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Supplementary Materials for

Targeting GLUT1 and the Warburg Effect in Renal Cell Carcinoma by Chemical Synthetic Lethality

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The PDF file includes:

SUPPLEMENTAL MATERIALS AND METHODS

Reagents

In vitro kinase activities were performed by Millipore KinaseProfiler.

Cell viability

For 2, 3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assays, five thousand cells were plated in 96-well plates. The next day, vehicle (DMSO) or drug was added by serial dilution. Four days later, media were aspirated, XTT solution (0.3 mg/ml of XTT (Sigma), 2.65 μg/ml N-methyl dibenxopyrazine methyl sulfate (Sigma) in phenol red-free media) was added, and the plates were incubated at 37°C for 1-2 hours. Metabolism of XTT was quantified by measuring the absorbance at 450 nm. IC_{50}s were calculated using linear interpolation. For clonogenic survival assays, three hundred cells were plated per 60 mm tissue culture dish. The cells were allowed to attach overnight and then treated with vehicle or drug for 14 days. Colonies were fixed and stained with crystal violet (0.1% crystal violet in 95% ethanol). All conditions were measured in triplicate and each experiment was done in duplicate or triplicate.

Necrosis

To determine necrosis, media and cells were collected following drug treatment, centrifugated, and resuspended in 0.4% trypan blue (Invitrogen). Live and dead cells were counted on a hematocytometer.

Quantitative real-time PCR

Total RNA was extracted from cells (TRIzol, Invitrogen) as per manufacturer's directions. Total RNA (1.5 μg) was reversed transcribed with random hexamers and MMLV-RT. Power SYBR

Green PCR reactions were performed in triplicate for each sample and analyzed using the ABI Prism 7900HT sequence detection system. Data were normalized to TBP levels.

Primers

ARNT/HIF-1β

Forward: 5'-CTGCCAACCCCGAAATGACAT-3'

Reverse: 5'-GCCGCTTAATAGCCCTCTGG-3'

GLUT1/SLC2A1:

Forward: 5'-GGCCAAGAGTGTGCTAAAGAA-3'

Reverse: 5'-ACAGCGTTGATGCCAGACAG-3'

GLUT2/SLC2A2:

Forward: 5'-GTCACTGGGACCCTGGTTTTC-3'

Reverse: 5'-AGTTGTTGATAGCTTTTCGGTCA-3'

HK1:

Forward: 5'-TGGCCTATTACTTCACGGAGC-3'

Reverse: 5'-GGAATGGACCTTACGAATGTTGG-3'

HK2:

Forward: 5'-TTTGACCACATTGCCGAATGC-3'

Reverse: 5'-GGTCCATGAGACCAGGAAACT-3'

PAI-1/Serpine1:

Forward: 5'-CATCCCCCATCCTACGTGG-3'

Reverse: 5'-CCCCATAGGGTGAGAAAACCA-3'

PDK:

Forward: 5'-CTGTGATACGGATCAGAAACCG-3'

Reverse: 5’-TCCACCAAACAATAAAGAGTGCT-3’

PGK:

Forward: 5’-CCTGGGCGGAGCTAAAGTTG-3’

Reverse: 5’-TCTCAGCTTTGGACATTAGGTCT-3’

VEGF:

Forward: 5’-CAACATCACCATGCAGATTATGC-3’

Reverse: 5’-CCCACAGGGATTTTCTTGTCTT-3’

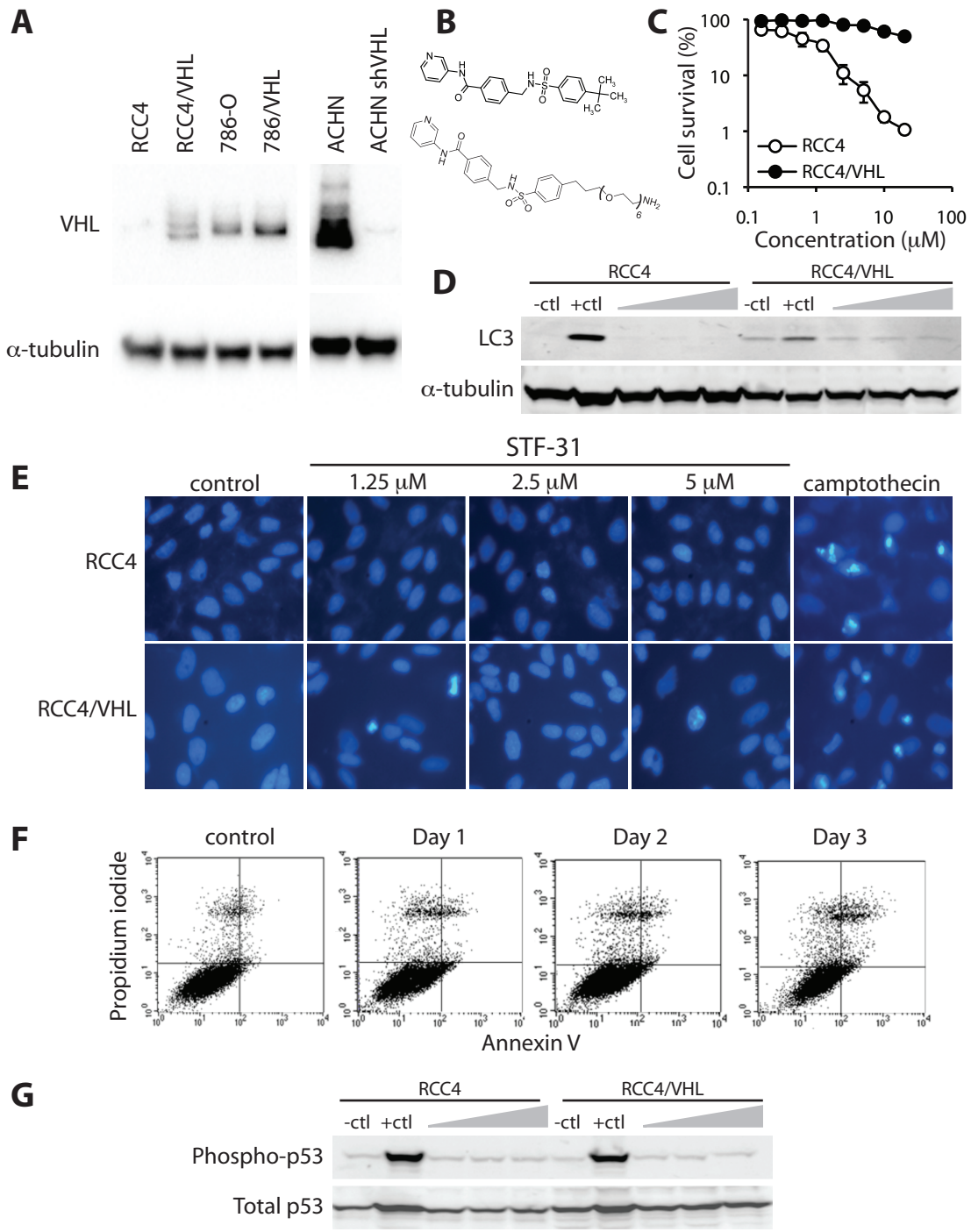

Fig. S1. STF-31 does not induce autophagy, apoptosis, or DNA damage.
(A) Western blot of VHL in RCC4, RCC4/VHL, 786-O, 786/VHL, ACHN, and ACHN shVHL cells. α-tubulin is used as a loading control. **(B)** Structure of STF-31 and a STF-31 analog with a linker. **(C)** XTT validation of STF-31, which was identified from chemical synthetic lethal screen of renal carcinoma cells that have lost VHL. Cells were treated for 4 days. All error bars represent the standard error of the mean (n=3)(*p<0.0005). **(D)** RCC4 and RCC4/VHL cells were treated with increasing concentrations of STF-31 (1.25, 2.5 and 5 μM), a negative control (DMSO) and a positive control (STF-62247). Cells were lysed and probed for LC3, a marker of autophagy, or α-tubulin (loading control). **(E)** RCC4 and RCC4/VHL cells were treated with vehicle, increasing concentrations of STF-31, and camptothecin. Cells were stained with DAPI and nuclear condensation was examined by fluorescence microscopy. **(F)** RCC4 cells were treated with STF-31 (5 μM) for the indicated time and stained with Annexin V and propidium iodide and subjected to FACS analysis. **(G)** RCC4 and RCC4/VHL cells were subjected to increasing concentrations of STF-31 (1.25, 2.5, and 5 μM), a negative control (DMSO), and a positive control (doxorubicin). Cells were lysed and subjected to Western blot with the indicated antibodies.

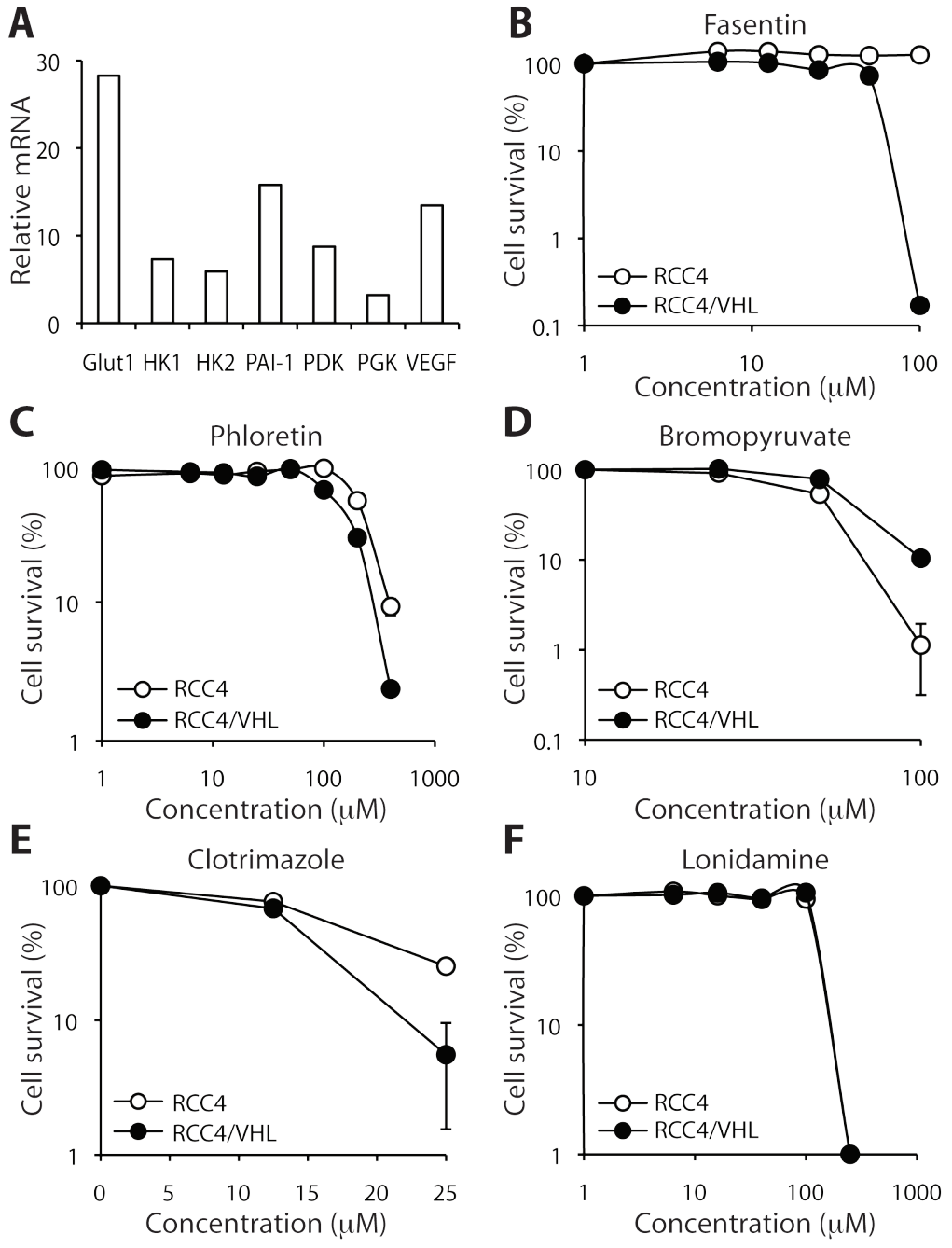

Fig. S2. Hexokinase and other glucose transport inhibitors do not affect VHL-deficient cell survival. **(A)** Relative mRNA expression levels for different genes involved in glucose metabolism in RCC4 cells relative to RCC4/VHL cells. **(B)** Clonogenic survival of RCC4 and RCC4/VHL cells grown in the presence of fasentin. **(C)** XTT assay of RCC4 and RCC4/VHL cells grown in the presence of phloretin. **(D)** Clonogenic survival of RCC4 and RCC4/VHL cells grown in the presence of bromopyruvate. **(E)** Clonogenic survival of RCC4 and RCC4/VHL cells grown in the presence of clotrimazole. **(F)** Clonogenic survival of RCC4 and RCC4/VHL cells grown in the presence of lonidamine. All error bars represent the standard error of the mean (n=3).

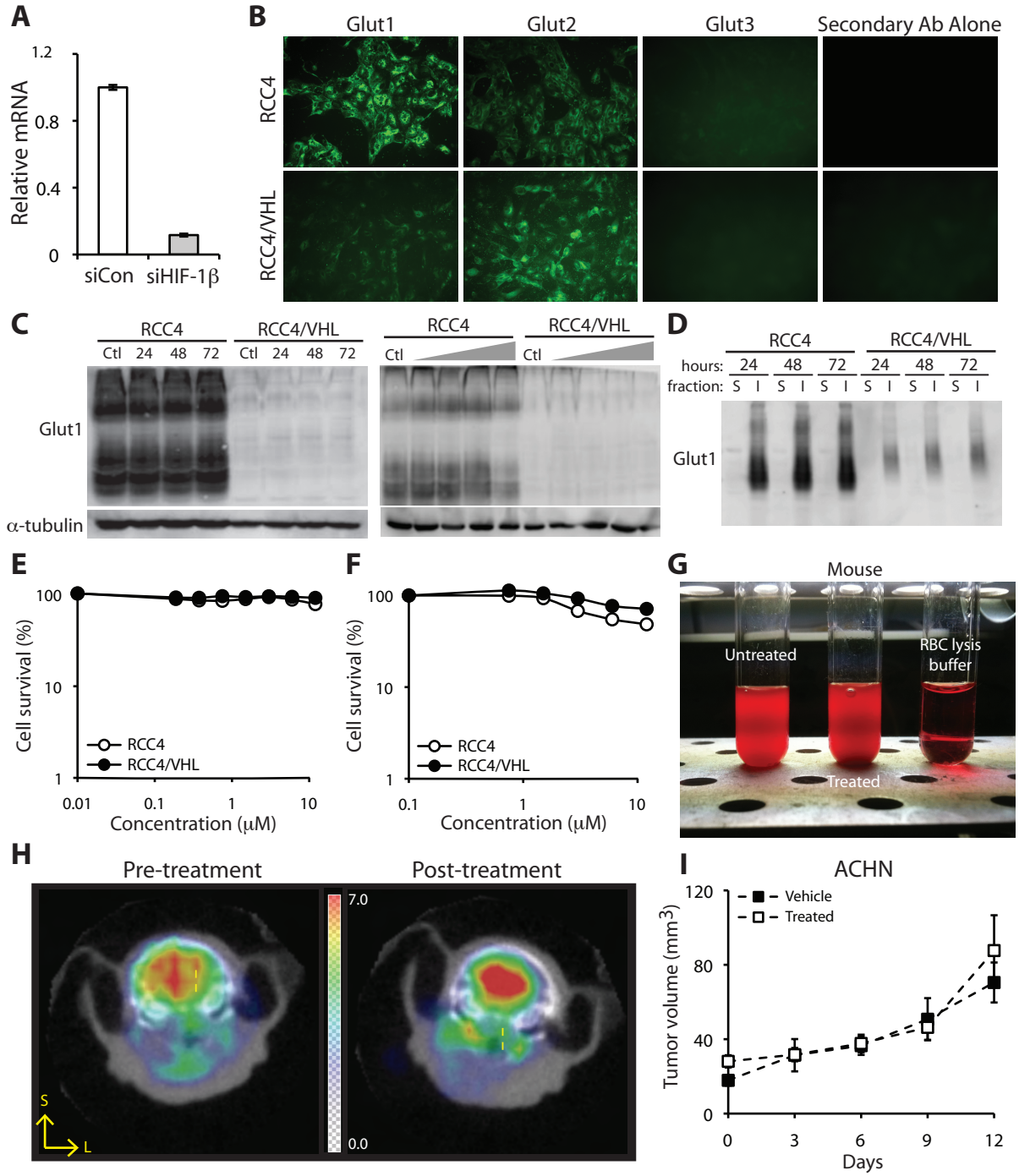

Fig. S3. GLUT1 protein levels are unaffected by STF-31.

(A) Quantitative RT-PCR confirming knockdown of HIF-1β in RCC4 cells, following transfection with indicated siRNA. **(B)** Immunofluorescence staining of GLUT1, GLUT2, or GLUT3 in RCC4 or RCC4/VHL cells, demonstrating high levels of GLUT1 in RCC4, high levels of GLUT2 in RCC4/VHL cells, and low expression of GLUT3 in both RCC4 and RCC4/VHL cells. **(C)** GLUT1 protein levels in cells treated with STF-31 (5 μM) for the indicated time (left panel). GLUT1 protein levels with increasing concentrations (1.25, 2.5, 5 or 10 μM) of STF-31. Cells were treated for 3 days (right panel). **(D)** RCC4 and RCC4/VHL cells were fractionated into soluble and insoluble fractions, following treatment with STF-31 for the indicated times. Fractions were then probed for GLUT1 protein. **(E)** XTT assay of RCC4 and RCC4/VHL cells grown in the presence of 2-deoxyglucose. **(F)** Clonogenic survival assay of RCC4 and RCC4/VHL cells grown in the presence of 2-deoxyglucose. **(G)** Representative photos of mouse red blood cells were treated with vehicle, soluble STF-31 analog (2.5 μM or 5 μM), or red blood cell lysis buffer. **(H)** Representative cranial cross section of a mouse prior to treatment (left) and following three daily i.p. injections with a soluble STF-31 analog (11.6 mg/kg)(right), overlaid with CT scan. **(I)** ACHN tumors with wild-type VHL are insensitive to soluble STF-31 analog treatment. ACHN cells were implanted subcutaneously into the flanks of immunocompromised mice. Once tumors reached an average of >20 mm3, mice were treated daily with 3-series (7.8 mg/kg) or vehicle. All error bars represent the standard error of the mean.

Abl	108	IRAK1	105
AMPK	92	JAK2	113
ASK1	129	JNK1α1	101
Aurora-A	108	MAPKAP-K2	87
Axl	87	MEK1	99
CaMKI	87	Met	105
CDK1/cyclinB	96	MKK4	128
CDK6/cyclinD3	105	MLK1	97
CHK1	114	MSK1	107
CK1γ1	85	mTOR	101
cKit(D816H)	99	NEK2	103
CSK	99	PAK2	97
c-RAF	103	PDK1	105
cSRC	104	PI3K	97
DAPK1	92	Pim-2	112
DYRK2	94	PKA	96
EphA1	102	PKBα	99
FGFR1	107	PKCδ	116
Flt3	111	Plk3	104
Fyn	91	ROCK-I	74
GSK3α	134	Rsk1	120
Hck	84	SAPK2a	127
IGF-1R	108	Syk	94
IKKα	102	Tie2	111
IR	121	TrkA	124

Table S1. STF-31 does not inhibit a broad range of kinases.

	Vehicle	3-series
WBC	3.8 ± 1.8	5.5 ± 0.6
RBC	8.2 ± 1.1	8.1 ± 0.7
Hemoglobin	12.3 ± 1.5	12.1 ± 1.1
Hematocrit	39.6 ± 4.7	38.4 ± 3.7
MCV	49.6 ± 1.0	47.6 ± 2.1
MCH	15.4 ± 0.1	15.1 ± 0.7
MCHC	31.0 ± 0.7	31.6 ± 0.2
Neutrophils	36.8 ± 14.3	66.2 ± 14.3
Lymphocytes	57.8 ± 16.8	26.2 ± 14.5
Monocytes	5.2 ± 3.4	6.0 ± 3.1
Absolute Neutrophils	1372.0 ± 902.5	3599.8 ± 702.8
Absolute Lymphocytes	2241.8 ± 1169.1	1475.6 ± 871.9
Absolute Monocytes	174.2 ± 105.6	322.0 ± 147.2

Table S2. Complete blood counts from control- and STF-31-treated mice (10 days).

SUPPLEMENTAL REFERENCES

S1. W. G. Kaelin, Jr., The concept of synthetic lethality in the context of anticancer therapy, *Nat Rev Cancer* **5**, 689-698 (2005).

S2. M. B. Atkins, D. E. Avigan, R. M. Bukowski, R. W. Childs, J. P. Dutcher, T. G. Eisen, R. A. Figlin, J. H. Finke, R. C. Flanigan, D. J. George, S. N. Goldberg, M. S. Gordon, O. Iliopoulos, W. G. Kaelin, Jr., W. M. Linehan, A. Lipton, R. J. Motzer, A. C. Novick, W. M. Stadler, B. T. Teh, J. C. Yang and L. King, Innovations and challenges in renal cancer: consensus statement from the first international conference, *Clin Cancer Res* **10**, 6277S-6281S (2004).

S3. T. Ahmad and T. Eisen, Kinase inhibition with BAY 43-9006 in renal cell carcinoma, *Clin Cancer Res* **10**, 6388S-6392S (2004).

S4. R. J. Motzer and P. Russo, Systemic therapy for renal cell carcinoma, *J Urol* **163**, 408-417 (2000).

S5. M. Zimmer, D. Doucette, N. Siddiqui and O. Iliopoulos, Inhibition of hypoxia-inducible factor is sufficient for growth suppression of VHL-/- tumors, *Mol Cancer Res* **2**, 89-95 (2004).

S6. R. J. DeBerardinis, J. J. Lum, G. Hatzivassiliou and C. B. Thompson, The biology of cancer: metabolic reprogramming fuels cell growth and proliferation, *Cell Metab* **7**, 11-20 (2008).

S7. G. L. Semenza, Hypoxia-inducible factor 1: master regulator of O2 homeostasis, *Curr Opin Genet Dev* **8**, 588-594 (1998).

S8. C. J. Hu, L. Y. Wang, L. A. Chodosh, B. Keith and M. C. Simon, Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation, *Mol Cell Biol* **23**, 9361-9374 (2003).

S9. H. Shim, Y. S. Chun, B. C. Lewis and C. V. Dang, A unique glucose-dependent apoptotic pathway induced by c-Myc, *Proc Natl Acad Sci U S A* **95**, 1511-1516 (1998).

S10. P. Sonveaux, F. Vegran, T. Schroeder, M. C. Wergin, J. Verrax, Z. N. Rabbani, C. J. De Saedeleer, K. M. Kennedy, C. Diepart, B. F. Jordan, M. J. Kelley, B. Gallez, M. L. Wahl, O. Feron and M. W. Dewhirst, Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice, *J Clin Invest* **118**, 3930-3942 (2008).

S11. R. Fukuda, H. Zhang, J. W. Kim, L. Shimoda, C. V. Dang and G. L. Semenza, HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells, *Cell* **129**, 111-122 (2007).

S12. H. Zhang, P. Gao, R. Fukuda, G. Kumar, B. Krishnamachary, K. I. Zeller, C. V. Dang and G. L. Semenza, HIF-1 Inhibits Mitochondrial Biogenesis and Cellular Respiration in VHL-Deficient Renal Cell Carcinoma by Repression of C-MYC Activity, *Cancer Cell* **11**, 407-420 (2007).

S13. Y. Yang, V. A. Valera, H. M. Padilla-Nash, C. Sourbier, C. D. Vocke, M. A. Vira, M. S. Abu-Asab, G. Bratslavsky, M. Tsokos, M. J. Merino, P. A. Pinto, R. Srinivasan, T. Ried, L. Neckers and W. M. Linehan, UOK 262 cell line, fumarate hydratase deficient (FH-

/FH-) hereditary leiomyomatosis renal cell carcinoma: in vitro and in vivo model of an aberrant energy metabolic pathway in human cancer, *Cancer Genet Cytogenet* **196**, 45-55 (2010).

S14. Y. H. Ko, P. L. Pedersen and J. F. Geschwind, Glucose catabolism in the rabbit VX2 tumor model for liver cancer: characterization and targeting hexokinase, *Cancer Lett* **173**, 83-91 (2001).

S15. J. Penso and R. Beitner, Clotrimazole and bifonazole detach hexokinase from mitochondria of melanoma cells, *Eur J Pharmacol* **342**, 113-117 (1998).

S16. A. Floridi, M. G. Paggi, M. L. Marcante, B. Silvestrini, A. Caputo and C. De Martino, Lonidamine, a selective inhibitor of aerobic glycolysis of murine tumor cells, *J Natl Cancer Inst* **66**, 497-499 (1981).

S17. G. V. Thomas, C. Tran, I. K. Mellinghoff, D. S. Welsbie, E. Chan, B. Fueger, J. Czernin and C. L. Sawyers, Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer, *Nat Med* **12**, 122-127 (2006).

S18. D. A. Mankoff, J. F. Eary, J. M. Link, M. Muzi, J. G. Rajendran, A. M. Spence and K. A. Krohn, Tumor-specific positron emission tomography imaging in patients: [18F] fluorodeoxyglucose and beyond, *Clin Cancer Res* **13**, 3460-3469 (2007).

S19. Z. Weihua, R. Tsan, W. C. Huang, Q. Wu, C. H. Chiu, I. J. Fidler and M. C. Hung, Survival of cancer cells is maintained by EGFR independent of its kinase activity, *Cancer Cell* **13**, 385-393 (2008).