Supplementary Information

Metastatic Colorectal Cancer Rewrites Metabolic Program Through a Glut3-YAP-dependent Signaling Circuit

Chih-Chia Kuo[1,2*], Hsiang-Hsi Ling[1,2*], Ming-Chen Chiang[1,2*], Chu-Hung Chung[1,2], Wen-Ying Lee[3], Cheng-Ying Chu[4], Yu-Chih Wu[1,5], Cheng-Hsun Chen[1], Yi-Wen Lai[1], I-Lin Tsai[1,2], Chia-Hsiung Cheng[1,2], Cheng-Wei Lin[1,2,5#]

[1]*Department of Biochemistry and Molecular Cell Biology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan.*

[2]*Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan.*

[3]*Department of Cytopathology, Chi Mei Medical Center, Tainan, Taiwan.*

[4]*TMU Research Center of Cancer Translational Medicine, Taipei Medical University, Taipei, Taiwan.*

[5]*Center for Cell Therapy and Regeneration Medicine, Taipei Medical University, Taipei, Taiwan.*

[#]**Corresponding author:** Cheng-Wei Lin; Department of Biochemistry and Molecular Biology, School of Medicine, College of Medicine, Taipei Medical University, 250 Wu-Xing Street, Taipei 110, Taiwan Email: cwlin@tmu.edu.tw; Phone: 886-2-27361661 ext 3160; Fax: 886-2-27356689

[*]Chih-Chia Kuo, Hsiang-Hsi Ling, and Ming-Chen Chiang contributed equally to this work

Supplementary figures & legends

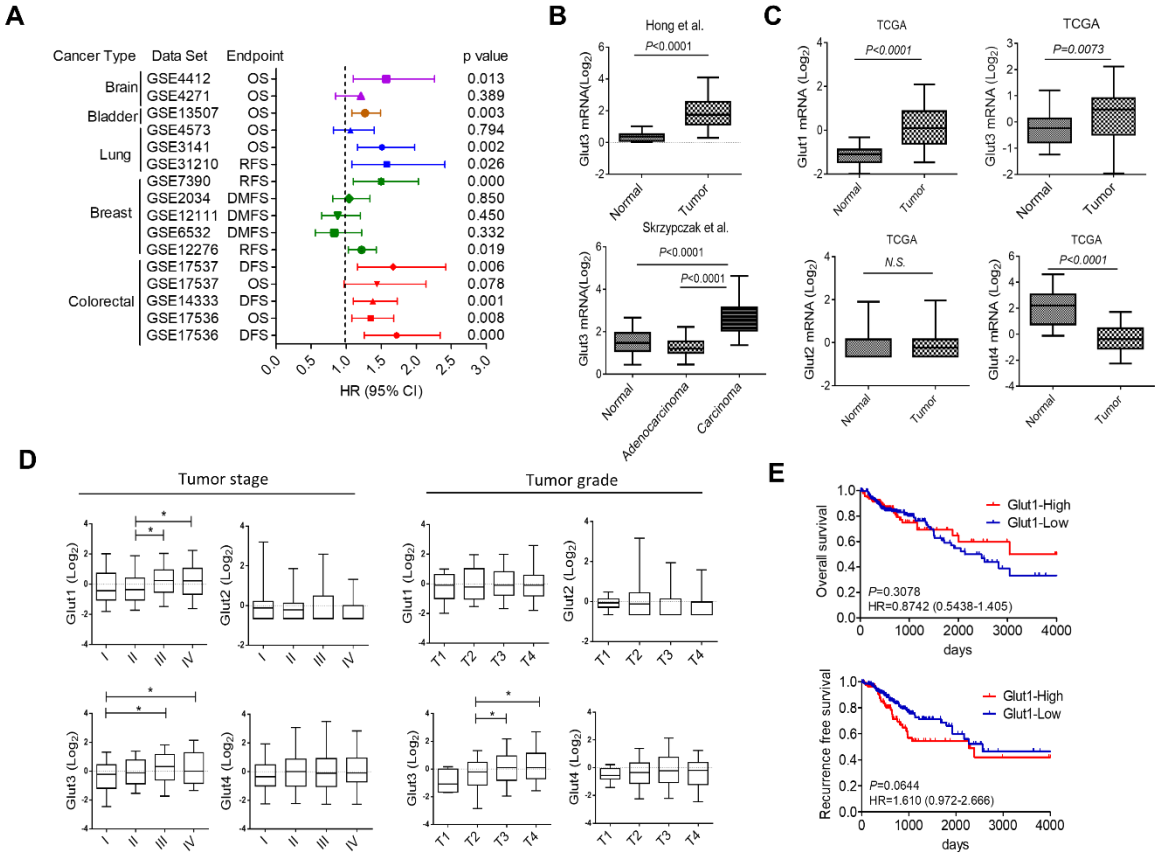

Figure S1. Clinicopathological correlation of Glut family in colorectal cancer patients. (A) Survival outcome of Glut3 in different cancer types. OS: overall survival; DFS: disease free survival; RFS: recurrence free survival; DMFS: distant metastasis free survival. HR: hazard ratio. (B) Comparison of Glut3 expression in normal and cancerous tissues in Hong colorectal and Skrzypczak datasets by using Oncomine database. P value was analyzed by unpaired *t*-test. (C) Analysis of Glut 1-4 isoforms in normal and cancerous tissues by using TCGA database. P value was analyzed by unpaired *t*-test. N.S. no significant difference. (D) Clinicopathological correlation of Glut family and tumor stage and tumor grade in colorectal cancer patients. P value was analyzed by unpaired *t*-test. (E) Kaplan-Meier analysis shows no correlation between Glut1 and survival prognosis in colorectal cancer patients by using TCGA database.

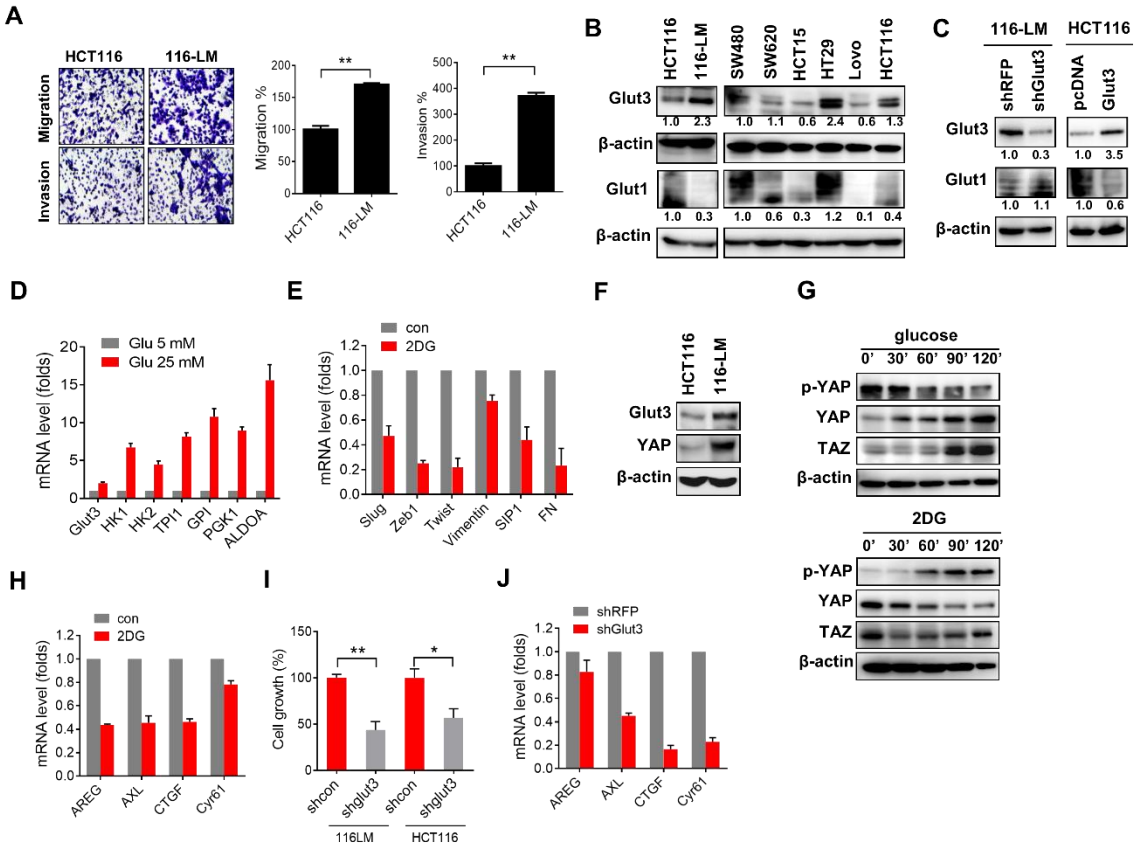

Figure S2. Upregulations of Glut3 and the YAP signaling in 116-LM cells. (A) Transwell analysis of tumor migration and invasion in HCT116 and 116-LM cells, Representative images are presented (left panel) and the relative percentage of migratory and invasive cells were counted (right panel). ** P value<0.01 was analyzed by unpaired *t*-test. (B) Western blot analysis of Glut3 and Glut1 in HCT116, 116-LM, and colorectal cancer cell lines. (C) Western blot analysis of Glut3 and Glut1 in 116-LM/shRFP and 116-LM/shGlut3 (left panel) and HCT116/mock and HCT116/Glut3 (right panel) cells. Quantifications of Western blots were performed using ImageJ software. (D) 116-LM cells were exposed to low (5 mM) or high (25 mM) concentrations of glucose for 48 h, and the expression of glycolysis-related genes was analyzed by quantitative PCR. (E) Blockage of glycolysis by 2-deoxyglucose (2DG; 25 mM) suppresses expression of EMT genes. (F) Western blot analysis of Glut3 and YAP in HCT116 and 116LM cells. (G) 116-LM cells were

treated with glucose (25 mM) or 2-deoxyglucose (2DG; 25 mM) for different time intervals, and protein expression of YAP/TAZ was analyzed by Western blot. (H) 116-LM cells were treated with 25 mM 2DG for 24 h, and the expression of YAP downstream targets was analyzed by quantitative PCR. (I) Trypan blue exclusion analysis of cellular growth in Glut3-silencing HCT116 and 116-LM cells, respectively. * P value <0.05; ** P value <0.01. (J) Quantitative PCR analysis of YAP downstream genes in Glut3-silenced 116-LM cells.

Figure S3. Validation of Glut3 knockout in 116LM cells. (A) Western blot analysis of Glut3 protein level in CRISPR/Cas9-mediated Glut3 knockout clones. Asterisks denote homozygous deletion of Glut3 which confirmed by DNA sequencing. (B) Alignment of Glut3 coding sequences in parental and Glut3 knockout 116LM cells. Asterisks denote stop codon.

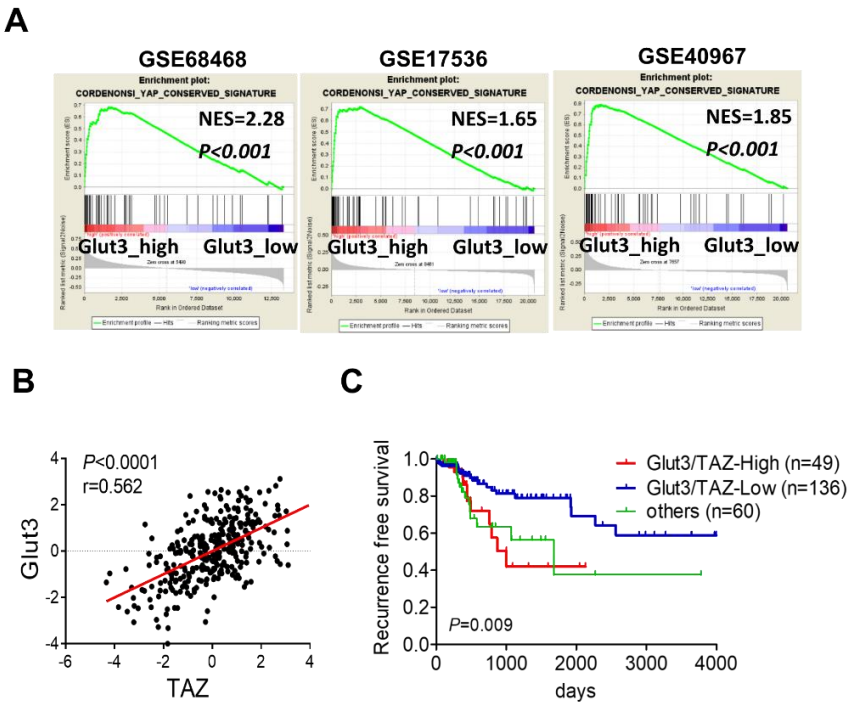

Figure S4. Glut3 associated with YAP signature in colorectal cancer. (A) GSEA of the YAP signature in colon cancer patients (GSE68468; GSE17536; GSE40967) stratified by Glut3 expression. NES; normalized enrichment score. (B) Positive correlation of Glut3 and TAZ mRNA levels in TCGA colon cancer patients. The correlation coefficient was analyzed by Pearson's correlation. (C) Kaplan-Meier curve analysis of survival probability of colon cancer patients stratified by both Glut3/TAZ high expression and otherwise. P value was analyzed by log-rank Mantel–Cox.

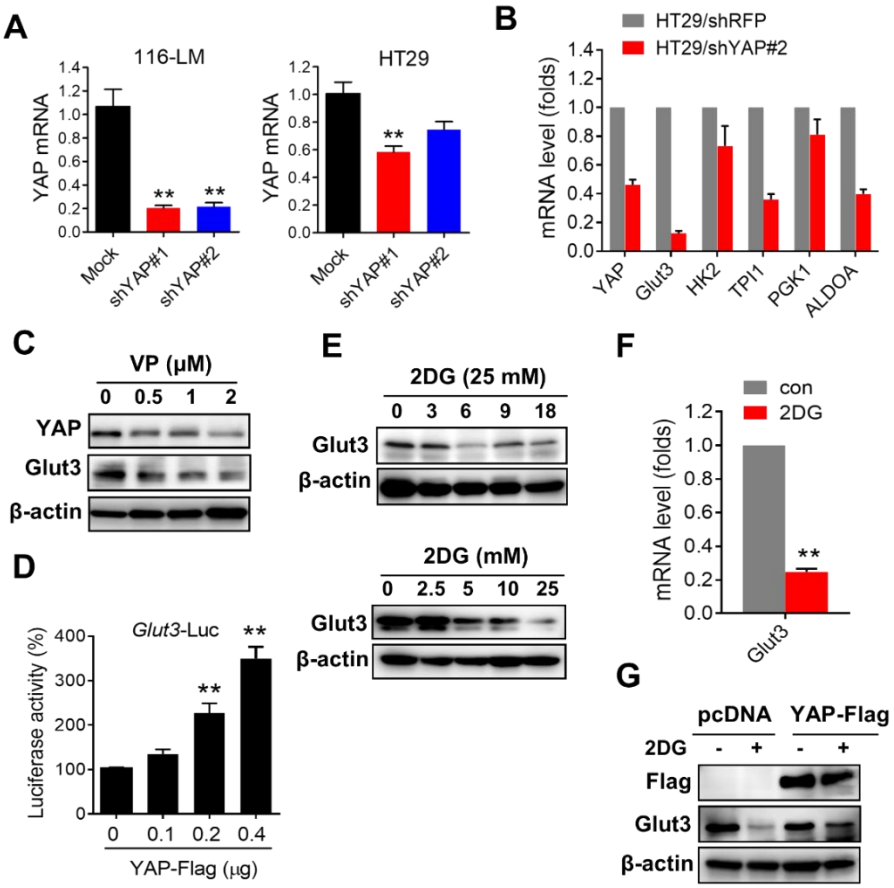

Figure S5. YAP regulates Glut3 expression. (A) Real-time PCR analysis of YAP mRNA in YAP knockdown 116-LM and HT29 cells. ** *P* value <0.01 was analyzed by unpaired *t*-test. (B) Real-time PCR analysis of glycolytic genes in YAP knockdown HT29 cells. (C) Cells were treated with different concentrations of vertporfin (VP) for 24 h. Expressions of YAP and Glut3 were measured by Western blot. (D) Luciferase reporter assay of Glut3 promoter activity in pcDNA-YAP-flag transfected HEK293 cells. ** *P* value <0.01 was analyzed by unpaired *t*-test. (E and F) Inhibition of glycolysis by 2-DG suppressed Glut3 expression. (E) Time- and concentration-dependent analyses of Glut3 protein level in the presence of 2DG in 116-LM cells. (F) Real-time quantitative PCR analysis of Glut3 mRNA in 116-LM cells exposure to 2DG (25 mM) for 24 h. ** *P* value <0.01 was analyzed by unpaired *t*-test. (G) Overexpression of YAP restored Glut3 level in 2-DG (25 mM) treated 116-LM cells.

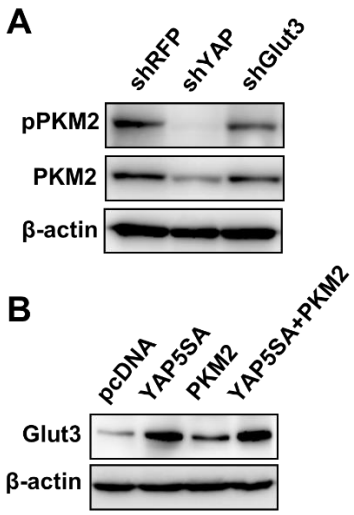

Figure S6. Effects of YAP and Glut3 inhibitions on PKM2. (A) Western blot analysis of phospho- and total PKM2 protein levels in YAP and Glut3 knockdown 116-LM cells. (B) Western blot analysis of Glut3 level in YAP5SA, PKM2, YAP5SA+PKM2 overexpressed HCT116 cells.

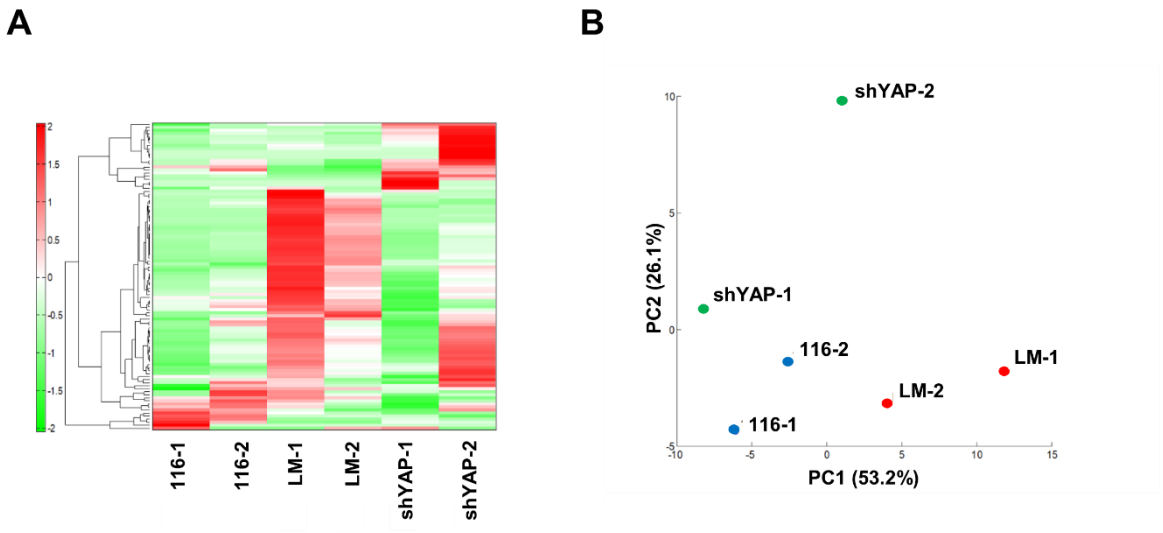

Figure S7. Statistical analysis of overall metabolomics profiles in colon cancer cells.

(A) Heat map representation of metabolome profiles analyzed by hierarchical clustering analysis. (B) Principal component analysis was conducted in order to compare the overall metabolomic profiles in colon cancer cells. PC1 vs PC2 plot based on the result of principal component analysis.

Table S1. Clinicopathological correlation of Glut3 (*SLC2A3*) in colorectal cancer patients from TCGA COADREAD dataset

Features	Number of patients	SLC2A3 expression		P-value
		Low	High	
Age(years)				
<65	138	94(68.12%)	44(31.88%)	0.4712
>65	188	135(71.81%)	53(28.19%)	
Gender				
Male	147	108 (73.47%)	39(26.53%)	0.1748
Female	173	115(66.47%)	58(33.53%)	
Lymphatic invasion				
NO	196	144(73.47%)	52(26.53%)	0.003***
YES	88	49(55.68%)	39(44.32%)	
Venous invasion				
NO	214	154(71.96%)	60(28.04%)	0.0781
YES	63	38(60.32%)	25(39.68%)	
Tumor grade				
T1,T2	56	47(83.93%)	9(16.07%)	0.0102*
T3,T4	263	175(66.54%)	88(33.46%)	
Lymph node metastasis				
N0	191	146(76.44%)	45(23.56%)	0.0012***
N1,N2	128	76(59.37%)	52(40.63%)	
Distant metastasis				
M0	212	151(71.23%)	61(28.77%)	0.1128
M1	44	26(59.06%)	18(40.91%)	
Tumor stage				
I,II	178	134(75.28)	44(24.72%)	0.0066***
III,IV	133	81(60.90%)	52(39.10%)	

*P value <0.05; *** P value <0.001, as determined by chi-square test.

Table S2 Absolute concentration of cellular metabolites

HMT DB	Concentration (pmol/10^6 cells)					
Sample	**116-1**	**116-2**	**LM-1**	**LM-2**	**KD-1**	**KD-2**
ADP-ribose	1.0	1.3	1.3	0.9	0.9	1.7
Glucose 6-phosphate	19	50	39	39	59	103
Ribose 5-phosphate	4.0	5.5	2.2	2.1	19	17
Dihydroxyacetone phosphate	39	43	12	3.9	41	47
GMP	17	22	29	22	19	30
AMP	33	35	87	73	45	44
GDP	49	60	172	132	41	73
ADP	301	308	837	648	234	353
GTP	497	662	1,459	1,062	430	855
ATP	2,315	3,071	6,403	4,565	1,636	3,258
Glycerol 3-phosphate	57	118	101	74	50	67
Gly	10,011	10,787	17,648	12,697	6,885	12,512
Ala	8,495	10,241	15,254	11,274	8,006	14,357
Ser	4,576	4,931	4,164	3,581	2,629	4,116
Pro	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Val	2,575	3,328	4,327	3,410	2,303	4,672
Thr	6,990	8,455	13,578	10,364	5,009	8,551
Cys	27	47	55	35	88	43
Leu	2,279	3,077	4,043	3,141	2,216	4,482
Ile	2,582	3,320	4,399	3,523	2,471	4,654
Asn	429	536	1,665	1,118	304	685
Asp	1,918	2,152	6,389	5,064	1,451	2,716
Gln	17,210	21,539	29,271	23,149	18,824	31,965
Lys	745	1,126	1,112	792	1,332	3,272
Glu	18,939	22,527	34,328	26,568	14,706	23,325
Met	337	407	570	756	295	532
His	750	914	1,314	957	650	1,305
Phe	1,231	1,631	2,361	1,793	1,172	2,327
Arg	206	318	277	214	527	1,315
Tyr	1,222	1,543	2,436	1,834	1,112	2,278
Trp	231	303	436	339	245	466
Glutathione (GSSG)	735	761	1,397	1,237	1,082	4,512
Glutathione (GSH)	9,018	11,365	22,116	16,048	8,111	7,336
Adenylate Energy Charge	0.9	0.9	0.9	0.9	0.9	0.9

Total Adenylate	2,649	3,414	7,327	5,287	1,915	3,655
Guanylate Energy Charge	0.9	0.9	0.9	0.9	0.9	0.9
Total Guanylate	563	744	1,659	1,216	490	959
GSH/GSSG	12	15	16	13	7.5	1.6
Total Glutathione	10,489	12,887	24,910	18,521	10,275	16,360
Glycerol 3-phosphate/DHAP	1.5	2.8	8.8	19	1.2	1.4
Total Amino Acids	80,752	97,183	143,630	110,609	70,226	123,575
Total Essential Amino Acids	17,720	22,562	32,142	25,076	15,694	30,262
Total Non-essential Amino Acids	63,032	74,621	111,487	85,533	54,532	93,313
Total Glucogenic Amino Acids	77,729	92,980	138,475	106,675	66,678	115,821
Total Ketogenic Amino Acids	15,280	19,457	28,367	21,787	13,557	26,030
Total BCAA	7,436	9,726	12,770	10,075	6,990	13,807
Total Aromatic Amino Acids	2,684	3,477	5,234	3,967	2,529	5,071
G6P/R5P	4.8	9.0	17	19	3.1	5.9

N.D. indicates not detected

Table S3. Metabolic Parameters and Their Relevance to Cell Metabolism and Physiology (Human Metabolome Technologies, Inc.).

Metabolic parameter	Equation	Relevance
Adenylate Energy Change	([ATP])+0.5*[ADP])/([ATP]+[ADP]+[AMP])	Energy status
Total Adenylate	[ATP]+[ADP]+[AMP]	Purine synthesis/ degradation
Guanylate Energy Charge	([GTP])+0.5*[GDP])/([GTP]+[GDP]+[GMP])	Energy status
Total Guanylate	[GTP]+[GDP]+[GMP]	Purine synthesis/ degradation
Glutathione Redox Ratio	[GSH]/[GSSG]	Oxidative stress
Total Glutathione	[GSH]/2*[GSSG]	Glutathione synthesis/ degradation
Glycerol 3-phosphate/DHAP	[Glycerol 3-phosphate]/[DHAP]	Redoxpotential
Total Amino Acids	Sum of all 20-proteinogenic amino acid	Amino acid synthesis/ degradation, influx/efflux
Total Essential Amino Acids	Sum of [His],[Ile],[Leu],[Lys],[Met],[Phe], [Thr],[Trp] and [Val]	Essential Amino acid degradation, influx/efflux
Total non-Essential Amino Acids	Sum of [Ala],[Arg],[Asn],[Asp],[Cys],[Gln], [Glu],[Pro],[Ser] and [Tyr]	Non-Essential Amino acid synthesis /degradation, influx/efflux
Total Glucogenic Amino Acids	Sum of all 20-proteinogenic amino acids except [Leu] and [Lys]	Glucogenic Amino acid degradation, influx/efflux, Gluconeogenesis
Glucose 6-phosphate / Ribose 5-phosphate	[Glucose 6-phosphate] / [Ribose 5-phosphate]	Glycolysis, Pentose phosphate pathway

Table S4: List of shRNA target sequences and gRNA sequences

Name	Clone ID	Sequences 5'→3'
shYAP#1	TRCN0000107265	CCCAGTTAAATGTTCACCAAT
shYAP#2	TRCN0000107266	GCCACCAAGCTAGATAAAGAA
shPKM2#1	TRCN0000195581	CAACGCTTGTAGAACTCACTC
shPKM2#2	TRCN0000195588	GTTCGGAGGTTTGATGAAATC
shRFP	TRCN0000072203	CGCGTGATGAACTTCGAGGAC

Table S5: List of primer sequences for real-time PCR and ChIP

Genes		Sequences 5'→3'
18srRNA	Forward	GCAATTATTCCCCATGAACG
	Reverse	GGGACTTAATCAACGCAAGC
ABCG2	Forward	GAAGTCCCTGAGAAACTCCT
	Reverse	CACAGAATTCATCACAAACG
ALDOA	Forward	CAGCTCCCGGACTGACTG
	Reverse	ATTCCACGGGCTAGAGGAG
Amphregulin	Forward	GGGAGTGAGATTTCCCCTGT
	Reverse	AGCCAGGTATTTGTGGTTCG
BMI	Forward	TGAAGATAGAGGAGAGGTTGC
	Reverse	CTGCTGGGCATCGTAAGTAT
E-cadherin	Forward	GGA ACT ATG AAA AGT GGG CTT G
	Reverse	AAA TTG CCA GGC TCA ATG AC
FN1	Forward	GAACTATGATGCCGACCAGAA
	Reverse	GGTTGTGCAGATTTCCTCGT
Glut1	Forward	CGG GCC AAG AGT GTG CTA AA
	Reverse	TGA CGA TAC CGG AGC CAA TG
Glut2	Forward	GCT GCT CAA CTA ATC ACC ATG C
	Reverse	TGG TCC CAA TTT TGA AAA CCC C
Glut3	Forward	TTG CTC TTC CCC TCC GCT GC
	Reverse	ACC GTG TGC CTG CCC TTC AA
Glut4	Forward	ATC CTT GGA CGA TTC CTC ATT GG
	Reverse	CAG GTG AGT GGG AGC AAT CT
GPI	Forward	GAAGTGCTGGTCCATCCAGT
	Reverse	AACATGTTCGAGTTCTGGGA
HK1	Forward	CACCTGTGAGGTTGGACTCA
	Reverse	CCACCATCTCCACGTTCTTC
HK2	Forward	AAGCCCTTTCTCCATCTCCT

	Reverse	CTTCTTCACGGAGCTCAACC
Nanog	Forward	GTCCCGGTCAAGAAACAGAA
	Reverse	TGCGTCACACCATTGCTATT
Oct3/4	Forward	ATTCAGCCAAACGACCATCT
	Reverse	ACACTCGGACCACATCCTTC
PGK1	Forward	CTTGGGACAGCAGCCTTAAT
	Reverse	CAAGCTGGACGTTAAAGGGA
SNAI1	Forward	GCTGCAGGACTCTAATCCAGA
	Reverse	ATCTCCGGAGGTGGGATG
SOX2	Forward	ATGGGTTCGGTGGTCAAGT
	Reverse	ATGTGTGAGAGGGGCAGTGT
TPI1	Forward	GGCGAAGTCGATATAGGCAG
	Reverse	AGTTCTTCGTTGGGGGAAAC
TWIST	Forward	GGCATCACTATGGACTTTCTCTATT
	Reverse	GGCCAGTTTGATCCCAGTATT
Vimentin	Forward	TGTCCAAATCGATGTGGATGTTTC
	Reverse	TTGTACCATTCTTCTGCCTCCTG
YAP	Forward	CGCTCTTCAACGCCGTCA
	Reverse	AGTACTGGCCTGTCGGGAGT
Zeb1	Forward	GGGAGGAGCAGTGAAAGAGA
	Reverse	TTTCTTGCCCTTCCTTTCTG
Glut3_ChIP (-1164~-1155)	Forward	CACCAGCTTCTTGGAGAGTACC
	Reverse	GGCATTTGCCATATCACCCAT
Glut3_ChIP (-127~-118)	Forward	GAGAGAGTGGAAGGATGTGGTT
	Reverse	AATCTCCGCAAAGGGTGGAG
Glut3_ChIP (-1791~-1782)	Forward	ACTCTCAACCTGGAACCACTC
	Reverse	AGTTATGGACTGTGAGTTTCTTGA