RUNX1*⁺ BCP-ALL



Jurkat: Tetraploid T-ALL



697: *E2A-PBX1*⁺ BCP-ALL



Supplemental Figure 12.

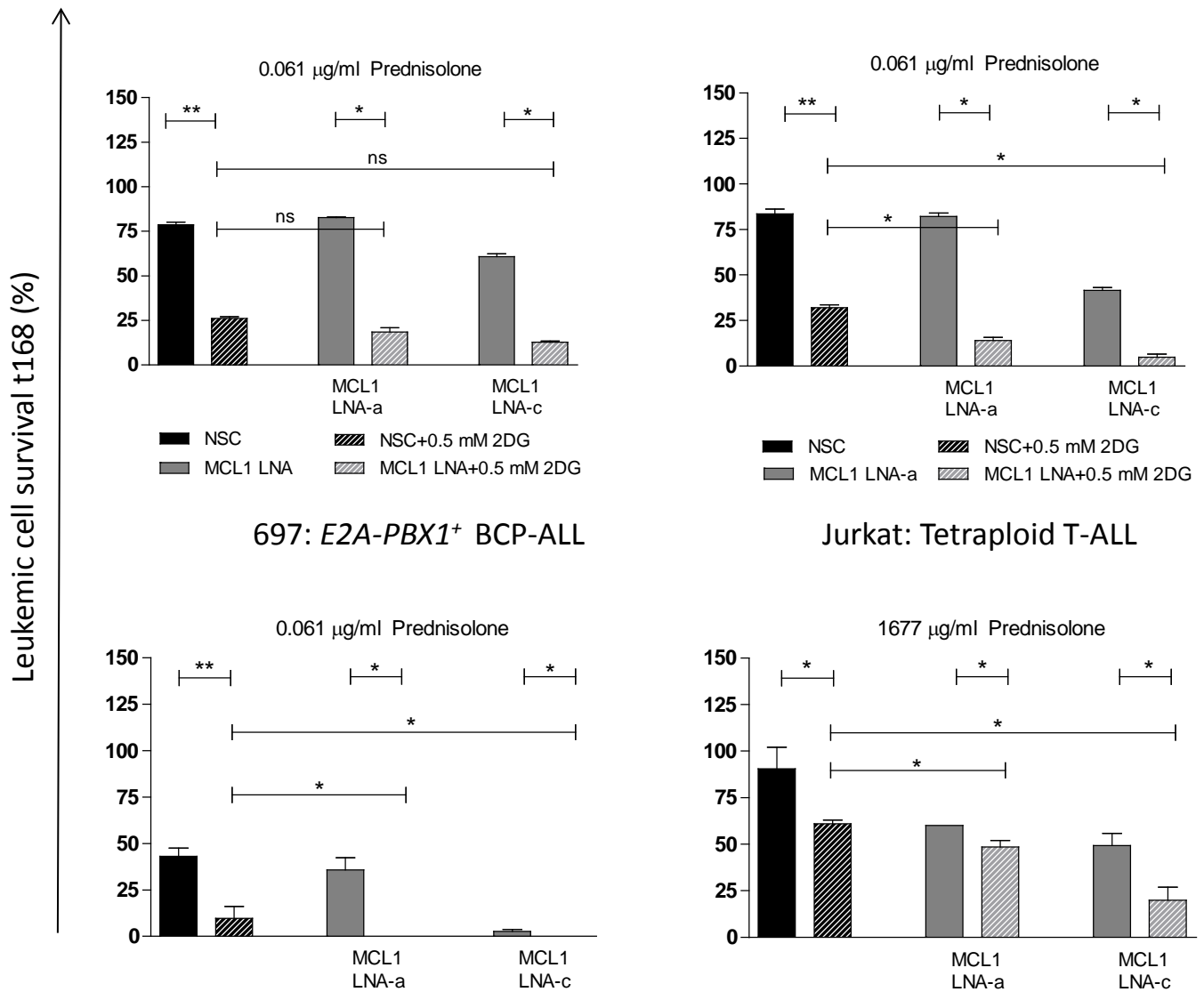
MCL1 silencing and glycolysis inhibition synergistically inhibits leukemic cell survival.

Leukemic cell survival of five distinct leukemic cell lines after treatment with either MCL1 LNA alone or in combination with 0.5mM 2-DG was determined with a trypan blue exclusion assay and analyzed with the MACSQuant. Data are presented as means plus SEM of three (*ETV6-RUNX1*⁺ and Tetraploid) or two independent experiments (*p<0.05, ** p<0.01). Fsyn represents the synergy factor, where Fsyn<1 is synergistic.

Supplemental Figure 13.

SEM: *MLL-AF4*⁺ BCP-ALL

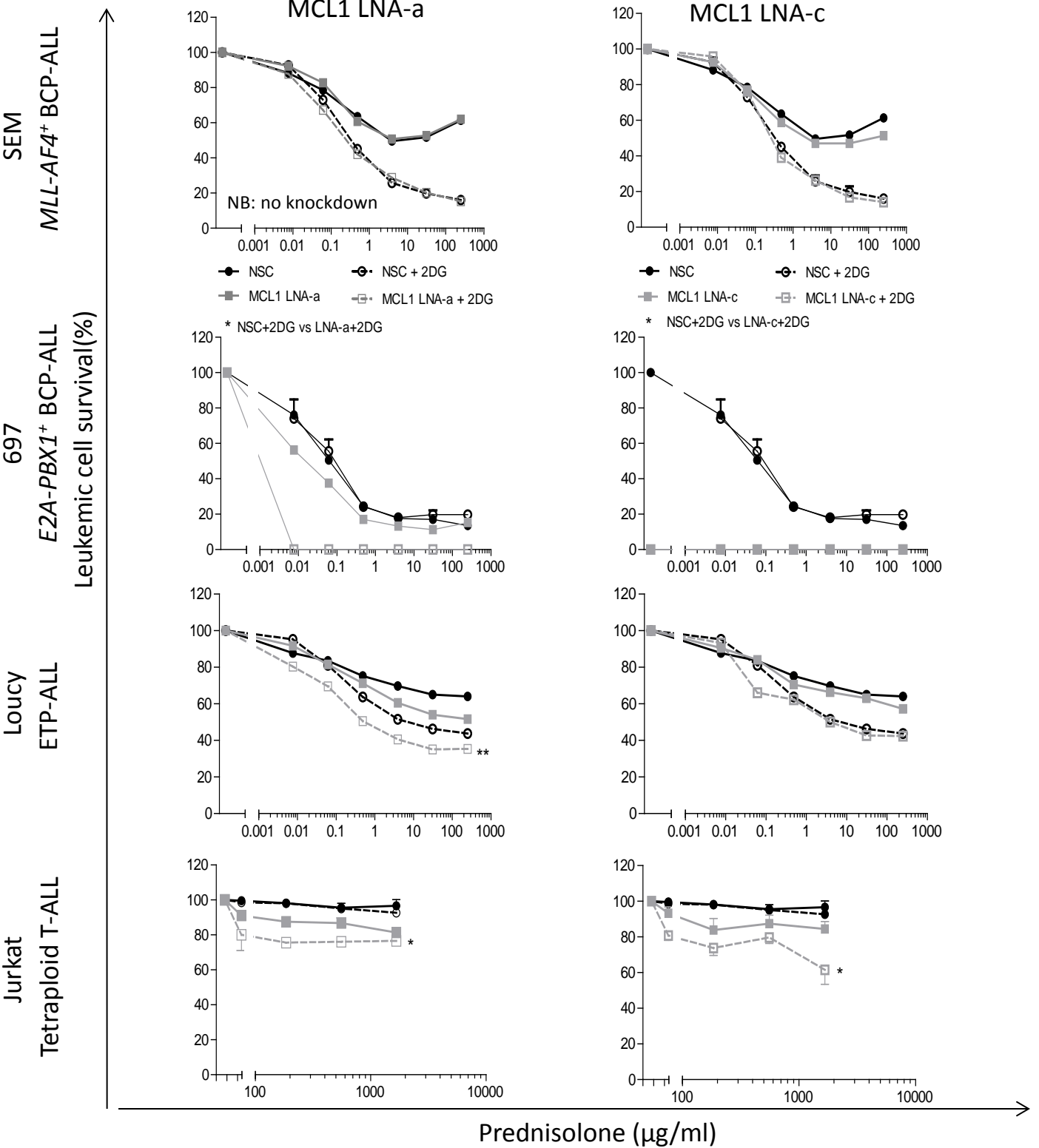
Loucy: ETP-ALL



Supplemental Figure 13.

MCL1 knockdown, glycolysis inhibition and prednisolone treatment concomitantly inhibit leukemic cell survival. Leukemic cell survival after 3-day, i.e. from t96 until t168, prednisolone exposure of *MLL-AF4*⁺ BCP-ALL, *E2A-PBX1*⁺ BCP-ALL, ETP-ALL and tetraploid T-ALL cells with either MCL1 LNA-b or 0.5mM 2-DG alone or in combination. Data were compared to NSC control without prednisolone, to visualize the total effect on cell survival of prednisolone, MCL LNA and 2-DG together. Data are presented as means plus SEM of three (*ETV6-RUNX1*⁺ and Tetraploid) or two independent experiments (* $p < 0.05$, ** $p < 0.01$).

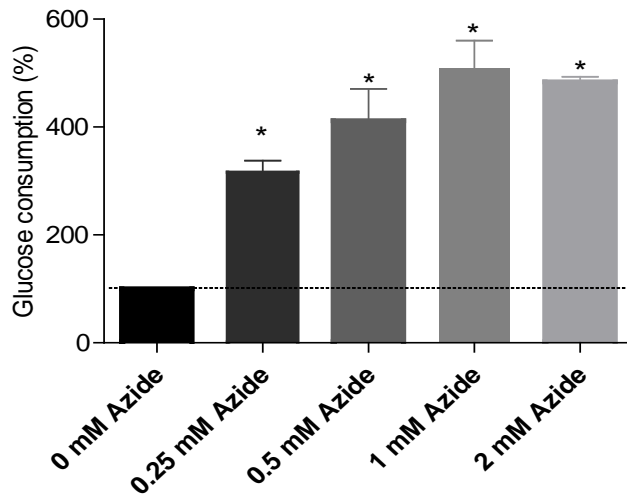
Supplemental Figure 14.



Supplemental Figure 14.

MCL1 silencing together with glycolysis inhibition concomitantly reverses drug resistance. Sensitivity of distinct leukemic cell lines to prednisolone after treatment with mock LNA control, MCL1 LNA –a and LNA-b and 0.5mM 2-DG alone or in combination was measured in a 3-day MTT assay. Sensitivity was corrected for cell death induced by *MCL1* knockdown and 0.5mM 2-DG itself in the absence of prednisolone. Data are presented as means plus SEM of three or two independent experiments (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Supplemental Figure 15.



Supplemental Figure 15.

Inhibition of oxidative phosphorylation by Azide augments glycolysis.

Glucose consumption was calculated after treatment with a concentration range of Azide, an inhibitor of oxidative phosphorylation, relative to untreated *ETV6-RUNX1*⁺ BCP-ALL cells (REH), and corrected for cell growth. Data are presented as means plus SEM of two independent experiments (* $p < 0.05$).