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## Supplemental Information

# Resistance to Antiangiogenic Therapies by Metabolic Symbiosis in Renal Cell Carcinoma PDX Models and Patients

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**Figure S1**

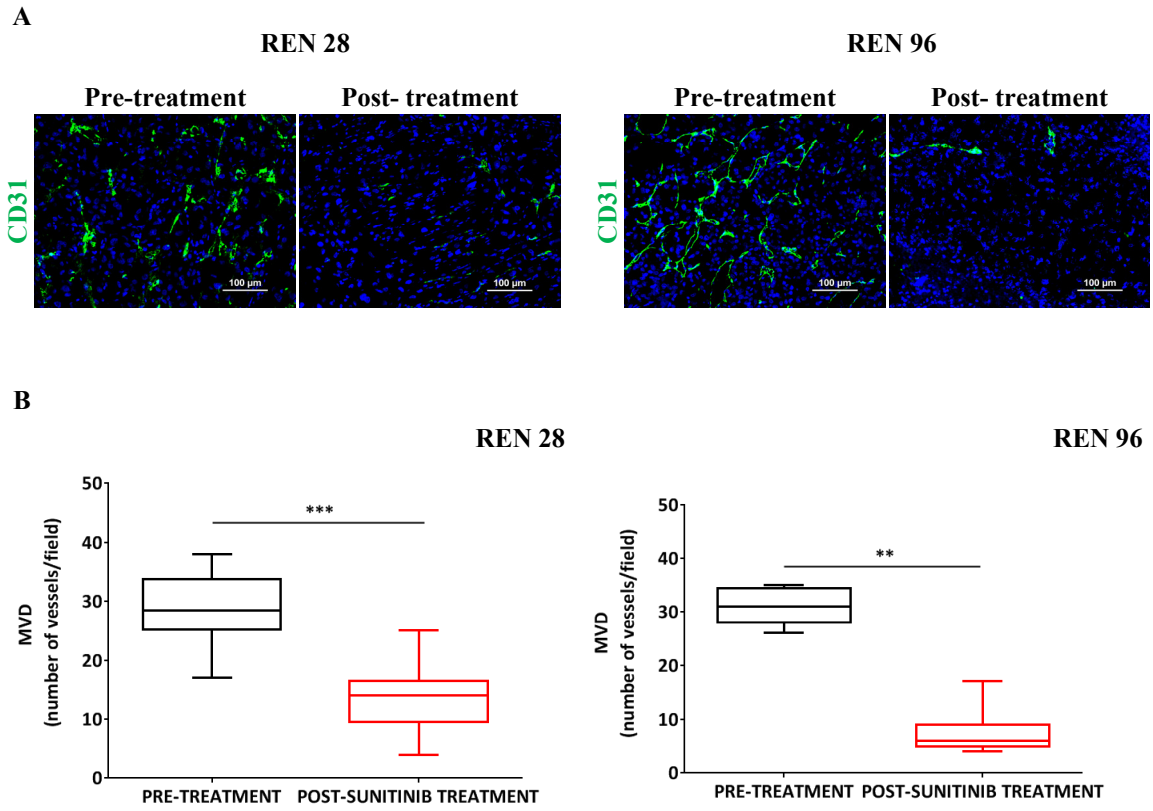



**Figure S1. Vessel density evaluation before and after treatment.** Related to Figure 1.
**A)** Representative images of CD31 immunohistofluorescence staining in patient-derived orthoxenograft tumors, 20X (Nucleus, DAPI).
**B)** Quantification of microvessel density graphed as box plots (mean+s.d.) from from 4 animals per treatment group, 4 images each, 20x (Nucleus, DAPI).

**Figure S2**

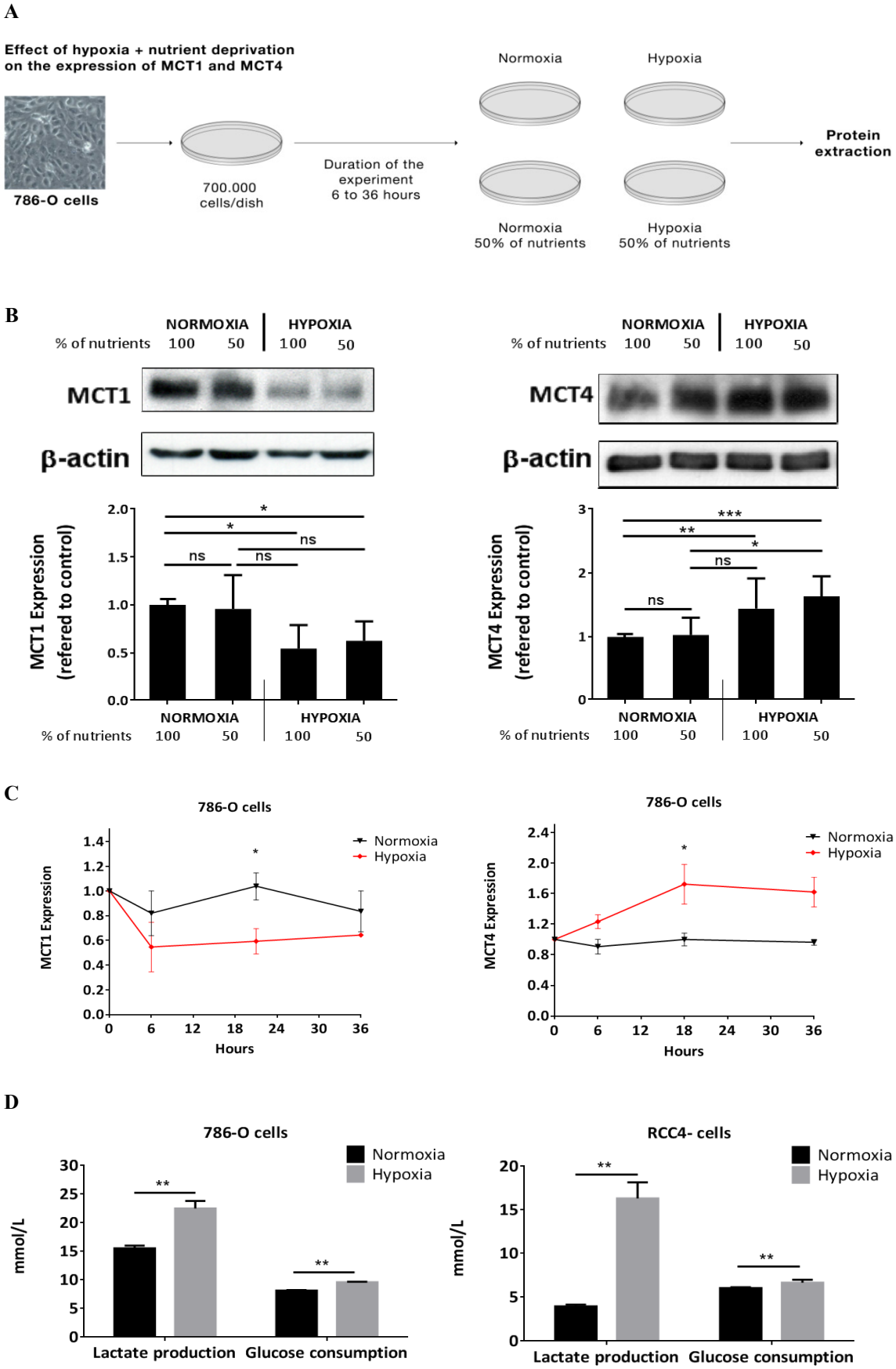

A
Effect of hypoxia + nutrient deprivation on the expression of MCT1 and MCT4
786-O cells
700.000 cells/dish
Duration of the experiment 6 to 36 hours
Normoxia
Hypoxia
Normoxia 50% of nutrients
Hypoxia 50% of nutrients
Protein extraction
B
NORMOXIA
HYPOXIA
% of nutrients 100 50 100 50
MCT1
β-actin
MCT1 Expression (refered to control)
MCT4
MCT4 Expression (refered to control)
C
786-O cells
Normoxia
Hypoxia
MCT1 Expression
MCT4 Expression
Hours
D
786-O cells
RCC4- cells
mmol/L
Lactate production
Glucose consumption

**Figure S2. *In vitro* analysis of hypoxia/nutrient response.** Related to Figure 2.
**A)** Schematic illustration of hypoxia and nutrient deprivation (50%) in 786-O cells. 786-O cells were maintained in normoxia at 21% $O_2$, 5% $CO_2$, 37ºC and in hypoxia at 3% $O_2$, 5% $CO_2$, 37ºC and full reconstituted RPMI medium (100% nutrients) or a dilution of 50% PBS (50% nutrients) from 6 until 36 hours. **B)** Protein expression of 786-O cell markers evaluated by western blot after hypoxia and nutrient deprivation (50%). **(B left)** MCT1, **(B right)** MCT4. Cells were incubated with standard or low levels of nutrients under normoxic or hypoxic conditions. The expression levels relative to the β-actin expression levels were compared. Columns show mean ± SD values of 4 independent experiments, (Mann-Whitney test). **C)** Time course of 786-O cells in normoxia and hypoxia. Protein expression of 786-O cell markers **(C left)** MCT1, **(C right)** MCT4 evaluated by western blot after hypoxia from 6 to 36 hours. **D)** Quantification of glucose consumption and lactate production *in vitro* in 786-O and RCC4- cells under hypoxic compared with normoxic conditions. Cells were plated in P6 wells at 2.0 x $10^5$ cells/well, incubated for 48 h *in vitro* under normoxic or hypoxic conditions ($pO_2$ = 0.5%) and metabolic parameters quantified by an automatic analyser. Columns show mean ± SD values of 5-6 independent experiments, $^{**}P < 0.005$, compared to normoxic values (Mann-Whitney test).

**Figure S3**

**A**

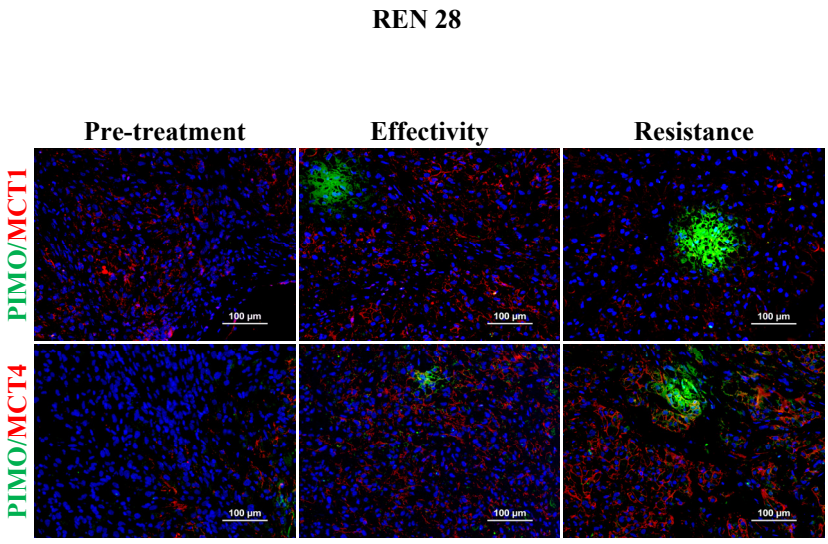



**Figure S3. Localization of MCT1/MCT4 regions relative to hypoxia.** Related to Figure 2.
**A)** Representative images of pimoniazole adducts in green (PIMO) and MCT1/MCT4 immunohistofluorescence staining in red, contrarested with DAPI (20X) in patient-derived orthoxenograft tumors before and after sunitinib treatment.

**Figure S4**

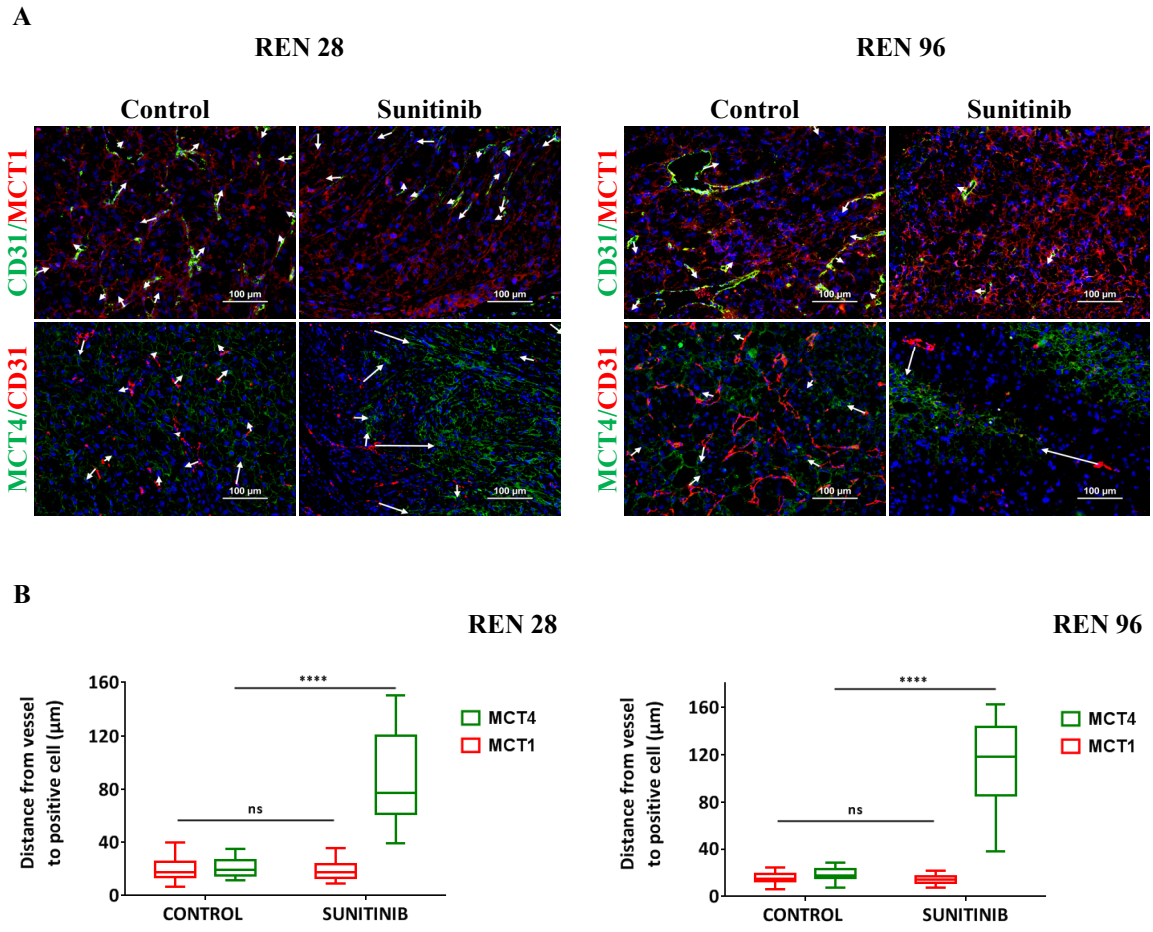



**Figure S4. Quantification of distance of MCT1/MCT4 to vessels.** Related to Figure 2.
**A)** Representative images of CD31/MCT4 and CD31/MCT4 immunohistofluorescence staining in patient-derived orthoxenograft tumors, 20X (Nucleus, DAPI). White arrows indicate distance from blood vessels to MCT1 or MCT4 positive areas.
**B)** Quantification of distance from blood vessels to MCT1 or MCT4 positive areas, graphed as box plots (mean+s.d.), from 4 animals per treatment group, 3 to 4 images each. 20x (Nucleus, DAPI).

**Figure S5**

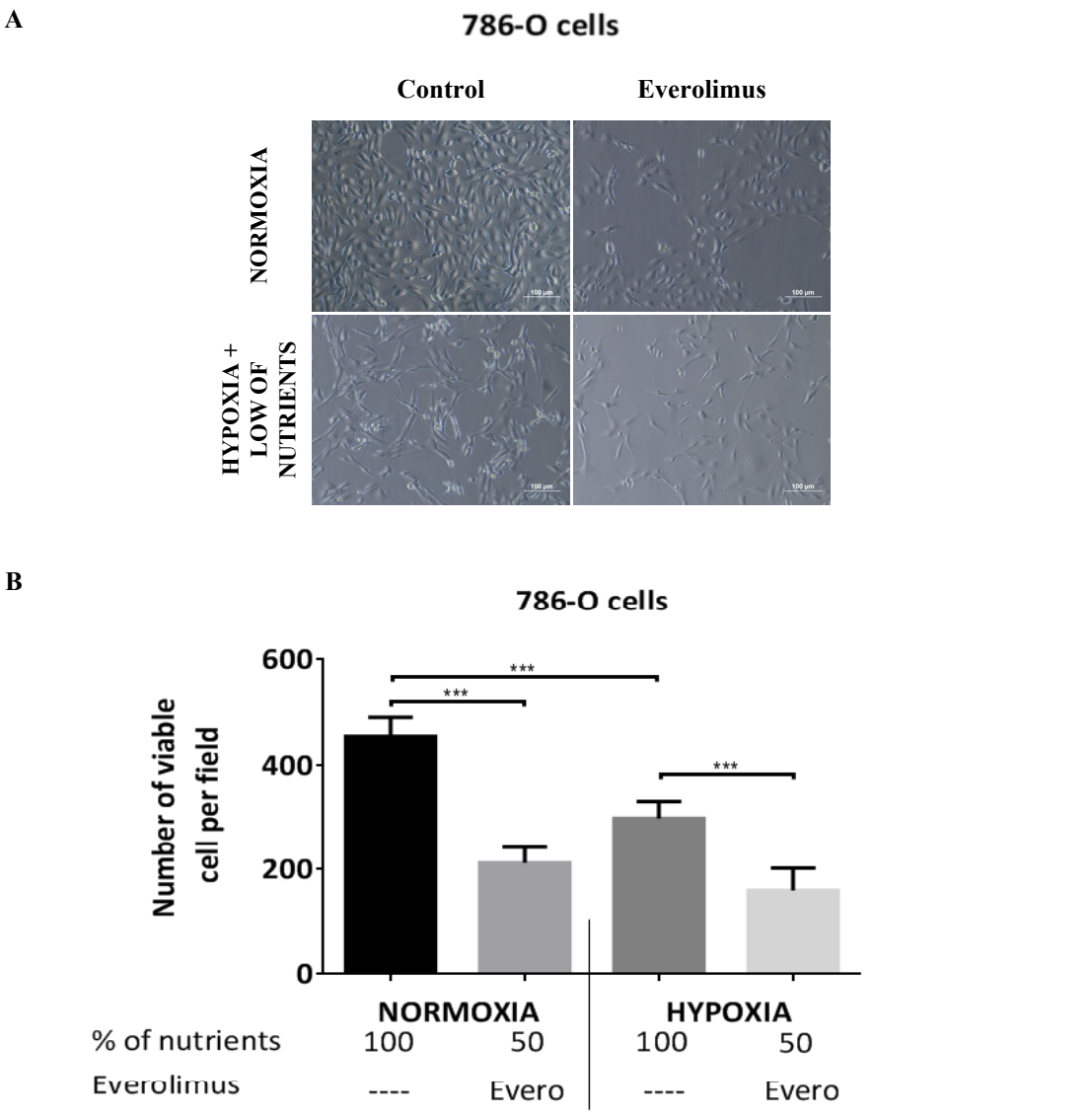



**Figure S5. In vitro analysis of mTOR inhibition in cell viability.** Related to Figure 3.
**A)** Representative images of hypoxia, nutrient deprivation and everolimus treated in 786-O cells, 10X magnification. 786-O cells were maintained in normoxia at 21% $O_2$, 5% $CO_2$, 37ºC, in hypoxia at 3% $O_2$, 5% $CO_2$, 37ºC and full reconstituted RPMI medium (100% nutrients) or a dilution of 50% PBS (50% nutrients) or treated with everolimus 10nM during 36 hours.
**B)** Quantification of number of viable cells per field from 4 pictures per treatment group, 10X magnification. Columns show mean ± SD values of 2 independent experiments, (Mann-Whitney test).

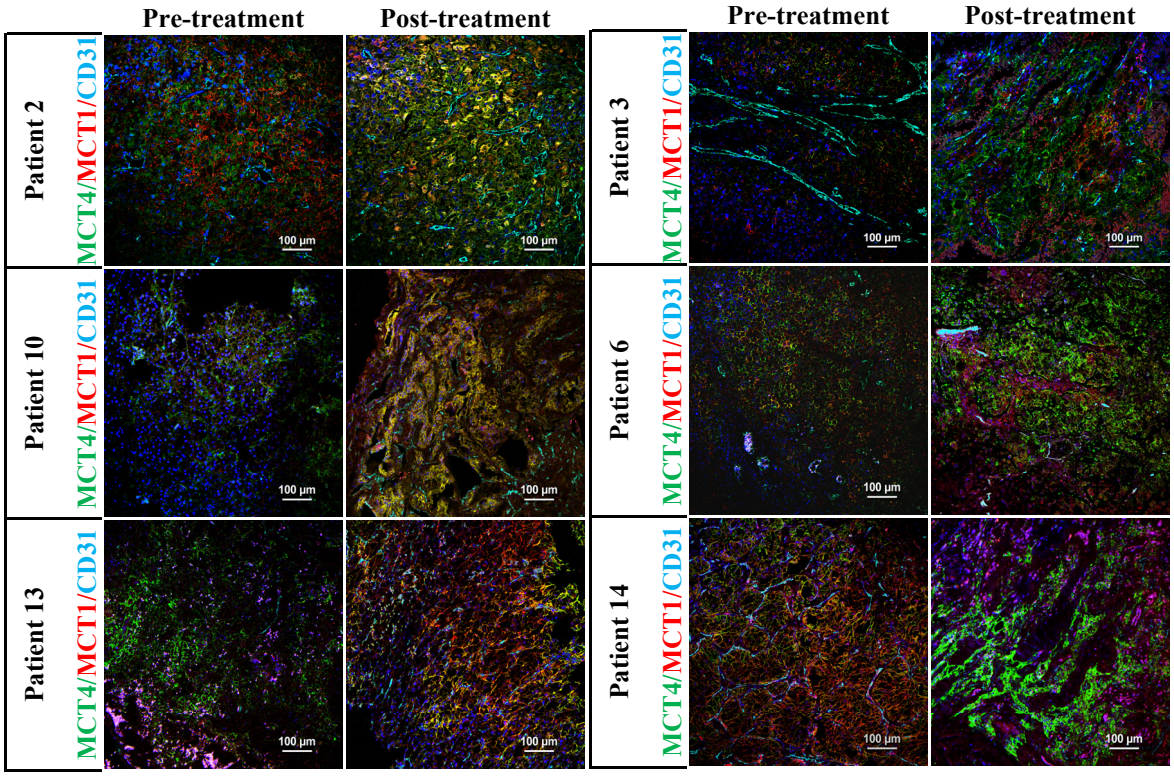
## Figure S6

**A**

| Patient | Gender | Duration of antiangiogenic treatment | Clinical response | Overall survival |
|---|---|---|---|---|
| 1 | *Female* | 21 days. | *SD* | 3 years. |
| 2 | *Male* | 1.9 years. | *SD* | 2.1 years. |
| 3 | *Male* | 2 years. | *PD* | 4.9 years. |
| 4 | *Female* | 1.6 years. | *PD* | 2.4 years. |
| 5 | *Female* | 4 months. | *PD* | 1 year. |
| 6 | *Female* | 6 months. | *PD* | 11 months. |
| 7 | *Male* | 6 months. | *PD* | 4.2 years. |
| 8 | *Male* | 1.6 years. | *PD* | 5.5 years. |
| 9 | *Male* | 1.3 years. | *SD* | 5.2 years. |
| 10 | *Male* | 8.1 years. | *SD* | Still alive (10 years and 6 months). |
| 11 | *Female* | 6 months. | *SD* | 2.4 years. |
| 12 | *Female* | 1.8 years + mTOR inhibitor 3.2 years. | *PD (first line); SD (second line)* | 5.2 years. |
| 13 | *Male* | 4.8 years. | *SD* | Still alive (4.8 years). |
| 14 | *Male* | 1.5 years. | *PD* | 3.7 years. |
| 15 | *Male* | 3.2 years. | *PD* | Still alive (3.8 years). |

***CR** (Complete Response), **PD** (Progressive Disease), **PR** (Partial Response), **SD** (Stable Disease)*

**B**




**C**

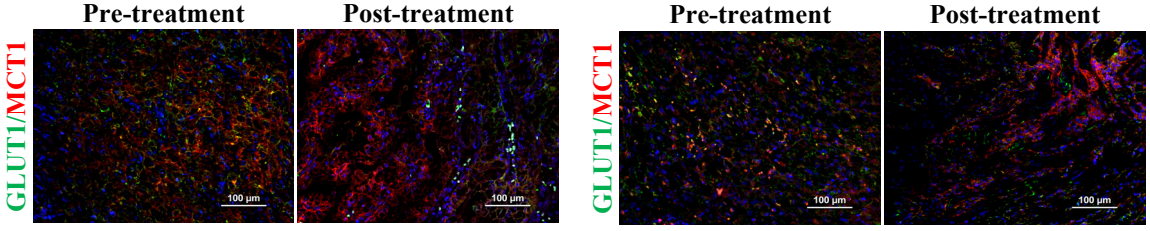


**Figure S6. Validation of metabolic symbiosis in patient samples.** Related to Figure 4.
**A)** Table of patient characteristics according to treatment duration, clinical response and overall survival. CR (Complete Response), PD (Progressive Disease), PR (Partial Response), SD (Stable Disease). **B)** Representative images of MCT1, MCT4 and CD31 immunohistofluorescence staining in RCC patients, 20x (Nucleus, DAPI).
**C)** Representative images of Glut1 and MCT1 immunohistofluorescence staining in RCC patients, 20X (Nucleus, DAPI).

**Supplemental Experimental Procedures**

**Cell lines**

pVHL-deficient 786-O (786-O-) and RCC4 (RCC4-) cell lines were kindly provided by B. Jimenez (Instituto de Investigaciones Biomédicas CSIC-UAM, Madrid, Spain). Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 50 units/mL penicillin, 50 µg/mL streptomycin sulfate, and 2 mmol/L glutamine (all Gibco, Life technologies, California, USA).

**Hypoxia and western blot analyses**

786-O and RCC4- cells were maintained in normoxia at 21% $O_2$, 5% $CO_2$, 37ºC and in hypoxia from 0,5 to 3% to $O_2$, 5% $CO_2$, 37ºC and full reconstituted RPMI medium (100% nutrients) or a dilution of 50% PBS (50% nutrients) from 6 until 48 hours.

Western blot analysis utilized monoclonal anti-β-actin (A5441, Sigma-Aldrich), anti-vinculin (V9131, Sigma-Aldrich), rabbit anti-MCT1 (H-70) (Santa Cruz Biotechnology, Cat. No SC-50324), rabbit anti-MCT4 (H-90) (Santa Cruz Biotechnology, Cat. No SC-50329). For western blots in Figure S2, quantification was performed using Quantity One software (version 4.6.1; Bio-Rad). Statistical comparison was done using Mann-Whitney U test (2-tailed) performed in GraphPad Prism (GraphPad Software, Inc. USA). Differences were considered statistically significant at $p<0.05$. (*, $p<0.05$; **, $p< 0.01$; ***, $p< 0.001$, ****, $p< 0.0001$).

**Glucose and lactate measurements**

Glucose and lactate concentrations in supernatants media of cultured cancer cells were determined by colorimetric methods on an automated analyzer (Dimension RxL, Dade Behring). Glucose consumption and lactate production in 786-O and RCC4- cells under hypoxic compared with normoxic conditions. Cells were plated at $2.0 \times 10^5$ cells/well, incubated for 48 h in vitro under normoxic or hypoxic conditions ($pO_2 = 0.5\%$) and metabolic parameters quantified. Statistical comparison was done using Mann-Whitney U test (2-tailed) performed in GraphPad Prism (GraphPad Software, Inc. USA). Differences were considered statistically significant at $p<0.05$. (*, $p<0.05$; **, $p< 0.01$; ***, $p< 0.001$, ****, $p< 0.0001$).

**Cell viability**

To examine the cell viability, 786-O cells were seeded at the density of ($5x10^5$) per plate and cultured with everolimus 10nM during 36 hours. 786-O cells were maintained in normoxia at 21% $O_2$, 5% $CO_2$, 37ºC, or in hypoxia at 3% $O_2$, 5% $CO_2$, 37ºC and full reconstituted RPMI medium (100% nutrients) or a dilution of 50% PBS (50% nutrients). Cell viability was quantified at 36 hours, counting the number of viable cell per plate. The cells were photographed at 36 hours with a Leica digital camera microscope (Leica). Statistical comparison was done using Mann-Whitney U test (2-tailed) performed in GraphPad Prism (GraphPad Software, Inc. USA). Differences were considered statistically significant at $p<0.05$. (*, $p<0.05$; **, $p< 0.01$; ***, $p< 0.001$, ****, $p< 0.0001$).