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Supplemental Information

Lactate Dehydrogenase A Depletion Alters MyC-CaP Tumor Metabolism, Microenvironment, and CAR T Cell Therapy

Mayuresh M. Mane, Ivan J. Cohen, Ellen Ackerstaff, Khalid Shalaby, Jenny N. Ijoma, Myat Ko, Masatomo Maeda, Avi S. Albeg, Kiranmayi Vemuri, Jaya Satagopan, Anna Moroz, Juan Zurita, Larissa Shenker, Masahiro Shindo, Tanner Nickles, Ekaterina Nikolov, Maxim A. Moroz, Jason A. Koutcher, Inna Serganova, Vladimir Ponomarev, and Ronald G. Blasberg

Lactate Dehydrogenase A depletion alters MyC-CaP tumor metabolism, the microenvironment and CAR T cell therapy

Short Title: LDH-A depletion affects tumor growth and CAR T cell therapy

Supplemental Information

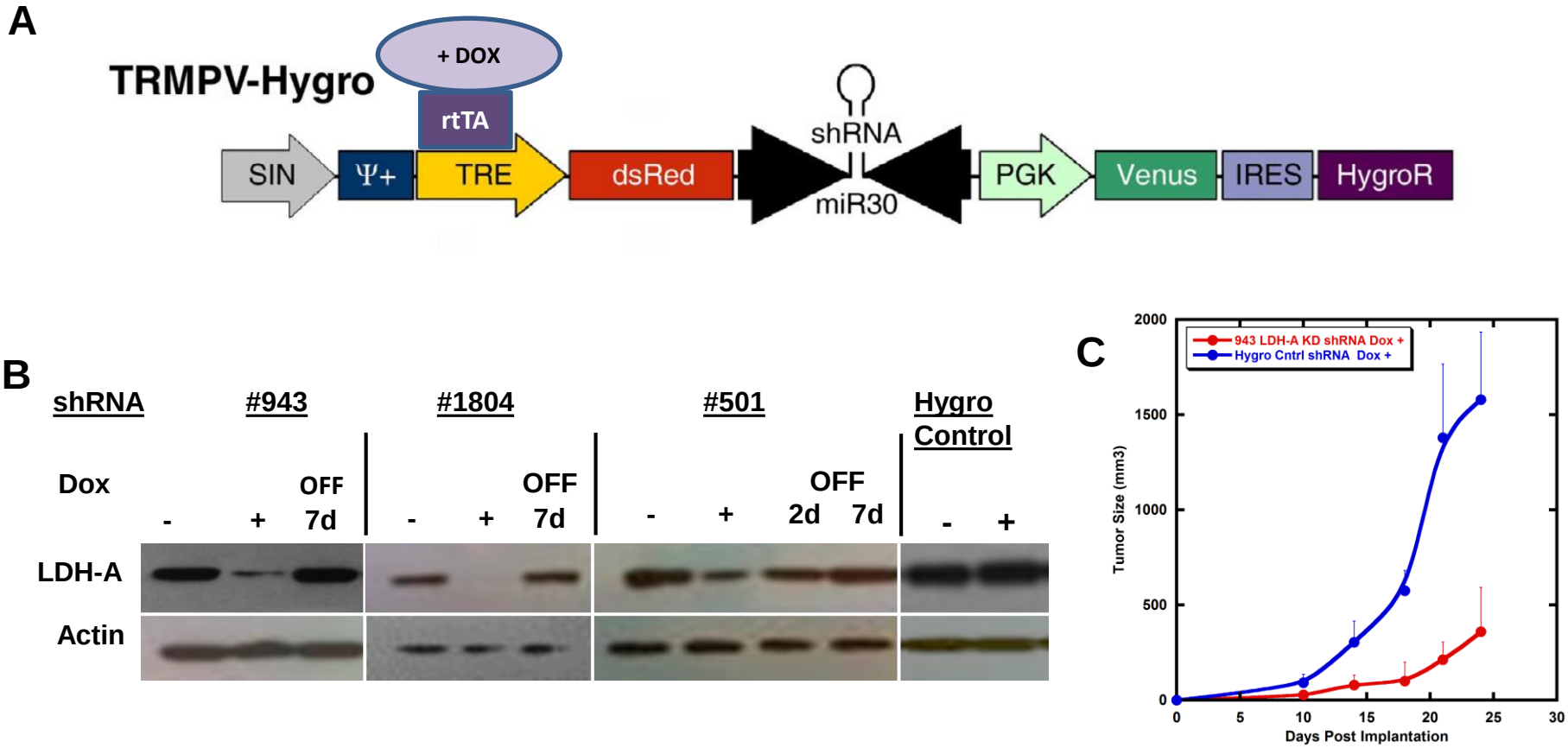

Figure S-1. **Doxycycline inducible system for LDH-A KD and its characterization.**

The doxycycline inducible reporter construct for LDH-A knock-down is shown in panel **(A)**. Western blotting for LDH-A protein expression; there is a consistent LDH-A KD effect in the presence of doxycycline across the different LDH-A shRNA plasmids (#943, #1804, #501), when compared with a Hygromycin control plasmid **(B)**. *In vivo* growth of MyC-CaP hPSMA(+) tumors transduced with the #943 construct shows significantly slower growth rate in SCID mice compared to the Hygromycin control tumor **(C)**. (Mean ± SEM).

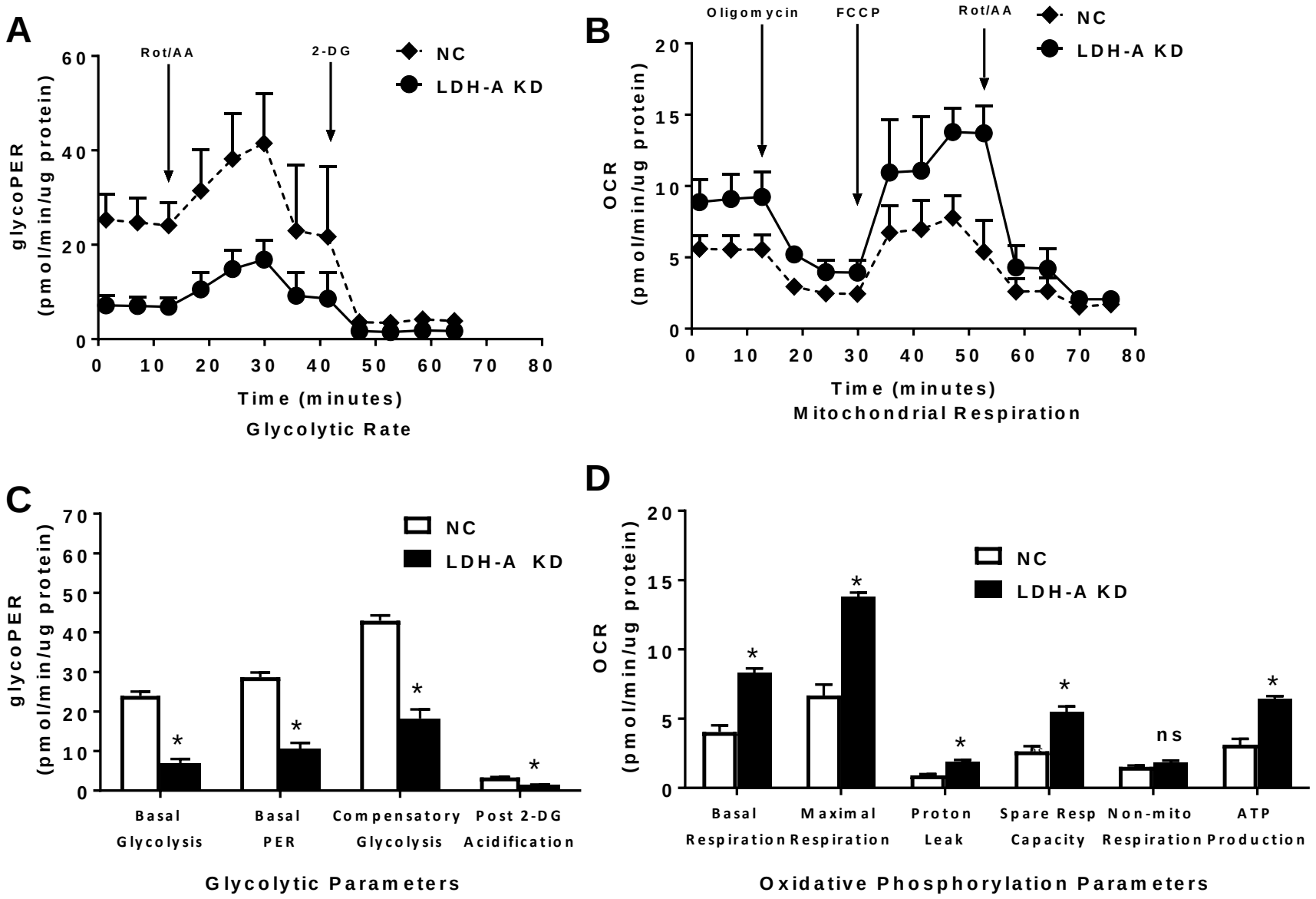

Figure S-2. Metabolic activity profile of MyC-CaP:hPSMA(+) LDH-A-KD and NC cells *in vitro.*

(**A,B**) Metabolic profiles of MyC-CaP hPSMA(+) NC and LDH-A KD cells measured on an Agilent Seahorse XF96 Extracellular Flux Analyzer. (**C**) Lower levels of basal ($p<0.0001$) and compensatory glycolysis ($p<0.0001$), and (**D**) higher oxidative phosphorylation, including levels of basal ($p=0.0011$) and maximum respiration ($p=0.0003$) were observed in LDH-A KD compared to the NC cells. (Mean ± SEM).

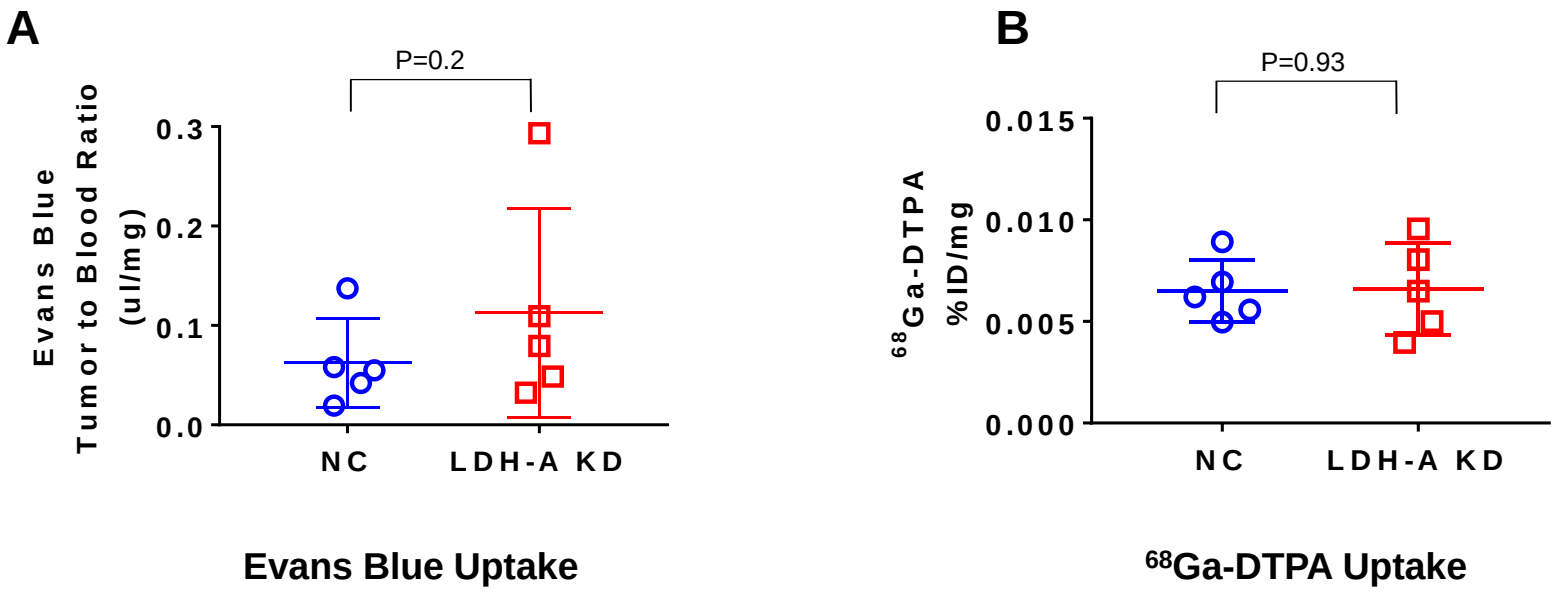

Figure S-3. Comparative vascular permeability between LDH-A KD and control NC MyC-CaP hPSMA(+) tumors. Whole-tumor uptake of Evans Blue (**A**) and ^{68}Ga-DTPA (**B**) revealed no differences in vascular permeability. (Mean ± SEM).

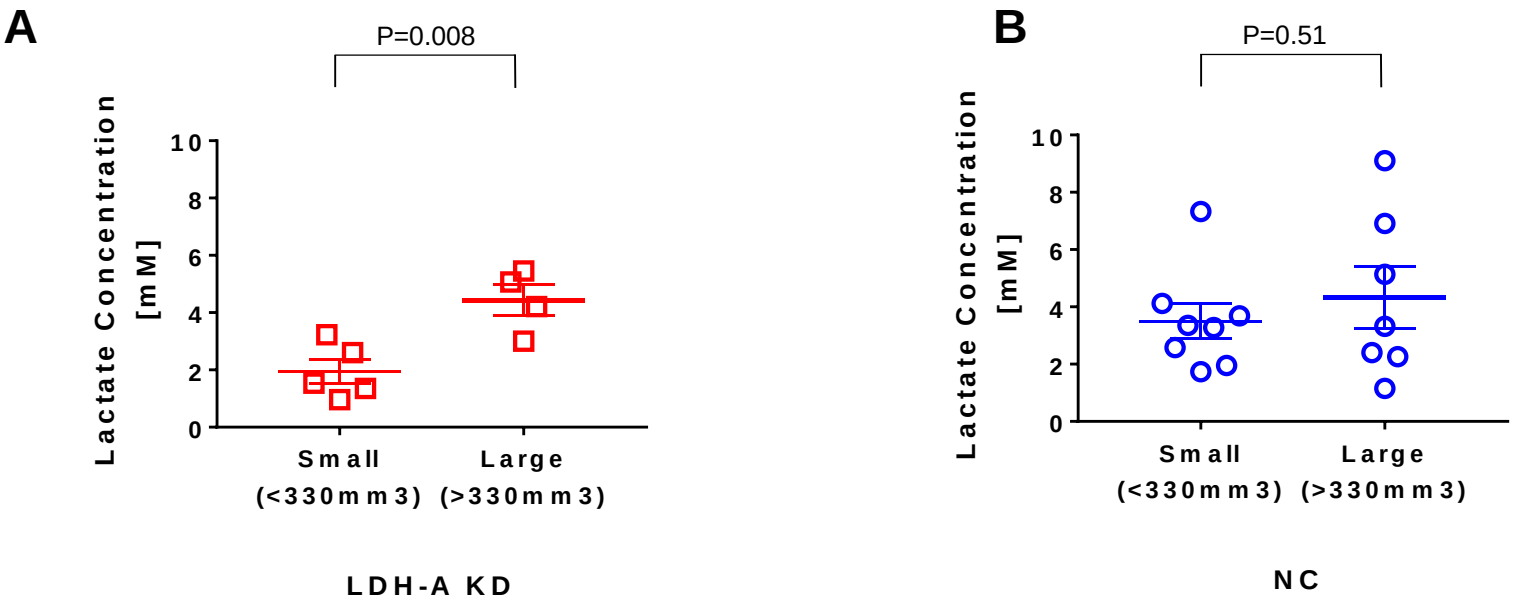

Figure S-4. Comparing whole-tumor lactate concentrations in small and large tumors by MRS.

Lactate concentration in small and large LDH-A KD tumors were different (p=0.008) (**A**); but not for NC tumors (p=0.51) (**B**). (Mean ± SEM).

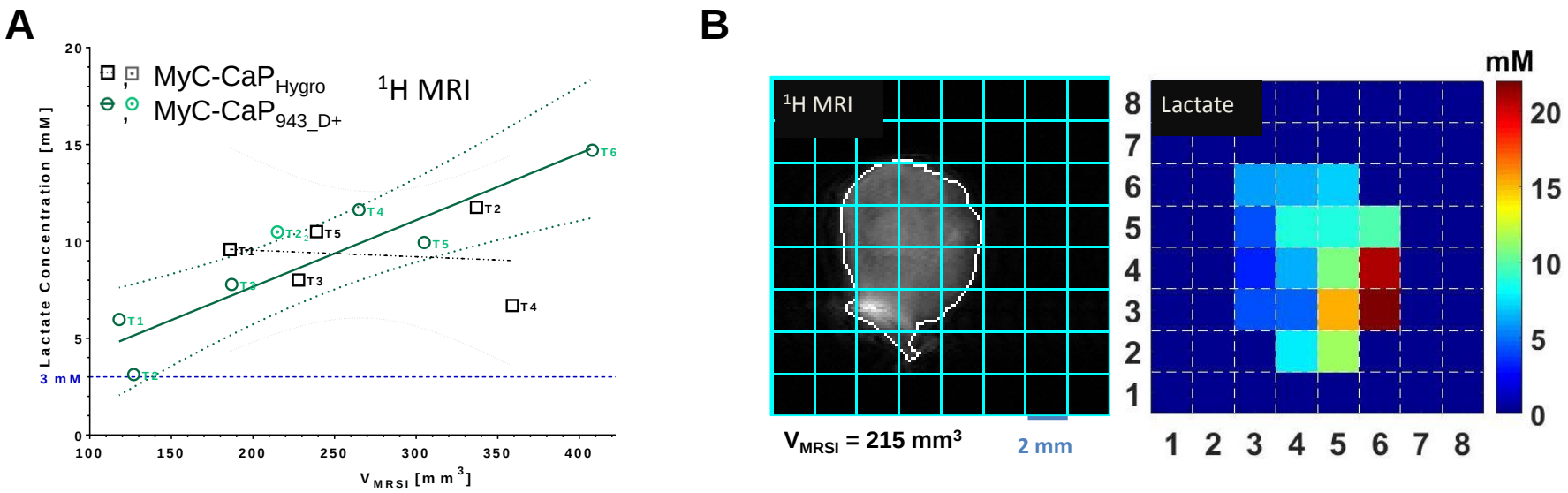

Figure S-5. Spatial distribution of tumor lactate and influence of tumor volume.

Whole-tumor lactate concentration plotted as a function of tumor volume in the lactate measurement slice (V_{MRSI}) in MyC-CaP$_{Hygro}$ (open squares, NC_{Mir}) and MyC-CaP$_{943_D+}$ tumors exposed to Doxycycline (open circles, KD_{Mir}) **(Panel A)**. The number beside each value depicts the numbering for each independent tumor, a subscript 2 and a central dot in the symbol identifies tumors that were measured a second time at a larger tumor size (T5 for MyC-CaP$_{Hygro}$ and T2 for MyC-CaP$_{943_D+}$). For MyC-CaP$_{943_D+}$ tumors, a linear regression analysis shows a significant linear relationship between the whole-tumor lactate concentration [Lac] and tumor size V_{MRSI} over the tumor volumes assessed: [Lac] = (0.0344±0.0064) mM/mm^3 • V_{MRSI} + (0.77±1.6) mM.. T2 MR image of a MyC-CaP$_{943_D+}$ tumor, with tumor tissue outlined (white) and a pixel grid overlay (cyan) (**Panel B, left**). The corresponding ^{1}H quantitative lactate map of the MyC-CaP$_{943_D+}$ tumor (**Panel B, right**). The color scale bar limits are set from 0 mM to 22 mM lactate to improve visibility of tumor lactate variation and comparison between the maps of the MyC-CaP:hPSMA(+) NC and LDH-A KD tumors shown in **Fig. 4.** The lactate concentration map demonstrates that tumor lactate is heterogeneous in this tumor, as observed for the MyC-CaP:hPSMA(+) NC and LDH-A KD tumors.

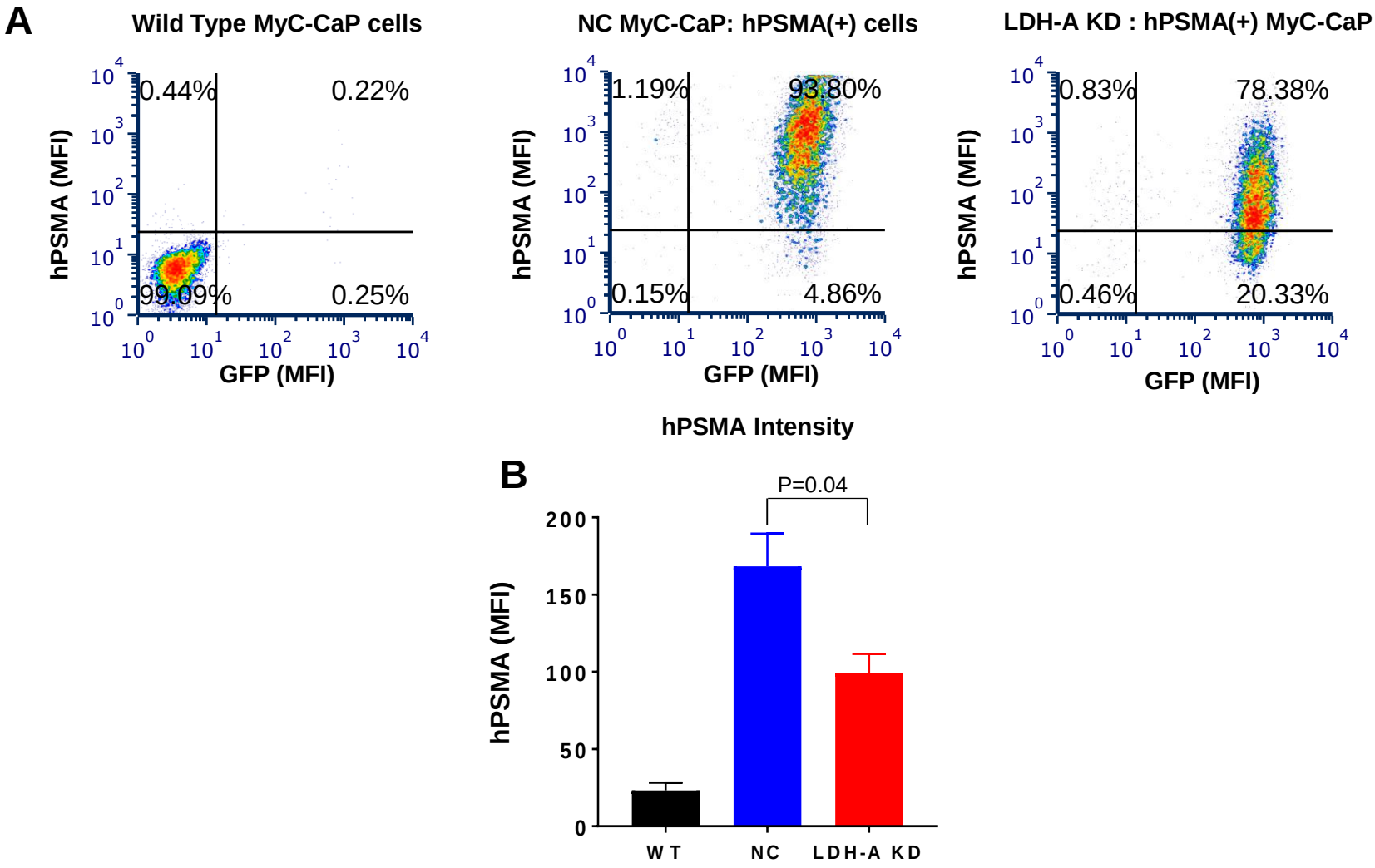

Figure S-6. Characterization of MyC-CaP:hPSMA(+) RLuc-IRES-GFP cells prior to animal implantation.

FACS analysis of hPSMA expression in wild-type, NC and LDH-A KD MyC-CaP:hPSMA(+) tumor cells (**A**); hPSMA staining intensity of FACS profiles in Panel A (Mean ± SEM) (**B**).

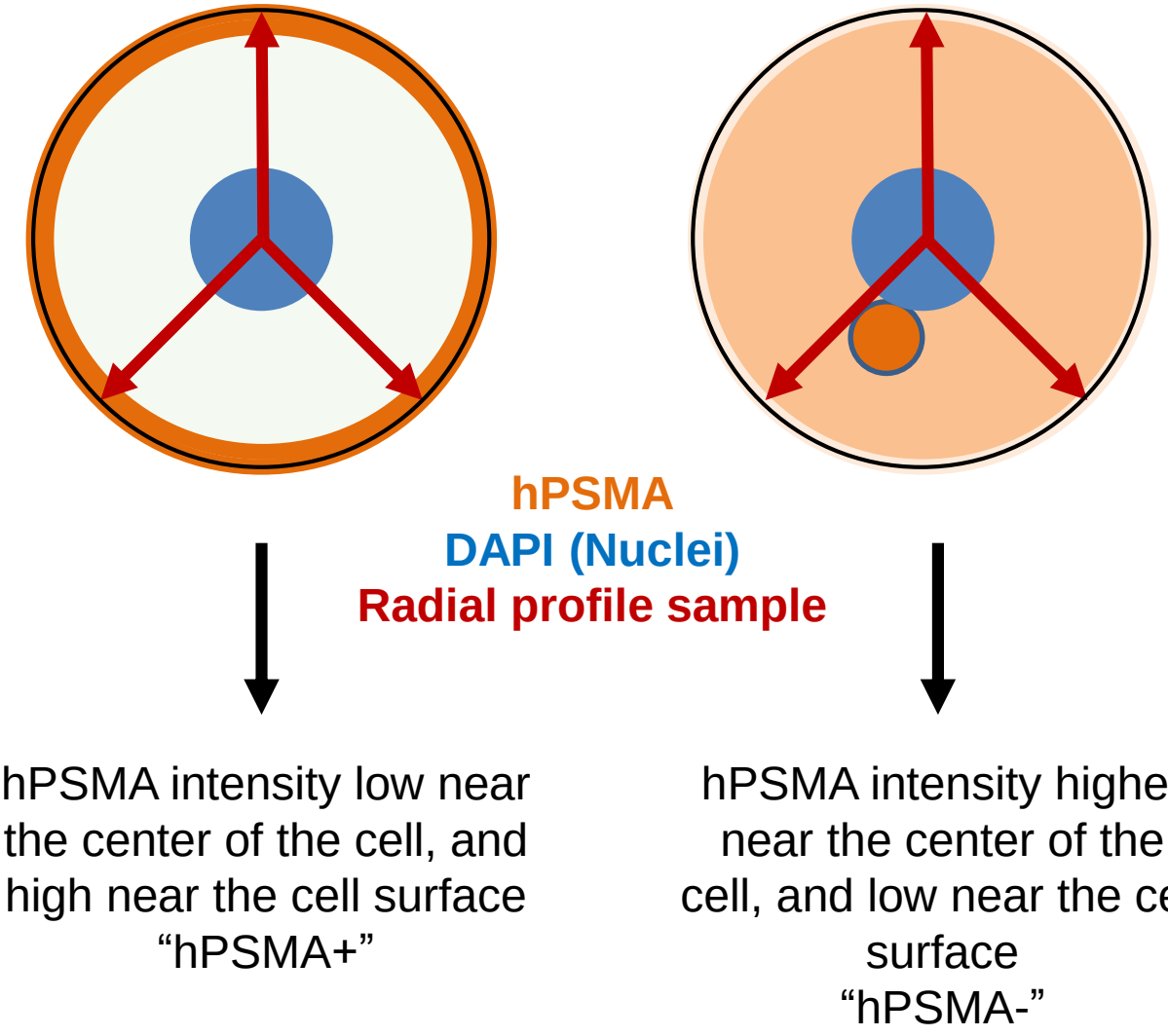

Figure S-7. Radial density analysis to assess tumor cell membrane expression of hPSMA.

To determine the density and percentage of hPSMA(+) cells, individual cells were first identified by DAPI staining. If cells were determined to have hPSMA expression above a specified threshold, radial intensity profiles were drawn to assess the subcellular localization of hPSMA (i.e., closer to the nucleus vs. closer to the membrane). If the slope of the radial intensity profile was higher than 2.0 (greater expression closer to the cell membrane), cells were considered to be hPSMA(+), and the density and percentage of hPSMA(+) cells per tumor were calculated.

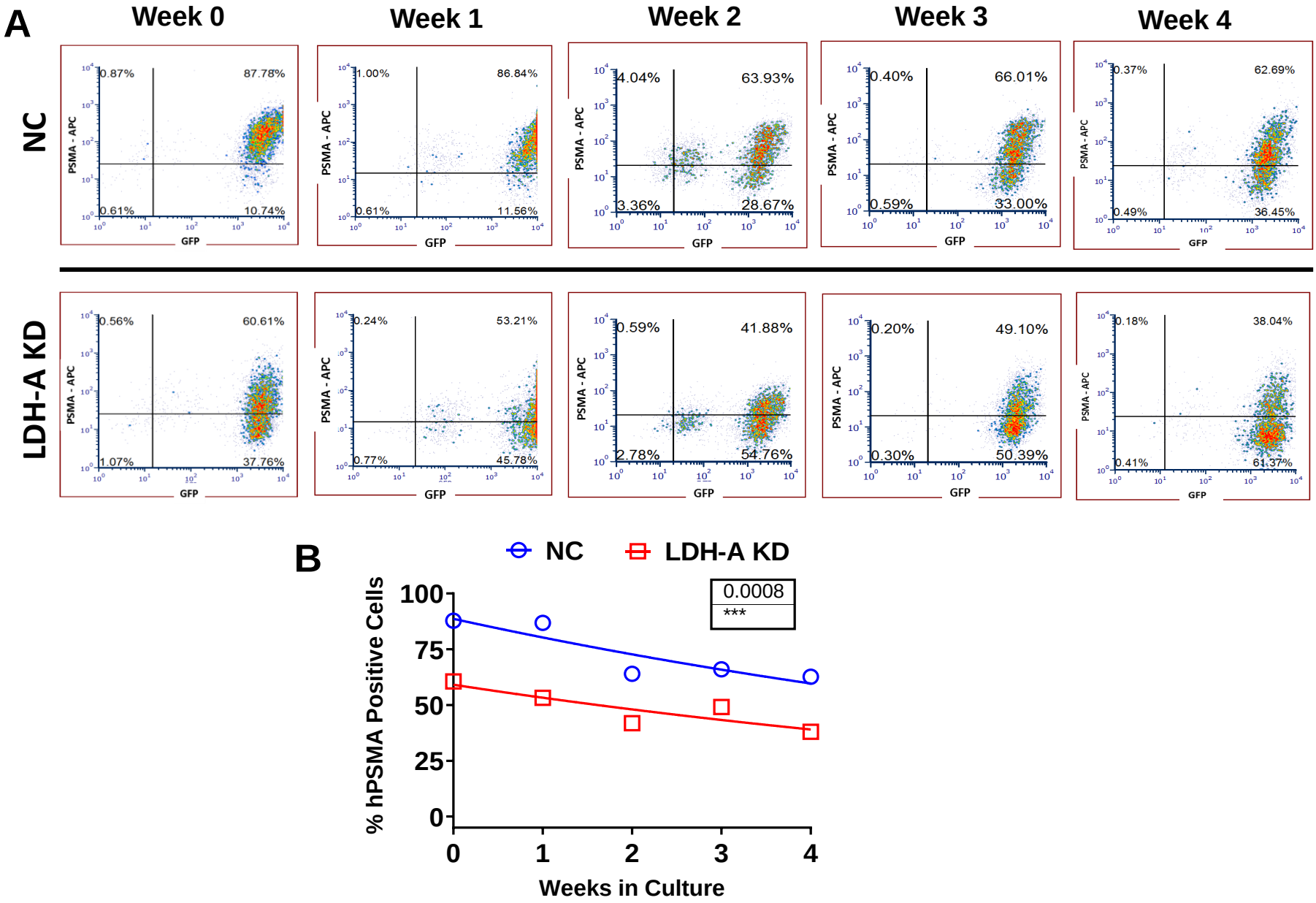

Figure S-8. **Loss of cell membrane hPSMA expression from MyC-CaP hPSMA(+) RLuc-IRES-GFP cells over time *in vitro*.**

FACS analysis of hPSMA expression in NC and LDH-A KD MyC-CaP:hPSMA(+) tumor cells in culture over 4 weeks (**A**); Percentage of hPSMA positive cells measured each week (NC vs LDH-A KD; p=0.0008) (**B**)

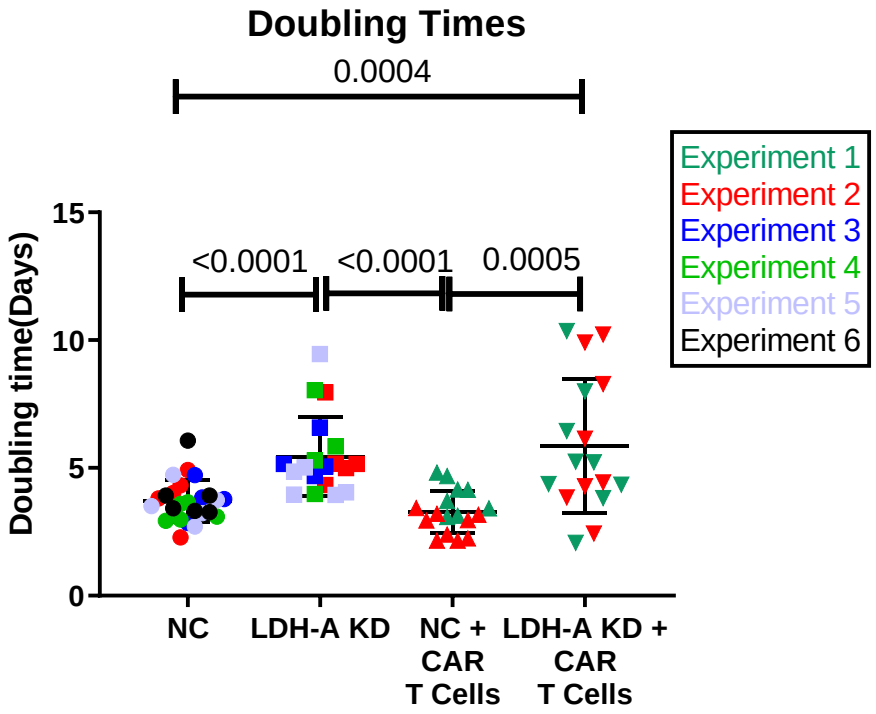

Figure S-9. **Tumor Doubling Times.** LDH-A KD tumors showed significantly longer tumor doubling times than NC tumors, in both CAR T cell treated (p=0.0005) and CAR T cell untreated (p<0.0001) groups. (Mean ± SEM).

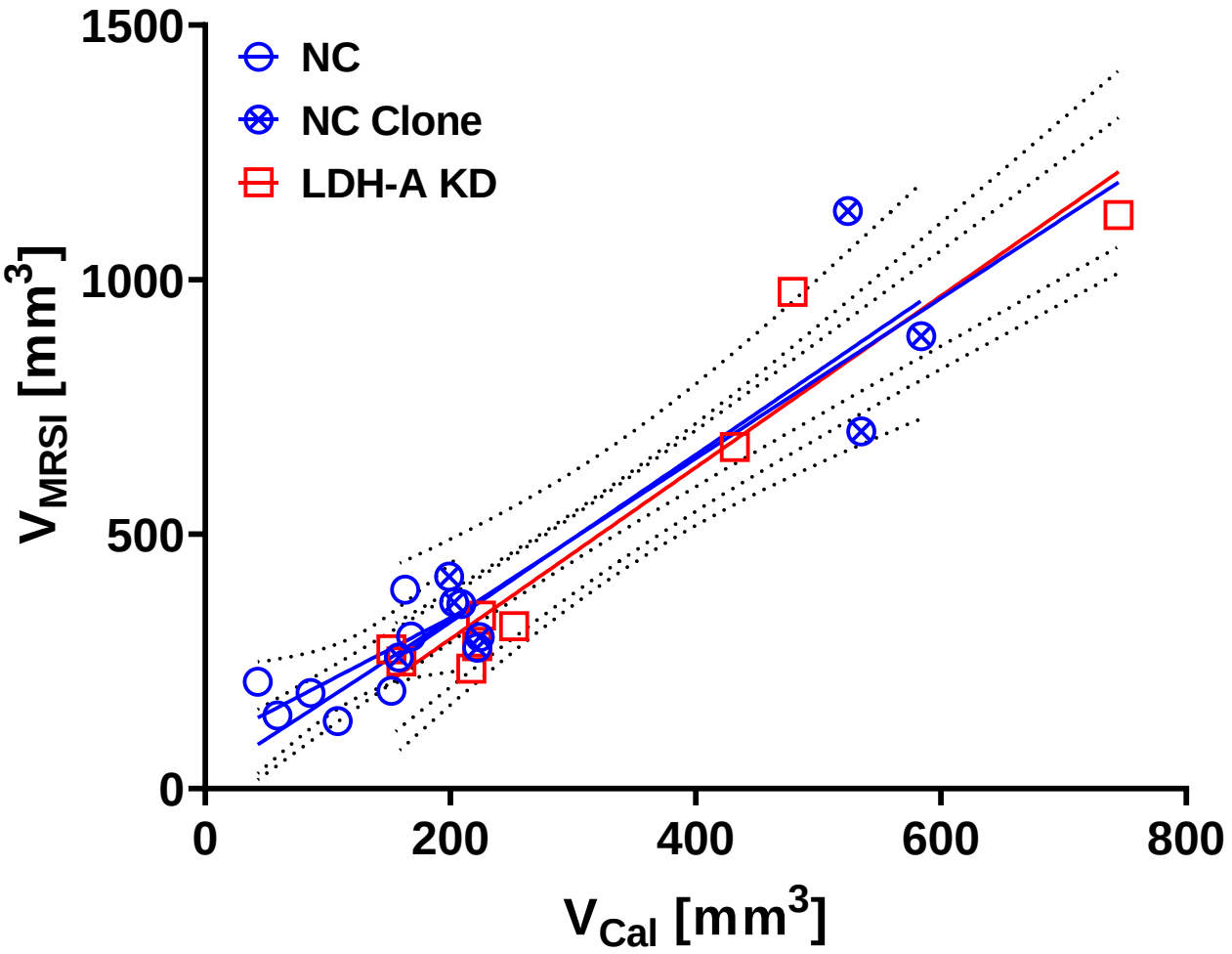

Figure S-10. **MRSI to Caliper Volume Comparison.** Caliper volume measurements while consistently lower were correlated with MRSI volume measurements.

Table S1. *In vitro* growth and metabolic studies comparing MyC-CaP:hPSMA(+) LDH-A KD and NC cells.

Cell Line	Doubling Time (in 25 mM glucose, full media)	Media pH (full media) (over 3 days)	Media Lactate Production (over 3 days)	Glucose Remaining in the Media (after 3 days)	Total Glucose Remaining in the Media (after 3 days)
Units	**h**	**pH**	**mmoles/L/3d**	**mmoles/L/10^5 cells**	**mmoles/L**
hPSMA(+) MyC-CaP NC	**14.7 ± 0.5**	**7.46 ± 0.09**	**7.90 ± 0.30**	**(0.6± 0.1) * 10^{-5}**	**15.2 ± 0.2**
hPSMA(+) MyC-CaP LDHA-KD	**17.2 ± 0.6**	**7.64 ± 0.09**	**4.57 ± 0.52**	**(1.1± 0.1) * 10^{-5}**	**18.5 ± 0.4**
NC vs LDH-A KD	**p=0.009**	**p=0.011**	**p=0.0007**	**p=0.0003**	**p=0.0002**
n	**6**	**11**	**3**	**8**	**8**

Table S-1. *In vitro* growth and metabolic studies comparing hPSMA(+) MyC-CaP LDH-A KD and NC cells.

Growth of MyC-CaP NC and KD cells were measured with Thermo Scientific Invitrogen Countess Automated Cell Counter and metabolic studies were measured with pH detection test, Eton Bioscience L-lactate Colorimetric Assay Kit and Accutrend glucometer. Following LDH-A knockdown, MyC-CaP KD cells showed an inhibition of the proliferation of cancer cell growth, demonstrated by lower doubling times (p=0.009). Additionally, MyC-CaP KD cells showed a reduction of lactate media production (p=0.0007), extracellular acidification (p=0.011), and glucose utilization (p=0.0002). (Mean ± SEM).

Table S-2. Metabolic (Seahorse) profile of MyC-CaP:hPSMA(+) NC and KD cells

Cell Line	In Vitro Doubling Time	Basal Glycolysis	Basal PER	Compensatory Glycolysis	Post 2DG Acidification	Basal Respiration	Maximum Respiration	Proton Leak	Spare Respiratory Capacity	Non-Mitochondrial O2 Consumption	ATP Production
Units	h	pmol/min/ ug protein	pmol/min/ ug protein	pmol/min/ ug protein	pmol/min/ ug protein	pmol/min/ ug protein	pmol/min/ ug protein	pmol/min/ ug protein	pmol/min/ ug protein	pmol/min/ ug protein	pmol/min/ ug protein
hPSMA(+) NC	14.7 ± 0.5	24.0 ± 0.9	28.7 ± 2.1	43.0 ± 2.3	3.3 ± 0.2	4.0 ± 0.8	6.7 ± 1.4	0.9 ± 0.2	2.6 ± 0.7	1.5 ± 0.2	3.1 ± 0.7
hPSMA(+) LDH-A KD	17.2 ± 0.6	6.9 ± 1.9	10.6 ± 2.5	18.3 ± 3.9	1.4 ± 0.2	8.3 ± 0.6	13.8 ± 0.6	1.9 ± 0.2	5.5 ± 0.7	1.8 ± 0.3	6.4 ± 0.4
P value (2-sided)	0.0093	<0.0001	<0.0001	<0.0001	<0.0001	0.0011	0.0003	0.0001	0.0004	0.025	0.005
Mean, ± SD, n=3 independent experiments											

Table S-2. *In vitro* Metabolic profile of MyC-CaP:hPSMA(+) NC and KD cells.

Metabolic profiles of MyC-CaP NC and KD cells were measured on an Agilent Seahorse XF96 Extracellular Flux Analyzer. Following LDH-A knockdown, MyC-CaP KD cells showed less dependence on glycolysis than control MyC-CaP NC cells, demonstrated by lower levels of basal glycolysis ($p<0.0001$), basal PER ($p<0.0001$) and compensatory glycolysis ($p<0.0001$). In contrast, KD cells were shown to exhibit a higher rate of oxidative phosphorylation as compared to the NC. This is especially evident in the higher levels of basal respiration ($p=0.0011$) and maximum respiration ($p=0.0003$) in KD cells. (Mean ± SEM).