Appendix

Branched-chain ketoacids secreted by glioblastoma cells via MCT1 modulate macrophage phenotype

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Supplementary Figure legends

Appendix Figure S1 – MCT1 inhibition does not impact on glioblastoma cell proliferation.

A, B Effect of MCT1 inhibitor AR-C155858 on cell proliferation of U87nt and U87shBCAT1 **(A)** and of U251nt and U251shBCAT1 **(B)** cells determined using the Click-iT proliferation assay. n=2-3 technical replicates.

Appendix Figure S2 – Combination of MCT1 inhibition and MCT4 knockdown does not result in an additive effect on BCKA excretion.

A BCKAs (KIV, KIC, KMV) levels are determined by Ultra Performance Liquid Chromatography (UPLC) coupled to fluorescence detection in supernatants from U87 cells expressing normal MCT4 levels (sint) or low MCT4 levels (siMCT4) treated with AR-C155858 for 24 hours at 37°C 10%CO_2. BCKAs levels are normalized to total protein content and to the detected levels in cells expressing normal MCT4 levels (sint) treated with the MCT1 inhibitor. n=3 technical replicates.

Appendix Figure S3 – *BCAT1* expression after pharmacological or genetic perturbations of MCT1 or MCT4 in glioblastoma cells.

A-D Effect of MCT1 inhibitor AR-C155858 **(A)**, MCT1 knockdown **(B)** or MCT4 knockdown **(C, D)** on *BCAT1* expression is determined using qRT-PCR. *ARF1* (ADP-ribosylation factor 1) and *TBP (*TATA-box binding protein) are used as housekeeping genes. Expression levels are normalized to the expression levels of cells treated with DMSO (control) or expressing normal MCT1 or MCT4 levels (sint). n=3 technical replicates.

Appendix Figure S4 – *In situ* proximity ligation assay controls.

A Representative images of the negative controls of *in situ* proximity ligation assay (PLA) in U87nt cells. Anti-BCAT1 antibody (rabbit polyclonal antibody, Insight Biotechnology limited (Wembley, UK)), Anti-MCT1 antibody (ab90582, Abcam) was used at 1:100 dilution. Anti-MCT4 antibody (376140, Santa Cruz), anti-PGK1 (GTX107614) and anti-LDHB (PA527505, Invitrogen) antibodies are used at 1:200 dilution. Anti-LDHA antibody (SAB1100050, Sigma) was used at 1:2400. Magnification: 200x. Red, PLA signal; blue, DAPI

B Representative images of *in situ* proximity ligation assay (PLA) in the U87nt and U251nt cells. Anti-MCT1 antibody (ab90582, Abcam) was used at 1:100 dilution and Anti-MCT4 antibody (376140, Santa Cruz) and Anti-PGK1 antibody (GTX107614) are used at 1:200 dilution. Magnification: 200x. Red, PLA signal; blue, DAPI.

Appendix Figure S5 – MCT1 (*SLC16A1)* and MCT4 (*SLC16A3)* expression in macrophages.

A MCT1 (*SLC16A1)* and MCT4 (*SLC16A3)* expression in M-CSF- or U87nt or U87shBCAT1 conditioned medium-differentiated macrophages was determined using qRT-PCR. *ARF1* (ADP-ribosylation factor 1) and *TBP (*TATA-box binding protein) are used as housekeeping genes. Expression levels are normalized to the expression levels of U87-MG cells expressing normal BCAT1 levels (U87nt). n=3 technical replicates.

Appendix Figure S6 – Effects of BCKAs on macrophage phagocytosis of fluorescent beads. Human-derived monocytes are differentiated to macrophages in the presence of M-CSF for 7 days. Monocyte-derived macrophages previously incubated with or without 300µM BCKAs at 37°C for 24 hours are incubated with 1µm diameter fluorescent biotin-labeled beads at 37°C for 2 hours. Phagocytosis of fluorescent beads (green) was examined by fluorescence microscopy. Representative pictures from two independent blood donors, run in triplicates are

shown. Streptavidin Phycoerythrin (PE) (red) binds to non-engulfed beads resulting in an orange staining. Hoechst stains nuclei (blue). Scale bar: 40µm.

Appendix Figure S7 – *In situ* proximity ligation assay.

A-D Representative images of *in situ* proximity ligation assay (PLA) in the U87-MG **(A, C)** and U251-MG **(B, D)** cells expressing normal BCAT1 levels (U87nt, U251nt respectively) are shown. Anti-LDHA antibody (SAB1100050, Sigma) was used at 1:2400 dilution, anti-LDHB (PA527505, Invitrogen) was used at 1:200 dilution, anti-MCT1 antibody (ab90582, Abcam) was used at 1:100 dilution and anti-MCT4 antibody (376140, Santa Cruz) was used at 1:200 dilution.

E, F Quantification of PLA signals per cell in U87nt **(E)** and U251nt cells **(F). (E)** n > 170; **(F)** n> 270. Mann-Whitney test; ns: not significant; * $p<0.05$; **** $p< 0.0001$. Quantification of PLA signals per cell for the combination of BCAT1 with MCT1 or MCT4 antibodies already shown in figure 6E and 6F is shown here to simplify the direct comparison with the combination of LDH and MCT1 or MCT4 antibodies.

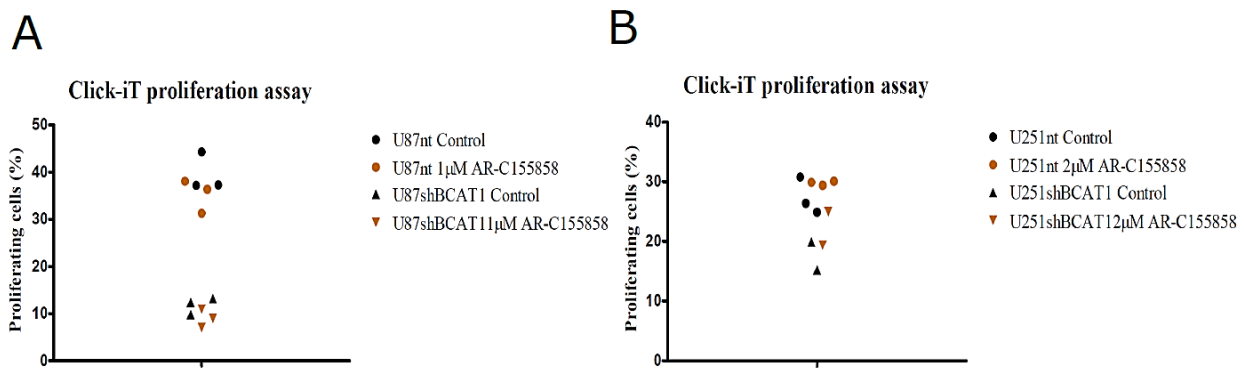
G Western blot analysis of LDHA levels in U87-MG cells and U251-MG expressing normal levels of BCAT1 (U87nt and U251nt). Anti-LDHA (SAB1100050, Sigma) antibody was used at 1:2000 dilution. α-tubulin was used as loading control.

Appendix Figure S8 – Breast cancer cells produce and excrete BCKAs.

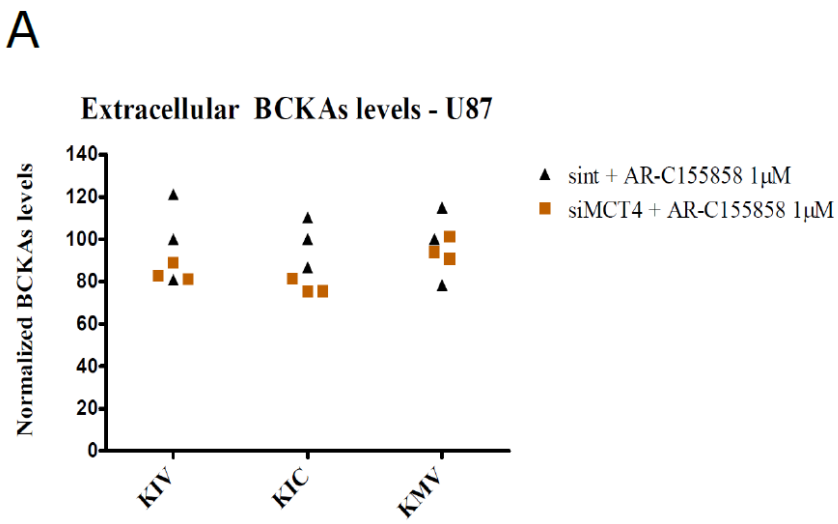
A, B BCKAs (KIV, KIC, KMV) are detected by Ultra Performance Liquid Chromatography (UPLC) coupled to fluorescence detection in cell extracts **(A)** or in cell culture supernatants **(B)** from SUM149, SUM159 and U87nt and U251nt cells. BCKAs levels are normalized to total protein content. n=3 technical replicates. KIV: α-ketoisovalerate. KIC: α-ketoisocaproate. KMV: α-keto-β-methylvalerate.

Supplementary Figures

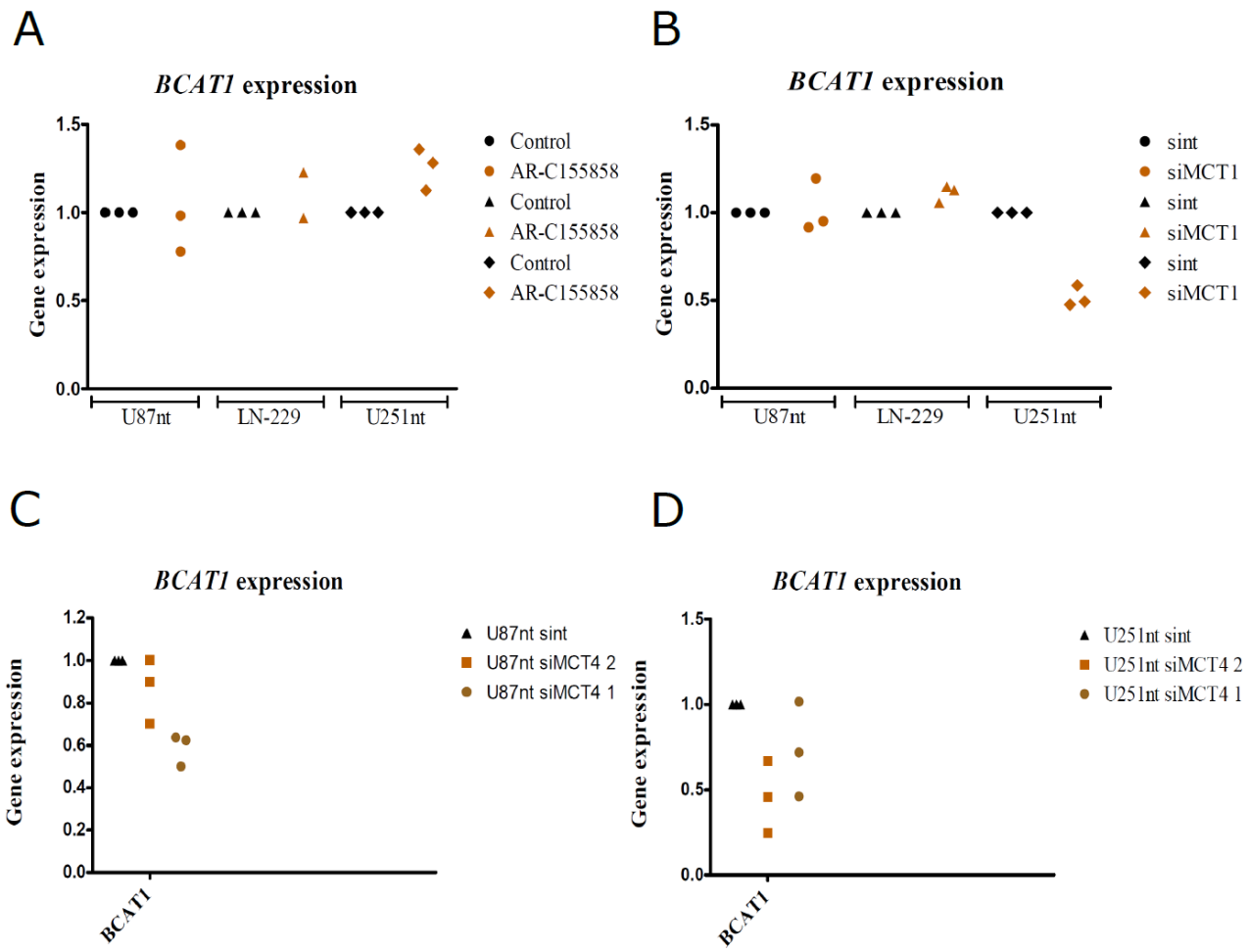
Appendix Figure S1



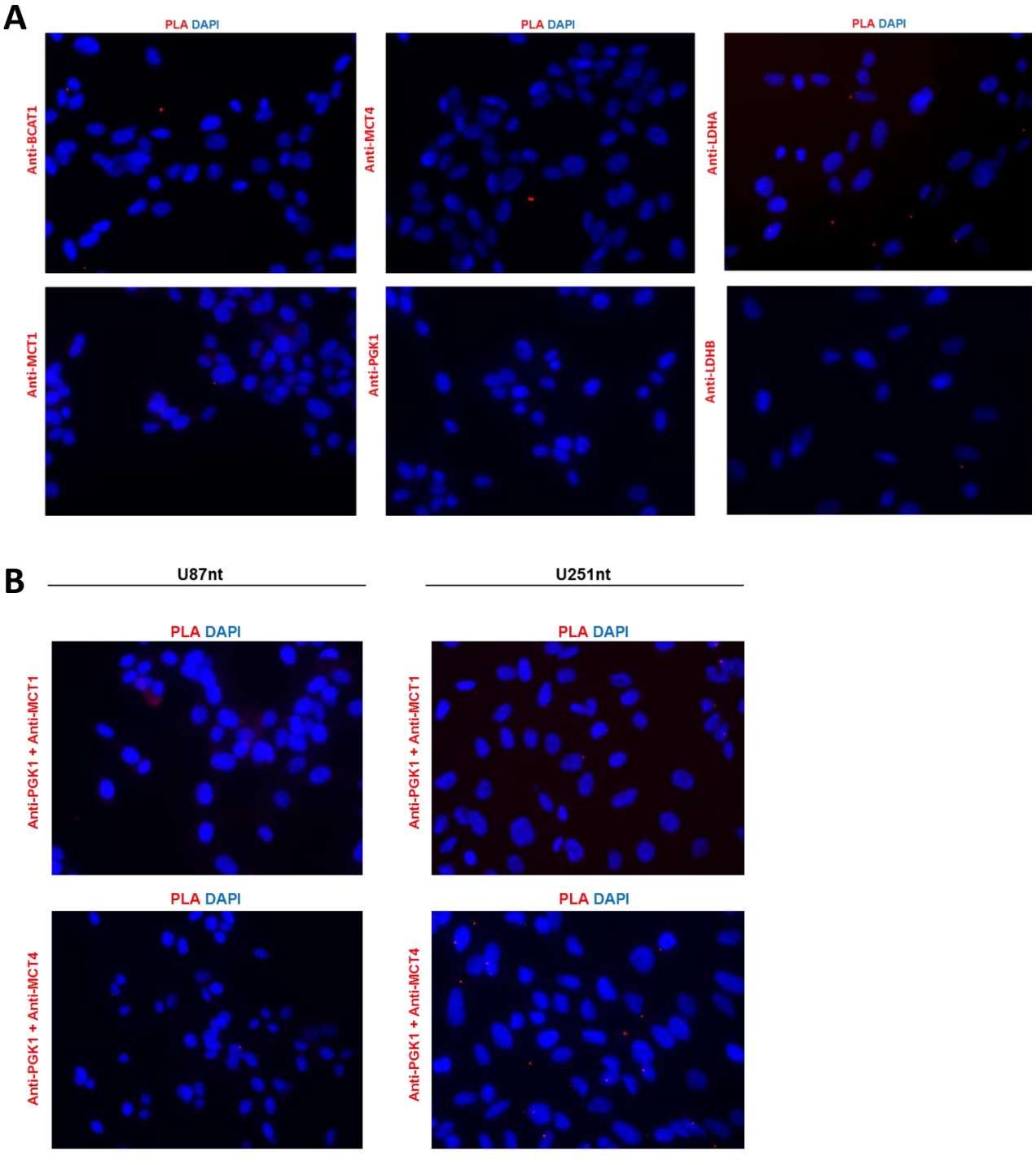
Appendix Figure S2



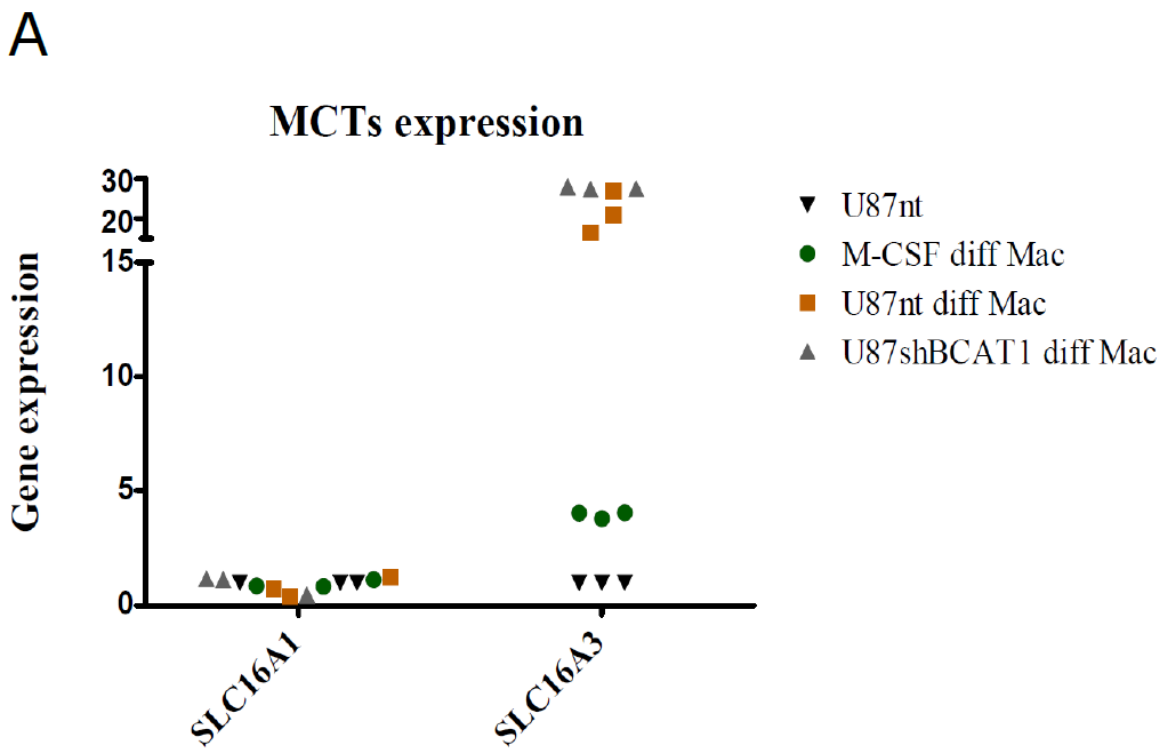
Appendix Figure S3



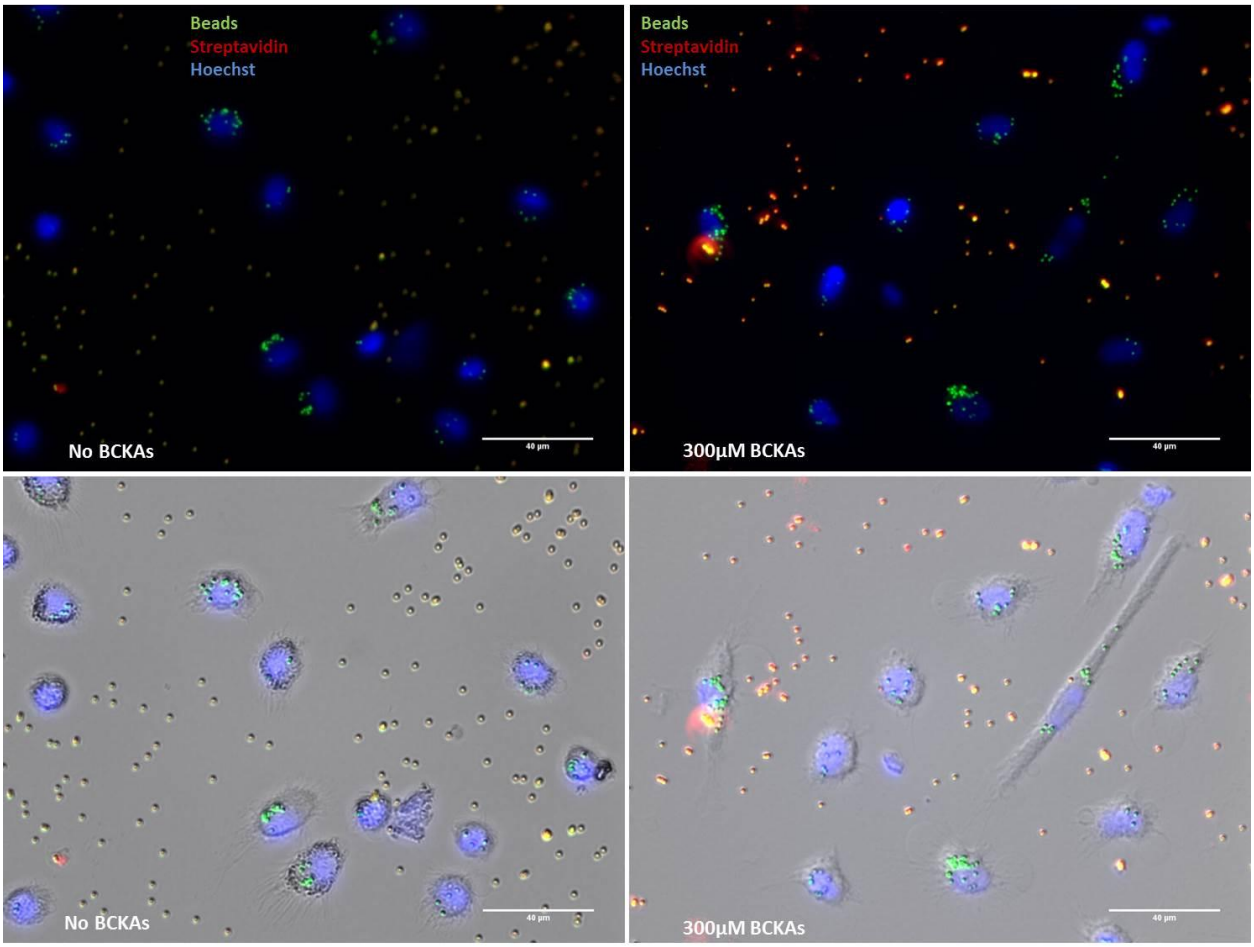
Appendix Figure S4



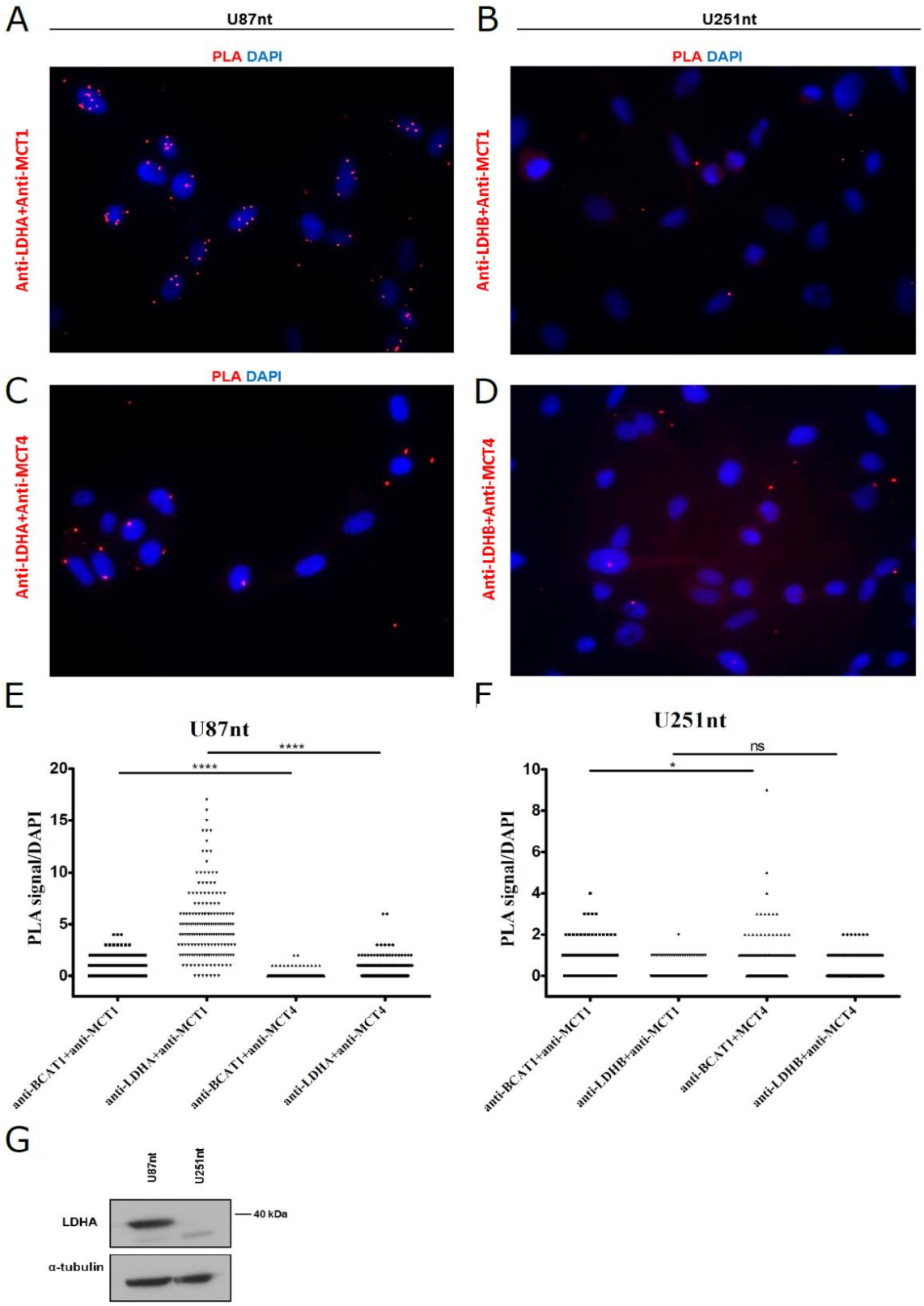
Appendix Figure S5



Appendix Figure S6

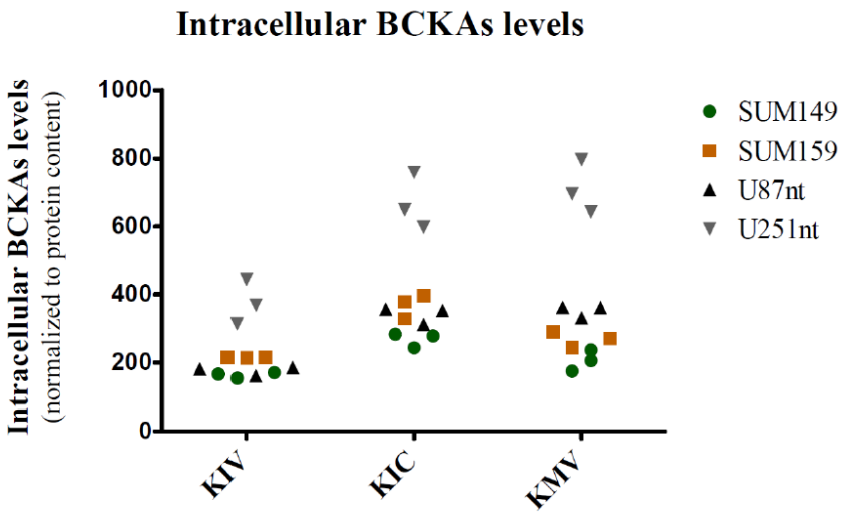


Appendix Figure S7



Appendix Figure S8

A



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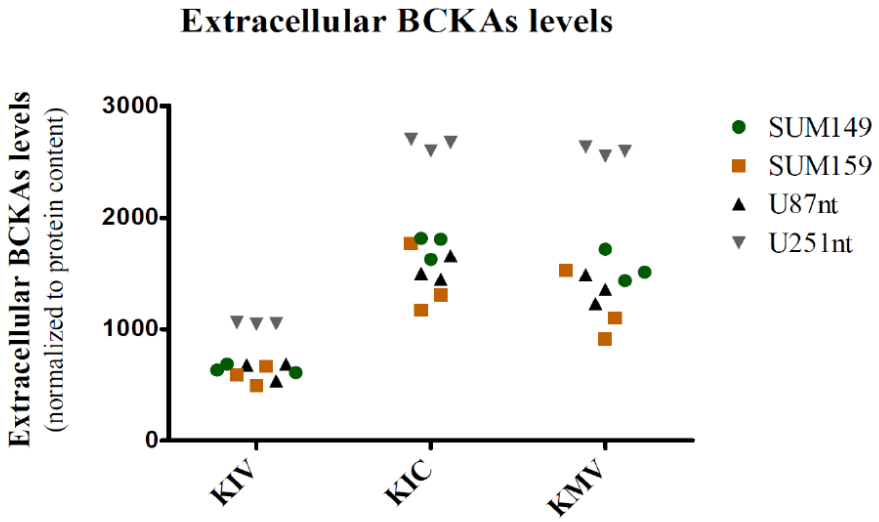

Supplementary tables

Appendix table S1 – Sequences of the primers used for qRT-PCR

Application	Oligo Name	Sequence 5' to 3'
qRT-PCR	**MCT1-F3**	TTGTGGAATGCTGTCCTGTC
	MCT1-R3	ACATGTCATTGAGCCGACCT
	MCT4-F3	TACCTCACCACTGGGGTCAT
	MCT4-R3	TTCAGCATGATGAGCGAGGG
	BCAT1_h_rt_fwd1	CAACTATGGAGAATGGTCCTAAGCT
	BCAT1_h_rt_rev1	TGTCCAGTCGCTCTCTTCTCTTC
	ARF_h_rt_fwd	GACCACGATCCTCTACAAGC
	ARF_h_rt_rev	TCCCACACAGTGAAGCTGATG
	TBP_h_rt_fwd	GAACCACGGCACTGATTTTC
	TBP_h_rt_rev	CCCCACCATGTTCTGAATCT

Appendix table S2 – List of intracellular metabolites analyzed by GC-MS after incubation of monocyte-derived macrophages with ^{13}C-αKIC (1,2-13C2) and ^{13}C-αKIV (13C5) for 48 hours.

Metabolite
2-Hydroxyglutarate
a-Ketoglutarate
Alanine
Aspartate
Citrate
Fumarate
Glutamate
Glycine
Isoleucine
Lactate
Leucine
Malate
Methionine
Ornithine
Pyruvate
Serine
Succinate
Valine

Appendix table S3 - Metabolite levels detected by Ultra Performance Liquid Chromatography (UPLC) in cell extracts (pmol/million cells) using the DMB derivatization method and in cell culture supernatants and in commercial DMEM (D5921, Sigma) (µM) using the OPD derivatization method.
KIV: α-ketoisovalerate. KIC: α-ketoisocaproate. KMV: α-keto-β-methylvalerate; n.d not detected

	DMB method	**OPD method**	
Metabolite	**Levels (pmol/mio cells) - cell extracts**	**Levels (µM) - cell culture supernatants**	**Levels (µM) - DMEM**
KIV	3-16	20-40	n.d
KIC	5-35	40-85	n.d
KMV	7-28	40-85	n.d
Pyruvate	high background	30-130	2-3.5