Supplementary content

Nicotinamide nucleotide transhydrogenase (NNT) deficiency dysregulates mitochondrial retrograde signaling and impedes proliferation

Hung-Yao Ho, Yu-Ting Lin, Gigin Lin, Pei-Ru Wu, and Mei-Ling Cheng

Supplemental Materials and Methods Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, glutamine, pyruvate, trypsin-EDTA, and trypan blue were acquired from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Fluorogenic dyes, namely Mitotracker Red 580FM, 5,5',6,6'-tetrachloro-1,19,3,39-tetraethylbenz- imidazolocarbocyanine iodide (JC-1), and MitoSOX Red, were purchased from Thermo Fisher Scientific (Waltham, MA, USA). [U-$^{13}C_5$] glutamine (CLM-1166, chemical purity >98%) and [U-$^{13}C_6$] glucose (CLM-1396, chemical purity >98%) were purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Antibodies to NNT (ab57153; Abcam, Cambridge, MA, USA), porin (MSA03; Abcam, Cambridge, MA, USA), histone H3 (05-928; Merck Millipore, Billerica, MA, USA), acetylated-lysine (9814; Cell Signaling Technology, Danvers, MA, USA), p53 (sc-1311; Santa Cruz Biotechnology, Dallas, Texas, USA), acetyl-p53(Lys373) (06-916; Merck Millipore, Billerica, MA, USA), HIF-1α (3716S; Cell Signaling Technology, Danvers, MA, USA), β-actin (A5441; Sigma-Aldrich, St. Louis, MO, USA), Total OXPHOS Human WB Antibody Cocktail (ab110411; Abcam, Cambridge, MA, USA), Membrane Integrity WB Antibody Cocktail (ab110414; Abcam, Cambridge, MA, USA) and HDAC1(GTX100513; GeneTex, Inc., CA, USA) were purchased from respective vendors.

Cell culture

SK-Hep-1 cells (ATCC catalog number: HTB-52) were maintained in DMEM, supplemented with 10% (v/v) FCS, 25 mM glucose, 2 mM pyruvate, 6 mM glutamine, and 100 units/ml penicillin 0.1 mg/ml streptomycin at 37^{o}C in a humidified atmosphere containing 5% CO_2. Retrovirus packaging cell lines BOSC23, PT67 and SD3443 cells were routinely maintained in DMEM supplemented with 10% (v/v) FCS, 25 mM glucose, 4 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

For determination of growth curves for SK-shNNT and SK-Sc cells, 10^4 cells were seeded in 24-well culture plate, and maintained in complete DMEM. At daily intervals, cells were fixed in 3.7% formaldehyde for 10 min, and stained with 5 μg/ml of Hoechst 33342 for 15 min. The cell number was determined using IN Cell Analyzer 1000 (GE Healthcare Life Sciences, USA).

For studies comparing the effect of glucose or glutamine on cell growth, cells were similarly seeded in complete medium, and after attachment, were cultured in medium containing indicated combinations of 0-25 mM glucose or 0-6 mM glutamine for 4 days. The cell number was determined using Hoechst 33342 staining as described above.

For studies comparing the effect of glucose, glutamine and pyruvate on cell growth, cells were similarly seeded in complete medium, and after attachment, were cultured in medium containing

indicated combinations of 25 mM glucose, 2 mM pyruvate, or 6 mM glutamine for 4 days. The cell number was determined using Hoechst 33342 staining as described above.

For studies involving pharmacological treatment, 2×10^4 cells were seeded in 24-well culture plate, and after attachment, treated with indicated concentrations of rotenone, 3-BrPA or DMS for indicated periods. The cell number was determined using Hoechst 33342 staining as described above.

Construction of expression vectors encoding shNNT and tdTomato

A mini-cassette encoding shRNA against NNT include overhangs that allow cloning into Bgl II and Hind III of pSuper Retro vector, target sense and antisense sequences with an intervening loop, and a transcription terminator sequence. The target sequence was selected as previously described [1]. The sequence of NNT shRNA (shNNT) cassette (sense strand) was 5'-GATCCCCGCAGGAGAACTCTACATTCATATTCAAGAGATATGAATGTAGAGTTCTCCTGTTTTTTGCT-3'; its antisense counterpart was 5'-AGCTAGCAAAAAACAGGAGAACTCTACATTCATATCTCTTGAATATGAATGTAGAGTTCTCCTGCGGG-3'. The sequence of scrambled control shRNA (Sc) cassette (sense strand) was 5'-GATCCCCGCTAACGACATATACTACGTATTCAAGAGATACGTAGTATATGTCGTTAGCTTTTTTGCT-3'; the antisense counterpart was 5'-AGCTAGCAAAAAAGCTAACGACATATACTACGTATCTCTTGAATACGTAGTATATGTCGTTAGCGGG-3'. Oligonucleotides were synthesized and purified using polyacrylamide gel (Integrated DNA Technologies, IA). They were dissolved in annealing buffer (10 mM Tris-Cl, pH 8.0/50 mM NaCl/1 mM EDTA) and annealed by heating to 95°C and stepwise cooling as follows: 95°C, 4 min; 90°C, 4 min; 85°C, 4 min; 80°C, 4 min; 75°C, 4 min; 70°C, 4 min; 65°C, 4 min; 60°C, 4 min; 55°C, 4 min; 50°C, 4 min; 45°C, 4 min; 40°C, 4 min; 35°C, 4 min; 30°C, 4 min; 25 C, 4 min. The oligonucleotide duplexes coding for NNT and scrambled shRNAs were ligated to Bgl II- and Hind III-cleaved pSuper.retro.puro to generate pSuper.retro-shNNT and pSuper.retro-Sc, respectively. The expression vector encoding fluorescent protein tdTomato, pRSET-B.tdTomato, was kindly provided by Prof. Roger Tsien. The pRSET-B.tdTomato vector was digested with Eco RI and Bam HI. The fragment containing tdTomato coding sequence was blunt-ended with Klenow fragment (Thermo Fisher Scientific,Waltham, MA, USA), and cloned into Hpa I-cleaved pLXIN vector to yield pLXIN-tdTomato.

Retroviral delivery of shNNT and Sc expression vectors

Retroviral packaging and infection were accomplished as previously described [2]. The constructs pSuper.retro-shNNT and pSuper.retro-Sc were transiently transfected into ecotropic packaging BOSC23 cells using Polyfect (Qiagen, Valencia, CA, USA) according to manufacturer's instruction. The culture medium containing retroviral particles was harvested 48 h later, and was filtered through a 0.45 μm

syringe filter before its subsequent use for infection of amphotropic packaging PT67 cells. About 5×10^5 PT67 cells were infected with the retrovirus-containing supernatant in the presence of 4 μg/ml polybrene. Forty-eight hours later, the cells were selected in a medium containing 4 μg/ml puromycin. High titer virus-producing clones were picked for production of amphotropic virus. The culture supernatants from these clones were collected, filtered, and used for transduction of SK-Hep1 cells. The transduced cells, namely SK-shNNT and SK-Sc cells were grown in the presence of 4 μg/ml puromycin for 5 days.

Tagging of cells with tdTomato

The cells for IVIS study were retrovirally marked with tdTomato. Briefly stated, the pLXIN-tdTomato construct was transiently transfected into the packaging SD3443 cells using Polyfect. The culture medium containing retroviral particles was collected, filtered. It was used for transduction of SK-shNNT and SK-Sc cells to generate Tomato-SK-shNNT and Tomato-SK-Sc cells, respectively. The cells were cultured in the presence of 1 mg/ml G418 for 2 weeks.

In vivo tumor growth assessment and fluorescent imaging

All animal study was approved by the Institutional Animal Care and Use Committee of Chang Gung University (IACUC No. CGU10-035). Eight weeks old specific pathogen-free immunodeficient BALB/cAnN.Cg-Foxn1nu/CrlNarl (BALB/c nude mice) were employed. For tumor weight measurement, about 2×10^6 Tomato-SK-shNNT and Tomato-SK-Sc cells were subcutaneously injected into right and left dorsal flanks of nude mice, respectively. The tumor growth was monitored by in vivo fluorescent imaging. At weekly interval, the fluorescence emission was captured by Xenogen IVIS 100 system (PerkinElmer, Waltham, MA. USA), equipped with DsRed filter set. An exposure time of 1 s was used. The gray-scale photographic image was automatically superimposed on the pseudocolor fluorescent image so as to locate the optical signals on mice. The optical images were analyzed using Xenogen Living Image Software. The signal from tumors was quantified as photons flux (in the unit of photons/s). After 10th week, mice were scarified, and the tumors were retrieved for weighing.

Measurement of cellular OCR, ΔΨm, mitochondrial mass and superoxide

ΔΨm, mitochondrial mass and superoxide production was assayed as previously described (Cheng et al., 2014). Cellular oxygen consumption was assayed as previously described (Cheng et al., 2014). In brief, about 10^6 cells were seeded in 10 cm tissue culture dish, and harvested 2 days later in HBSS medium. The cells were pelleted, and then resuspended in respiration buffer (20 mM $NaKPO_4$, pH 7.2/65 mM KCl/125 mM sucrose/2 mM $MgCl_2$). The cell suspension was transferred to the respiratory chamber of Mitocell equipped with Clark-type electrode, which was connected to Strathkelvin 928 6-Channel Oxygen System (Strathkelvin Instruments, Glasgow, UK). Oxygen consumption rate was measured.

Isolation of mitochondria and OCR determination

About 3×10^6 cells were seeded in 15 cm tissue culture dish. After 2 days of cultivation, the cells were rinsed with SEH buffer (3 mM HEPES, pH 7.2/0.5 mM EGTA/250 mM sucrose), which was supplemented with cOmplete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The cells were scraped in ice-cold SEH buffer, and centrifuged at 200 × g for 5 min at 4°C. The cell pellet was resuspended in SEH buffer and homogenized using a Dounce homogenizer with 15-20 strokes. After centrifugation at 1000 × g for 10 min at 4°C, the supernatant was retained and centrifuged at 10000 × g for 10 min at 4°C. The mitochondrial pellet was resuspended in SEH buffer.

The OCR of mitochondria was measured using a Clark-type oxygen electrode as previously described with modifications [3]. An aliquot of mitochondria (equivalent to 50 μg of mitochondrial proteins) was assayed in 100 μl respiration buffer, using Mitocell MT200 connected to Strathkelvin 928 6-Channel Oxygen System (Strathkelvin Instruments, Glasgow, UK). The oxygen concentration of buffer was continuously monitored. For measurement of OCR associated with complex I, mitochondria were incubated in respiration buffer for 2 min; sodium pyruvate and malate were added to final concentrations of 10 mM, and the reaction mixture was left for 2 min; ADP was then added to a final concentration of 1 mM, and the reaction mixture was incubated for 2 min; and KCN was added to a final concentration of 3 mM, and the reaction proceeded for 2 more min. The OCR of KCN-insensitive respiration was subtracted from ADP-stimulated respiration of reaction mixture. The difference represents the respiratory activity associated with complex I.

Quantification of cellular and mitochondrial NADPH, $NADP^+$, NADH and NAD^+

For quantification of cellular NADP(H) and NAD(H), 10^6 cells were seeded in 6 well-plate, and after attachment, maintained in complete medium for 1 day prior to extraction. Cell extract was prepared as previously described [4], and the sample was dried under nitrogen gas. For quantification of NADP(H) and NAD(H) in respiring mitochondria, 1 mg of liver mitochondria (equivalent to 1 mg of mitochondrial proteins) and 0.3 mg of cellular mitochondria were resuspended in 1 ml and 0.3 ml of respiration buffer, respectively. The sample was incubated for 2 min before addition with 2 mM glutamate; 2 min later, ADP was added to a final concentration of 1 mM; the sample was incubated for 2 min before extraction. The reaction mixture was centrifuged at 10000 × g for 10 min at 4 °C. The mitochondrial extract was prepared as previously described [5], and the sample was dried under nitrogen gas.

The sample was analyzed by Acquity UPLC System (Waters Corp., Milford, MA, USA) equipped with photodiode array detector. The sample was chromatographed on an Acquity HSST3 reversed phase C18 column (2.1 mm × 150 mm, particle size of 1.8 μm) adapted to a guard column (2.1 mm × 5 mm). The mobile phase was constituted of 25 mM potassium phosphate buffer, pH 5.8 (solvent A) and 100% methanol (solvent B). The mobile phase condition was as follows: solvent A, 2 min; gradient from 0 to

3% solvent B, 0.5 min; gradient from 3% to 4% solvent B, 2.5 min; gradient from 4% to 15% solvent B, 2.5 min; 15% solvent B, 1 min; gradient from 15% to 100% solvent B, 1 min; solvent B, 1.5 min. The column temperature was maintained at 37 °C. The flow rate was set at 0.38 ml/min. NADH and NADPH were measured by the absorbance at 340 nm, while NAD^+ and $NADP^+$ were measured by the absorbance at 260 nm. The relative levels of NAD(H) and NADP(H) are presented as $\frac{[NADPH]}{[NADPH]+[NADP^+]}$ (abbreviated as $\frac{NADPH}{NADP(H)}$) and $\frac{[NADH]}{[NADH]+[NAD^+]}$ (abbreviated as $\frac{NADH}{NAD(H)}$).

Quantification of cellular ATP and extracellular lactate

About 2×10^5 cells were seeded in 10 cm petri dish, and after attachment, cultivated for 1, 3 and 5 days prior to extraction. Culture medium was also collected for measurement of lactate. Cell extract was prepared as previously described [4], and the sample was dried under nitrogen gas. The sample was subject to UPLC analysis as described above. ATP was measured by the absorbance at 260 nm. For measurement of lactate, the medium collected was centrifuged at 200 × g for 10 min at 4°C. The supernatant was analyzed using lactate ELISA kit (Randox Laboratories, UK) according to the manufacturer's protocol.

Determination of HDAC activity

Nuclear extract was prepared as described previously [6], with the only exception that another nucleus extraction buffer (20 mM HEPES, pH 7.9/1.5 mM MgCl/300 mM KCl/0.4 mM EDTA/20% glycerol/ /0.5 mM DTT) supplemented with cOmplete™ EDTA-free protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) was used.

Recombinant HDAC1 was expressed from a pQC-3×FLAG-HDAC1 vector, which has a human HDAC1 cDNA insert (Clone ID: BC000301). The vector was transfected into 4×10^6 cells of 293T cells using JetPrime transfection reagent (Polyplus transfection, Illkirch, France) according to the manufacturer's instruction. Two days later, the transfected cells were rinsed twice with PBS, and lysed using CelLystic M (Sigma-Aldrich, St. Louis, MO, USA). After clarification of lysate by centrifugation, the recombinant HDAC1 was purified from lysate using FLAG M purification kit (CELLMM2; Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instruction. The FLAG-tagged protein was eluted with 3×FLAG peptide.

The in vitro non-isotopic HDAC assay was performed as previously described [7]. In brief, nuclear extract (200 μg) or recombinant HDAC1 (50 μl of eluate) was added to reaction buffer (1.4 mM NaH_2PO_4/18.6 mM Na_2HPO_4, pH 7.9/ 0.25 mM EDTA/10 mM NaCl/10% (v/v) glycerol/10 mM mercaptoethanol) in the presence and absence of 5 μM trichostatin A (TSA). The substrate BOC-Ac-Lys-AMC (12.6 μM) and internal control 7-hydroxycoumarin (50 μM) (Sigma-Aldrich, St. Louis, MO, USA) were added. The reaction mixture was incubated at 37 °C for 90 min, and terminated

by addition of stop solution (1 M HCl/0.4 M sodium acetate). It was extracted with ethyl acetate, and the upper phase was retained and dried under nitrogen gas. The sample was dissolved in 40 % acetonitrile, and analyzed by HPLC system (Waters Alliance 2690 separation module)(Waters Corp., Milford, MA, USA) with a Waters 2475 multi λ fluorescence detector. The sample was chromatographed on Waters Xbridge C18 Column (4.6 mm × 250 mm, particle size of 5 μm). The mobile phase was acetonitrile/water (40:60, v/v), and the flow rate was 1.2 ml/min. Excitation and emission wavelengths were set at 330 nm and 395 nm, respectively. The concentration of substrate was normalized to that of internal standard. The activity can be calculated from the substrate concentration of sample minus that of sample with TSA divided by the reaction time.

Western blotting

The cells were rinsed with cold PBS, scraped, and collected by centrifugation. They were immediately lysed in RIPA lysis buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 % NP-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Protein concentration of the lysate was determined by Bradford method. Cellular protein (50 μg), mitochondria (20 μg) or nuclear proteins (20 μg) was subject to SDS-PAGE and immunoblotting with antibodies to NNT, porin, histone H3, acetylated lysine, p53, acetyl-p53 (Lys373), HIF-1α, actin, total OXPHOS proteins, mitochondria membrane integrity proteins and HDAC1 according to manufacturers' instruction.

[U-$^{13}C_6$] glucose and [U-$^{13}C_5$] glutamine labeling and isotopologue analysis

About 2×10^5 cells were seeded in complete medium, and cultured at 37 °C for 72 hr. They were washed twice with serum-free DMEM (without glucose and glutamine)(D5030; Sigma-Aldrich, St. Louis, MO, USA). The medium was replaced with the [U-$^{13}C_6$] glucose labeling medium, that is DMEM (D5030) supplemented with 10% dialyzed FCS, 25 mM [U-$^{13}C_6$] glucose, 6 mM glutamine and 2 mM pyruvate, or with [U-$^{13}C_5$] glutamine labeling medium, that is DMEM (D5030) supplemented with 10% dialyzed FCS, 25 mM glucose, 6 mM [U-$^{13}C_5$] glutamine and 2 mM pyruvate. The labeled cells were rinsed twice with ice-cold PBS, and scraped in 1 ml of 80% ice-cold methanol. The sample was then dried under nitrogen gas, and dissolved in water prior to analysis. The dissolved sample was subject to liquid-chromatography time-of-flight mass spectroscopic (LC-TOF-MS) analysis using Agilent 1200 rapid resolution liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) as previously described [8]. The mass accuracy was automatically calibrated by continuous introduction of internal reference mass ions such as trifluoroacetic acid ($[M - H]^-$ = 112.985587) and hexakis-(1H, 1H, 3H-tetrafluoropropoxy) phosphazine trifluoroacetate ($[M - H]^-$ = 1033.988109). Data were collected using Agilent MassHunter Workstation Data acquisition software. The targeted metabolites, together with their isotopologues, were extracted according to their m/z values, and the corresponding peak areas

were calculated. The abundances of metabolites were corrected for natural abundance, and normalized to the abundance of an internal control, that is 2.5 nmoles of 4-morpholineethanesulfonic acid. The relative abundances of metabolites were further normalized to that of phenylalanine to correct for any difference in cell number. It was observed that the concentration of phenylalanine correlated well with cell number. Arbitrary units (a.u.) is relative to peak area from LC–TOF-MS analyses.

Amino acid analysis

The cell extract was prepared as described in the preceding section. The sample dried under nitrogen gas was re-dissolved in water. It was then mixed 4: 1 with 5 mM norvaline, and derivatized using Waters AccQTag ultra derivatization kit (Waters Corp., Milford, MA, USA) according to the manufacturer's instruction. Chromatographic separation was performed on an Acquity UPLC BEH reversed phase C18 column (2.1 mm × 100 mm, particle size of 1.7 μm), using an Acquity UPLC system (Waters Corp, Milford, MA). The mobile phase consists of 20 mM ammonium formate/1% acetonitrile/0.6% formic acid (solvent A) and 100 % acetonitrile (solvent B). The mobile phase condition was as follows: 0.1% solvent B, 0.54 min; gradient from 0.1 to 9.1% solvent B, 5.3 min; gradient from 9.1% to 21.2% solvent B, 2 min; gradient from 21.2% to 59.6% solvent B, 0.06 min; gradient from 59.6% to 90% solvent B, 0.05 min; 90% solvent B, 0.59 min; gradient from 90% to 0.1% solvent B, 0.09 min; 0.1% solvent B, 0.77 min. The flow rate was 0.7 ml/min. The column temperature was maintained at 60 °C. The PDA detector was set at 260 nm, with a sampling rate of 20 points/s.

Quantification of gene expression by reverse-transcription quantitative PCR (RT-qPCR)

Total RNA was isolated from cells using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA). First-strand cDNA was synthesized from 5 μg total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). One microlitre of a 1:100 dilution of cDNA was mixed with SsoFast EvaGreen supermix (BioRad Laboratories, Hercules, CA, USA) and primer pairs listed in below. The reactions were performed in CFX96 real-time PCR detection system under the following conditions: 30 s hold at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 5 s. Human gene for ribosomal protein L13 (RPL13) serves as normalization control.

Primer list

RPL10 (NM_033251): Forward 5'-CTGAAGGAGTACCGCTCCAAACT-3';
Reverse 5'-CAGCAGAACTGTCTCCCTTCTTG-3'

HDAC1 (NM_004964): Forward 5'-AGCCAAGAGAGTCAAAACAGA-3';
Reverse 5'-GGTCCATTCAGGCCAACT-3'

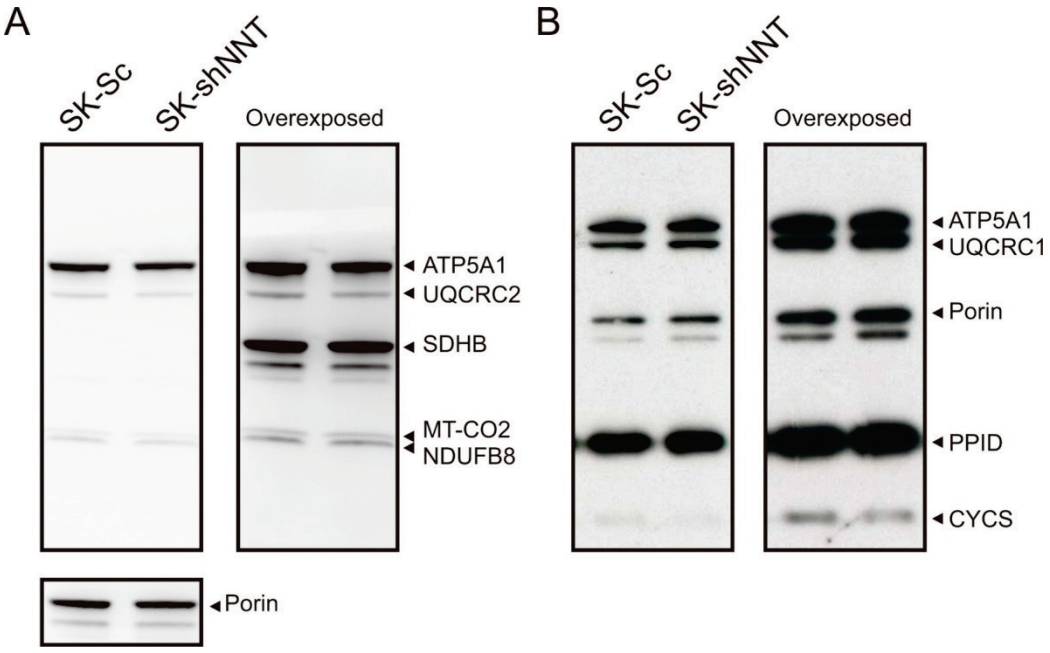
IGF1R (NM_000875): Forward 5'-CTCAGTTAATCGTGAAGTGGAACC -3';
Reverse 5'-GCAGTAATTGTGCCGGTAAAGG-3'

STC2 (NM_003714): Forward 5’-CTTACATGGGATTTGCATGACTT-3’;
Reverse 5’-AATGGATCATCTCCACTATCACC-3’
VEGFA (NM_001025366): Forward 5’-GAGGGCAGAATCATCACGAAGT-3’;
Reverse 5’-TCCTATGTGCTGGCCTTGGTGA-3’

Statistical Methods

Data are presented as mean±SD. The mass spectrometry data were analyzed as mentioned in the preceding section. Other data were analyzed by one-way analysis of variance (ANOVA) and Student’s *t*-test where appropriate. A *p* value of less than 0.05 is considered significant.

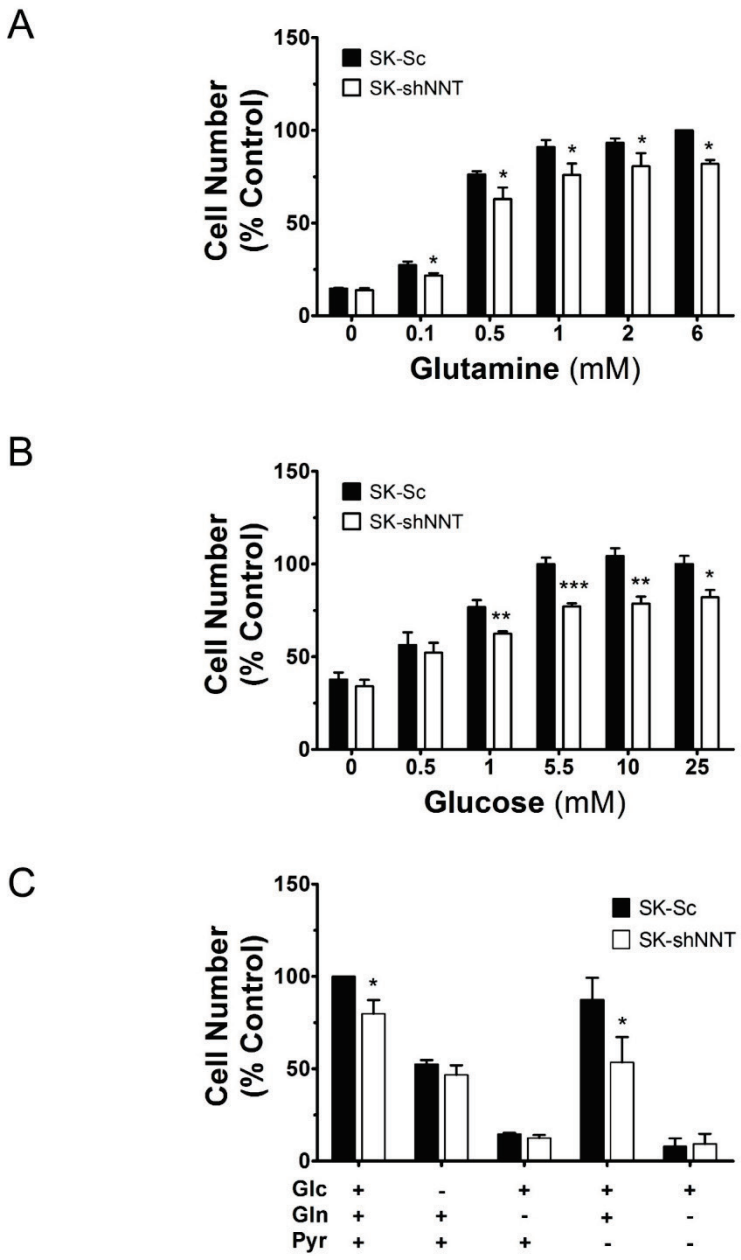
Supplemental Figures



Supplemental Figure 1., related to Figure 3. Expression of mitochondrial protein in SK-shNNT and SK-Sc cells.

Mitochondria isolated from SK-shNNT and SK-Sc cells were subject to immunoblotting with antibodies to mitochondrial proteins, ab110411 (Abcam, Cambridge, MA, USA) (A) and ab110414 (Abcam, Cambridge, MA, USA) (B), and with antibody to porin, MSA03. Immunoblot was exposed to film for 10 s or 60s (overexposed). A representative experiment out of three is shown.

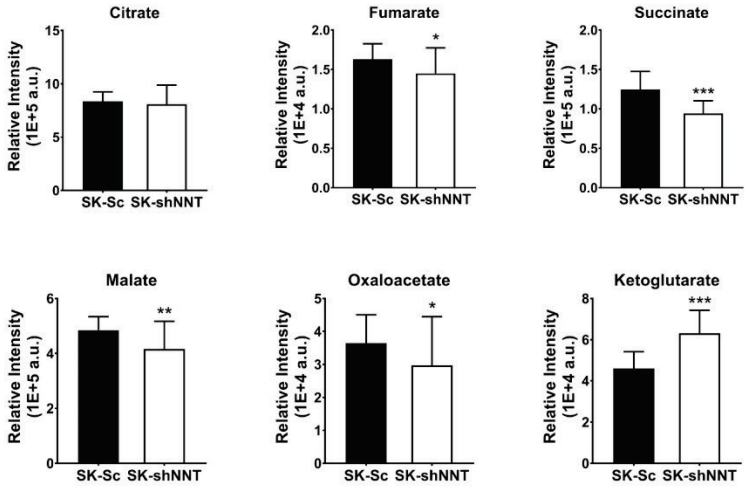
ATP5A: ATP synthase (F1-ATPase) α subunit
UQCRC2: Ubiquinol-cytochrome-C reductase complex core protein 2
SDHB: succinate dehydrogenase complex iron sulfur subunit B
MT-CO2: Mitochondrially encoded cytochrome C oxidase II
NDUFB8: NADH:ubiquinone oxidoreductase subunit B8
UQCRC1: Ubiquinol-cytochrome-C reductase complex core protein 1
PPID: Peptidylprolyl isomerase D;
CYCS: Cytochrome C
Porin as internal control of immunoblot



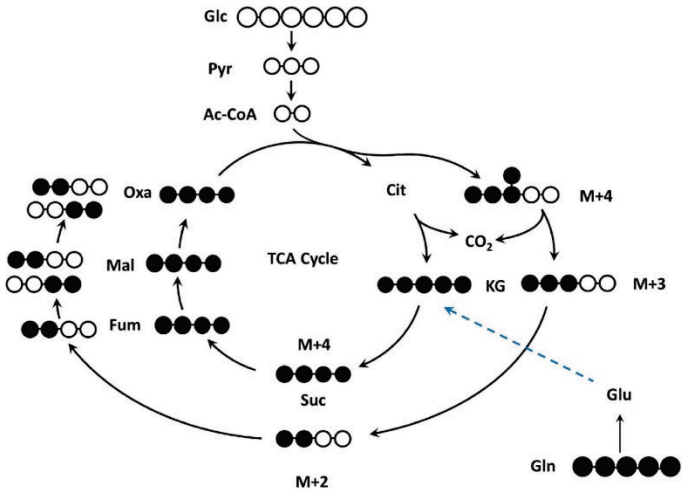
Supplemental Figure 2. Effect of glucose and glutamine on proliferation of SK-shNNT and SK-Sc cells.

Cells were cultured with indicated concentrations of glutamine (A) or glucose (B) for 4 days. Data are expressed as the percentage of SK-Sc cells cultured in medium supplemented with glucose, glutamine and pyruvate. Data are mean±SD, n=6. *p<0.05, **p<0.01 ***p<0.005 vs. SK-Sc cells at indicated concentration. (C) Cells were cultured in medium containing combinations of 25 mM glucose (glc), 2 mM pyruvate (pyr), or 6 mM glutamine (gln) for 4 days. Data are expressed as the percentage of SK-Sc cells cultured in medium supplemented with glucose, glutamine and pyruvate. Data are mean±SD, n=6. *p<0.05 vs. SK-Sc cells.

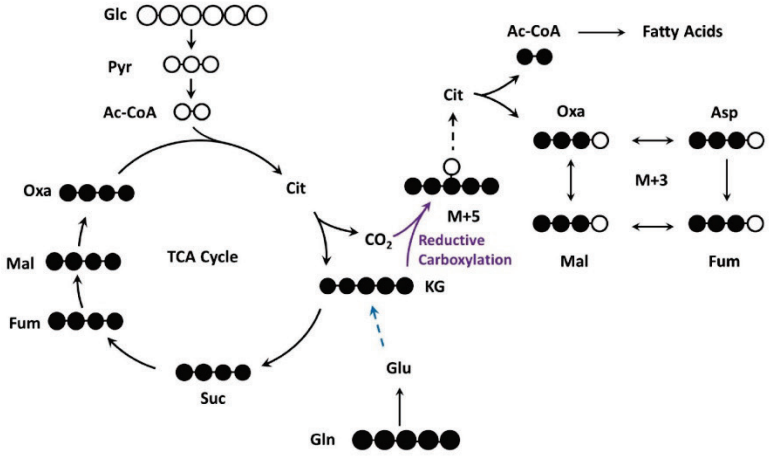
A


Citrate
Relative Intensity (1E+5 a.u.)
SK-Sc SK-shNNT
Fumarate
Relative Intensity (1E+4 a.u.)
*
SK-Sc SK-shNNT
Succinate
Relative Intensity (1E+5 a.u.)
***
SK-Sc SK-shNNT
Malate
Relative Intensity (1E+5 a.u.)
**
SK-Sc SK-shNNT
Oxaloacetate
Relative Intensity (1E+4 a.u.)
*
SK-Sc SK-shNNT
Ketoglutarate
Relative Intensity (1E+4 a.u.)
***
SK-Sc SK-shNNT

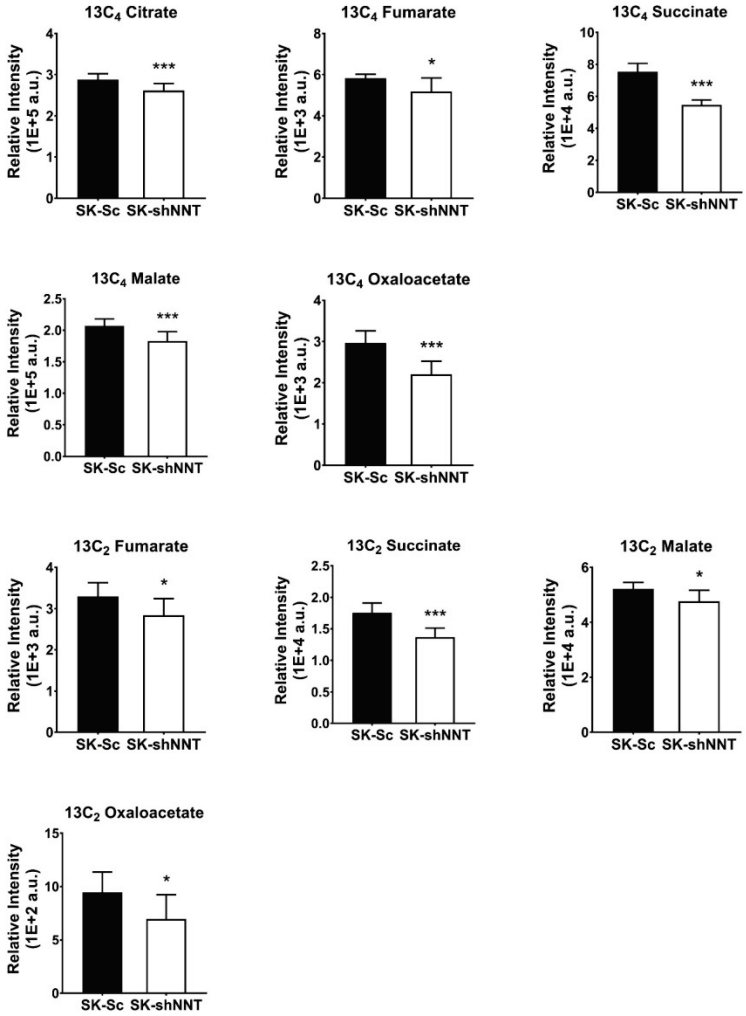
B


Glc
Pyr
Ac-CoA
Oxa
Cit
M+4
CO2
Mal
TCA Cycle
KG
M+3
Fum
M+4
Suc
Glu
M+2
Gln

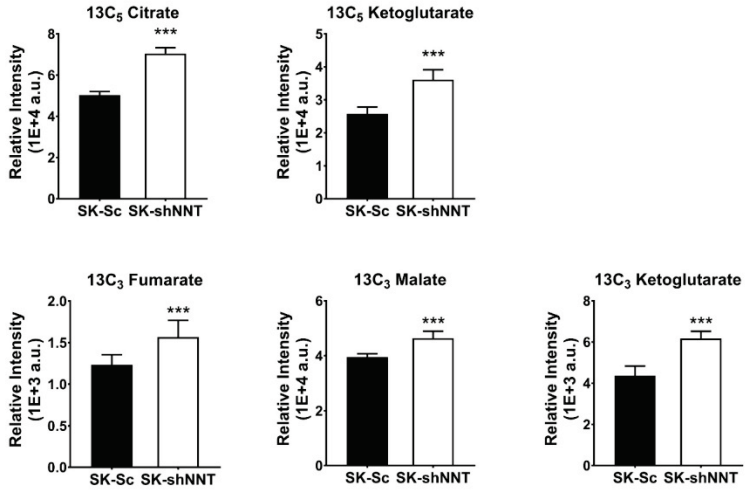
C


Glc
Pyr
Ac-CoA
Ac-CoA
Fatty Acids
Cit
Oxa
Asp
M+3
Oxa
Cit
M+5
CO2
Reductive Carboxylation
Mal
TCA Cycle
KG
Mal
Fum
Fum
Glu
Suc
Gln

D


13C4 Citrate
Relative Intensity (1E+5 a.u.)
***
SK-Sc SK-shNNT
13C4 Fumarate
Relative Intensity (1E+3 a.u.)
*
SK-Sc SK-shNNT
13C4 Succinate
Relative Intensity (1E+4 a.u.)
***
SK-Sc SK-shNNT
13C4 Malate
Relative Intensity (1E+5 a.u.)
***
SK-Sc SK-shNNT
13C4 Oxaloacetate
Relative Intensity (1E+3 a.u.)
***
SK-Sc SK-shNNT
13C2 Fumarate
Relative Intensity (1E+3 a.u.)
*
SK-Sc SK-shNNT
13C2 Succinate
Relative Intensity (1E+4 a.u.)
***
SK-Sc SK-shNNT
13C2 Malate
Relative Intensity (1E+4 a.u.)
*
SK-Sc SK-shNNT
13C2 Oxaloacetate
Relative Intensity (1E+2 a.u.)
*
SK-Sc SK-shNNT

E


13C5 Citrate
Relative Intensity (1E+4 a.u.)
***
SK-Sc SK-shNNT
13C5 Ketoglutarate
Relative Intensity (1E+4 a.u.)
***
SK-Sc SK-shNNT
13C3 Fumarate
Relative Intensity (1E+3 a.u.)
***
SK-Sc SK-shNNT
13C3 Malate
Relative Intensity (1E+4 a.u.)
***
SK-Sc SK-shNNT
13C3 Ketoglutarate
Relative Intensity (1E+3 a.u.)
***
SK-Sc SK-shNNT

Supplemental Figure 3., relative to Figure 4. NNT regulates TCA cycle and reverse carboxylation in vitro

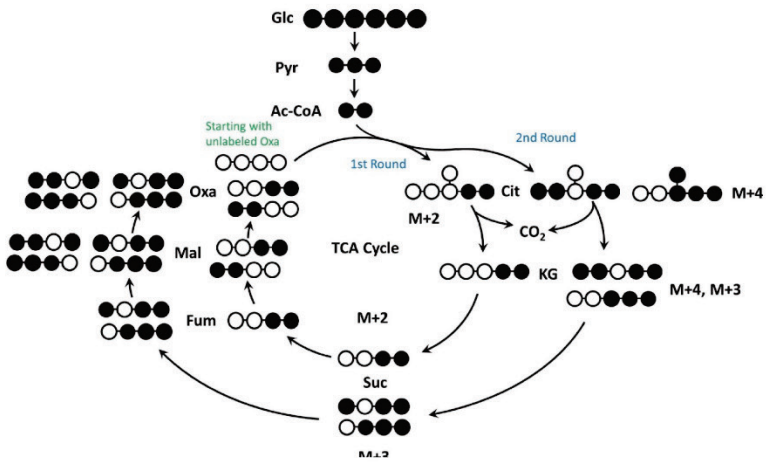
(A) The levels of total citrate (Cit), fumarate (Fum), succinate (Suc), malate (Mal), oxaloacetate (Oxa), and α-ketoglutarate (KG) in labeled cells are shown. Data are mean±SD, n=27. *p<0.05, **p<0.01, ***p<0.005 vs. SK-Sc cells.

(B) Schematic for metabolism of [U-$^{13}C_5$] glutamine. The labeling pattern of metabolites derived from [U-$^{13}C_5$] glutamine during glutaminolysis and TCA cycle is shown. Glutamine with five ^{13}C atoms (black) is converted to α-ketoglutarate (KG), and then to M+4 isotopologues of fumarate (Fum), malate (Mal) and oxaloacetate (Oxa). The M+4 Oxa reacts with unlabeled acetyl-CoA to form successively M+4 citrate (Cit), M+3 KG, M+2 succinate (Suc), and M+2 Fum. The M+2 Fum is converted to M+2 isotopomers of Mal and Oxa.

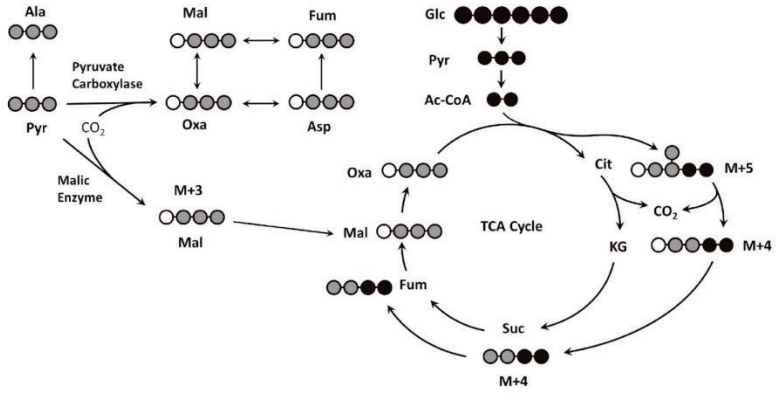
(C) The labeling pattern of metabolites derived from [U-$^{13}C_5$] glutamine during reductive carboxylation. The M+5 KG derived from labeled glutamine can be converted to M+5 Cit by isocitrate dehydrogenases (IDHs). It is then converted to M+3 four carbon-compounds (Oxa, Mal, Asp and Fum). (D) Relative intensities of the MS peaks corresponding to M+4 TCA cycle metabolites, after correction for natural isotope abundance, are shown. Data are presented as mean±SD, n=9. *p<0.05, ***p<0.005 vs. SK-Sc cells.

(E) Relative intensities of the MS peaks corresponding to M+3 and M+5 RC intermediates, after correction for natural isotope abundance, are shown. Data are presented as mean±SD, n=9. ***p<0.005 vs. SK-Sc cells.

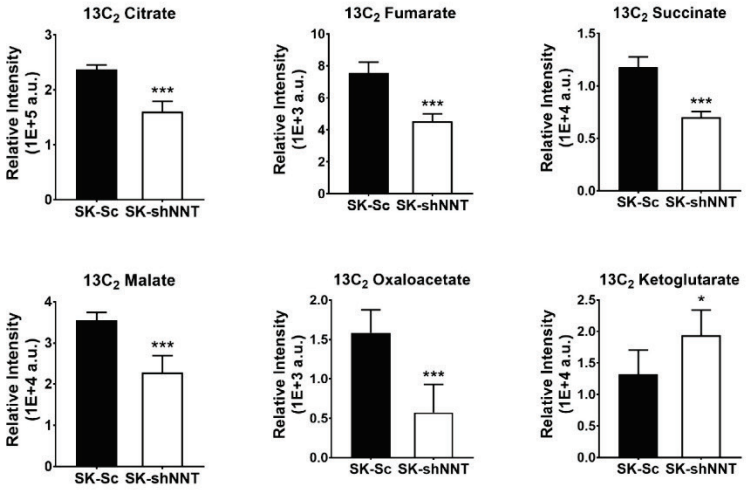
A


Glc
Pyr
Ac-CoA
Starting with unlabeled Oxa
1st Round
2nd Round
Oxa
Cit
M+4
M+2
CO_2
Mal
TCA Cycle
KG
M+4, M+3
Fum
M+2
Suc

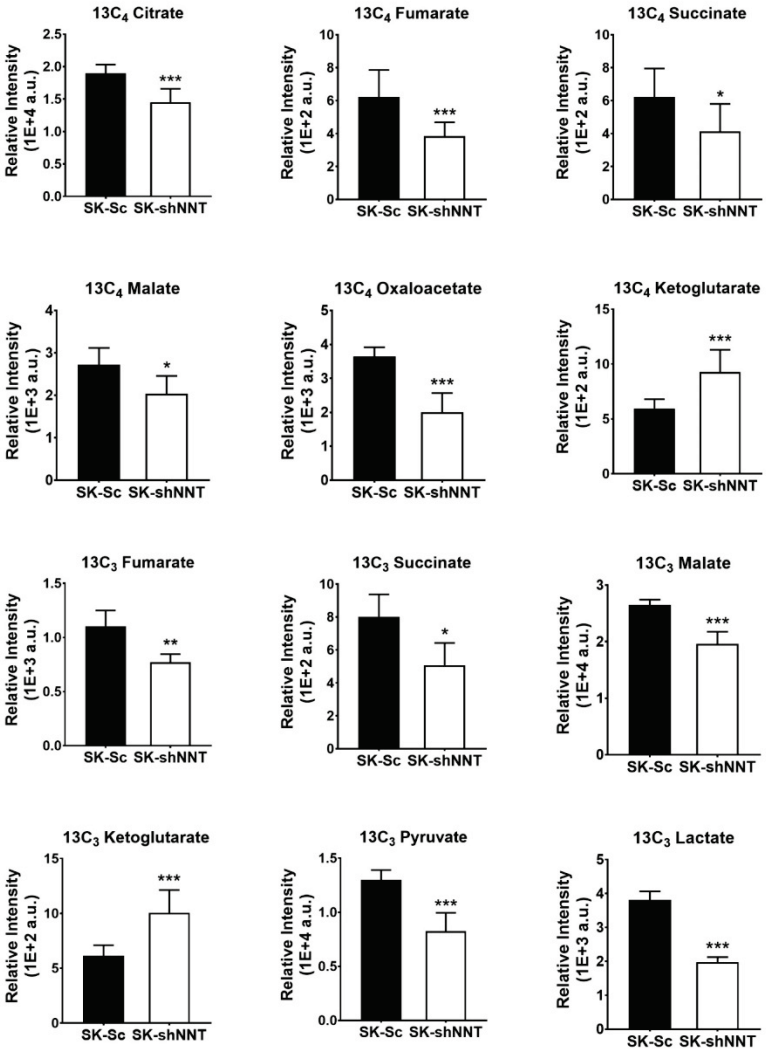
B


Ala
Mal
Fum
Glc
Pyr
Pyruvate Carboxylase
Ac-CoA
Pyr
CO_2
Oxa
Asp
Oxa
Cit
M+5
Malic Enzyme
M+3
CO_2
Mal
Mal
TCA Cycle
KG
M+4
Fum
Suc
M+4

C


$13C_2$ Citrate
Relative Intensity (1E+5 a.u.)
***
SK-Sc SK-shNNT
$13C_2$ Fumarate
Relative Intensity (1E+3 a.u.)
***
SK-Sc SK-shNNT
$13C_2$ Succinate
Relative Intensity (1E+4 a.u.)
***
SK-Sc SK-shNNT
$13C_2$ Malate
Relative Intensity (1E+4 a.u.)
***
SK-Sc SK-shNNT
$13C_2$ Oxaloacetate
Relative Intensity (1E+3 a.u.)
***
SK-Sc SK-shNNT
$13C_2$ Ketoglutarate
Relative Intensity (1E+3 a.u.)
*
SK-Sc SK-shNNT

D

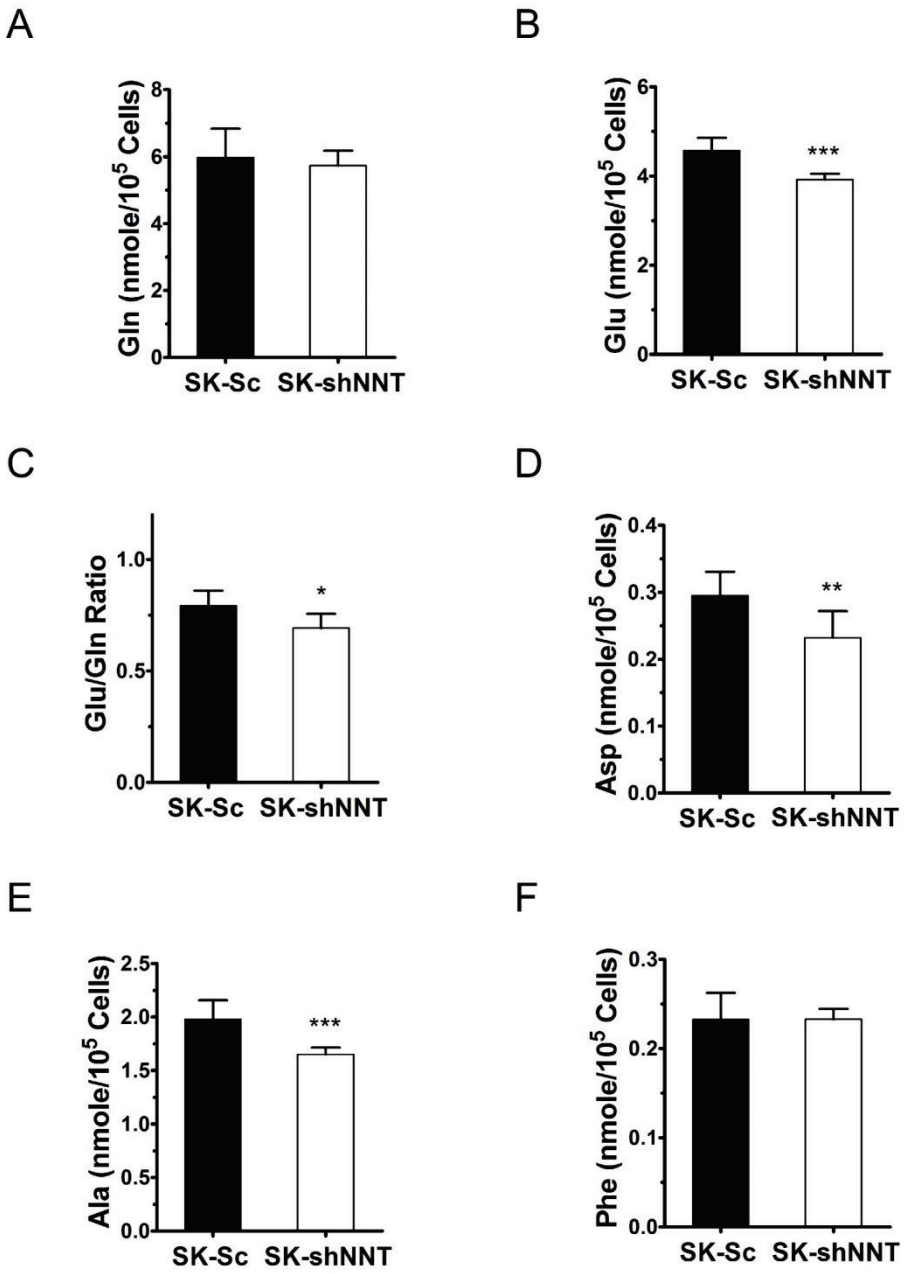


Supplemental Figure 4., relative to Figure 4. NNT deficiency slows down TCA cycle in vitro

(A) Schematic for metabolism of [U-$^{13}C_6$] glucose. The labeling pattern of metabolites derived from [U-$^{13}C_6$] glucose during TCA cycle is shown. Glucose with six ^{13}C atoms (black) is converted to acetyl-CoA, which reacts with unlabeled Oxa. The M+2 isotopologue of Cit formed is converted to M+2 Suc, Fum, Mal and Oxa. There are 2 isotopomers for Mal and Oxa. These metabolites are converted to M+4 isotopomer of Cit, which are converted to M+4 and M+3 isotopologues of KG, which further react to form different isotopomers of Fum, Mal and Oxa.

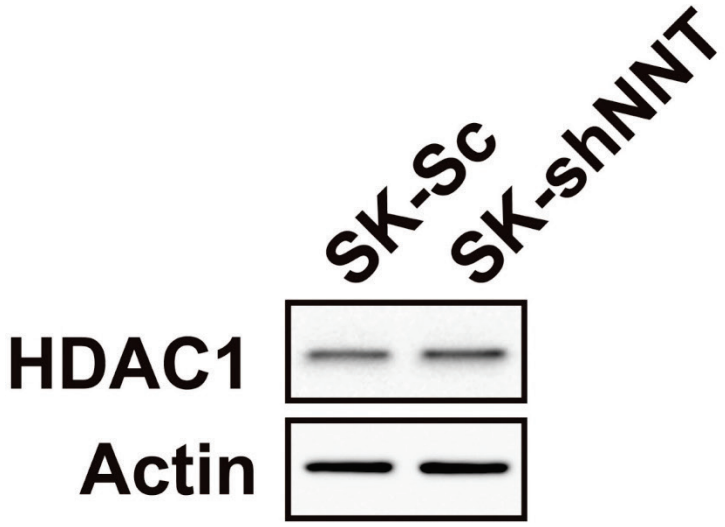
(B) The labeling pattern of metabolites derived from [U-$^{13}C_6$] glucose during reactions catalyzed by pyruvate carboxylase and malic enzyme. Pyruvate with three ^{13}C atoms (grey) can be converted to malate, which enters mitochondria and react with [U-$^{13}C_2$] acetyl-CoA (^{13}C atom in black). The M+5 Cit formed is converted successively to M+4 KG, Suc and Fum.

(C) Relative intensities of the MS peaks corresponding to M+2 TCA cycle metabolites, after correction for natural isotope abundance, are shown. Data are presented as mean±SD, n=9. *p<0.05, ***p<0.005 vs. SK-Sc cells.
(E) Relative intensities of the MS peaks corresponding to M+3 and M+4 metabolites, after correction for natural isotope abundance, are shown. Data are presented as mean±SD, n=9. *p<0.05, ***p<0.005 vs. SK-Sc cells.



Supplemental Figure 5. Changes in amino acids in SK-shNNT and SK-Sc cells.

SK-shNNT and SK-Sc cells were cultured for 3 days, and extracted for analysis of amino acids. The levels of glutamine (Gln)(A), glutamate (Glu)(B), aspartate (Asp)(D), alanine (Ala)(E) and phenylalanine (Phe)(F) are shown. The ratio of Glu to Gln was calculated, and is shown in (C). The results, expressed in nmole/10^5 cells, are mean±SD, n=9. *$p<0.05$, **$p<0.01$, ***$p<0.005$ vs. SK-Sc cells.



Supplemental Figure 6., relative to Figure 5A. Expression of HDAC1 in SK-shNNT and SK-Sc cells. Whole cell lysates of SK-shNNT and SK-Sc cells were prepared for western analysis of HDAC-1 and actin. A representative experiment out of three is shown.

Supplemental References

[1] H.Y. Ho, M.L. Cheng, Y.H. Wang, D.T. Chiu, Flow cytometry for assessment of the efficacy of siRNA, Cytometry A 69(10) (2006) 1054-61.

[2] L.P. Gao, M.L. Cheng, H.J. Chou, Y.H. Yang, H.Y. Ho, D.T. Chiu, Ineffective GSH regeneration enhances G6PD-knockdown Hep G2 cell sensitivity to diamide-induced oxidative damage, Free Radic Biol Med 47(5) (2009) 529-35.

[3] M.L. Cheng, L.M. Chi, P.R. Wu, H.Y. Ho, Dehydroepiandrosterone-induced changes in mitochondrial proteins contribute to phenotypic alterations in hepatoma cells, Biochem. Pharmacol. 117 (2016) 20-34.

[4] M.L. Cheng, H.Y. Ho, H.Y. Lin, Y.C. Lai, D.T. Chiu, Effective NET formation in neutrophils from individuals with G6PD Taiwan-Hakka is associated with enhanced NADP(+) biosynthesis, Free Radic. Res. 47(9) (2013) 699-709.

[5] M.A. Lorenz, C.F. Burant, R.T. Kennedy, Reducing time and increasing sensitivity in sample preparation for adherent mammalian cell metabolomics, Anal. Chem. 83(9) (2011) 3406-14.

[6] F. Hoppe-Seyler, K. Butz, C. Rittmuller, M. von Knebel Doeberitz, A rapid microscale procedure for the simultaneous preparation of cytoplasmic RNA, nuclear DNA binding proteins and enzymatically active luciferase extracts, Nucleic Acids Res. 19(18) (1991) 5080.

[7] B. Heltweg, J. Trapp, M. Jung, In vitro assays for the determination of histone deacetylase activity, Methods 36(4) (2005) 332-7.

[8] M.L. Cheng, M.S. Shiao, D.T. Chiu, S.F. Weng, H.Y. Tang, H.Y. Ho, Biochemical disorders associated with antiproliferative effect of dehydroepiandrosterone in hepatoma cells as revealed by LC-based metabolomics, Biochem. Pharmacol. 82(11) (2011) 1549-61.