EVI1 triggers metabolic reprogramming associated with leukemogenesis and increases sensitivity to L-asparaginase

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Supplementary Appendix

Methods

Cell lines

UCSD/AML1 and Kasumi-3 cells were cultured in RPMI1640 supplemented with 10% fetal calf serum (FCS) and 1 ng/mL human granulocyte-macrophage colony-stimulating factor (hGM-CSF). Kasumi-1, Nomo-1, NB4, THP-1 and MOLM1 cells were cultured in RPMI1640 supplemented with 10% FCS. MOLM13 and MOLM14 were a kind gift from Dr. Yusuke Furukawa (Jichi Medical University, Japan). Kasumi-1, Nomo-1 and THP-1 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB). Detailed information about these cell lines was published previously. [1,2]

Flow cytometry

BM cells were flushed from long bones (tibias and femurs) using Hank's buffered salt solution lacking calcium and magnesium but supplemented with 2% heat-inactivated bovine serum. To isolate lineage^{low}Sca-1^{-}c-kit^{+}CD16/32^{+}CD34^{+}, Leukemic

granulocyte/macrophage progenitor (L-GMP) cells, BM cells were incubated with eFluor660-conjugated CD34 (RAM34, eBioscience, San Diego, CA), PE-Cy7-conjugated anti-CD16/32 (93, BioLegend, San Diego, CA), APC/Cy7-conjugated c-kit (2B8, BioLegend), or PE-conjugated Sca-1 (D7, BioLegend), in addition to PE-conjugated antibodies specific for the following lineage markers: Ter119 (TER-119), B220 (RA3-6B2), Gr1 (RB6-8C5), CD2 (RM2-5), CD3 (145-2C11), CD8 (53-6.7), and CD127 (A7R34) (all from TONBO Bioscience). To measure levels of reactive oxygen species (ROS), the L-GMP stain was modified to enable the APC or PE channel to detect the MitoTracker Deep Red FM (Life Technologies, Carlsbad, CA) or CellROX Deep Red Reagent (Life Technologies) or MitoSOX Red Mitochondrial Superoxide Indicator dye (Life Technologies). After antibody staining, cells were incubated with MitoTracker Deep Red FM or CellROX Deep Red or MitoSOX Red for 15 min at 37°C prior to flow cytometry. Flow cytometry and cell sorting were performed using a JSAN cell sorter (Bay bioscience, Kobe, Japan). Data were analyzed using FlowJo software (TreeStar).

Colony forming assay and histology

Leukemia cells were plated in MethoCult M3234 (StemCell Technologies, Vancouver, Canada) medium supplemented with 20 ng/ml SCF, 10 ng/ml GM-CSF, 10 ng/ml IL-3, and 10 ng/ml IL-6, according to the manufacturer's instructions, and used for colony forming assays.

Quantitative real-time PCR

GFP^+ AML cells and L-GMPs were sorted into TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH, USA), and RNA was isolated according to the manufacturer's instructions.[3] cDNA was synthesized using random primers and SuperScript VILO (Thermo Fisher Scientific, CA, USA). Quantitative PCR was performed using Step One Plus (Thermo Fisher Scientific, CA, USA). The primers are listed in Online Supplementary Table S1.

Microarray analyses

GFP^+ and L-GMP cells were sorted into TRI REAGENT and RNA purified according to the manufacturer's instructions. Hybridization of cRNA and scanning of the probe arrays

was performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA) using Affymetrix Mouse Gene 2.0 ST Arrays (Affymetrix). The microarray data have been deposited in the Gene Expression Omnibus (GEO) (GSE118096). The normalized expression value for each array was calculated using GeneSpring software.

Lentiviral transduction of AML cells with shRNA

pLKO.1 puro was a gift from Bob Weinberg (Addgene plasmid # 8453). Control shRNA (scramble) was a gift from David Sabatini (Addgene plasmid # 1864). The sequences of the shRNA oligonucleotides are provided in the Supplementary Table S1. Plasmids pMD2.G-VSV-G and psPAX2-Gag-Pol were purchased from Addgene. On Day 2 post-transduction, cells were selected by exposure to puromycin (1 μg/ml; Sigma-Aldrich) for 4 days. Knock-down efficiency was analyzed by quantitative PCR analysis to detect Evi1, Idh1, Idh2 and Asns on Day 4.

Measurement of extracellular acidification rate (ECAR)

ECAR was measured using an XFp extracellular flux analyzer (Agilent Technologies).

Leukemia cells were suspended in XF Assay Medium supplemented with 2 mM glutamine. The Glycolysis Stress Test was performed as follows: (1) glucose was injected to measure glycolysis; (2) oligomycin (1 μM) was added to measure glycolytic capacity and glycolytic reserve; and (3) 2-DG was applied to measure non-glycolytic acidification.

AML xenograft tumors

Female NOG mice (8 weeks old) received a single subcutaneous injection of UCSD/AML1 cells (5×10^6) suspended in 100 μl phosphate-buffered saline and mixed with an equal volume of Matrigel (BD Matrigel, BD Biosciences, Bedford, MA, USA). Animals were treated daily with L-asp (Leunase, Kyowa Kirin, Japan; 1,000 U/kg, intraperitoneally) or vehicle. Tumor growth was measured three times per week using a caliper. Tumor volume was calculated using the modified ellipsoid formula: 1/2 (length × width^2). All experiments were approved by the University of Miyazaki Animal Care and Use Committee.

Metabolome analyses

Metabolites were analyzed by capillary electrophoresis time-of-flight mass spectrometry (CE-MS), as previously described. [4]

Supplementary figure legends

Supplementary Figure S1. *LSK cells*-specific mEVI1-expressing *transgenic mice.*

(A) Schematic representation showing the structure and production of the murine EVI1 transgene. mEvi1 cDNA was inserted into the *EcoR1*-subcloning site of the eR1-mhsp68p vector. (B) The numbers of white blood cells, red blood cells, and platelets in peripheral blood from Evi1-TG mice were not significantly different from those in WT mice. (C) EVI1-TG had no effect on HSCs (hematopoietic stem cells), MPPs (multipotent progenitors), GMPs (granulocyte/macrophage progenitors), MEPs (megakaryocyte/erythroid progenitors), or lineage cells in mice aged 8 to 12 weeks.

Supplementary Figure S2.

Heat map showing genes encoding mitochondrial-related factors differentially expressed by WT/MF9 or Evi1/MF9 leukemia cells (n=3).

Supplementary Figure S3.

Expression of several enzymes involved in the glycolytic pathways (as determined by qRT-PCR). Expression levels were normalized to that of *β-actin* and to that of WT/MF9 whole AML cells (n=4).

Supplementary Figure S4.

(A) The maximal respiratory capacity of cells transfected with shEvi1 or treated with EPZ004777 was significantly lower than that of control cells. (B) Evi1/MF9-shEvi1 cells were more resistant to glutamine starvation than Evi1/MF9-shScr cells. EPZ004777 had no effect on glucose and glutamine dependency. (C) Expression of Glut-1, Idh1 and Idh2 operating in the glycolytic pathways and TCA cycle, as measured by qRT-PCR. Expression of Idh2 by shEvi1 or treated with EPZ004777 cells was significantly lower than that of control cells. (D) The maximal respiratory capacity of cells transfected with shIdh2 was lower than that of cells transfected with shScr or shIdh1. *, $p<0.05$; **, $p<0.005$; and ***, $p<0.0005$ (Student's t test).

Supplementary Figure S5.

(A) Mac-1, Gr-1, CD3, and B220 cells within the WT/MF9 and Evi1/MF9 leukemia cell populations were detected by flow cytometry. (B) L-asp does not suppress mitochondrial oxidation in WT progenitor cells. L-asp suppressed basal and maximum OCR in the mitochondria of Evi1-TG normal progenitor cells. (C) Analysis of normal progenitor cells revealed that there was no difference in the IC_{50} of L-asp between WT and Evi1-TG mice. (D) The effects of high glutamine concentrations on the OCR. There was no change in the OCR of WT/MF9 cells in the presence of different glutamine concentrations, whereas high glutamine concentrations attenuated the OCR-suppressing effect of Evi1/MF9 cells.

Supplementary Figure S6.

(A) Schematic outline of the WT/MF9 mouse model treated with L-asp. Treatment began 5 days post-transplantation. (B) L-asp treatment did not reduce the number of WT/MF9 cells in the peripheral blood (n=5). (C) L-asp treatment did not extend the survival of recipient mice significantly (n=5).

Supplementary Figure S7.

WT/MF9 shAsns cells were more sensitive to L-asp than WT/MF9 shScr cells.

Supplementary Figure S8.

(A) Expression of IDH2, ASNS, and SLC1A5 (as measured by qRT-PCR) in AML cell lines. Expression was normalized to that of *β-actin* and to that in MOLM13 cells.

(B) Expression of ASNS by $EVI1^+$ primary AML cells was significantly lower than that by $EVI1^-$ cells. *, $p<0.05$; **, $p<0.005$; and ***, $p<0.0005$ (Student's t test).

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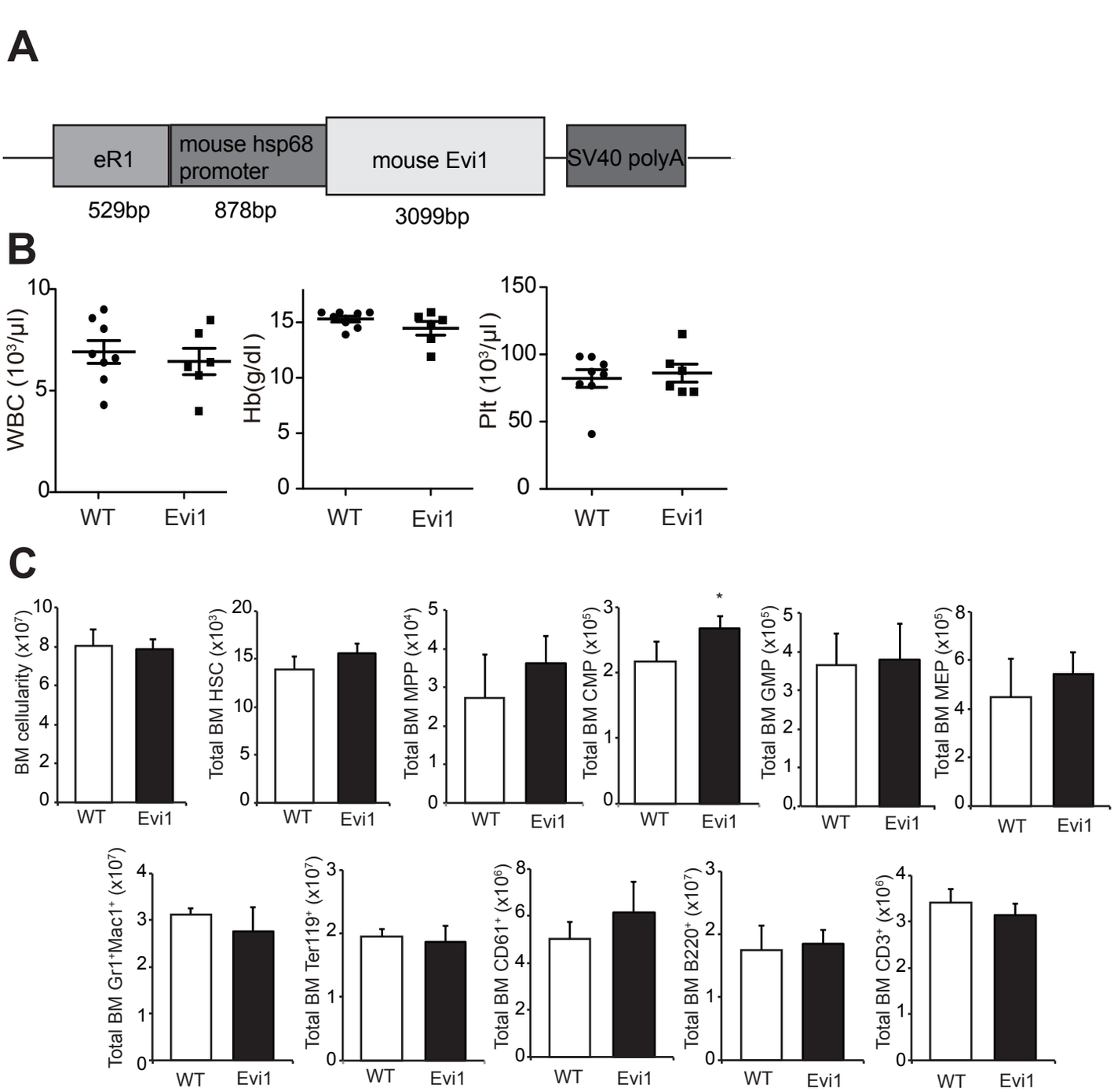
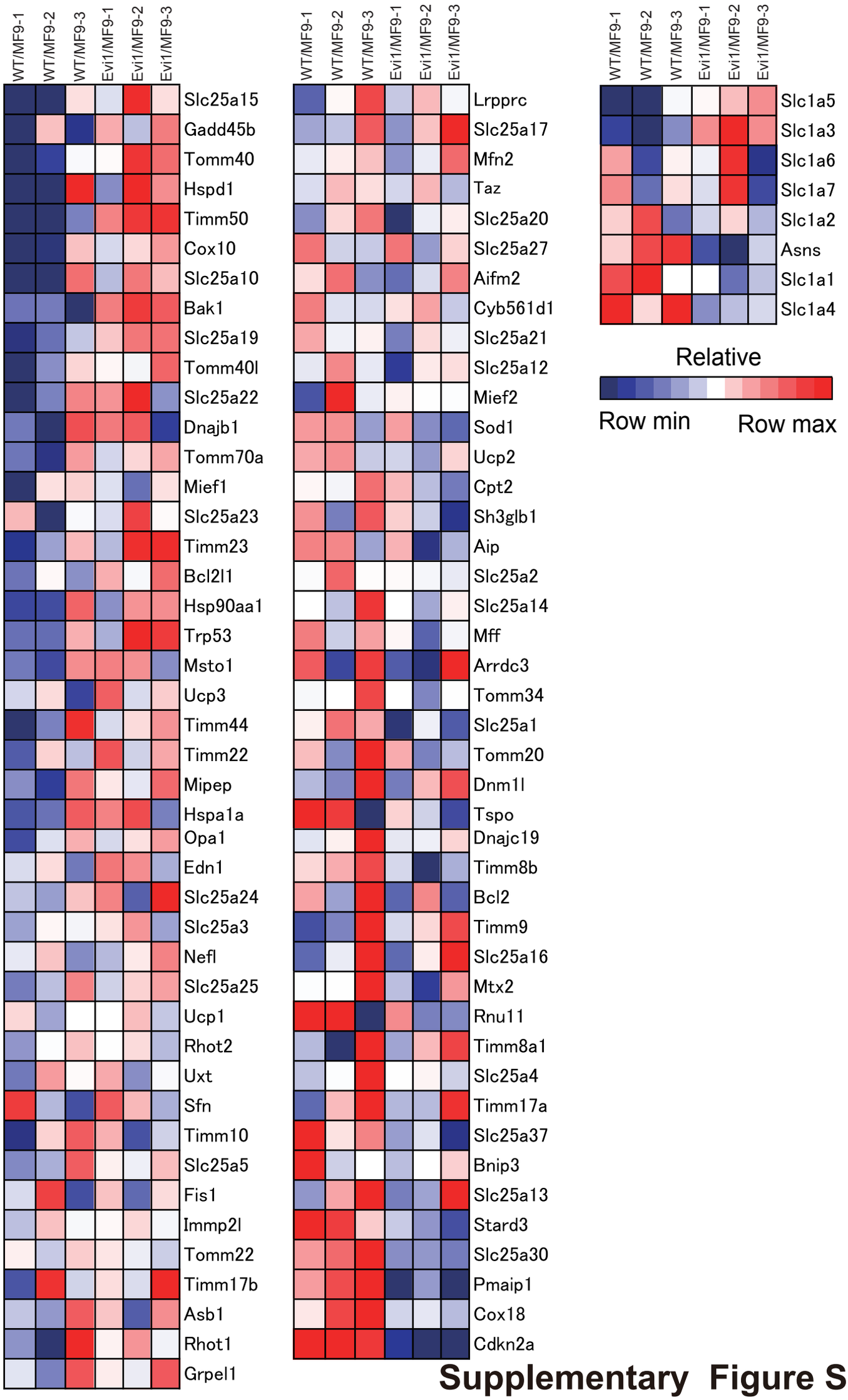


Supplementary Figure S1



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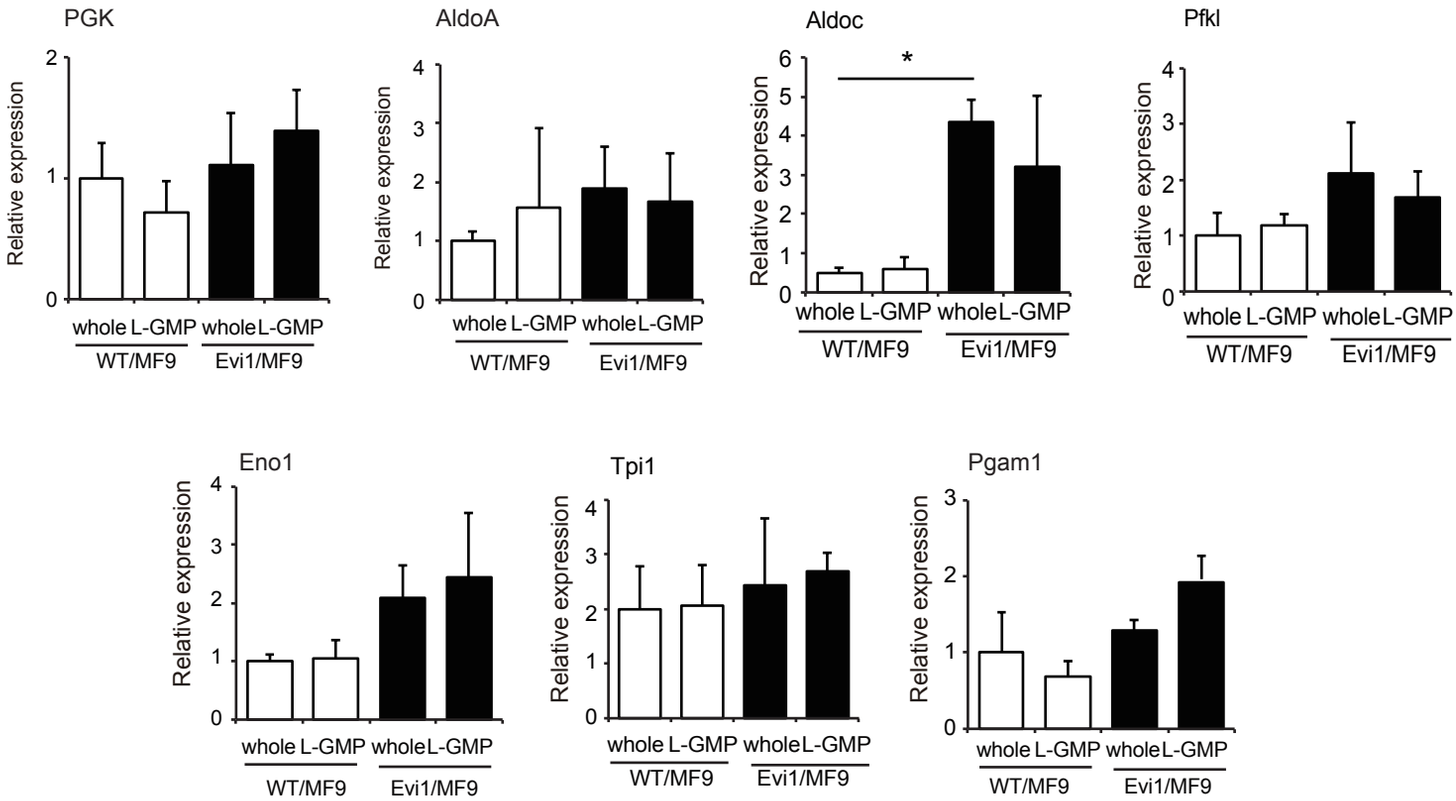

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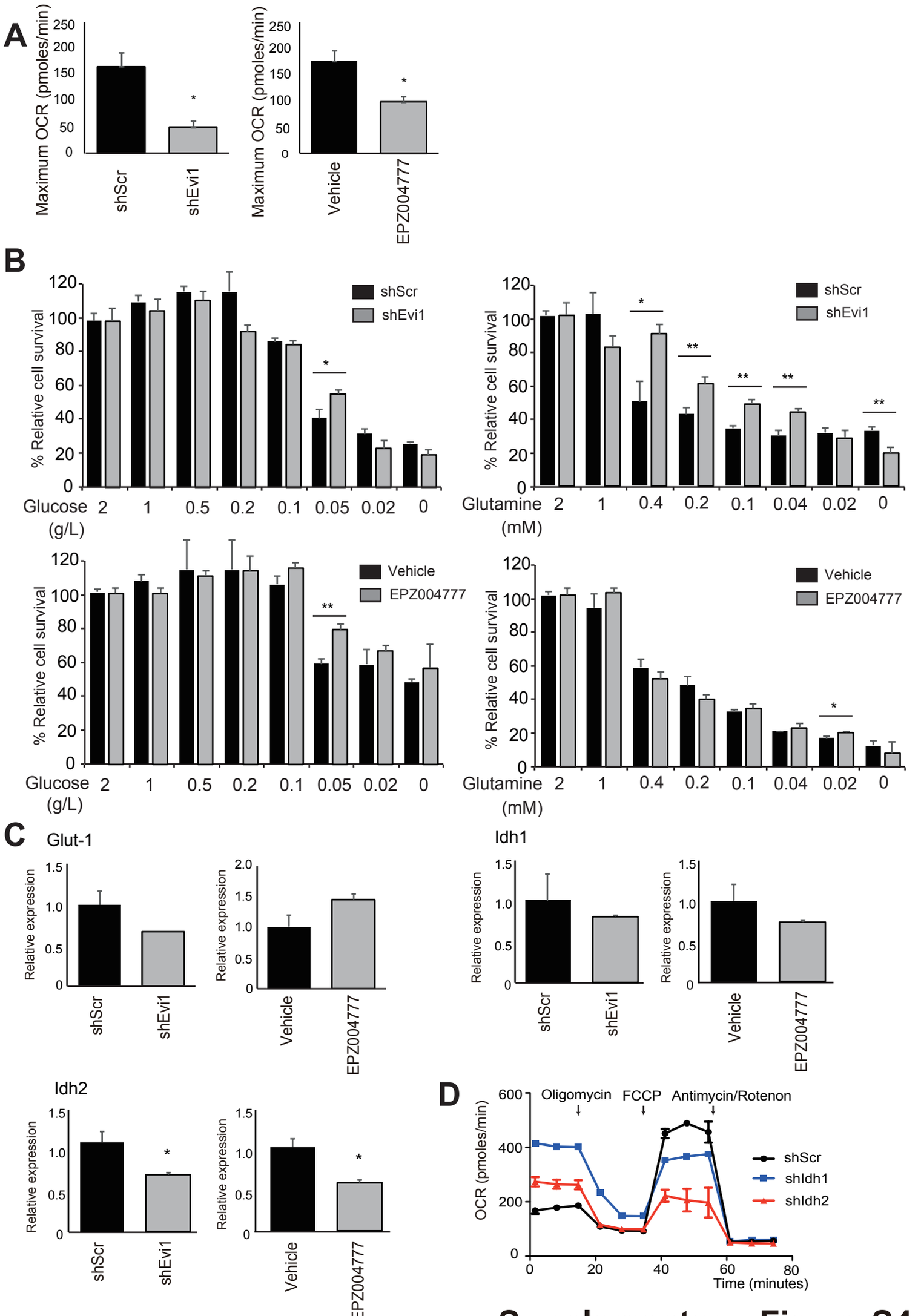

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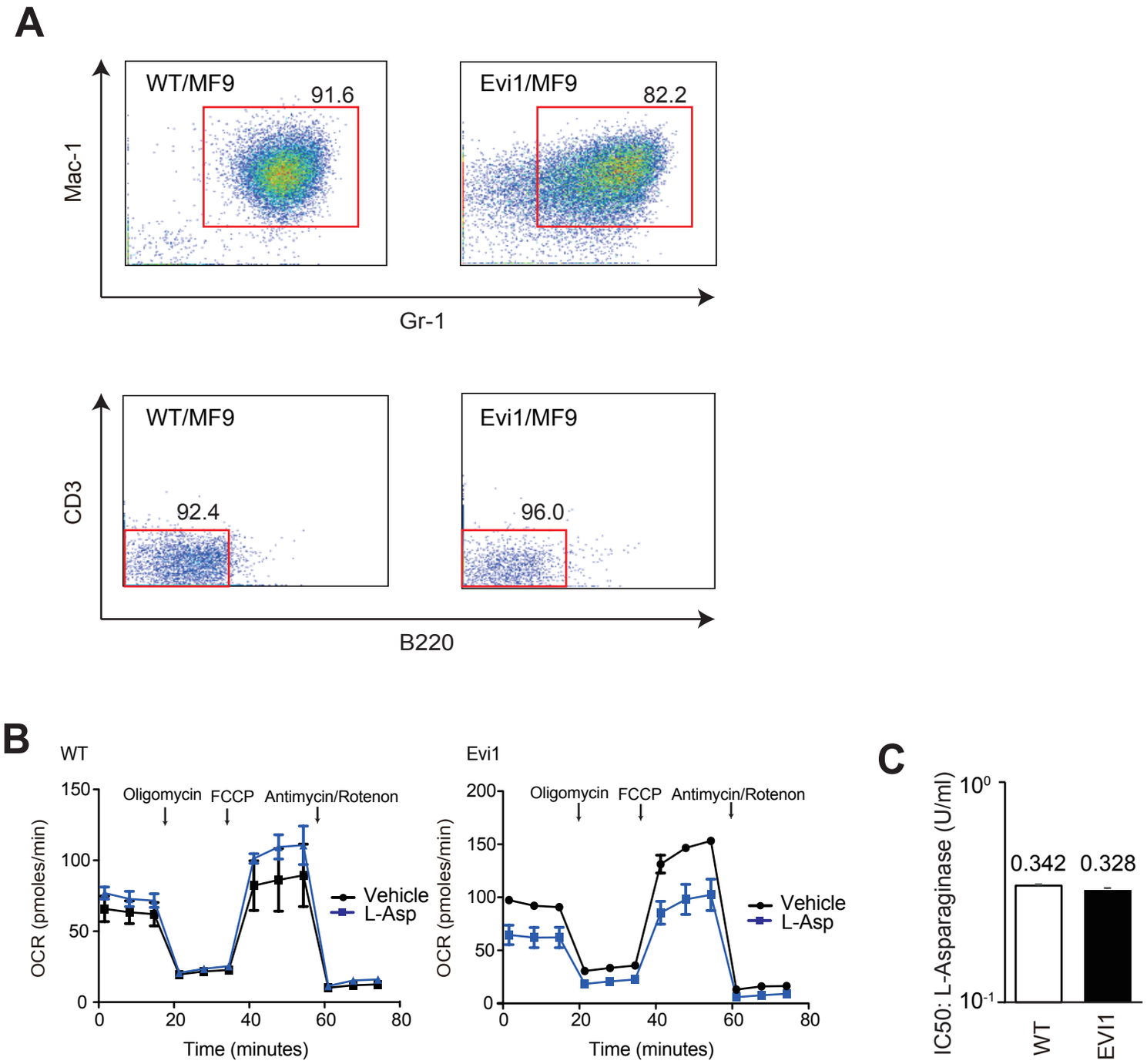

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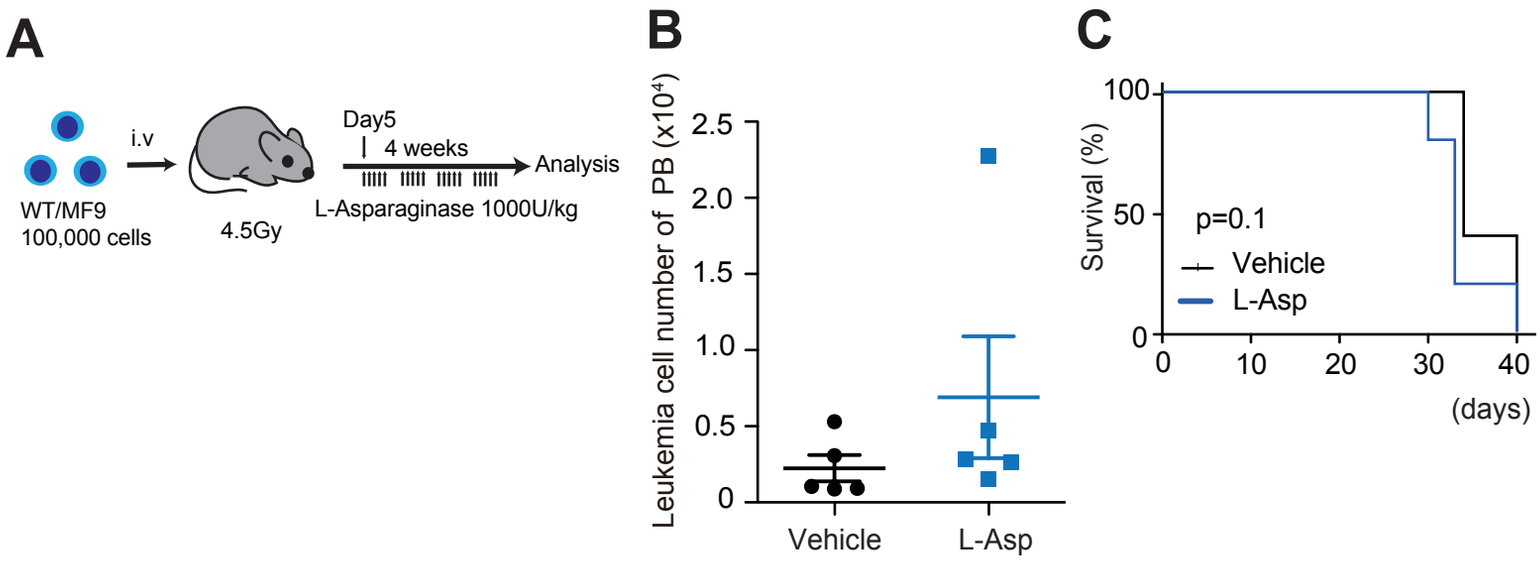

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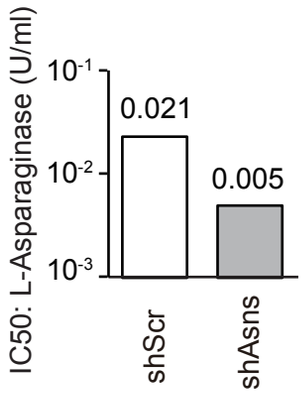

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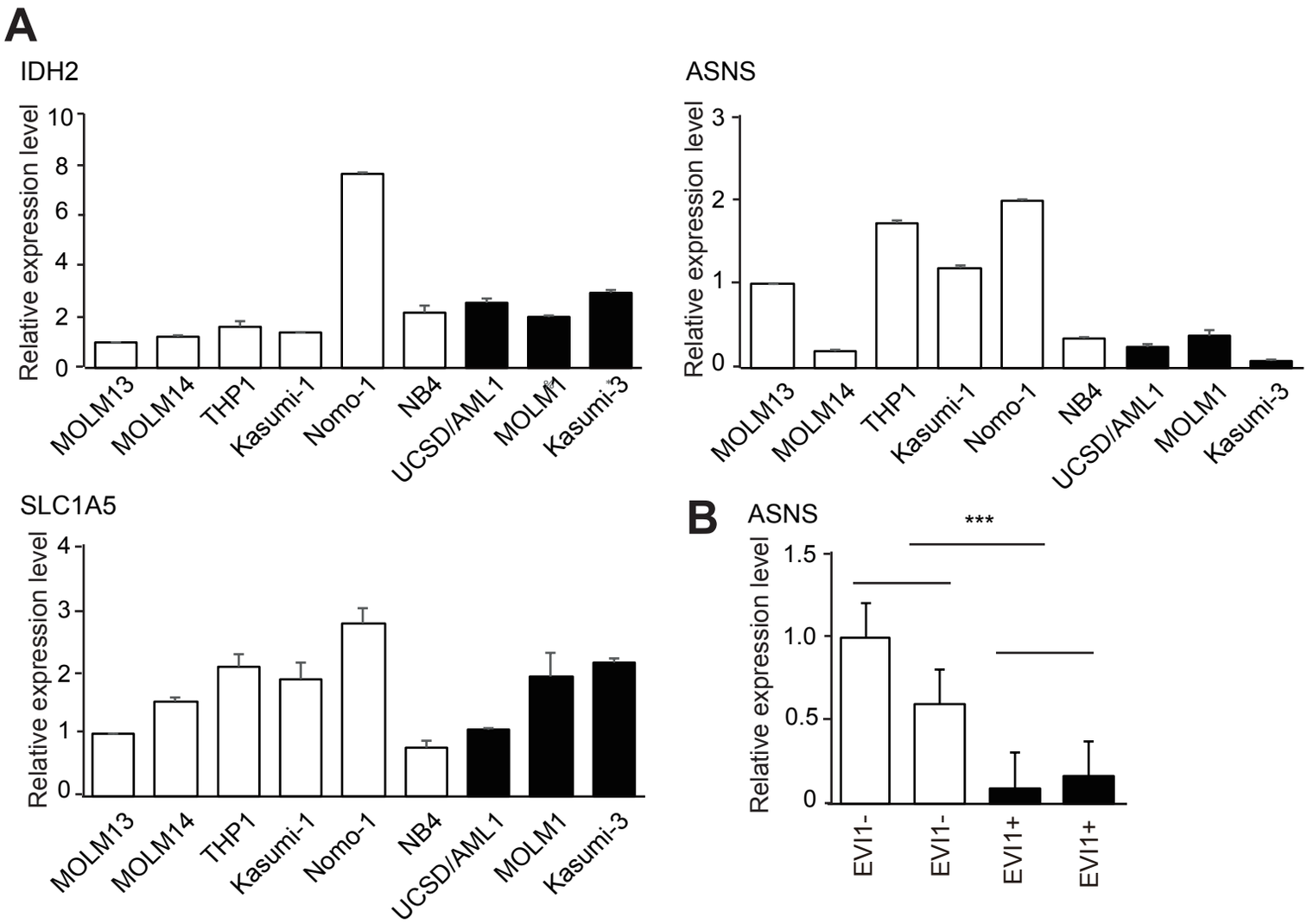

Supplementary Figure S8

Supplementary Table S1

Genes	Forward Sequence	Reverse Sequence
Glut1	5'-AAACATGGAACCACCGCTAC-3	5'-AGGCCAACAGGTTCATCATC-3'
Hk1	5'-GAGGGATGCTGTAAAAAGGAGA-3'	5'-TTCATAAGCACAAGTCATCATGG-3'
Hk2	5'-GCTGAAGGAAGCCATTCG-3	5'-TCCCAACTGTGTCATTTACCAC-3'
Pfkl	5'-TCTCATCCAGCTACGTGAAGG-3'	5'-CCTCCTCGCTGTACATGACC-3'
Aldoa	5'-CAGCAACAGACAGAGTTAGGAAAG-3'	5'-AATGCGGTGAGCGATGTC-3'
Aldoc	5'-CGTAGGCATCAAGGTTGACA-3'	5'-GAGCACAGCGTTCCAAGAG-3'
Tpi1	5'-ACCGAGAAGGTCGTGTTCGA-3'	5'-GGCCAGGACCACCTTGCT-3'
Pgk1	5'-CCAAGGCTTTGGAGAGTCC-3'	5'-GATCAGCTGGATCTTGTCTGC-3'
Pgam1	5'-CCTCATGGTGATTTTTAACCCTAA-3'	5'-AAGATTGATCCCAACCTTCTAGG-3'
Eno1	5'-GGGTGATGAGGGTGGATTC-3'	5'-ATCCATGCCAATGACAACCT-3'
Idh1	5'-TCAGACTCAGTCGCCCAAG-3'	5'-GGTAGTGACGTGTGACAGTGC-3'
Idh2	5'-CAGGTCACCATTGACTCTGC-3'	5'-CCACATTTTCTTCAGCTTGAACT-3'
Aco2	5'-AGGTTGGGGGTGAGAAAGAC-3'	5'-GAAGCCCACACCATACTTGG-3'
Sdha	5'-CCATGCCAGGGAAGATTACA-3'	5'-TCCAGTGTTCCCCAAACG-3'
Slc1a5	5'-CATCATCCTGGAAGCAGTCA-3'	5'-CCTTCCACGTTGAGGACAGT-3'
Asns	5'-TCAGATGAACTTACACAGGGCTAT-3'	5'-GAGTTCCTTCAGCAGTCTCTCAC-3'
Evi1	5'-ATCGGAAGATCTTAGATGAGTTTT-3'	5'-CTTCCTACATCTGGTTGACTGG-3'
β -actin	5'-CGTCGACAACGGCTCCGGCATG-3'	5'-GGGCCTCGTCACCCACATAGGAG-3'

Genes	Forward Sequence	Reverse Sequence
IDH1	5'-GGTGACATACCTGGTACATAACTTTG-3'	5'-GTGTGCAAAATCTTCAATTGACTT-3'
IDH2	5'-TGACCCGTATTATCTGGCAGT-3'	5'-GGAGCCCGAGGTCAAAATAC-3'
SLC1A5	5'-GATTCGTTCCTGGATCTTGC-3'	5'-TCCTCTCTTCATAGGTGGTAGAGTATG-3'
ASNS	5'-ATCACCCTGACCTGCTTACG-3'	5'-CAAACAGCGCCCAAATGC-3'
β -actin	5'-GACAGGATGCAGAAGGAGATCACT-3'	5'-TGATCCACATCTGCTGGAAGGT-3'

Genes	Forward Sequence	Reverse Sequence
IDH1	5'-GGTGACATACCTGGTACATAACTTTG-3'	5'-GTGTGCAAAATCTTCAATTGACTT-3'
IDH2	5'-TGACCCGTATTATCTGGCAGT-3'	5'-GGAGCCCGAGGTCAAAATAC-3'
SLC1A5	5'-GATTCGTTCCTGGATCTTGC-3'	5'-TCCTCTCTTCATAGGTGGTAGAGTATG-3'
ASNS	5'-ATCACCCTGACCTGCTTACG-3'	5'-CAAACAGCGCCCAAATGC-3'
β -actin	5'-GACAGGATGCAGAAGGAGATCACT-3'	5'-TGATCCACATCTGCTGGAAGGT-3'

shRNA

Genes	Forward Sequence	
Evi1	5'-CCGGTGAGGTATAAAGAGGAA-3'	TRCN0000096096
Idh1	5'-CCCAGTTTGAAGCTCAGAATA-3'	TRCN0000041714
Idh2	5'-CAAGGAGTGGGAGGTGTATAA-3'	TRCN0000437043
Asns	5'-GCCAGATATGAGAATTCCAAA -3'	TRCN0000031700