SUPPLEMENTAL DATA:

MATERIALS AND METHODS:

Isolation of rat primary pancreatic β cells: Pancreatic islets were isolated from Wistar rats [1]. Collagenase P enzyme solution (1.2–1.4 mg/ml; Roche Diagnostics) was injected into the distal end of the donor pancreas. After digestion, islets were gradient purified, handpicked and cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS and 1% PenStrep and cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air. Single cells from islets were prepared as described with modifications [2]. Briefly, after 24-48 hr islets were collected at 1100 rpm for 5 min at 4^{o}C followed by washing in PBS (see above) twice. Collected islets were treated with 0.05% trypsin-EDTA (see above) for 3 min and then triturated gently to separate cells. Trypsin activity was stopped by adding RPMI1640 medium containing FBS before triturating. Cells were seeded at the indicated density for respirometry measurements as described below. We have used the term primary β-cells for dispersed islet cells throughout the text because they constitute the majority of the islet cell population [3]. All procedures were performed following approval by the Baystate Medical Center Institutional Animal Care and Use Committee (IACUC # protocol number 132688) in compliance with the U.S. Public Health Service regulation.

NAD(P) measurements: Mitochondria were isolated as described [4]. Mitochondrial content was measured using a microplate-based BCA protein assay kit (Thermo Fisher). 20 μg of mitochondria were incubated with glutamate + malate (10 mM each) in the presence of 2 μM rotenone for 15 min at 37^{o}C. FLUOstar Omega fluorimeter (BMG Labtech) was used to measure NAD(P)H fluorescence at 460 nm following excitation at 355 nm. An NADH standard curve was used to determine concentrations of NAD(P)H. Additionally, the Fluoro NAD™ kit (#FLNADH-100) from Cell Technology, Inc. was used to measure NAD and NAD^+ separately. 500,000 cells/well were seeded in 24-well culture plates and grown as described above for 48 hours. Cells were permeabilized with 1 nM rPFO and then treated with glutamate + malate or pyruvate + malate (10 mM each) for 10 minutes at room temperature in the presence of 2 μM rotenone. All treatments were made in LKB buffer containing 0.4% BSA. NAD^+ and NADH were extracted and quantified (by using an NADH standard curve) according to the manufacturer's instructions. Data were normalized on a per unit protein basis. Graph Pad Prism 5 was used to determine significance using the Student *t* test.

Pyruvate carboxylase (PC) knock-down in INS1E cells: PC was knocked down using siRNAs as described by Xu et al. [40]. PC_siRNA oligonucleotides were: 5'-CCAUCAAGAAAGUAAUGGUGGCCAA-3' (forward) and 5' UUGGCCACCAUUACUUUCUUGAUGG-3' (reverse). The Control (or scrambled) siRNA oligonucleotides were 5'-CCAAGGUGAGAAUGAAGUACCUCAA-3' (forward) and 5'-UUGAGGUACUUCAUUCUCACCUUGG-3' (reverse). About 70-80% confluent INS1E cells were transfected with siRNAs using Stealth RNA transfection system (Invitrogen, USA). After 72 hours of transfection the reduction in PC protein level was confirmed by Western blotting with an anti-PC antibody (sc-67021; Santacruz,

USA) [41]. For respirometry, 100,000 INS1E cells after 6 hour of transfection were seeded in V7 culture PS plate and grown (2 days) and starved (1 day) as described above.

FIGURE LEGENDS:

Figure S1: **Assay of mitochondrial function with digitonin-permeabilized cells.** Cells were grown as described in Materials and Methods. About 60 min before assays they were incubated in Ca^{2+}-free LKB buffer containing 2 mM (A) or 16.7 mM glucose (B, C). Succinate was either present in medium from the beginning (A) or added after permeabilization (B, C). Downward arrows indicate injections of indicated compounds. **A)** Assay with overnight starved INS1E cells. *Injections*- DIG: 0.01% (w/v) digitonin; ADP/CC: buffer (control); 1 mM ADP with (ADP+CC) or without 10 μM cytochrome c (ADP). **B)** Digitonin titration with non-starved INS1E cells. *Injections*- DIG: indicated concentrations of digitonin (%DIG); Succ+ADP: 10 mM succinate and 1 mM ADP with (0.01% DIG+CC) or without (0.0025-0.01% DIG) 10 μM cytochrome c. **C)** Respiratory response of primary β cells permeabilized with nPFO vs. digitonin**.** *Injections*- Succ+ADP: 10 mM succinate and 1 mM ADP with 25 nM nPFO or DIG in the presence of 10 μM cytochrome c; Oligo: 2 μg/ml oligomycin. Data mean ± SD (n=4-5 wells/group) are shown. CC: cytochrome c; DIG: digitonin, Succ: succinate. They are representative of > 3 replicates.

Figure S2: **Glutamine and monomethyl sodium succinate (MMS)-stimulated respirations in INS1E cells. A**) Glutamine-stimulated respiration compared to glucose. Cells were starved for 24 hr in low glucose medium. About 1 hr before assay the medium was replaced with regular LKB buffer containing 2 mM glucose. *Injections*- Glu/Gln: 14.7 mM glucose (Glu) or 10 mM glutamine (Gln). **B**) Effect of different carbon substrates that induce respiration in INS1E cells. Respiration of non starved cells was measured in 16.7 mM glucose containing regular LKB buffer without added $CaCl_2$. *Injections*- Oligo: 2μg/ml oligomycin (all groups); Subs/PSF/PG3SF:10 mM of pyruvate (Pyr) or glutamine (Gln) or monomethyl succinate (MMS) to intact cells; 1 nM rPFO and 2 μM FCCP with 10 mM of succinate (PSF) or glycerol-3-phospahte (PG3SF). *P, S, G3P, FCCP* and *Subs* denote PFO, succinate, glycerol-3-phospahe, FCCP and substrates respectively. Data mean ± SD (n= at least 4 wells/group) are shown. They are representative of 3 replicates.

Figure S3: Effect of oligomycin pretreatment on CO_2 evolution in permeabilized HEK293 cells. Assays were performed in Ca^{2+}-free MLKB containing 15 mM glucose and respiration (OCR) and CO_2 evolution (ECAR) were measured simultaneously. **A)** Pyruvate and malate supported respiration in HEK293 cells pretreated with oligomycin before permeabilization. *Injections*- Oligo: 2 μg/ml oligomycin; P/Sub/F: 1 nM rPFO (all groups), 10 mM pyruvate with (Pyr+Mal) or without malate (Pyr), 3 μM FCCP (Pyr); Mal/F: 10 mM malate (Pyr), 3 FCCP (Pyr+Mal), buffer (PFO). Data mean ± SD of 3-4 wells/group are shown.

Figure S4: **Relative level of NADH in INS1E vs. HEK293 cells.** The NADH and NAD^+ levels were determined using the Fluoro NAD™ kit as described in Materials and

Methods. NADH (A), NADH/NAD^+ ratio (B) and total (NADH+NAD^+) levels in INS1E vs. HEK293 cells are shown. Data from one representative experiment are shown (n=3). Significance was calculated using student t test at p values *<0.05; **<0.01; and ***<0.001.

Figure S5: Effect of pyruvate carboxylase (PC) knockdown on the decline of oligomycin-insensitive respiration. INS1E cells transected with control and PC_siRNAs were prepared for respirometry as described in Materials and Methods. Respiration was measured in regular LKB containing 2 mM glucose before injections with indicated compounds. *Injections*- Glu: 14.6 mM glucose; Oligo: 2 μM oligomycin. Inlet shows the relative level of PC protein in control and PC-siRNA treated cells by Western blotting. Data from one of the two independent experiments are shown.

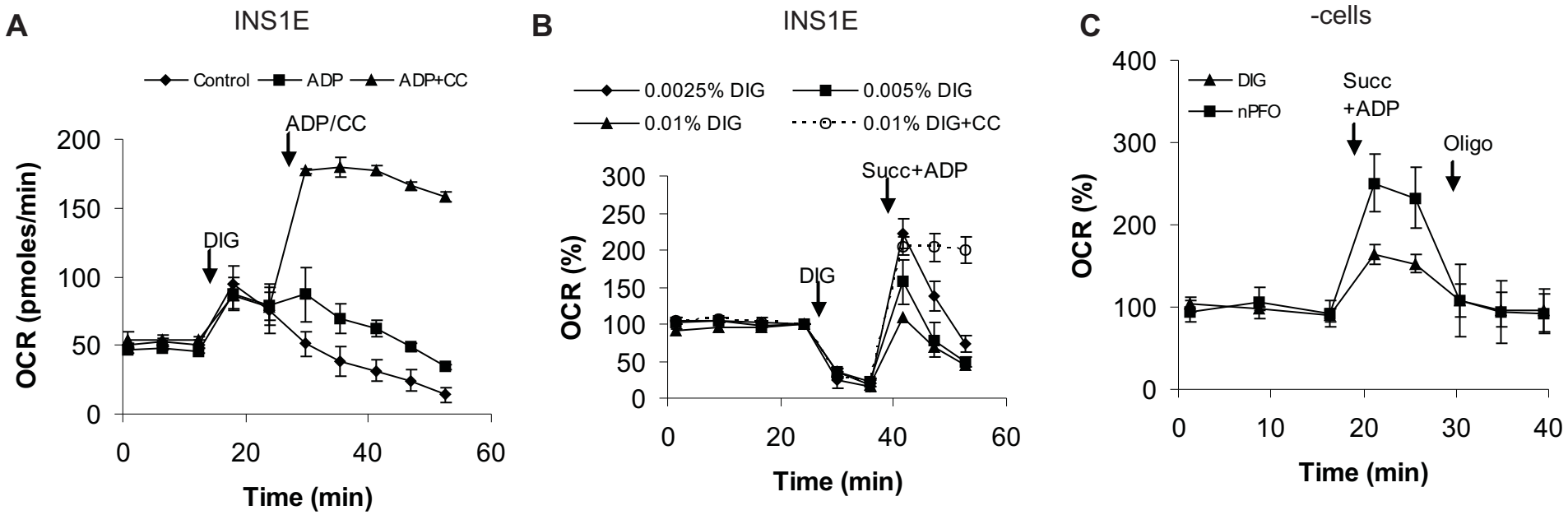
Reference List

1. Brown ML, Bonomi L, Ungerleider N, Zina J, Kimura F, Mukherjee A, Sidis Y, Schneyer A (2011) Follistatin and Follistatin Like-3 Differentially Regulate Adiposity and Glucose Homeostasis. Obesity (Silver Spring) . oby201197 [pii];10.1038/oby.2011.97 [doi].

2. Xu J, Han J, Long YS, Lock J, Weir GC, Epstein PN, Liu YQ (2008) Malic enzyme is present in mouse islets and modulates insulin secretion. Diabetologia 51: 2281-2289. 10.1007/s00125-008-1155-0 [doi].

3. Elayat AA, El-Naggar MM, Tahir M (1995) An immunocytochemical and morphometric study of the rat pancreatic islets. J Anat 186 (Pt 3): 629-637.

4. Affourtit C, Brand MD (2009) Measuring mitochondrial bioenergetics in INS-1E insulinoma cells. Methods Enzymol 457: 405-424. S0076-6879(09)05023-X [pii];10.1016/S0076-6879(09)05023-X [doi].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ADP
ADP+CC
ADP/CC
DIG
OCR (pmoles/min)
Time (min)
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0.005% DIG
0.01% DIG
0.01% DIG+CC
Succ+ADP
DIG
OCR (%)
Time (min)
C
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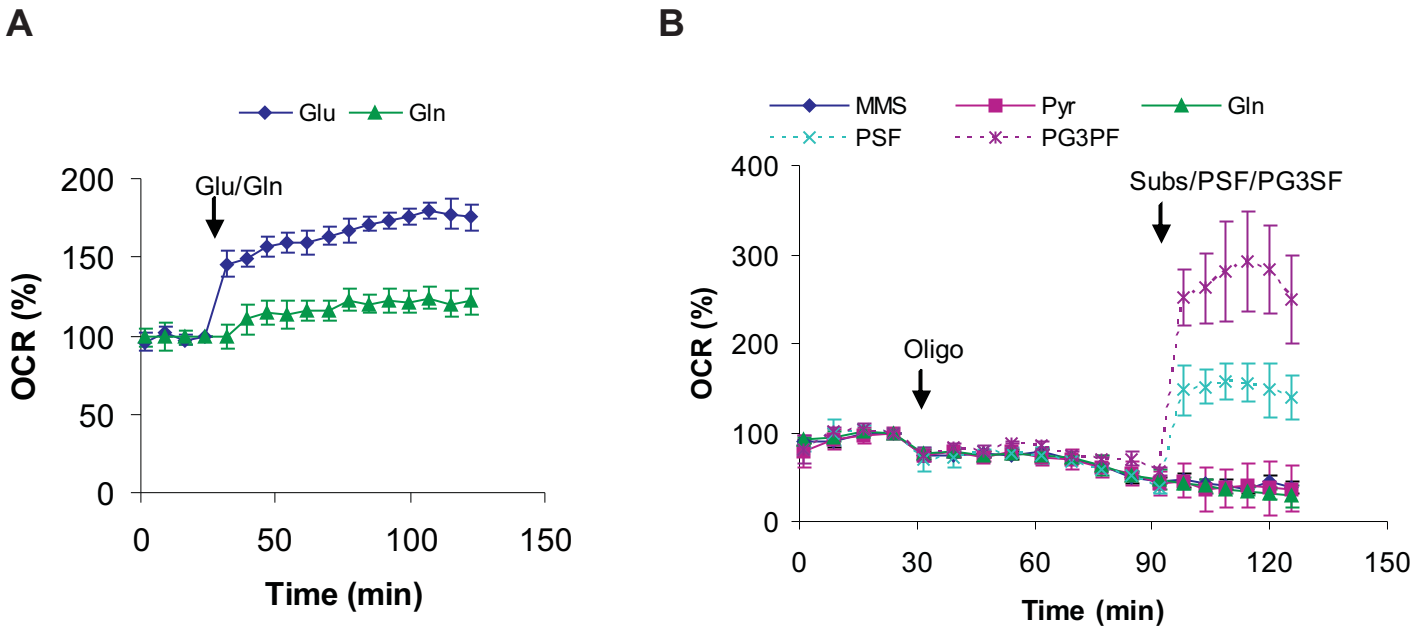
DIG
nPFO
Succ
+ADP
Oligo
OCR (%)
Time (min)

Figure S1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Gln
PSF
PG3PF
Subs/PSF/PG3SF
Oligo
OCR (%)
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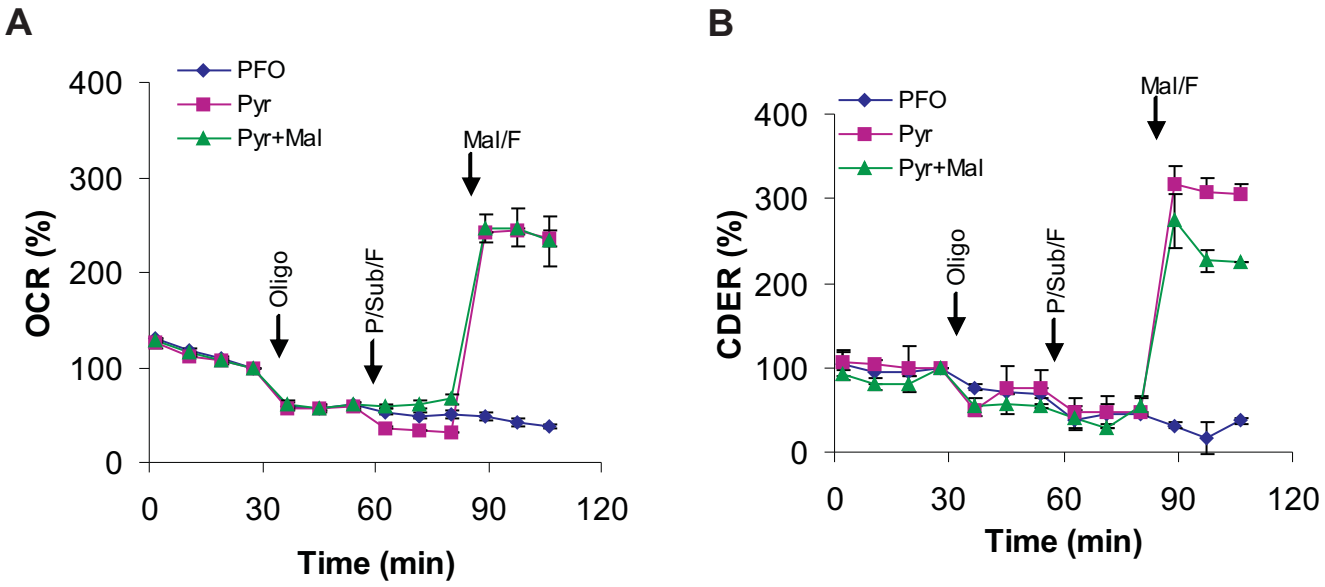
120
150
Time (min)

Figure S2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Pyr+Mal
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Oligo
P/Sub/F
OCR (%)
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100
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60
90
120
Time (min)
B
PFO
Pyr
Pyr+Mal
Mal/F
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P/Sub/F
CDER (%)
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0
30
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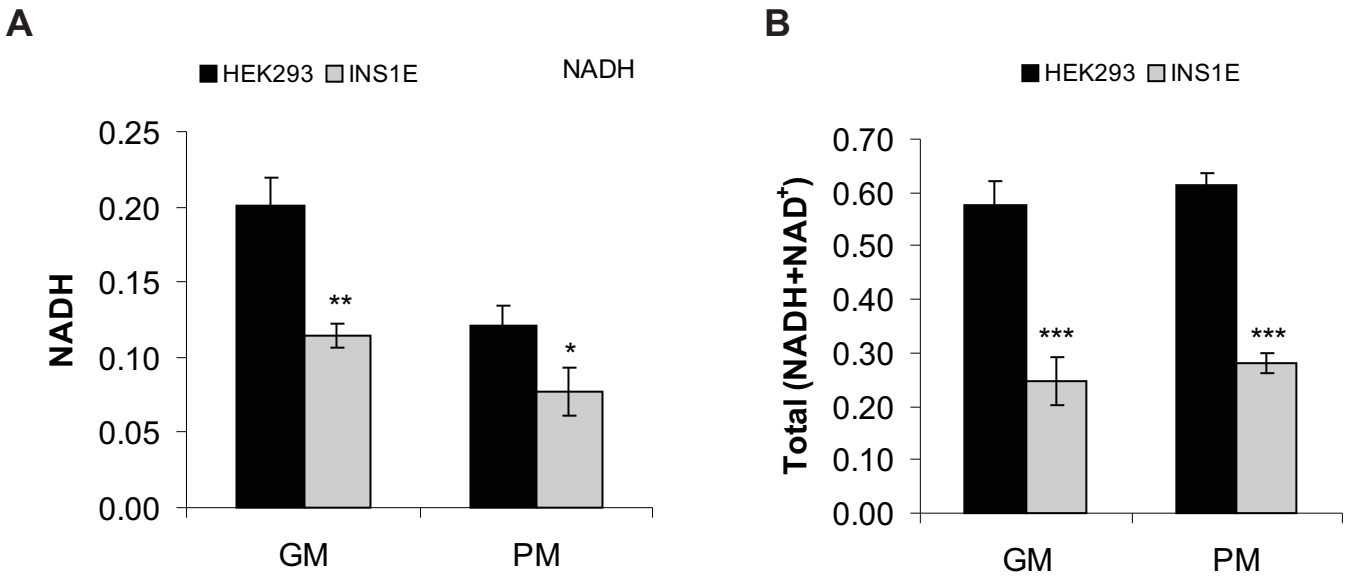
120
Time (min)

Figure S3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PM
B
HEK293
INS1E
Total (NADH+NAD+)
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0.40
0.30
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0.00
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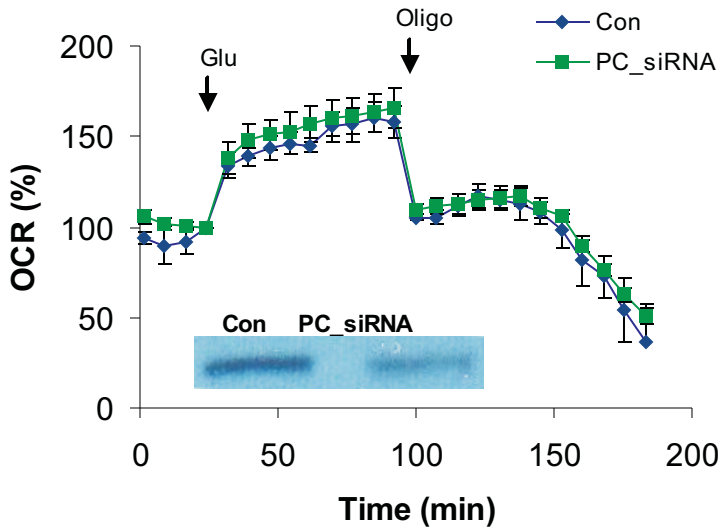
***
GM
PM

Figure S4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Glu
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OCR (%)
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PC_siRNA
0
50
100
150
200
Time (min)

Figure S5