Targeting the Warburg effect in cancer cells through ENO1 knockdown rescues oxidative phosphorylation and induces growth arrest

Supplementary Materials

Supplementary Figure S1: ENO1 gene silencing in a panel of human cancer cells. Western blot analysis of ENO1 (left panels), all enolase isoforms (assessed by using a pan-enolase polyclonal antibody, middle left panels) and c-Myc expression (middle panels) in CFPAC-1 (A), PT45 (B), T3M4 (C), MDA-MB-231 (D) and NCI-H441 (E) non-transduced (parental) and stably transduced with an shRNA targeting ENO1 (shENO1) or a scrambled shRNA (shCTRL) as a control. β-actin was used as loading control. qRT-PCR analysis (middle right panels) of ENO1, ENO2, ENO3 and c-Myc and analysis of enolase activity measured as the rate of NADH oxidation (right panels) in CFPAC-1 (A), PT45 (B), T3M4 (C), MDA-MB-231 (D) and NCI-H441 (E) shCTRL and shENO1 cells. Results are the mean of three independent experiments ± SEM of triplicates. ***p < 0.001 relative to shCTRL. ENO1 knockdown also affects ENO2 and ENO3 expression, most probably through post-translational mechanisms, as ENO2 and ENO3 mRNA was unaffected. The expression of c-Myc, which is transcriptionally repressed by the ENO1 alternative transcript MBP-1, was not influenced by ENO1 knockdown, ruling out any functional effects related to this gene in ENO1-silenced cells.
Supplementary Figure S2: The polyol and pentose phosphate pathways increase the concentration of intracellular reactive oxygen species (ROS) in ENO1-silenced cells. (A) Heat map of differentially-expressed proteins in shENO1 compared to shCTRL CFPAC-1 cells. Based on the spectra count label-free quantitation approach, LC-MS/MS analysis identified 32 up-regulated (red) and 28 down-regulated (green) proteins. Proteins are classified according to their function: cell cycle/signaling, protein transport, protein biosynthesis, cell adhesion/migration and others/unknown. See also Figure 1A for other identified proteins. (B–C) Analysis of aldose reductase (ALDR) activity measured as the rate of NADPH oxidation (B), and NADPH oxidase activity assessed by the isoluminol-chemiluminescence assay (C) in shCTRL and shENO1 PT45 and T3M4 cell lines. Chemiluminescence was expressed as relative luminescence unit (RLU)/mg cell proteins. (D–E) Analysis of ROS concentration measured by DCFDA-AM assay (D) and of [1-14C] glucose flux through the Pentose Phosphate Pathway (PPP) assessed through 14CO2 release (E) after selective inhibition of mitochondrial chain (rotenone), NADPH oxidase (apocynin) and ALDR (zopolrestat) in shCTRL and shENO1 PT45 (left panels) and T3M4 (right panels) cell lines. (F) Analysis of PPP activity, as described above, after selective inhibition of the PPP (DHEA) in CFPAC-1 (left panels), MDA-MB-231 (middle panel) and NCI-H441 (right panel) cell lines. All the graphs illustrate the mean result of three independent experiments ± SEM *p < 0.05; **p < 0.01; ***p < 0.001 relative to shCTRL.
Supplementary Figure S3: mRNA expression analysis of mass spectrometry identified proteins. (A–B) Transcript analysis of proteins identified as up-regulated or down-regulated through semi-quantitative tandem mass spectrometry analysis in CFPAC-1 (red), PT45 (orange), T3M4 (purple), MDA-MB-231 (blue) and NCI-H441 (green) shENO1 compared to shCTRL (white) cells. Genes were divided according to their function: glycolysis-related (A) and catabolic pathways (B). Results are the mean ± SEM of triplicates. *p < 0.05; **p < 0.01; ***p < 0.001 relative to shCTRL-transduced cells. See Table S2 and S3 for extended gene names.
Supplementary Figure S4: ENO1 silencing enhances catabolic pathway adaptations. (A–C) Cells were exposed to [1-\(^{14}\)C] palmitic acid and total palmitate oxidation (sum of \(^{14}\)C-acid soluble metabolites and \(^{14}\)CO\(_2\) production) of palmitic acid in shCTRL and shENO1 PT45 and T3M4 cell lines (A) was measured. \(^{14}\)C-acid soluble metabolites production (B) and \(^{14}\)CO\(_2\) production (C) were measured in shCTRL and shENO1 CFPAC-1, PT45, T3M4, MDA-MB-231 and NCI-H441 cell lines. (D–E) Analysis of phenylalanine (D) and acetoacetate (E) concentration in shCTRL and shENO1 PT45 and T3M4 cell lines. (F) The TCA cycle rate was evaluated measuring CO\(_2\) emission after radiolabelling cells with [1-\(^{14}\)C] acetylcoenzyme A in shCTRL and shENO1 PT45 and T3M4 cell lines. All the graphs illustrate the mean result of three independent experiments ± SEM *\(p < 0.05\); **\(p < 0.01\); ***\(p < 0.001\) relative to shCTRL. (G) Schematic diagram of ENO1 silencing-induced catabolic pathway adaptations.
Supplementary Figure S5: ENO1 knockdown promotes oxidative phosphorylation. (A–C) Analysis of glutaminase (GLS) (A), glutamine amidoprophosyltransferase (GPAT) (B) and carbamoyl phosphate synthetase II (CPSII) (C) activity in shCTRL and shENO1 PT45 and T3M4 cell lines. (D) Analysis of mitochondrial respiratory chain complex I and complexes II–IV activity in shCTRL and shENO1 PT45 and T3M4 cells, expressed as nmol NAD+/min/mg mitochondrial protein for complex I, nmol Cyt c reduced/min/mg mitochondrial protein for complexes II–III and nmol Cyt c oxidized/min/mg mitochondrial protein for complex IV. (E) Analysis of ATP production in shCTRL and shENO1 PT45 and T3M4 cell lines. All the graphs illustrate the mean result of three independent experiments ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 relative to shCTRL. (F) Transcript analysis of proteins involved in catabolic pathways/mitochondrial biogenesis in CFPAC-1 (red), PT45 (orange), T3M4 (purple), MDA-MB-231 (blue) and NCI-H441 (green) shENO1 compared to shCTRL (white) cells. Results are the mean ± SEM of triplicates. *p < 0.05; **p < 0.01; ***p < 0.001 relative to shCTRL-transduced cells.
Supplementary Figure S6: Analysis of ENO1-silenced cell metabolic adaptations under low glucose conditions. (A–F)

Analysis of aldose reductase (ALDR) (A), glutaminase (GLS) (B), glutamine amidophosphoribosyltransferase (GPAT) (C), carbamoyl phosphate synthetase II (CPSII) (D), mitochondrial respiratory chain complex I and complexes II-IV (E) activity and ATP production (F) in shCTRL (white bars) or shENO1 (black bars) CFPAC-1 cells after 24 h culture in low (1 g/L) or high (4.5 g/L) glucose media. Results are mean of three independent experiments ± SEM.
Supplementary Figure S7: ENO1 silencing impairs cancer cell growth. (A) Cell proliferation analysis of PT45 (left panel) and T3M4 (right panel) after shCTRL or shENO1 infection. Cells were starved and counted every 24 hr after serum replenishment. ***p < 0.001 was assessed by two-way ANOVA with Sidak’s post hoc test. (B) Cell survival assessed by MTT assay. Cells were starved and MTT solution was added 48 hr after serum replenishment. OD values were measured at 570 nm. (C) Colony forming assay in soft agar. Cells were plated in 0.45% agarose overlaying a 0.9% agar layer. Colonies were counted by optical microscope after 3 weeks. T3M4 cell line was unable to form colonies in soft agar. (D–E) Flow cytometry cell cycle analysis of serum-starved shCTRL and shENO1 NCI-H441 (D) and MDA-MB-231 (E) cells at the indicated time points after serum replenishment. Data are expressed as the percentage of cells at each phase. (F) Senescence-associated β-galactosidase staining. Senescent CFPAC-1 and T3M4 cells were colored blue upon X-gal staining at pH 6. Parental and shCTRL CFPAC-1 cells showed positive β-galactosidase staining even in the absence of a senescent morphology. One representative out of three independent experiments is shown. Results are mean of three independent experiments ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 relative to shCTRL.
Supplementary Figure S8: Analysis of ENO1-silenced cell metabolic adaptations under antioxidant treatment.

(A) Analysis of aldose reductase (ALDR) activity measured through the rate of NADPH oxidation in shCTRL (white bars) and shENO1 (black bars) cell lines after 24 h treatment with NAC or TROLOX-C. (B) Oxidation of palmitic acid in CFPAC-1, MDA-MB-231 and NCI-H441 cell lines transduced with shCTRL (white bars) or shENO1 (black bars) after 24 h treatment with antioxidants. Results are mean of three independent experiments ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 relative to untreated cells.
Supplementary Figure S9: Analysis of ENO1-silenced cell metabolic adaptations under antioxidant treatment. (A–C) Analysis of mitochondrial respiratory chain complex I and complexes II–IV activity in CFPAC-1 (A), MDA-MB-231 (B) and NCI-H441 (C) cell lines after 24 h treatment with NAC or TROLOX-C. Activity is expressed as nmol NAD+/min/mg mitochondrial protein for complex I; nmol Cyt c reduced/min/mg mitochondrial protein for complexes II–III and nmol Cyt c oxidized/min/mg mitochondrial protein for complex IV. (D) The TCA cycle rate was evaluated measuring CO₂ emission after radiolabelling cells with [1-¹⁴C] acetylcoenzyme A in CFPAC-1, MDA-MB-231 and NCI-H441 cell lines treated for 24 h with antioxidants. TCA cycle activity is expressed as pmol CO₂/min/mg protein. Results are mean of three independent experiments ± SEM.
**Supplementary Figure S10:** (A) Effects of GLS inhibitor BPTES (bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl) ethyl sulfide) on shENO1 cells after 96 h treatment as assessed by MTT assay. (B) Dose-dependent analysis of ENO1 enzymatic activity in CFPAC-1 cells after 6 h treatment with PhAH. Results are mean of three independent experiments ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 relative to untreated. (C) Pearson’s correlation analysis between CFPAC-1 cell enolase enzymatic activity shown in panel B and the percentage of viable cells compared to the untreated control after treatment with different doses of PhAH (range 6.25 – 100 μM, shown in Figure 6G) for the indicated time.
**Supplementary Table S1: Complete MS analysis and criteria (Excel file)**

**Supplementary Table S2: Upregulated proteins**

| Protein name                                                                 | Functional annotation          | Accession^a | Parental | shCTRL | shENO1 | Relative Difference (%)^b |
|------------------------------------------------------------------------------|--------------------------------|-------------|----------|--------|--------|--------------------------|
| aldo-keto reductase family 1, member C2 (AKR1C2)                             | metabolism                     | 4503285     | 14       | 6      | 29     | 131                      |
| aldo-keto reductase family 1, member C1 (AKR1C1)                             | metabolism                     | 5453543     | 2        | 2      | 11     | 138                      |
| nicotinamide N-methyltransferase (NNMT)                                      | metabolism                     | 5453790     | 2        | 2      | 6      | 100                      |
| casein kinase II alpha 1 subunit isoform a (CSNK2A1)                        | metabolism/cell cycle           | 29570791    | 1        | 1      | 5      | 133                      |
| aldo-keto reductase family 1, member B1 (AKR1B1)                             | metabolism                     | 4502049     | 0        | 0      | 5      | 200                      |
| inorganic pyrophosphatase 2 isoform 1 precursor (PPA2)                       | metabolism                     | 29171702    | 1        | 1      | 4      | 120                      |
| ilvB (bacterial acetolactate synthase)-like (ILVBL)                          | metabolism                     | 21361361    | 0        | 0      | 3      | 200                      |
| carbonyl reductase 1 (CBR1)                                                  | metabolism/antioxidant          | 4502599     | 4        | 4      | 12     | 100                      |
| nitric oxide synthase interacting protein (NOSIP)                            | NO metabolism                  | 7705716     | 0        | 0      | 3      | 200                      |
| hexokinase 2 (HK2)                                                           | glycolysis                     | 15553127    | 0        | 0      | 5      | 200                      |
| fumarylacetoacetase (FAH)                                                    | tyrosine catabolism             | 4557587     | 0        | 0      | 3      | 200                      |
| albumin preproprotein (ALB)                                                  | starvation response             | 4502027     | 0        | 0      | 6      | 200                      |
| fatty acid binding protein 5 (psoriasis-associated) (FABP5)                 | lipid transport                 | 4557581     | 5        | 3      | 16     | 137                      |
| leukocyte receptor cluster member 4 protein (MBOAT7)                         | lipid metabolism               | 23308572    | 0        | 1      | 6      | 143                      |
| sequestosome 1 isoform 1 (SQSTM1)                                            | autophagy/apoptosis             | 4505571     | 6        | 2      | 20     | 164                      |
| BH3 interacting domain death agonist isoform 2 (BID)                         | apoptosis                      | 4557361     | 1        | 0      | 4      | 200                      |
| DEAD (Asp-Glu-Ala-Asp) box polypeptide 47 isoform 1 (DDX47)                 | apoptosis/mRNA processing       | 20149629    | 0        | 0      | 3      | 200                      |
| NOL1/NOP2/Sun domain family, member 2 (NSUN2)                                | cell cycle                     | 39995082    | 1        | 1      | 6      | 143                      |
| erythropoietin 4 immediate early response (ZC3H15)                         | cell cycle                     | 118150660   | 1        | 1      | 4      | 120                      |
| COP9 signalosome subunit 4 (COPS4)                                          | cell cycle                     | 38373690    | 2        | 2      | 6      | 100                      |
| dual specificity phosphatase 3 (DUSP3)                                       | signaling                      | 4758208     | 1        | 1      | 6      | 143                      |
| ATPase, H^+ transporting, lysosomal V1 subunit A (ATP6V1A)                  | vacuolar H^+ ion transport     | 19913424    | 3        | 4      | 17     | 124                      |
| ATPase, Ca^{++} transporting, slow twitch 2 isoform 3 (ATP2A3)              | Ca^{++} ion transporter        | 209413709   | 1        | 0      | 6      | 200                      |
| USO1 homolog, vesicle docking protein (USO1)                                 | vesicular transport            | 4505541     | 1        | 0      | 5      | 200                      |
| Gene Name | Function | NCBI GI Number | MS2# shENO1 | MS2#_shCTRL | Relative Difference (%) |
|-----------|----------|----------------|-------------|-------------|-------------------------|
| lectin, mannos-binding, 1 precursor (LMAN1) | protein transport | 5031873 | 0 | 0 | 3 | 200 |
| Sec23 homolog B (SEC23B) | protein transport | 14591928 | 0 | 0 | 3 | 200 |
| signal recognition particle 68kDa (SRP68) | protein transport | 24497620 | 0 | 0 | 3 | 200 |
| transportin 1 isoform 1 (TNPO1) | protein transport | 133925811 | 0 | 0 | 3 | 200 |
| actin related protein 2/3 complex subunit 4 isoform a (ARPC4) | cytoskeleton organization | 5031595 | 1 | 0 | 4 | 200 |
| capping protein (actin filament) muscle Z-line, alpha 2 (CAPZA2) | cytoskeleton organization | 5453599 | 0 | 0 | 3 | 200 |
| secreted phosphoprotein 1 isoform a (SPP1) | cell adhesion | 91206462 | 0 | 0 | 4 | 200 |
| breast cancer anti-estrogen resistance 1 (BCAR1) | cell adhesion | 44662836 | 0 | 0 | 3 | 200 |
| tyrosyl-tRNA synthetase 2, mitochondrial (YARS2) | protein synthesis | 94681057 | 0 | 0 | 5 | 200 |
| MMS19 nucleotide excision repair homolog (MMS19) | transcription/DNA damage | 170763479 | 2 | 1 | 10 | 164 |
| ribosomal protein L10 (RPL10) | translation | 223890243 | 0 | 0 | 4 | 200 |
| ribosomal protein L37a (RPL37A) | translation | 4506643 | 0 | 0 | 3 | 200 |
| ribosomal protein L13a (RPL13) | translation | 6912634 | 0 | 0 | 3 | 200 |
| transducin (beta)-like 1 X-linked receptor 1 (TBL1XR1) | transcription | 19913371 | 0 | 0 | 4 | 200 |
| heterochromatin protein 1-beta (CBX1) | heterochromatin | 187960037 | 0 | 0 | 4 | 200 |
| H2A histone family, member Y isoform 1 (HIST1H2AY) | nucleosome assembly | 20336746 | 0 | 0 | 3 | 200 |
| heat shock 70kDa protein 4-like (HSPA4L) | stress response | 31541941 | 0 | 0 | 4 | 200 |
| barrier to autointegration factor 1 (BANF1) | antiviral response | 4502389 | 0 | 0 | 3 | 200 |
| bolA-like protein 2B (BOLA2) | ND | 85797673 | 0 | 1 | 4 | 120 |
| hypothetical protein LOC23065 | ND | 22095331 | 0 | 0 | 4 | 200 |
| hypothetical protein LOC25940 (FAM98A) | ND | 56699482 | 0 | 0 | 4 | 200 |

aNCBI GI Number.
bRelative difference (%) = (MS2# shENO1-MS2#_shCTRL)/[(MS2#_shENO1+ MS2#_shCTRL)/2]*100.
| Protein name                                      | Functional annotation | Accession\(^a\) | Parental | shCTRL | shENO1 | Relative Difference (\%\(^b\)) |
|--------------------------------------------------|-----------------------|-----------------|----------|--------|--------|---------------------------------|
| enolase 1 (ENO1)                                 | glycolysis            | 4503571         | 124      | 117    | 23     | -134                            |
| enolase 2 (ENO2)                                 | glycolysis            | 5803011         | 145      | 151    | 8      | -180                            |
| liver phosphofructokinase (PFKL)                 | glycolysis            | 48762920        | 3        | 5      | 0      | -200                            |
| thiosulfate sulfurtransferase (TST)              | metabolism            | 17402865        | 4        | 8      | 0      | -200                            |
| EH-domain containing 2 (EHD2)                    | endocytosis/ metabolism| 21361462        | 6        | 7      | 2      | -111                            |
| enolase 2 (ENO2)                                 | lipid metabolism      | 68051721        | 6        | 7      | 2      | -111                            |
| acyl-Coenzyme A dehydrogenase, very long chain isoform 2 precursor (ACADVL) | lipid metabolism      | 76496475        | 4        | 6      | 0      | -200                            |
| acyl-CoA thioesterase 7 isoform hBACHa (ACOT7)   | lipid metabolism      | 75709208        | 4        | 4      | 0      | -200                            |
| platelet-activating factor acetylhydrolase, isoform 1b, gamma subunit (PAFAH1B3) | lipid metabolism      | 4505587         | 3        | 4      | 0      | -200                            |
| sideroflexin 3 (SFXN3)                           | iron homeostasis      | 31621303        | 4        | 5      | 0      | -200                            |
| 3-hydroxyisobutyryl-Coenzyme A hydrolase isoform 1 (HIBCH) | Amino acid catabolism | 37594471        | 4        | 4      | 0      | -200                            |
| AHNAK nucleoprotein isoform 1 (AHNAK)            | cell adhesion         | 61743954        | 95       | 110    | 35     | -103                            |
| anterior gradient 2 homolog (AGR2)               | cell adhesion/ proliferation | 5453541     | 55       | 82     | 22     | -115                            |
| catenin, delta 1 isoform 1ABC (CTNND1)           | cell adhesion/ signaling | 146231940   | 8        | 10     | 2      | -133                            |
| theoretical protein LOC64855 isoform 2 (MINERVA) | cell adhesion         | 79750824        | 8        | 10     | 1      | -164                            |
| galectin 3 (LGALS3)                              | cell adhesion         | 115430223       | 13       | 9      | 1      | -160                            |
| catenin, alpha 1 (CTNNA1)                        | cell adhesion         | 55770844        | 6        | 7      | 1      | -150                            |
| integrin alpha-V isoform 1 precursor (ITGAV)     | cell adhesion         | 4504763         | 5        | 4      | 1      | -120                            |
| galectin-4 (LGALS4)                              | cell adhesion         | 5453712         | 10       | 13     | 0      | -200                            |
| Golgi apparatus protein 1 isoform 1 (GLG1)       | cell adhesion/ signaling | 54633312     | 8        | 13     | 0      | -200                            |
| PREDICTED: similar to mucin 5, partial           | cell adhesion         | 169202626       | 7        | 10     | 0      | -200                            |
| mucin 5AC (MUC5AC)                               | cell adhesion         | 161019170       | 5        | 6      | 0      | -200                            |
| serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 5 (SERPINb5) | cell migration       | 167860126       | 5        | 4      | 1      | -120                            |
| PDZ and LIM domain 1 (PDLIM1)                    | cytoskeleton regulation | 13994151     | 9        | 16     | 2      | -156                            |
| cysteine-rich protein 1 (intestinal) (CRIP1)      | cytoskeleton regulation | 4503047     | 5        | 3      | 0      | -200                            |
| PDGFA associated protein 1 (PDAP1)               | cell proliferation    | 7657441         | 5        | 3      | 0      | -200                            |
| Gene Name                                      | Function                  | GI Number | Fold Change | Regulation |
|-----------------------------------------------|---------------------------|-----------|-------------|------------|
| myxovirus resistance protein 2 (MX2)          | cell cycle/protein transport | 11342664  | 7           | 8          | 2          | −120 |
| cytokine induced protein 29 kDa (SARNP)       | cell cycle                | 32129199  | 4           | 4          | 1          | −120 |
| signal transducer and activator of transcription 1 isoform beta (STAT1) | transcription regulation | 21536301  | 21          | 20         | 10         | −67  |
| promyelocytic leukemia protein isoform 5 (PML) | transcription regulation   | 109637788 | 4           | 8          | 0          | −200 |
| major vault protein (MVP)                     | mRNA/protein transport    | 19913412  | 23          | 27         | 11         | −84  |
| splicing factor 3a, subunit 1, 120kDa isoform 1 (SF3A1) | mRNA processing | 5032087   | 3           | 5          | 0          | −200 |
| thymopoietin isoform beta (TMPO)              | nuclear envelope organization | 73760405 | 4           | 4          | 0          | −200 |
| N-acylaminoacyl-peptide hydrolase (APEH)      | proteolysis               | 23510451  | 4           | 4          | 0          | −200 |
| D-dopachrome tautomerase-like (DDTL)          | lyase                     | 145386559 | 4           | 4          | 0          | −200 |
| ISG15 ubiquitin-like modifier (ISG15)         | antiviral response        | 4826774   | 4           | 4          | 0          | −200 |
| 2′-5′ oligoadenylate synthetase 3 (OASL)      | antiviral response        | 45007007  | 4           | 3          | 0          | −200 |
| beta-2-microglobulin precursor (BZMP)         | antigen presentation      | 4757826   | 4           | 3          | 0          | −200 |

aNCBI GI Number.
bRelative difference (%) = (MS2#_shONO1-MS2#_shCTRL)/(MS2#_shONO1+ MS2#_shCTRL)/2]*100.
MATERIAL AND METHODS

Viral Transduction

Lentiviral infections were performed using HEK293T cells as producers of viral supernatants. HEK-293T cells were grown on 10-cm plates to 70% confluence and co-transfected with shRNA lentiviral DNA and the helper vectors pCMV∆8.74 and pVSV-G (Clonetech) using the calcium phosphate precipitation method. The medium was harvested 24 hr post-transfection and filtered through a 0.45 μm filter. Supernatants from HEK-293T cultures were used to cross-transduce cancer cells in the presence of 8 μg/mL Polybrene (Sigma-Aldrich), and subsequent clones were selected by puromycin (2 μg/mL).

shRNAs

Short-hairpin RNAs (shRNAs) targeting the human ENO1 mRNA and cloned into the pLKO.1-puro vector were obtained from the human library MISSION® TRC-Hs 1.0. Five shRNAs targeting ENO1 were purchased as glycerol stocks from Sigma-Aldrich (SHCLNG-NM_001428). The efficiency of each shRNA in decreasing the ENO1 mRNA level was assessed by real-time qRT-PCR in transiently transfected cells. The best shRNA (TRCN0000029324) with the targeted sequence 5′-CCGGCTACCGCTTCCTTAGAACTTCTCGAGA-AGTTCTAAGGAAGCGGTACGTTTTT-3′ was chosen for the generation of stable cell lines. The control shRNA (SHC002, Sigma-Aldrich) generates a scrambled shRNA sequence that does not target any known human gene.

Quantitative RT–PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen) and reverse transcription was performed from 2 μg of total RNA using iScript cDNA synthesis kit (BioRad) according to the manufacturer’s instructions. Quantitative RT–PCR was performed using SYBR Green dye (Life Technologies) on a Thermal iCycler (BioRad). PCR reactions were performed in triplicate and the relative amount of cDNA was calculated by the comparative C_ T method using β-actin RNA sequences as a control. Data are represented as mean ± SEM of three or more independent experiments.

Oligonucleotide primer sequences for SybrGreen qRT-PCR

| Gene   | Sense         | Antisense                   |
|--------|---------------|-----------------------------|
| ENO1   | GCCCTCTGCTCAAAAGTCAAC | AACGATGAGACACCATGACG       |
| ENO2   | AGTGGACACACATCAACTCCACCAT | ACTCCAGCATCAAGTTGTCGAGTT   |
| ENO3   | CTCCCATGCTGGAAACAAC | TGGCATCCTTCCCATGACT         |
| C-MYC  | CTCCACTCGGAAGGACTAT | TTGTGTTGCGCTCTTGT         |
| AKR1C2 | GGTTGCGCAGCTTATGCTCCTTAT | AATCCAGGACAGGCGAATGTA      |
| AKR1C1 | GCTT TAGAGGCGACAAATGCGA | ACTGCAATCTGAACCTTGCTCG     |
| AKR1B2 | ACACAGAAAGCAGCTTGTGGGAGAC | AGGTGACGCTCAACAAGGCACGACA |
| HK2    | TGACACGCACTCAAGGAGAAAGAAG | ACGGTCTTATGAGCCGCTTGAGCA  |
| PFKL   | AGTATACGTGGTGCGACATGCTTT | TGCGGAGTGTTCCTCACAATGACT  |
| EDH2   | CGGTGCGAAGCATTAGAG | GATGACGAGGATGATAGT       |
| NNMT   | ATGCCTCAAGAGCAGCTACTACA | CTTGGTGGCCATGTTGGAAGAAT    |
| CSNK2A1| AGGCAGGAAGAAAGGAGGAAGG | AGACACACTTCCCAAGAGCCACT   |
| FAH    | TCAGGAAGTGTGCTCATCTCTCCCA | TCAACGATTCTTCTTGCCCTGA   |
| FABP5  | ACAGTGTCAGCTGCAAGGAGAAG | ATACACTGCTGGTCCCATTGCG  |
| MBOAT7 | TGCCGCAATTCTCCCTTCT | CTTGGTGGCCTAGGGTGAATA    |
| SQSTM1 | AAATGGTGGCACCAGGAACCTGGA | TCAAGCTATATGGCAGGCTGAGTA |
| NCEH1  | CGGTATGTCATGTTGAAGATGT | CAAAGAGGAGTGGATCCTGAAT    |
| ACADVL | AGAGGACATATTGCTAAGGCGGATA | TCTCTGTGAAGTGGGCAAATGTTG |
| PFAH1B3| CGTGCGTGCAGCAAGAAGATAAGGA | CCGTCACCAACATGGCAGAAGTGA |
| HIBCH  | ATAAAAGGGAAGGAGGAAAGGCT | GGTTGGGGTGCAGAACCACAAGCAG |
| AMPK1  | CGTGTCAGGAGAGGAATCC | GAGTAGCAGTCCCTGATTG       |
| PGC1alpha | GGAGGAAAGTGGACAGGATTAG | GTGAGGCTAGTGTGACTC      |
| LKB1   | GTACACACCTCACAAATCAC | GTACTCAAGCCTCCCTCTCA     |
| beta ACTIN | CGCCGCGACTCACCATG | CACCATGAGGAGGAGGACGCG  |
Western blot analysis

After SDS–PAGE, proteins were transferred to Hybond-N Nitrocellulose membrane (GE Healthcare Bio-Sciences). Membranes were blocked in Tris-buffered saline (TBS) containing 5% non-fat dry milk and 0.1% Tween 20 (TBS-T), before incubation with the primary antibody overnight at 4°C. Membranes were then washed with TBS-T followed by exposure to the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hr, and then visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare Bio-Sciences) by ProXPRESS 2D (PerkinElmer) scanning. The following antibodies were used: α-enolase mAb clone 72/1 (1), enolase (H300, Santa Cruz Biotechnology), β-actin (A2066, Sigma-Aldrich), c-Myc (GT168, GeneTex), p62/SQSTM1 (N3C1, GeneTex), ATG4B (GeneTex), ATG4D (N3C3, GeneTex), Sirt1 (19A7AB4, Abcam) phospho-Tyr15-cdc2 (9111, Cell Signaling), phospho-Ser807/811-Rb (8516, Cell Signaling), phospho-Ser345-Chk1 (2348, Cell Signaling), phospho-Thr68-Chk2 (2661, Cell Signaling), phospho-Ser15-p53 (9284, Cell Signaling), Cyclin D1 (2926, Cell Signaling), Cyclin D3 (2936, Cell Signaling). LC3-II was enriched and quantified by Western blot analysis using LC3-II Enrichment Kit (Merck Millipore) according to the manufacturer’s instructions.

Trypsin digestion and desalting

Cell pellets were resuspended in 200 µL of 8 M urea, and the protein concentration was measured by the Bradford Assay (BioRad). Proteins were transferred to a 1.5-mL eppendorf tube, reduced by 10 mM dithiothreitol (DTT) for 30 min at 37°C, and then alkylated by 50 mM iodoacetamide for 20 min at room temperature. The concentrated urea in the sample was diluted to a final concentration of 2 M, and the proteins were digested by trypsin at 37°C for 6 hr in a buffer containing ammonium bicarbonate (50 mM, pH 9). The digestion mixture was then acidified by adding glacial acetic acid to a final concentration of 2% and desalted by ZipTip (Millipore).

Tandem mass spectrometry analysis

Peptides were analyzed by highly sensitive reversed-phase liquid chromatography coupled nanospray tandem mass spectrometry (LC-MS/MS) using an LTQ-Orbitrap mass spectrometer (Thermo Fisher) (2). Briefly, the reversed-phase LC column was slurry-packed in-house with 5 μm, 200 Å pore size C18 resin (Michrom BioResources) in a 100 μm i.d. × 10 cm long piece of fused silica capillary (Polymicro Technologies) with a laser-pulled tip. After sample injection, the column was washed for 5 min with mobile phase A (0.1% formic acid), and peptides were eluted using a linear gradient of 0% mobile phase B (0.1% formic acid, 80% acetonitrile) to 50% B in 160 min at 200 nL/min, then to 100% B in an additional 10 min for proteomics analysis. The LTQ-Orbitrap mass spectrometer was operated in a data-dependent mode in which each full MS scan (60,000 resolving power) was followed by eight MS/MS scans where the eight most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35%. The Dynamic Exclusion Time was 30 s, and the Dynamic Exclusion Size was 200. The “FT master scan preview mode”, “Charge state screening”, “Monoisotopic precursor selection”, and “Charge state rejection” were enabled so that only the 1+, 2+, and 3+ ions were selected and fragmented by CID.

Mass spectrometry data analysis

Tandem mass spectra collected by Xcalibur (version 2.0.2) were searched against the NCBI human protein database using SEQUEST (Bioworks software from ThermoFisher, version 3.3.1) with full tryptic cleavage constraints, static cysteine alkylation by iodoacetamide, and variable methionine oxidation. Mass tolerance for precursor ions was 5 ppm and mass tolerance for fragment ions was 0.25 Da. The SEQUEST search results of proteomics data were filtered by the criteria “Xcorr versus charge 1.9, 2.2, 3.0 for 1+, 2+, 3+ ions; ∆Cn > 0.1; probability of randomized identification of peptide < 0.01”. Confident peptide identifications were determined using these stringent filter criteria for database match scoring followed by manual evaluation of the results. The "false discovery rate (FDR)" was estimated by searching a combined forward-reversed database as described by Elias (3). The SEQUEST search results were exported to Excel files and compared. Spectra count label-free quantitation analysis was performed applying the formula: Relative difference (%) = [(Total no. peptides in shENO1 + Total no. peptides in shCTRL) - (Total no. peptides in shENO1 - Total no. peptides in shCTRL)]/[2*(Total no. peptides in shENO1 + Total no. peptides in shCTRL)]*100.

Glucose uptake

The uptake of glucose was measured by radiolabeling cells with 0.3 µCi/ml 2-deoxy-D-[3H]glucose, as described earlier (4) insulin increases cyclic GMP production by inducing nitric oxide (NO). The non carrier-mediated glucose uptake was measuring by performing the assay in the presence of 10 µM cytochalasin B, a strong inhibitor of the facilitated glucose uptake. Results were expressed as pmol 2-deoxy-D-[3H]glucose/mg cell proteins.

Lactate

Analysis of lactate level was performed on 2 × 10⁶ cells with the L-Lactate Assay Kit (Abcam, Cambridge, MA, USA), following the manufacturer’s instructions. Results were expressed as pmol/10⁶cells.
**Aldose reductase activity**

Cells were washed with PBS, detached by gentle scraping, centrifuged at 13,000 × g for 5 min at 4°C, re-suspended in 0.4 mL of 50 mmol/L NaPO₄ buffer (pH 7.0). A 50 µL aliquot was sonicated and used for determining the cell proteins. The remaining sample was transferred to a 96-well plate, in the presence of 10 mmol/L glucose, dissolved in NaPO₄ buffer. 1.5 mmol/L NADPH was added. The rate of NADPH oxidation was followed for 6 min, monitoring the absorbance at 340 nm with a Packard microplate reader EL340 (Bio-Tek Instruments). Results were expressed as nmol NADPH produced/min/mg cell proteins.

**NADPH oxidase activity**

NADPH oxidase activity was carried out *in vitro* by the isoluminol-chemiluminescence assay. A total of 2 × 10⁶ cells was washed with PBS, detached with trypsin/EDTA (0.05/0.02% v/v), re-suspended in 1 mL PBS containing 7.5 mmol/L glucose, 0.9 mmol/L CaCl₂, 0.5 mM MgCl₂, and incubated for 10 min at 37°C, in the presence of 200 ng/mL phorbol myristate acetate (PMA) to activate the enzyme, 125 µmol/L isoluminol, 25 U/mL horseradish peroxidase. A 50 µL aliquot was sonicated and used for determining the cell proteins. Chemiluminescence of each sample derived from superoxide and lucigenin was detected using a Synergy HT microplate reader (Bio-Tek Instruments) and was expressed as relative luminescence unit (RLU)/mg cell proteins. For the negative control, in each experiment, one sample was treated with 450 µL of 80% w/v trichloroacetic acid. To remove the unincorporated ¹⁴CO₂, the tubes were heated at 85°C for 3 hr; the remaining samples, containing ¹⁴C-carbamoyl aspartate were analyzed by liquid scintillation.

**Tyrosine catabolism**

Determination of phenylalanine and acetooacetate levels was performed according to the instruction manuals of EnzyChrom TM phenylalanine and ketone body assay kits (BioAssay Systems).

**Glutamine catabolism**

Glutamine catabolism was measured as reported (5), with minor modifications. Cells were washed with PBS, detached by gentle scraping, centrifuged at 13,000 × g for 5 min at 4°C, re-suspended in 250 µL of buffer A (150 mmol/L KH₂PO₄, 63 mmol/L Tris/HCl, 0.25 mmol/L EDTA; pH 8.6) and sonicated. The intracellular protein content was measured using the BCA kit (Sigma-Aldrich). A volume of 100 µL of the whole cell lysates was incubated for 30 min at 37°C in a quartz cuvette, in the presence of 50 µL of 20 mmol/L L-glutamine and 850 µL of buffer B (80 mmol/L Tris/HCl, 20 mmol/L NAD⁺, 20 mmol/L ADP, 3% v/v H₂O₂; pH 9.4). The absorbance of NADH was monitored at 340 nm using a Lambda 3 spectrophotometer (PerkinElmer). The kinetics was linear throughout the assay. The results were expressed as µmol NADH/min/mg cell proteins, and were considered as an index of the activity of glutaminase (GLS) plus L-glutamic dehydrogenase. In a second series of samples, 20 µL of the GLS inhibitor bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide BTPES (30 µmol/L) was added after 15 min. This concentration was chosen as it produced 100% inhibition of glutaminase activity in our system (not shown). The absorbance of NADH was monitored for 15 min as described previously. The results, considered as an index of the activity of L-glutamic dehydrogenase, were expressed as µmol NADH/min/mg cell proteins. GLS activity was obtained by subtracting the rate of the second assay from the rate of the first one.

**Purine synthesis**

The activity of glutamine amidophosphoribosyltransferase (GPAT), considered as an index of the de novo synthesis of purine nucleotides, was measured as described previously (6). Cells were washed twice with PBS, detached with trypsin/EDTA (0.05/0.02% v/v), centrifuged at 13,000 × g for 5 min, re-suspended in 1 mL of 50 mmol/L potassium phosphate buffer (pH 7.4), containing 5 mmol/L diethiothreitol (DTT), C₁₀₀ µg/mL phenylmethanesulfonylfluoride (PMSF), 2 µg/mL aprotinin, and sonicated. Samples were then centrifuged at 40,000 × g for 20 min at 4°C; supernatants were used for protein quantification and GPAT assay. 0.5 mL of each sample was incubated for 1 hr at 37°C with 5 mmol/L 5-phosphoribosyl 1-pyrophosphate, 5 mmol/L MgCl₂, 1 mmol/L DTT, 0.75 µCi [¹⁴C]-L-glutamine (200 mCi/mmol, PerkinElmer). The [¹⁴C]-L-glutamate generated from the reaction was separated from [¹⁴C]-L-glutamine by ion exchange chromatography in a 2 mL column. The radioactivity of the eluate containing [¹⁴C]-L-glutamate was counted by liquid scintillation and expressed as pmol glutamate/h/mg cell proteins.

**Pyrimidine synthesis**

The activity of carbamoyl phosphate synthetase II (CPSII) was measured as an index of the de novo synthesis of pyrimidine nucleotides. Cells were washed twice with PBS, re-suspended in 0.5 mL of lysis buffer (20 mmol/L Tris/HCl, 1% v/v Triton X-100, 10% v/v glycerol, 137 mmol/L NaCl, 0.150 mmol/L NaVO₄, 0.250 mmol/L PMSF, 5 µg/mL leupeptin; pH 7.5), then centrifuged at 10,000 x g for 10 min at 4°C. A 50 µL aliquot was used for the protein content quantification; the remaining lysate was incubated with 0.5 mL of the assay buffer (87 mmol/L Tris/HCl, 87 mmol/L KCl, 25 mmol/L MgCl₂, 10 mmol/L ATP, 3.3 mmol/L-glutamine, 17.5 mmol/L-aspartate, 0.8 mmol/L DTT, 6.5% v/v DMSO, 2.2% v/v glycerol, 4 µCi [¹³C]-NaHCO₃ (54 mCi/mmol, PerkinElmer) for 30 min at 37°C. The reaction was stopped by adding 0.2 mL of 80% w/v trichloroacetic acid. To remove the unincorporated [¹³C]CO₂, the tubes were heated at 85°C for 3 hr; the remaining samples, containing [¹³C]-carbamoyl aspartate were analyzed by liquid scintillation.
Results were expressed as pmol carbamoyl aspartate/min/mg cell proteins.

Measurement of mitochondrial respiratory chain

Cells were washed twice in ice-cold PBS, then lysed in 0.5 mL buffer A (50 mmol/L Tris, 100 mmol/L KCl, 5 mmol/L MgCl₂, 1.8 mmol/L ATP, 1 mmol/L EDTA, pH 7.2), supplemented with protease inhibitor cocktail III [100 mmol/L AEBSF, 80 mmol/L aprotinin, 5 mmol/L bestatin, 1.5 mmol/L E-64, 2 mmol/L leupeptin and 1 mmol/L pepstatin (MerckMillipore) 1 mmol/L PMSF, 250 mmol/L NaF. Samples were clarified by centrifuging at 650 × g for 3 min at 4°C, and the supernatant was collected and centrifuged at 13,000 × g for 5 min at 4°C. The new supernatant was discarded, the pellet containing mitochondria was washed in 0.5 mL buffer A and re-suspended in 0.25 mL buffer B (250 mmol/L sucrose, 15 μmol/L K₂HPO₄, 2 mmol/L MgCl₂, 0.5 mmol/L EDTA, 5% w/v bovine serum albumin). A 50 µL aliquot was sonicated and used for the measurement of protein content. The activity of mitochondria respiration complexes was measured according to (7). Results were expressed as nmol NAD⁺/min/mg mitochondrial protein for complex I, nmol cyt c reduced/min/mg mitochondrial protein for complexes II-III, nmol cyt c oxidized/min/mg mitochondrial protein for complexes IV.

Enolase inhibitor studies

For enolase inhibitor studies, PhAH lithium salt was custom-synthesized by CAGE chemicals. PhAH was dissolved in PBS as a 50 mM stock and stored at −20°C until use.

Enolase enzymatic activity assay

Enolase activity was measured by coupling the reactions of enolase, pyruvate kinase and lactate dehydrogenase, according to (8). After PhAH treatment, cells were washed with PBS, detached by gentle scraping, centrifuged at 13,000 × g for 5 min at 4°C, re-suspended in 0.25 mL of 100 mmol/L Tris buffer (pH 8.0), 10 mmol/L MgCl₂, 100 mmol/L KCl, 1 mmol/L 2-phosphoglyceric acid, 0.4 mmol/L ADP, 6.8 U/mL pyruvate kinase, 9.9 U/mL lactate dehydrogenase. 0.2 mmol/L NADH was added. The rate of NADH oxidation was followed for 6 min, monitoring the absorbance at 340 nm with a Packard microplate reader EL340 (Bio-Tek Instruments). Results were expressed as nmol NAD⁺ produced/min/mg cell proteins.

Inhibitors and positive controls for biochemical tests

Zopolrestat: 2.5 µmol/L, 3 hr (pre-incubation) – aldose reductase inhibitor. Apocynin: 10 µmol/L, 3 hr (pre-incubation) – NADPH oxidase inhibitor. DHEA: 250 µmol/L, 3 hr (pre-incubation) – pentose phosphate pathway inhibitor. Rotenone: 100 µmol/L, 3 hr (pre-incubation) – mitochondrial chain inhibitor.

Proliferation assay

Cells were seeded in 6-well plates at a density of 1 × 10⁵ cells/well. After 24 hr of serum starvation, cells were grown in complete medium and then detached, stained with trypan blue, and counted every 24 hr. Results represent at least three independent experiments.

Cell survival assay

Cell survival was assessed by MTT assay. Cells were seeded in 96-well plate at 2 × 10⁵ cells/well and serum starved for 24 hr. After 48 hr of culture in complete medium, 20 µL of 5 mg/µL MTT solution (Sigma-Aldrich) was added to the medium and incubated at 37°C for a further 4 hr. Medium was removed and the insoluble formazan product was dissolved in 200 µl DMSO (Sigma-Aldrich) for 10 min at room temperature. OD values were measured at 570 nm in an ELISA microtiter plate reader (BioRad). Results represent at least three independent experiments.

Colonies forming assay

Briefly, a bottom layer of 2.5 mL culture medium containing 0.9% agarose type VII (Sigma-Aldrich) was initially solidified in a 6-well culture plate. Then, 2 mL of 0.45% agarose solution containing 3 × 10⁴ cells was layered on top of each well. Cells were fed twice a week with complete DMEM and incubated at 37°C for 3 weeks. Colonies were counted by optical microscope. Results represent at least three independent experiments.

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