Supplemental Information

Desnutrin/ATGL Activates PPARδ to Promote Mitochondrial Function for Insulin Secretion in Islet β cells

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Supplemental Experimental Procedures

Generation and maintenance of βcell-specific desnutrin knockout mice

Desnutrin-floxed mice were generated and backcrossed to C57BL/6 background as previously described (Ahmadian et al., 2011). RIP-Cre and RIP-CreER mice were from Jackson Laboratory. Mice were maintained on a HFD (45% fat, 35% carbohydrate, and 20% protein; Research Diets) or standard chow diet ad libitum at weaning at 4 wks of age. At 6-wks of age, RIP-CreER mice were injected intraperitoneally 1mg/day tamoxifen (Sigma) dissolved in ethanol and then in corn oil for 5 consecutive days. Eight-week old male mice were used for experiments.

Islet isolation and culture of INS 832/13 cells

Islets from 8-week old mice were isolated by collagenase (Roche) perfusion through the common bile duct into the pancreas as previously described (Li et al., 2009). The islets were either used immediately or cultured overnight in glutamine containing RPMI-1640 with 11 mM glucose, 1 mM pyruvate, 10 mM HEPES, pH 7.2, 50 μM β-mercaptoethanol, supplemented with 2 mM glutamine and 10% FBS prior to experiments. INS 832/13 cells were cultured in the same media as above and with 100 IU/ml penicillin and streptomycin.

Islet dispersion

Freshly isolated islets were dispersed into single cells with 1mg/ml trypsin and 30 μg/ml DNase (King et al., 2007). β cells were roughly separated from α cells by centrifuge at 800g according to their different density and sedimentation velocity (Pipeleers, 1987). The cells were then counted and aliquots were removed for glucose-stimulated insulin secretion and MitoTracker or Nile red staining.

Adenoviral overexpression and shRNA knockdown of desnutrin

Adenovirus for enhanced green fluorescent protein (GFP) or desnutrin-GFP was generated as previously described (Ahmadian et al., 2011). Lentiviral infection of INS 832/13 cells was carried out with shRNA constructs (Origene) with either desnutrin or scrambled sequence. Isolated islets, dispersed islet cells or INS 832/13 cells grown to 80% confluence were infected 48 hrs prior to experiments. Cells were grown to 70% confluence and were infected as we previously described (Wang and Sul, 2009). After 72 hrs, experiments were performed after verification of desnutrin expression levels.

Glucose and insulin tolerance tests

For GTT, mice were injected intraperitoneally with 2 mg/g BW of D-glucose following a 15 hr-fast. For ITT, insulin (humulin, Eli Lilly, 0.75 mU/g BW) was injected

intraperitoneally following a 5 hr-fast. Tail vein blood was collected at the indicated time points and glucose was measured using a glucometer and insulin and C-peptide were measured using ELISA (Alpco). Following a 15 hr-fast, tail vein blood was collected for measurements of NEFA (WAKO), TAG (Thermo) and glucose levels.

TAG extraction and quantification

Total neutral lipids were extracted according to the Folch method (Folch et al., 1957). Lipids were solubilized in 1% Triton X-100 and TAG levels were measured using Infinity Reagent (Thermo) and quantified by a colorimetric plate reader (Molecular Devices, Spectra Max 250) reading at 500 nm.

H&E staining, immunostaining, and Nile red staining

Pancreas samples from mice were embedded in OCT medium (TissueTek) and snap frozen in liquid nitrogen. Samples were sectioned while frozen into 8 μm slices in a cryotome and fixed with a 10% formaldehyde solution. H&E staining of pancreas were performed as described previously. Pictures were taken with a Hamamatsu Orca camera. Average islet size and frequency were calculated by NIH ImageJ software after measuring and counting 100 islets per pancreas cryosection.

For immunostaining, islets were handpicked under a stereomicroscope and fixed in a 10% formaldehyde solution. Islets were stained with primary antibodies against insulin (Cell Signaling) followed by Alexa Fluor 488 secondary antibody staining or glucagon (Abcam) followed by Alexa Fluor 495 secondary antibody and DAPI was used to visualize nuclei. Sections or islets were visualized with an AxioImager.

Nile red was used to stain isolated islets or INS 832/13 cells grown on coverslips and fixed in 10% formaldehyde.

Insulin secretion and insulin, glycerol, FFA levels

Insulin release was measured in the secretagogue buffer using ELISA (Alpco). For time-dependent glucose-stimulated insulin secretion, media was aliquoted and centrifuged for 5 min at 4 °C to remove any cell debris (Sun-Wada et al., 2006; Wang et al., 2005) at various time points. For insulin content measurement, insulin was extracted from whole pancreas or isolated islets by acid/ethanol extraction and insulin level was normalized to protein content determined by Bradford method (BioRad). Glycerol concentration was measured in the media from INS cells or isolated islets by using free glycerol reagent (Sigma) as previously described (Jaworski et al., 2009). Intracellular free FA concentrations were measured by free FA quantification fluorometric kit (BioVision) on a fluorescence plate reader (Molecular Devices Gemini EM) and normalized to protein levels.

RNA extraction and RT-qPCR

Total RNA was extracted from isolated islets using Trizol Reagent (Qiagen) and cDNA was synthesized from 1 μg of total RNA by reverse transcription (Promega). Gene expression levels were determined by RT-qPCR performed with an ABI PRISM 7700 sequence fast detection system (Applied Biosystems), quantified by measuring the threshold cycle normalized to GAPDH or SYBR Green detection system (Fermentas, Thermo), quantified by measuring the threshold cycle normalized to β-actin, and results

are expressed relative to control floxed mice. Primer ID or sequences for these genes are shown in Table S1.

Mitochondrial DNA quantification

Specific primers were designed for either Mitochondrial DNA or genomic DNA: Mitochondrial DNA; Cytochrome B, 5'-GCTTTCCACTTCATCTTACCATTTA-3', and 5'-CAGGATCAAACAACCCAACA-3' (Ylikallio et al., 2010); nuclear DNA; NDUFV1, 5'-CTTCCCCACTGGCCTCAAG-3'and 5'-GCTGGATCACTG GGTTTTGG-3' (Shende et al., 2011). Mitochondrial DNA to nuclear DNA ratio was quantified by SYBR green qPCR system.

Transmission electron microscopy

Isolated islets were fixed in 2% glutaraldehyde in 0.1M phosphate buffer overnight at 4°C. Islets were then postfixed in 1% OsO_4 and embedded in an Epon-Araldite mixture. Ultra thin sections (0.2 μm) were mounted on 150-mesh copper grids. Grids were stained with lead citrate and visualized under a FEI Tecnai 12 transmission electron microscope. Number of insulin granules per area was calculated from 10-12 different pictures taken randomly.

Immunoblotting

Cell lysates were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes and blocked in 5% BSA for 1 hr. Membranes were incubated with either anti-desnutrin (Ahmadian et al., 2011), anti-ATGL (Cell Signaling), or anti-GAPDH (Santa Cruz) antibodies followed by horseradish peroxidase conjugated secondary antibody (BioRad). Enhanced chemiluminescence substrate (Perkin Elmer) was used to visualize blots and images were captured using a Kodak Image Station 4000MM. ImageJ software was used for quantification.

ATP/ADP ratio and citrate synthase activity

Isolated islets or INS 832/13 cells were incubated in 1X SAB buffer containing 3 mM glucose at 37°C for 2 hrs. The reaction was terminated 3 min after the addition of 15 mM glucose by quick chilling on ice. The cells were lysed in ADP assay buffer and the lysates were deproteinized by using the sample preparation kit (BioVison). ATP and ADP contents were measured by fluorometric assay kit (BioVision) using a plate reader (Molecular Devices Gemini EM). Citrate synthase activity was measured with citrate synthase assay kit (Sigma) using lysates from isolated islets (Schuler et al., 2006). Briefly, 100 μM 5,5'-dithio-bis(2-nitrobenzoic acid) and 250 μM acetyl-CoA were added to lysates and reaction was initiated by adding 500 μM oxaloacetate to the mixture. Absorbance was measure by spectrophotometer at 412 nm. Activity was calculated as the absorbance rate per min per μg of protein.

Measurement of oxygen consumption rate

Isolated islets were washed with assay media (Seahorse Bioscience). 80-100 islets per well were plated and pre-incubated for 2 hrs at 37 ° without CO_2 in assay media containing 3 mM glucose, 0.8 mM Mg^{2+}, 1.8 mM Ca^{2+}, 143 mM NaCl, 5.4 mM KCl, 0.91 mM NaH_2PO_4, Phenol red 15 mg/ml and 1% FBS. To optimize the islet respiration

condition, 10 mM pyruvate and 2 mM glutamine were added into the assay media. Glucose at 20 mM was added to stimulate cellular oxygen consumption. Oligomycin (5 μM) was injected to determine the oligomycin-independent OCR. The mitochondrial respiration uncoupler FCCP (1 μM) was added to determine the electron transport capacity. Rotenone (5 μM) was added at the end of the assay period to block electron transfer. To minimize the variation, OCR of each well was normalized to the initial rates under basal conditions (Wikstrom et al., 2012).

PPAR binding

Briefly, nuclear extracts were prepared from approximately 30 islets using NE-PER Nuclear and cytoplasmic extraction reagents (Pierce). Nuclear extracts were added to a plate coated with PPAR response element. After wash, antibodies specific to either PPARδ or PPARα were added. The plate was read at 450 nm in a colorimetric plate reader after wash. Absorbance was normalized to the positive control for each antibody for the antibody efficiency and protein concentration.

MitoTracker red/green staining

For mitochondrial membrane potential measurements, dispersed islet cells were stained with MitoTracker reagents (Invitrogen). Briefly, isolated islets were dispersed and cultured overnight in RPMI media. Media were changed to SAB buffer with 3mM glucose and incubated for 2 hrs.

MitoTracker Red (20 nM) was added with 15 mM glucose to stain the active mitochondria (Tracker dry spontaneously forms complexes with more polarized mitochondrial with intense red fluorescence). After incubation at 37°C for 30 min, MitoTracker Red was washed off from the cells with SAB buffer and MitoTracker Green (20 nM) (tracker dry stains both polarized and depolarized mitochondria) was added and incubated for 5 min. Fluorescence was either read on Molecular Devices Gemini EM plate reader or visualized with a Zeiss Axio Observer microscope and pictures taken with a Hamamatsu 9100-13 camera. Mitochondrial activity was expressed as the density of staining of MitoTracker red/green ratio.

In desnutrin overexpressing INS 832/13 cells, because of the overlapping wavelength of MitoTracker Green and GFP, mitochondria were only stained with MitoTracker Red, and GFP fluorescence was used for cell number.

FA oxidation and FA uptake

Cell media were changed to SAB buffer with palmitic acid (20 μCi/ml). After 2 hrs of incubation at 37°C with gentle shaking, the buffer was harvested and acidified with perchloric acid, and the $^{14}CO_2$ was trapped on Whatman paper with ethanolamine. The radioactivity was quantified by scintillation counting and normalized to protein concentration.

Fatty acid uptake was measured by adding bodipy-labeled fatty acid (Invitrogen) as described previously (Varlamov et al., 2010) into INS 832/13 cells after 48-hr adenoviral infection. After 1 hr incubation, cells were washed and lysed. Bodipy-labeled fatty acid uptake was measured by fluorescence-activated cell sorting (BD FACSCalibur).

Supplemental References:

Ahmadian, M., Abbott, M.J., Tang, T., Hudak, C.S., Kim, Y., Bruss, M., Hellerstein, M.K., Lee, H.Y., Samuel, V.T., Shulman, G.I.*, et al.* (2011). Desnutrin/ATGL is regulated by AMPK and is required for a brown adipose phenotype. Cell Metab *13*, 739-748.

Folch, J., Lees, M., and Sloane Stanley, G.H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem *226*, 497-509.

Jaworski, K., Ahmadian, M., Duncan, R.E., Sarkadi-Nagy, E., Varady, K.A., Hellerstein, M.K., Lee, H.Y., Samuel, V.T., Shulman, G.I., Kim, K.H.*, et al.* (2009). AdPLA ablation increases lipolysis and prevents obesity induced by high-fat feeding or leptin deficiency. Nat Med *15*, 159-168.

Jensen, M.V., Joseph, J.W., Ilkayeva, O., Burgess, S., Lu, D., Ronnebaum, S.M., Odegaard, M., Becker, T.C., Sherry, A.D., and Newgard, C.B. (2006). Compensatory responses to pyruvate carboxylase suppression in islet beta-cells. Preservation of glucose-stimulated insulin secretion. J Biol Chem *281*, 22342-22351.

King, A.J., Fernandes, J.R., Hollister-Lock, J., Nienaber, C.E., Bonner-Weir, S., and Weir, G.C. (2007). Normal relationship of beta- and non-beta-cells not needed for successful islet transplantation. Diabetes *56*, 2312-2318.

Li, D.S., Yuan, Y.H., Tu, H.J., Liang, Q.L., and Dai, L.J. (2009). A protocol for islet isolation from mouse pancreas. Nat Protoc *4*, 1649-1652.

Pipeleers, D. (1987). The biosociology of pancreatic B cells. Diabetologia *30*, 277-291.

Schuler, M., Ali, F., Chambon, C., Duteil, D., Bornert, J.M., Tardivel, A., Desvergne, B., Wahli, W., Chambon, P., and Metzger, D. (2006). PGC1alpha expression is controlled in skeletal muscles by PPARbeta, whose ablation results in fiber-type switching, obesity, and type 2 diabetes. Cell Metab *4*, 407-414.

Shende, P., Plaisance, I., Morandi, C., Pellieux, C., Berthonneche, C., Zorzato, F., Krishnan, J., Lerch, R., Hall, M.N., Ruegg, M.A.*, et al.* (2011). Cardiac raptor ablation impairs adaptive hypertrophy, alters metabolic gene expression, and causes heart failure in mice. Circulation *123*, 1073-1082.

Sun-Wada, G.H., Toyomura, T., Murata, Y., Yamamoto, A., Futai, M., and Wada, Y. (2006). The a3 isoform of V-ATPase regulates insulin secretion from pancreatic beta-cells. J Cell Sci *119*, 4531-4540.

Varlamov, O., Somwar, R., Cornea, A., Kievit, P., Grove, K.L., and Roberts, C.T., Jr. (2010). Single-cell analysis of insulin-regulated fatty acid uptake in adipocytes. Am J Physiol Endocrinol Metab *299*, E486-496.

Wang, C.Z., Wang, Y., Di, A., Magnuson, M.A., Ye, H., Roe, M.W., Nelson, D.J., Bell, G.I., and Philipson, L.H. (2005). 5-amino-imidazole carboxamide riboside acutely potentiates glucose-stimulated insulin secretion from mouse pancreatic islets by KATP channel-dependent and -independent pathways. Biochem Biophys Res Commun *330*, 1073-1079.

Wang, Y., and Sul, H.S. (2009). Pref-1 regulates mesenchymal cell commitment and differentiation through Sox9. Cell Metab *9*, 287-302.

Wikstrom, J.D., Sereda, S.B., Stiles, L., Elorza, A., Allister, E.M., Neilson, A., Ferrick, D.A., Wheeler, M.B., and Shirihai, O.S. (2012). A novel high-throughput assay for islet respiration reveals uncoupling of rodent and human islets. PLoS One *7*, e33023.

Ylikallio, E., Page, J.L., Xu, X., Lampinen, M., Bepler, G., Ide, T., Tyynismaa, H., Weiss, R.S., and Suomalainen, A. (2010). Ribonucleotide reductase is not limiting for mitochondrial DNA copy number in mice. Nucleic Acids Res *38*, 8208-8218.

Figure S1

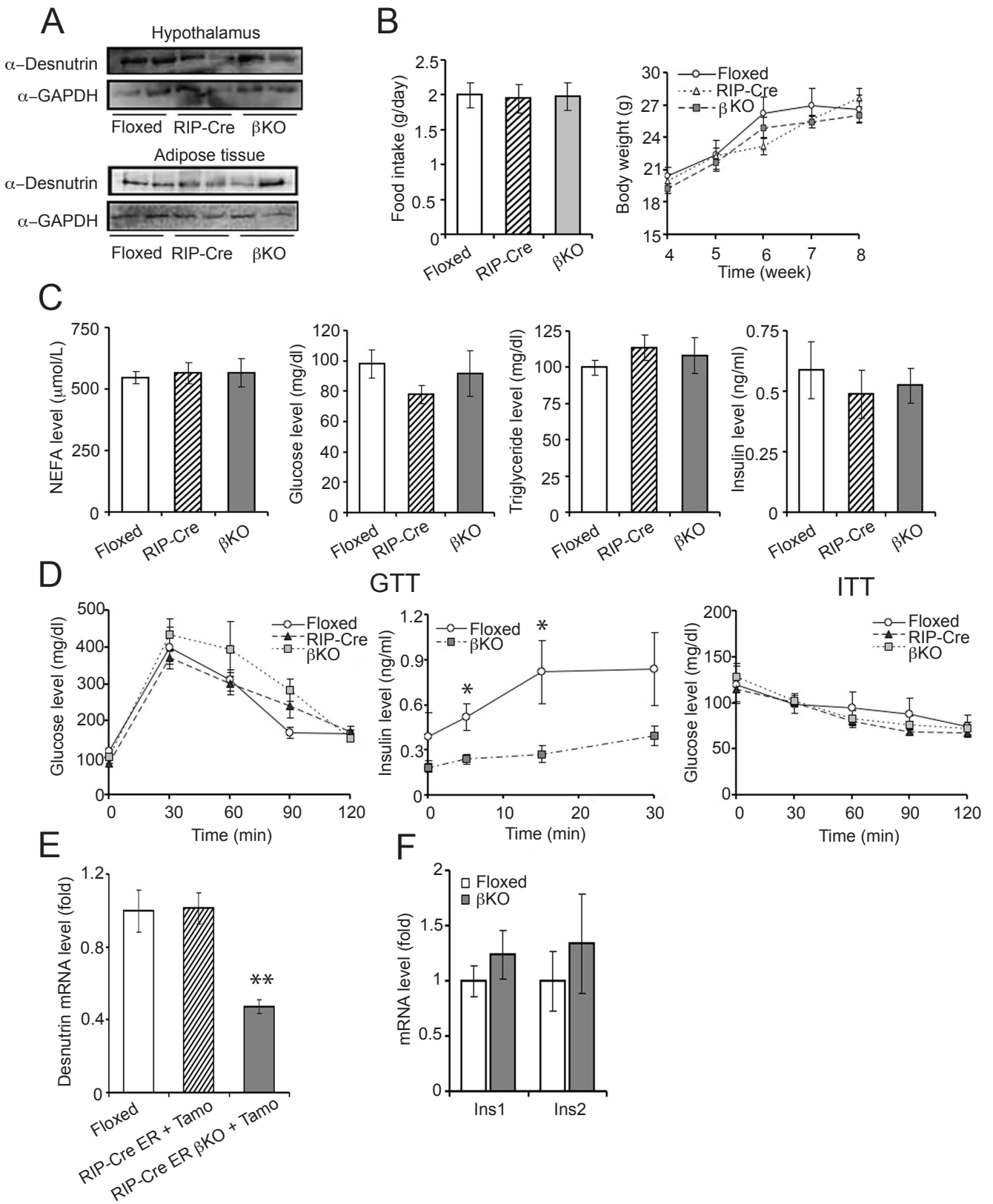
A
Hypothalamus
α–Desnutrin
α–GAPDH
Floxed RIP-Cre βKO
Adipose tissue
α–Desnutrin
α–GAPDH
Floxed RIP-Cre βKO
B
Food intake (g/day)
Floxed
RIP-Cre
βKO
Body weight (g)
Floxed
RIP-Cre
β KO
Time (week)
C
NEFA level (μmol/L)
Glucose level (mg/dl)
Triglyceride level (mg/dl)
Insulin level (ng/ml)
Floxed
RIP-Cre
βKO
D
GTT
ITT
Glucose level (mg/dl)
Floxed
RIP-Cre
βKO
Time (min)
Insulin level (ng/ml)
Floxed
βKO
Time (min)
Glucose level (mg/dl)
Floxed
RIP-Cre
βKO
Time (min)
E
Desnutrin mRNA level (fold)
Floxed
RIP-Cre ER + Tamo
RIP-Cre ER βKO + Tamo
F
mRNA level (fold)
Floxed
βKO
Ins1
Ins2

Figure S1. Characterization of desnutrin βKO mice, related to Figure 1. A) Desnutrin protein levels in hypothalamus and white adipose tissue. B) Total food intake of mice (left) and body weight of mice from 4 to 8 weeks on HFD (right). C) Fasting plasma non-esterified fatty acids, blood glucose, triaclyglycerol and insulin levels. D) Blood glucose (left) and plasma insulin levels (middle) during a GTT on standard chow diet. Blood glucose levels during an ITT (right) on standard chow diet (right). E) RT-qPCR of desnutrin gene expression in islets from floxed, RIP-CreER and RIP-CreER βKO mice after 2-wk tamoxifen injection. F) RT-qPCR for Insulin 1 and Insulin 2 in islets. Male mice, n=6-10; data are shown as mean ± SEM; *P<0.05, **P<0.01.

Figure S2

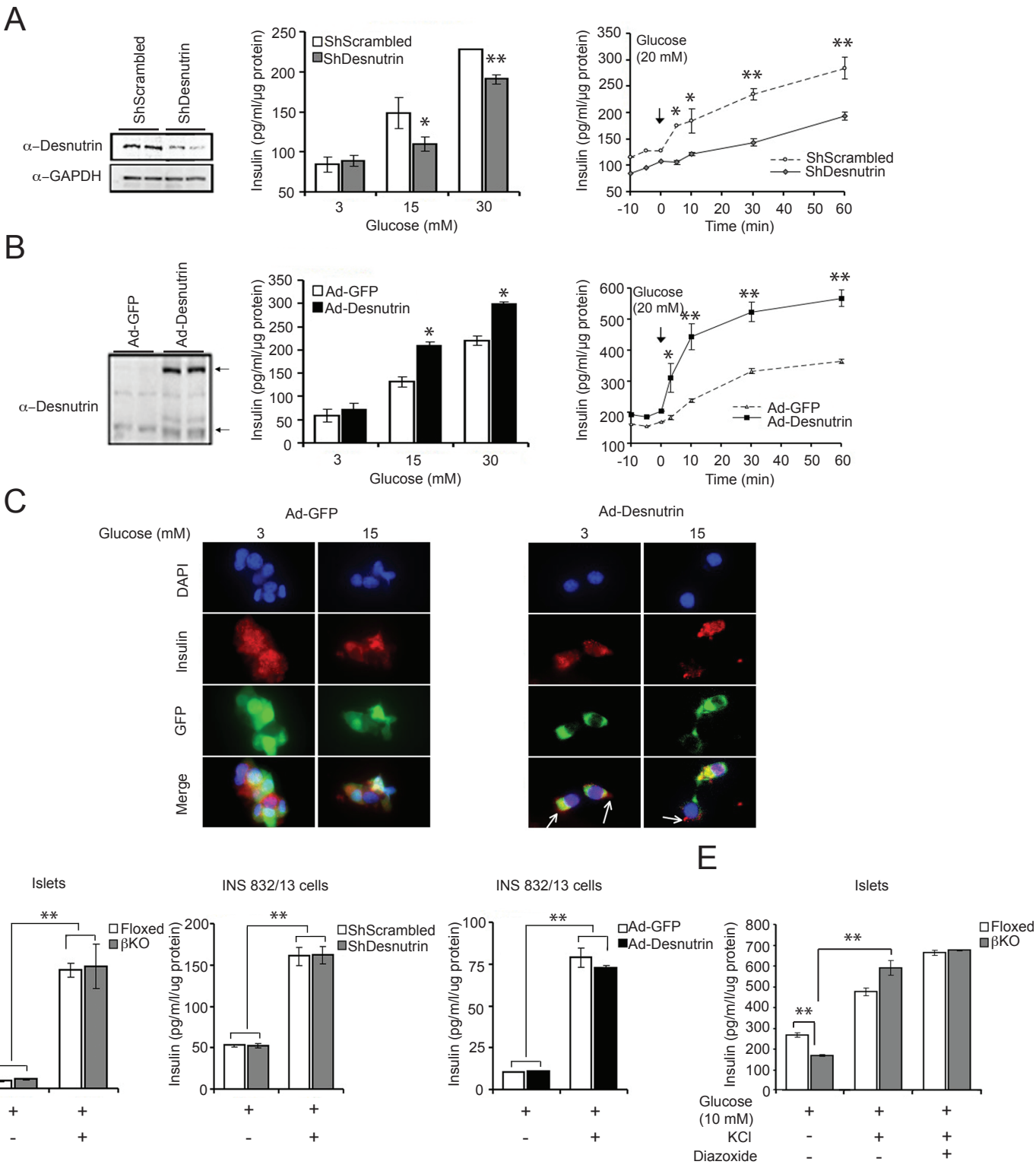
A
ShScrambled
ShDesnutrin
α–Desnutrin
α–GAPDH
Insulin (pg/ml/µg protein)
ShScrambled
ShDesnutrin
Glucose (mM)
Glucose (20 mM)
Time (min)
B
Ad-GFP
Ad-Desnutrin
α–Desnutrin
C
Ad-GFP
Ad-Desnutrin
Glucose (mM)
DAPI
Insulin
GFP
Merge
D
Islets
INS 832/13 cells
INS 832/13 cells
Floxed
βKO
Glucose (3 mM)
KCl
E
Islets
Glucose (10 mM)
KCl
Diazoxide

Figure S2. Desnutrin is required for glucose-stimulated insulin secretion in INS 832/13 cells, related to Figure 2. A) Lentiviral ShRNA desnutrin knockdown in INS 832/13 cells, as verified by immunoblotting (left), GSIS of INS 832/13 cells with desnutrin knockdown after treated with glucose (middle), and GSIS of INS 832/13 cells with desnutrin knockdown at 5, 10, 30 and 60 min after glucose treatment (right). B) Adenoviral desnutrin overexpression in INS 832/13 cells, as verified by immunoblotting, top arrow: desnutrin-GFP, bottom arrow: endogenous desnutrin (left), GSIS of INS 832/13 cells with desnutrin overexpression after glucose treatment (middle), and GSIS in INS 832/13 cells with desnutrin overexpression at 5, 10, 30 and 60 min after glucose treatment (right). C) Desnutrin-GFP does not association with insulin granules. Immunostaining of INS 832/13 cells after adenovirus-mediated GFP or desnutrin-GFP overexpression. Insulin (red) and desnutrin (green) are shown. White arrow: insulin granules. D) Insulin secretion in 3 mM glucose, in the presence or absence of 30 mM KCl in isolated islets (left), in shRNA desnutrin-knockdown INS 832/13 cells (middle), or in desnutrin-overexpressing INS 832/13 cells (right). E) Insulin levels in the incubation media were measured 5 min after addition of 10 mM glucose, in the presence or absence of 30 mM KCl and/or 250 μM diazoxide. Male mice, n=4-5, or INS 832/13 cells, n=3-4; data are shown as mean ± SEM; *P<0.05, **P<0.01.

Figure S3

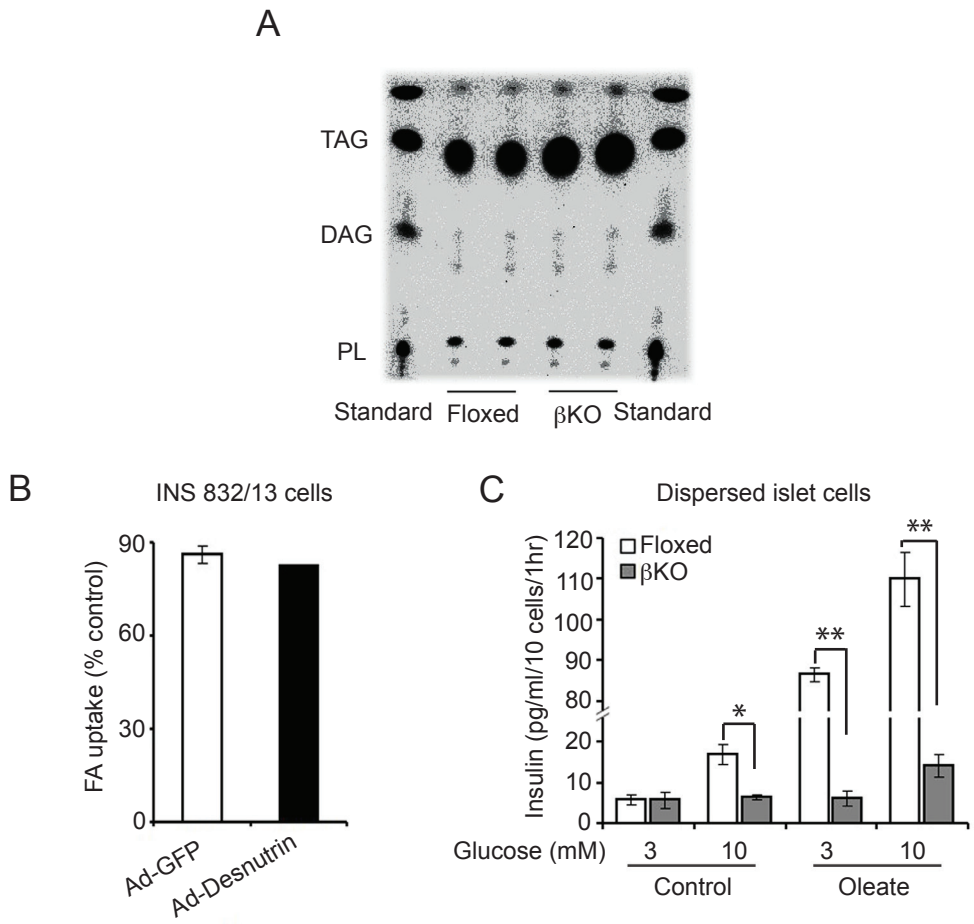
A
TAG
DAG
PL
Standard
Floxed
βKO
Standard
B
INS 832/13 cells
FA uptake (% control)
90
60
30
0
Ad-GFP
Ad-Desnutrin
C
Dispersed islet cells
Insulin (pg/ml/10 cells/1hr)
120
110
100
90
80
20
10
0
Floxed
βKO
*
**
**
Glucose (mM)
3
10
3
10
Control
Oleate

Figure S3. Desnutrin-catalyzed lipolysis is required for proper islet β cell function, related to Figure 3. A) Lipids extracted from islets were separated by TLC, TAG, DAG and Phospholipids were shown as labeled. B) Fatty acid uptake by INS 832/13 cells meusured by bodipy-labeled fatty acid, showing no difference between control and desnutrin overexpressing cells. C) Exogenous fatty acids (oleate) could not restore GSIS in desnutrin-ablated dispersed islet β cells. Male mice, n=5-6, or INS 832/13 cells, n=3-4; data are shown as mean ± SEM;*P<0.05, **P<0.01.

Figure S4

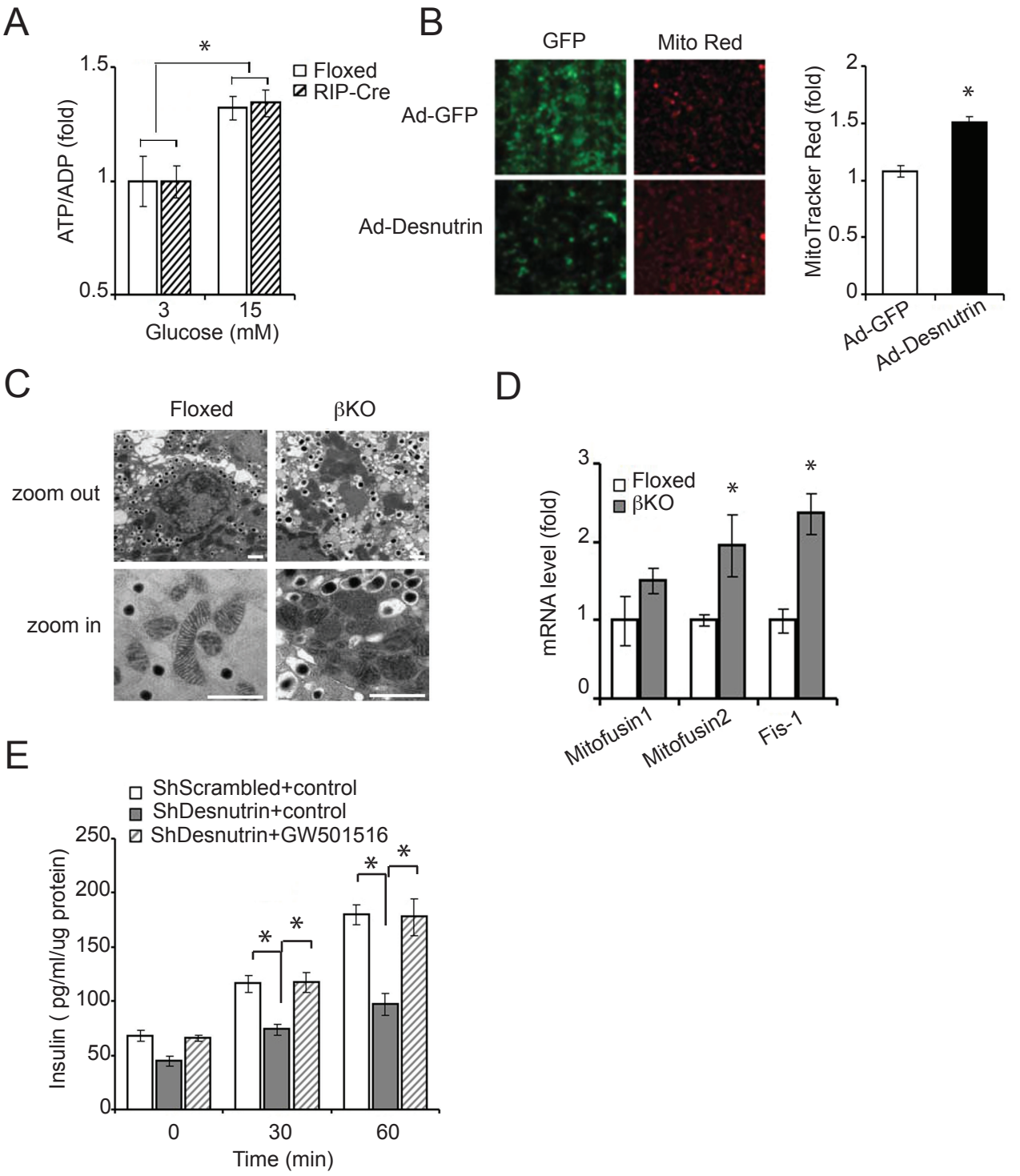
A
ATP/ADP (fold)
1.5
1
0.5
*
Floxed
RIP-Cre
3
15
Glucose (mM)
B
GFP
Mito Red
Ad-GFP
Ad-Desnutrin
MitoTracker Red (fold)
2
1.5
1
0.5
0
*
Ad-GFP
Ad-Desnutrin
C
Floxed
βKO
zoom out
zoom in
D
mRNA level (fold)
3
2
1
0
Floxed
βKO
*
*
Mitofusin1
Mitofusin2
Fis-1
E
ShScrambled+control
ShDesnutrin+control
ShDesnutrin+GW501516
Insulin (pg/ml/ug protein)
250
200
150
100
50
0
*
*
*
*
0
30
60
Time (min)

Figure S4. Desnutrin-catalyzed lipolysis promotes mitochondrial function in islets, related to Figure 4 and 5. A) ATP/ADP ratio in isolated islets from floxed and RIP-Cre at glucose level of 3 mM and 15 mM, no difference in the two control groups. B) MitoTracker red staining of mitochondria in control, GFP or desnutrin overexpressing INS 832/13 cells and quantification of relative fluorescence expressed as fold change over control cells and was normalized to protein concentration. C) Transmission electron microscopy of islets showing fused mitochondrial in βKO cells, scale: 1 μm. D) RT-qPCR for Mitofusin1, Mitofusin2, and Fis-1. E) Treatment with 1 μM PPARδ agonist GW501516 for 7 days restored GSIS in desnutrin knockdown INS 832/13 cells (cells treated with GW501516 were compared to two controls). Male mice, n=6-10, or INS 832/13 cells, n=3-4; data are shown as mean ± SEM; *P<0.05, **P<0.01.

Table S1

Gene Name	Taqman Primer and Probe ABI I.D.
GAPDH	Mm99999915_g1
PGC1α	Mm00447183_m1
Tfam	Mm00447485_m1
Nrf-1	Mm00447996_m1
INS-1	Mm01259683_g1
INS-2	Mm00731595_g1
Pnpla2	Mm00503040_m1
PPARα	Mm00440939_m1
PPARδ	Mm00803186_g1
Mfn-1	Mm00612599_m1
Mfn-2	Mm00500120_m1
Fis-1	Mm00481580_m1

Gene Name	SYBR Green Primer Sequences
Vamp2	F: TGAGGTTCCCATCACCTCTC
	R: CTGTGGGGTTTGCTTTTGTT
SNAP25	F: CAACGTGCAACAAAGATGCT
	R: GGGGGTGACTGACTCTGTGT
Synt1α	F: GAACAAAGTTCGCTCCAAGC
	R: ATTCCTCACTGGTCGTGGTC
Acadvl	F: AGATGCCTTTGAAACCGATG
	R: TTGGGCCTCTCTAATACCCA
CPT1b	F: GCTGCTTGCACATTTGTGTT
	R: TGAGTGACTGGTGGGAAGAA
CPT2	F: CCAATGCCGTTCTCAAAATC
	R: ATGCACTACCAGGACAGCCT
PDK4	F: CTTCTGGGCTCTTCTCATGG
	R: GATTGACATCCTGCCTGACC
Hadhb	F: TAT CAGAGAAGCCAGCTCCC
	R: TAGAGCTGCACTTTCGGGTT
Mdh2	F: TCCCAGGACAGCCACTTTAG
	R: CTCCTGCCAGTAGCTCCG
Sdhb	F: GTCTGTGCCCCTCGACAG
	R: TGACGTCAGGAGCCAAAAT

Table S1. Primer ID and sequences for RT-qPCR, related to Figure 1 and Figure S1 and S4.