Supporting Information

Banerjee et al. 10.1073/pnas.1424017112

SI Materials and Methods

Reagents and Cell Culture. All chemical reagents were obtained from commercial sources and used without purification unless otherwise noted. Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin, and 0.5% (vol/vol) Trypsin-EDTA was purchased from Gibco. FBS was purchased from HyClone. Primary Neonatal Rat Cardiomyocytes (NRCM) was prepared as per established protocol (1). NRCMs were plated onto 0.1% (wt/vol) gelatin-coated dishes and grown on DMEM containing 25 mM glucose media containing 5% (wt/vol) FBS and 1% pen-strep for 24 h, after which time the medium was changed to DMEM with 5 mM glucose containing 1% (wt/vol) FBS and 1% (wt/vol) pen-strep and the mediim was changed every 24 h. Antigen purified CTD 110.6 (2) was used for *O*-GlcNAc–specific immunoblots. Protein-specific antibodies were obtained from the following: complex II (Abcam; MS203), complex IV (Abcam; MS407), OGT (antigen-purified AL03) (3), OGA (antigen-purified 345) (4), tubulin (Sigma; T3526), iron sulfur subunit of succinate dehydrogenase (Abcam; 21A11AE7), and voltage-dependent anion channel (Sigma; V2139). A total of 50 nm gold-labeled anti-rabbit, anti-mouse, and anti-chicken antibodies were obtained from The Jackson Laboratories. Thiamet G was obtained via custom synthesis from SD Chem Molecules. Ac_4SGlcNAc was synthesized in laboratory via already published protocol (5).

Animals. All rats were treated in accordance with animal safety regulations according to The Johns Hopkins University School of Medicine. The 2- to 3-wk-old male (250 g) Sprague Dawley rats were purchased from Harlan Laboratories. Diabetes was induced by i.p. injection of 65 mg of streptozocin (STZ; Sigma-Aldrich) per kilogram of body weight as described previously (6). Control animals received injections of 100 mM citric acid (pH 4.5). Blood glucose was checked after 7 d and animals were killed after 4–5 wk and with their blood glucose levels over 600 ng/dL. Rat hearts were taken from both diabetic and control animals and used for mitochondrial purification.

Mitochondria Purification. Mitochondria were isolated by differential centrifugation protocol as described previously (7). All following operations were performed at 4 °C and under low light conditions. Briefly, about 250 mg of freshly obtained heart tissue were pulverized and then homogenized in 1 mL of modified isolation buffer (IS) (220 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L EDTA, 20 mmol/L Hepes, pH 7.4) plus EDTA-free protease inhibitor mixture tablet (Roche). The sample was centrifuged at 800 × *g* for 5 min at 4 °C and the pellet was resuspended in 1 mL of homogenization buffer and centrifuged as above. This step was repeated twice. Supernatants from each step were pooled and centrifuged at 8,000 × *g* for 10 min at 4 °C. The resulting pellet was resuspended in 1 mL of the isolation buffer and centrifuged at 8,000 × *g* for 15 min. The pellet was then resuspended in 1 mL IS. These pellets resuspended in IS buffer were further purified on a 17–35% histongenz gradient in an ultracentrifuge. The mitochondrial pellet at the junction between 35% and 17% histongenz was collected and used for Western blotting, activity assays, nucleotide sugar uptake, and electron microscopy. Mitochondria were stored at −80 °C as 100-μg aliquots until use.

For the isolation of mitochondria from NRCM cells, cells from 100-mm plates were washed twice with cold PBS and scraped into 2 mL of cold PBS supplemented with protease inhibitor mixture. The samples were centrifuged at 470 × *g* for 5 min at 4 °C and the pellets were homogenized in 1 mL of homogenization buffer (220 mmol/L mannitol, 70 mmol/L sucrose, 20 mmol/L Hepes, pH 7.4) with protease inhibitor mixture. The sample was centrifuged at 600 × *g* for 5 min at 4 °C and the pellet was resuspended in 100 μL of homogenization buffer and centrifuged as above. Supernatants from each step were pooled and centrifuged at 10,000 × *g* for 5 min. The final pellet was resuspended in 50 μL of homogenization buffer and protein concentration was determined by CB-X protein assay kit (G-Biosciences). Mitochondria were stored at −80 °C as 20-μg aliquots until use.

Electron Microscopy and Confocal Microscopy. Samples were fixed in 4% paraformaldehyde + 0.2% glutaraldeyde + 0.1 M phosphate buffer (Sorenson's) + 3 mM MgCl2 pH 7.2–7.4 overnight at 4 °C. Fixation and all subsequent steps up to infiltration were carried out at 4 °C, followed by curing at 50 °C. After a 45-min buffer rinse (0.1 M phosphate + 3% sucrose), samples were incubated with 0.12% tannic acid (Malinkrodt) in buffer for 1 h. Uncrosslinked glutaraldeyde was reduced for 30 min with 50 mM NH4Cl in buffer. Tissue was then en-bloc stained in 2% uranyl acetate (filtered) in maleate for 30 min. After a graded ethanol series dehydration, samples were infiltrated with a 2:1 90% ethanol:LR White resin (with catalyst), followed by 1:1, then 1:3 mixture for 40 min each. Samples were further infiltrated in pure LR White overnight at 4 °C. Finally, samples were polymerized in tightly sealed gelatin capsules at 50 °C, for 24 h. Polymerized blocks were trimmed and sectioned on a Reichert Ultracut E Microtome, using a low angle Diatome diamond knife. The 70- to 80-nm thin sections were collected on formvar-coated 200 mesh nickel grids (Pella) and processed for immunolabeling. Grids were floated, section side down in 50 mM NH4Cl in TBS for 10 min. Upon a 10-min block in 1% BSA/TBST, grids were incubated with primary antibodies (1:100-OGT, 1:100-OGA and CTD 110) in 1% BSA/TBST overnight at 4 °C. No primary antibody served as negative controls. Grids were allowed to come to room temperature for 1 h after primary antibody incubation, then incubated for 10 min on 1% BSA/TBST. After rinsing in TBS, grids were sequentially incubated in secondary Aby gold conjugate (6 nm Au-Goat anti-mouse, 1:40 dilution or 6 nm Au-Goat anti-rabbit in TBS; The Jackson Laboratories) for 1.5 h at room temperature. After rinsing in TBS then D-H_2O, grids were incubated in 2% glutaraldehyde (aqueous) for 5 min, then rinsed again in D-H_2O. After rinsing, grids were stained with 2% filtered Uranyl Acetate (aqueous) for 10 min and then allowed to dry. All grids were viewed with a Hitachi H-7600 TEM operating at 80 kV and images were captured with an XR 50–5 Megapixel CCD camera by AMT.

For immunofluorescence, NRCMs were plated on gelatin-coated coverslips, allowed to grow for at least 5 d in DMEM containing 5 mM glucose, and were treated with mito-tracker red dye (M7512; Life Technologies) for 30 min. The cells were then fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Following this step, the cells were blocked with 5% BSA and treated with antibodies for OGT (AL-03) and OGA (345) and subsequently with secondary antibodies (anti-IgG 488-conjugated Alexa Fluor; Invitrogen). The coverslips were then briefly stained for DNA with Dapi and mounted on a slide. Images were captured on a laser scanning Zeiss 510-Meta confocal microscope and acquired as a z stack of five 1-μm slices spanning a 2.8-μm range.

NRCM Mitochondrial Oxygen Consumption. Live cell oxygen consumption was measured by using a XF96 flux analyzer (Seahorse Biosciences). NRCM mitochondrial oxygen consumption assays were carried out in a seahorse 96-well cell culture plate with a XF96 sensor cartridge. The XF96-well plates were treated with 0.1% gelatin before the primary cardiomyocytes were plated at a density of 10,000 cells per well. Cells were allowed to grow for 5 d before treating them with Ac_4SGlcNAc and TMG, 24 h before oxygen consumption assays. Cells were incubated with unbuffered DMEM with 5 mM glucose and containing 200 mM glutamine, NaCl in a $C0_2$-free incubator at 37 °C for 1 h before loading the plate in the XF96 analyzer. The oxygen consumption rate was measured over a period of 100 min over which time oligomycin (0.5 μM), FCCP (0.3 μM), and both antimycin A (0.2 μM) and rotenone (0.1 μM) were sequentially added to each well at specified time points. The extracellular acidification rate (ECAR) was also measured over the same treatment and time.

Whole Cell ATP and Mitochondrial Membrane Potential Measurements. NRCMs treated with inhibitors of OGT and OGA were collected and pelleted. The cell pellets were ruptured using a homogenizer and mitochondria were prepared using differential centrifugation. The mitochondrial pellets were lysed and ATP levels determined using a luminescence assay as specified (ATP determination kit; Molecular Probes). For membrane potential determination, inhibitor-treated cells were incubated with mito-tracker dyes (M7512; Life Technologies) and JC-1 for 30 min. Cells treated with mito-tracker red (M7512; Life Technologies) were fixed with 4% paraformaldehyde and mounted on slides for immunofluorescence. Live cells treated with JC-1 (Life Technologies) were imaged directly on a laser scanning Zeiss 510-META confocal microscope. Relative membrane potential was assessed by the ratio of red to green fluorescence.

Complex IV, OGT, and OGA Activity Assays. For enzyme activity assays purified mitochondria from normal and STZ-treated rats were lysed using a 1% Triton lysis buffer. The mitochondrial lysate was desalted on a G50 desalting column and used for radioactive OGT activity assay (8). The lysate proteins were precipitated using an ammonium sulfate solution and the precipitated proteins resuspended in a 20 mM Hepes buffer containing 60 mM NaCl and used for OGA activity assay using 4MU as a substrate (9, 10). For mitochondrial complex IV activity assays, the lysates were added on to a 96-well plate complex IV activity assay kit (ab109911; Abcam,). After 3 h of incubation, the wells were washed with buffer and a solution of 50 mM reduced cytochrome *c* was added to the wells. Oxidation of cytochrome *c* was followed continuously for 30 min by measuring absorbance at 550 nm. The loss in absorbance was plotted against time and the initial slope was used to calculate activity of complex IV in the different mitochondrial samples.

Live Cell OGA Activity Assay. Cardiomyocytes were plated on glass-bottom dishes at concentrations of 5×10^4 cells per milliliter. Cells were allowed to grow in 5 mM glucose media for 5 d. Subsequently the cells were treated with either 2 mM TMG (10) (OGA inhibitor) or 2 mM M-31850 (lysosomal hexosaminidase inhibitor, EMD Biosciences). After 2 h of treatment GlcNAc-fluorescein was added to the cells at a final concentration of 2 μM. After 90 min of substrate addition, mito-tracker dye TMRM (Molecular Probes) was added to the cell at the recommended concentration. Finally within 30 min of TMRM addition, Hoesht was added to the media, just before imaging the cells. Imaging was carried out in a laser scanning Zeiss 710 NLO 2 photon confocal microscope and acquired as a z stack of seven 1-μm slices spanning a 2.8-μm range. Colocalization between the green channel (for hydrolyzed GlcNAc-fluorescein) and red channel (TMRM) was used to determine mito-specific activity of OGA.

Immunoprecipitation and Immunoblotting. Endogenous complex IV was immunoprecipitated by incubating 2 μg of antibody specific for Complex IV (Abcam; MS402) with 2 mg of purified rat heart mitochondrial lysate (2 mg/mL) overnight at 4 °C on a rotator. Samples were subsequently incubated with 5 μL GammaBind G-Sepharose beads (GE Healthcare) for 2 h at 4 °C on a rotator and subjected to five 5-min washes with 500 mM NaCl lysis buffer. For all experiments, washed immunoprecipitates were eluted with Laemli buffer.

For immunoblots, 20–60 μg of Laemli solubilized lysate or eluted immunoprecipitates were boiled, separated on Criterion precast SDS-polyacrylamide gels (Bio-Rad), and transferred to nitrocellulose (Millipore). Membranes were blocked for 1 h in Tris-buffered saline supplemented with 0.1% (vol/vol) Tween-20 (TBST) and 5% (wt/vol) nonfat dry milk or BSA and incubated with respective primary antibodies overnight at 4 °C. Membranes were washed, incubated with the appropriate HRP-linked secondary antibody for 1 h, washed again, developed using HyGLO quick spray (Denville Scientific) [or SuperSignal West Femto (Thermo Scientific) when appropriate] ECL detection reagent, and exposed to Hyperfilm ECL (GE Healthcare).

Clear and BN PAGE Analysis. Blue Native PAGE (BN-PAGE) was used to resolve the native, intact mitochondrial protein complexes (11). Briefly, mitochondria samples were solubilized in BN-PAGE sample buffer [50 mmol/L Tris·HCl, 50 mmol/L NaCl, 10% glycerol (wt/vol), 0.001% Ponceau S, pH 7.2 plus protease inhibitor mixture] with 2% digitonin (wt/vol) at about 5 μg/μL and incubated on ice for 30 min. Each sample was centrifuged for 30 min at 22,000 × *g* at 4 °C. The supernatant was collected and 50 μg of protein was mixed with 5% (wt/vol) Coomassie G250 to a final concentration of 0.4%, then loaded on a 3–12% NativePAGE Novex gel (1 mm, Invitrogen) and run according to the manufacturer's protocols. The gel was either transferred to PVDF membrane (Millipore; 45 μm) for Western blotting or separated by a second dimension SDS/PAGE. Upon completion of 1D BN-PAGE whole lanes were excised from the gel into 15-mL tubes for incubation in 1× NuPAGE LDS sample buffer [Invitrogen; 62 mmol/L Tris, 0.5% (wt/vol) LDS, 2.5% glycerol, 0.13 mmol/L EDTA, 0.55 mmol/L Coomassie G250 and 0.04 mmol/L phenol red, pH 8.5] with or without 50 mmol/L DTT, for reducing and nonreducing PAGE, respectively, at 37 °C for 30 min on a Nutator. Gel slices were placed on the top of an 18-cm 10% SDS/PAGE gel (1.5 mm, homemade) and overlaid with 0.5% agarose. The gels were run using Mes running buffer [50 mmol/L Mes, 50 mmol/L Tris–base, 0.1% (wt/vol) SDS, 1.0 mmol/L EDTA, pH 7.3] at 70 V (2 h) and 90 V (16 h). Gels were either fixed in 50% (vol/vol) methanol, 10% (vol/vol) acetic acid, or transferred to nitrocellulose membrane for Western blotting as described above.

Mitochondrial ^{3}H UDP-GlcNAc Transport Assay. To ascertain the possible influx of UDP-GlcNAc, substrate for protein *O*-GlcNAcylation, into the mitochondrial matrix, purified mitochondria from rat heart tissue was incubated with ^{3}H UDP-GlcNAc. Mitochondria from normal rat hearts was purified as described above and resuspended in a buffer used to study mitochondrial respiration (20 mM Hepes, 250 mM sucrose, and 10 mM $MgCl_2$, pH 7.02). The mitochondria were incubated with 1 μCi of ^{3}H UDP-GlcNAc for 5, 10, 15, or 30 min. Uptake was also analyzed in the presence of 500 μM of ATP, UTP, UDP, UMP, etc. Mitochondria incubated with radioactivity and containing 1% detergent was used as negative controls. Following incubation the mitochondria were pelleted by centrifugation at 10,000 × *g*. The pellet was then resuspended in buffer free of radioactivity and the mitochondria were again pelleted at 10,000 × *g*. This step was repeated three times. The remaining radioactivity within the mitochondrial pellet was then measured in a scintillation counter. Concentration of transported

UDP-GlcNAc per milligram of mitochondrial protein was plotted. Further analysis of nucleotide sugar uptake was carried out using purified mitoplasts in the same way as described above. Mitoplasts were prepared as described elsewhere (12). Control mitoplasts were also incubated with a solution of 10 mM streptolysin O.

For inhibition analysis of putative mitochondrial carriers, purified mitochondria was preincubated for 15 min with 20 mM butyl malonate, 50 mM bathophenanthroline, 20 mM pyridoxyl phosphate, 15 mM ethylmalemide, 20 mM phenyl succinate, and 20 mM cyanohydroxycinnamate followed by regular uptake assay as described above.

Cloning, Expression, and Purification of Pyrimidine Nucleotide Carrier and Deoxyribonucleotide Carrier. Plasmids expressing the dnc and pnc gene were obtained from Sarah O'Conner, National University of Ireland, Cork and Fernando Palmieri, University of Bari, Italy, respectively (13, 14). The proteins were expressed in bacteria using appropriate antibiotic selection and IPTG induction. The cell pellet was resuspended in 20 mM Tris containing 50 mM DTT and passed through a French press to lyse cells. The supernatant was removed and the pellet was again resuspended in 20 mM Tris, 1 mM DTT pH 7.02 buffer, and loaded on to a 30–70% sucrose density gradient to separate the inclusion bodies.

Generation of Proteoliposomes Containing Intermembrane PNC and DNC Proteins and Analysis of UDP-GlcNAc Uptake by These Liposomes. To 100 mg of powdered phospatidylcholine (PC) was added 1 mL of sterile water and the mixture was incubated for 3 h with periodic vortexing and bath sonication. Meanwhile the extruder was set up by putting a 0.1-μm filter between the two syringe units and residual air was removed by pushing water through the filter. The PC-water mix was extruded through the 0.1-μm filters 11 times such that the last extrusion completes in the syringe opposite to the one initially filled. Liposome prep was removed and stored in an Eppendorf tube at 4 °C for 5 d. A working mixture of liposome, protein, and detergent was prepared by taking 10 μg of purified protein (in inclusion bodies) and adding 60 μL of Triton X-114. To the mix 200 μL of PC liposome was added. The total solution was made up to 700 μL by adding a 20 mM Hepes buffer containing 20 mM NaCl and 10 mM nucleotide (UTP, UDP, etc.). This was vortexed a couple of times and passed through a XAD2 amberlite ion exchange resin for 13–20 consecutive times to exchange detergent and incorporate protein into liposome membrane (15). Subsequently, excess nucleotide was separated from liposome by passing the mixture through a G50 column. The resultant proteoliposome was used for DLS analysis. For uptake assay, 200 μL of the prepared proteoliposome was incubated with 1 μCi of ^{3}H UDP-GlcNAc for 20 min, at the end of which, 20 mM bathophenanthroline was added to stop uptake. The mixture was purified on a G50 column to remove excess radioactivity and the resultant ^{3}H UDP-GlcNAc in the liposome was measured in a scintillation counter (16, 17).

Knockout of SLC25A33 (pnc1) Gene in Cell Culture. Stealth siRNA against SLC25A33, the gene encoding for the pyrimidine nucleotide carrier, was obtained from Dharmacon, Invitrogen. Approximately 90% confluent plates of HeLa cells were transiently transfected with 500 pmol or 2 nmol of the siRNA and with 500 pmol of control siRNA using Lipofectamine 2000. The transfection reagent was removed after 6 h of incubation and the cells were allowed to grow for 48 h. Subsequently, cells were scraped, lysed, with a homogenizer, and crude mitochondria prepared using differential centrifugation technique. Both whole cell lysate and the mitochondrial prep were run on a 10% PAGE, transferred onto nitrocellulose, and blotted for CTD 110.6, tubulin, complex I, and SLC25A33.

The ^{3}H UDP-GlcNAc Transport Assay for PNC Knockdown Mitochondria. HeLa cells treated with PNC siRNA and control were used for enrichment of mitochondria as described above. A total of 5 mg of mitochondria was used to set up a transport assay in respiration buffer (20 mM Hepes, 250 mM sucrose, and 10 mM $MgCl_2$, pH 7.02). The mitochondria were incubated with 1 μCi of ^{3}H UDP-GlcNAc for 20 min. Following incubation, the mitochondria were pelleted by centrifugation at 10,000 × *g*. The pellet was then resuspended in buffer free of radioactivity and the mitochondria again were pelleted at 10,000 × *g*. This step was repeated three times. The remaining radioactivity within the mitochondrial pellet was then measured in a scintillation counter. Concentration of transported UDP-GlcNAc per milligram of mitochondrial protein was plotted.

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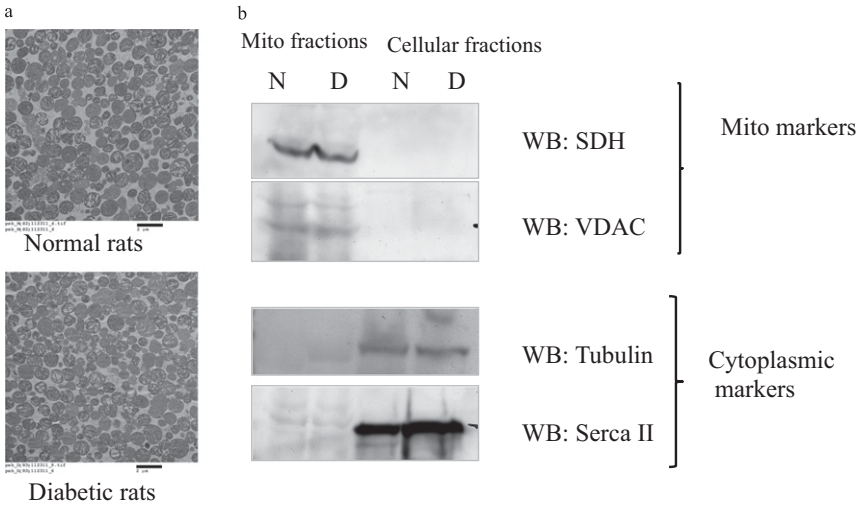


Fig. S1. TEM and immunoblotting analysis of purified rat heart mitochondria from normal and STZ (diabetic) rat hearts. (*A*) TEM images of purified mitochondria showing structurally intact well-resolved mitochondria with low levels of cellular impurities. (*B*) Western blotting of purified mitochondrial lysate and whole heart lysate to look at levels of mitochondrial proteins (SDH and VDAC) and cytoplasmic proteins (tubulin and Serca II).

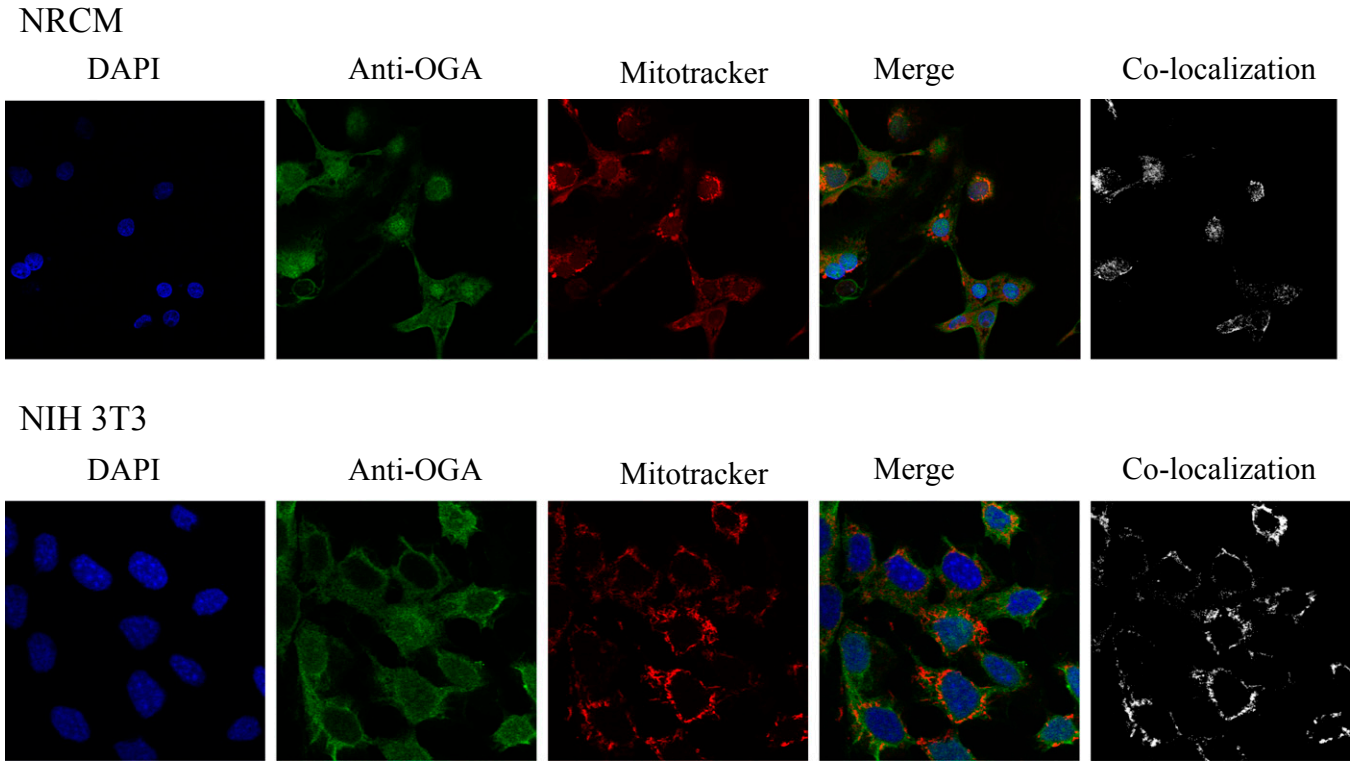
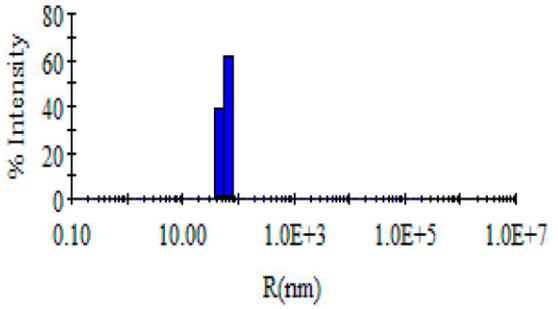


Fig. S2. Immunofluoresence imaging of neonatal rat cardiomyocytes and NIH 3T3 cells stained for nucleus (DAPI), OGA (anti-OGA antibody, 345), and mitochondria (mito-tracker red). Cells were treated with mito-tracker then fixed and permeablized after which they were stained for OGA. Images were taken in a confocal microscope. Colocalization analysis was done on z-stack images using Imaris software.

a Proteoliposomes with transporter



Item	R (nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	61.9	16.7	52376	100.0	100.0

b Control mito lysate Pnc_ko mito lysate

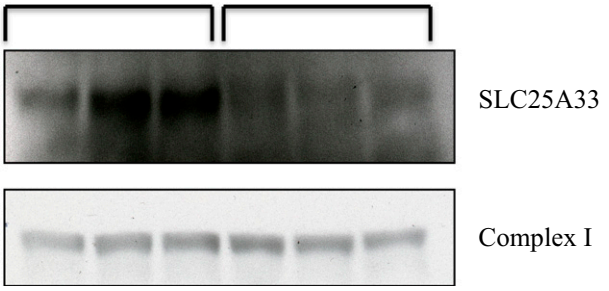


Fig. S3. (*A*) Size of proteoliposome as obtained by dynamic light scattering experiment. Showing liposomes of 62-nm size obtained in 16% polydispersity. (*B*) Western blotting of mitochondrial lysates from HeLa cells treated with stealth siRNA for PNC (SLC25A33) protein. Data show three individual treatments. Mitochondria from above preps were used for radioactive UDP-GlcNAc transport assay.

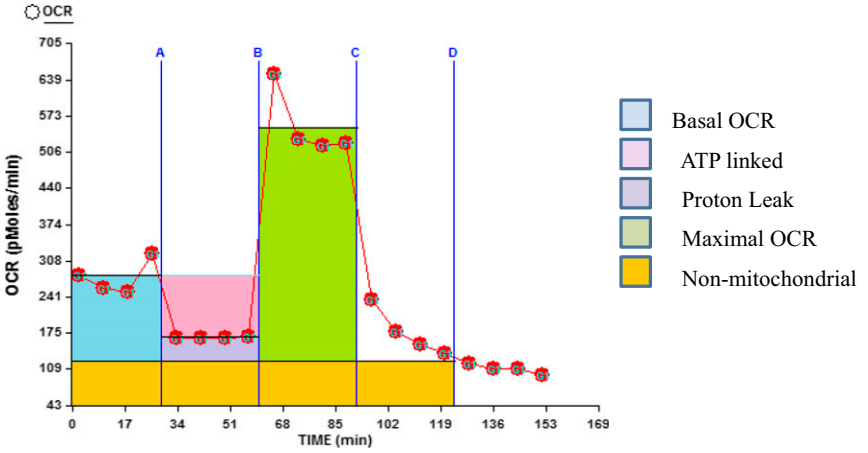


Fig. S4. Seahorse analysis of neonatal rat cardiomyocytes treated with OGT and OGA inhibitors. Representative graph showing mitochondrial oxygen consumption under different treatments. Color-coded areas represent values used to calculate basal, maximal OCR, etc. as reported in Fig. 6*E*.