Supplemental Information

Glucagon resistance and decreased susceptibility to diabetes in a model of chronic hyperglucagonemia

SUPPLEMENTAL MATERIALS

Islet Studies

Islet isolation was accomplished by collagenase digestion as described previously (1). Islets were cultured overnight in RPMI containing 5 mM glucose. Groups of 15 islets/mouse were placed in 8 μm cell culture inserts (Millicell), preincubated in HG KRBB (6 mM glucose) for 1-2 hours and incubated subsequently for 1 hour in each condition: LG KRBB (1 mM glucose), HG KRBB (6 mM, 12 mM and 24 mM glucose) or HG KRBB+Insulin (100nM) or LG KRBB+KCl (30 mM). Assessment of glucagon content of the islets was performed by extraction in 0.5 mL acid-alcohol per 15 islets/insert after each assay. Secreted glucagon levels and islet glucagon content were measured with an ELISA (Glucagon Duoset Elisa, R&D Systems). All data is represented as secreted glucagon in the culture medium normalized to islet glucagon content for each insert of islets and presented as fold change compared to control.

Flow Cytometry and FACS

Islets were isolated and incubated overnight in RPMI containing 5 mM glucose. The islets were dispersed into a single-cell suspension with trypsin-EDTA and fixed with BD Pharmingen Transcription Factor Phospho Buffer Set (BD Biosciences). The fixed cells were incubated with conjugated antibodies overnight, at 4 °C and gentle rotation. Dead cells were excluded by Ghost Dye Red 780 (Tonbo). Glucagon, insulin and pS6 (Ser 240) expression were analyzed by mean fluorescent intensity (MFI) per glucagon or insulin-positive cells using BD LSR II (BD Biosciences). The size of live glucagon and insulin-positive cells was analyzed by forward scatter area (FSC-A). Glucagon-positive cells were sorted as described previously (1). Glucagon content per cell was measured from 100 glucagon-positive cells sorted into 50 μl acid-ethanol. Antibody information is available in Supplemental Table 1.

Immunofluorescence and Cell Morphometry

Pancreata were fixed in 3.7% formaldehyde, embedded in paraffin and sectioned (5 μm). Fluorescent images were acquired using a microscope (Leica DM5500B) and a motorized stage using a camera (Leica DFC360FX) (Leica Microsystems). Cell mass was determined in 5 stained sections (5 μm) separated by 200 μm as described (1-3). The area of insulin, glucagon, and the area of each section were quantified with Image Pro Software (version 7; Media Cybernetics). The ratios of the hormone-stained area to the total pancreatic section area for each mouse were averaged and multiplied by the pancreas weight. Cell size was calculated

using NIH Image J Software (v1/49d available free at http://rsb.info.nih.gov/ij/index.html). PC2 stained sections were acquired with confocal microscope (SP5 upright laser-scanning confocal microscope, Leica Microsystems). Antibody information is available in Supplemental Table 1.

Assessment of Glucagon Signaling in Liver

After 6-hour fasting the mice were anesthetized with isoflurane, a liver biopsy was collected (Time 0). Glucagon (100 μg/kg; Sigma) was injected directly into the portal vein, and liver was collected after 5- and 10-minutes post injection. Protein was extracted from liver samples and analyzed for CREB phosphorylation by western blotting as described below.

Quantitative Real-Time PCR

For RNA expression, total RNA was extracted from liver samples using the RNeasy isolation kit (Qiagen). Gene expression was performed by quantitative real time RT-PCR using Power SYBR Green PCR Mix (Applied Biosystems) using StepOnePlus detection system (Applied Biosystems) with a standard protocol including a melting curve. Relative abundance for each transcript was calculated by a standard curve of cycle thresholds and normalized to 18S. Primers were purchased from IDT Technologies. Primer sequences are available in Supplemental Table 2.

Western Blotting

Liver was collected and homogenized in lysis buffer (125 mM Tris, pH7; 2% SDS, 1 mM DTT) containing a phosphatase (Roche Diagnostics) and protease (Sigma) inhibitor cocktails. Homogenates were boiled for 10 minutes, loaded and electrophoresed on 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Phospho-Akt (S473) and phospho-Creb (S133) antibodies were purchased from Cell Signaling. Images were acquired using Western Bright Sirius kit (BioExpress) and LI-COR Biosciences imaging system. Band densitometry was determined by measuring pixel intensity using NIH Image J software (v1/49d available free at http://rsb.info.nih.gov/ij/index.html) and normalized to actin in the same membrane. Antibody information is available in Supplemental Table 1.

Electron Microscopy

Islets were isolated and fixed with 2% glutaraldehyde overnight at 4 °C, dehydrated and embedded in Epon by the Microscopy & Image Analysis Laboratory Core (MiCORES). Ultrathin

sections were stained with uranyl acetate and lead citrate. Images were recorded digitally using electron microscope (JEM-1400 Plus).

REFERENCES

1. Bozadjieva N, Blandino-Rosano M, Chase J, Dai XQ, Cummings K, Gimeno J, et al. Loss of mTORC1 signaling alters pancreatic alpha cell mass and impairs glucagon secretion. *J Clin Invest.* 2017;127(12):4379-93.
2. Bernal-Mizrachi E, Wen W, Stahlhut S, Welling CM, and Permutt MA. Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. *J Clin Invest.* 2001;108(11):1631-8.
3. Bernal-Mizrachi E, Fatrai S, Johnson JD, Ohsugi M, Otani K, Han Z, et al. Defective insulin secretion and increased susceptibility to experimental diabetes are induced by reduced Akt activity in pancreatic islet beta cells. *J Clin Invest.* 2004;114(7):928-36.

Supplemental Table 1. Antibodies

Antibody	Specie	Source	Application
Glucagon	Mouse	Abcam	IFC
Glucagon	Rabbit	EMD Millipore	IFC
Glucagon-BV421	Human/Mouse	BD Biosciences	FC
Insulin	Guinea Pig	Dako	IFC
Insulin-APC	Human/Mouse/Bovine	R&D Systems	FC
pS6 (S240)	Rabbit	Cell Signaling	IFC
pS6 (S240) – PE600	Mouse	BD Biosciences	FC
Ghost Dye Red 780		Tonbo	FC
Ki67	Rabbit	Sigma	IFC
PC2	Rabbit	Lindberg Laboratory	IFC
PhosphoAKT (S473)	Rabbit	Cell Signaling	WB
PhosphoCreb (S133)	Rabbit	Cell Signaling	WB
Tubulin	Mouse	Sigma	WB

IFC = Immunofluorescent Chemistry

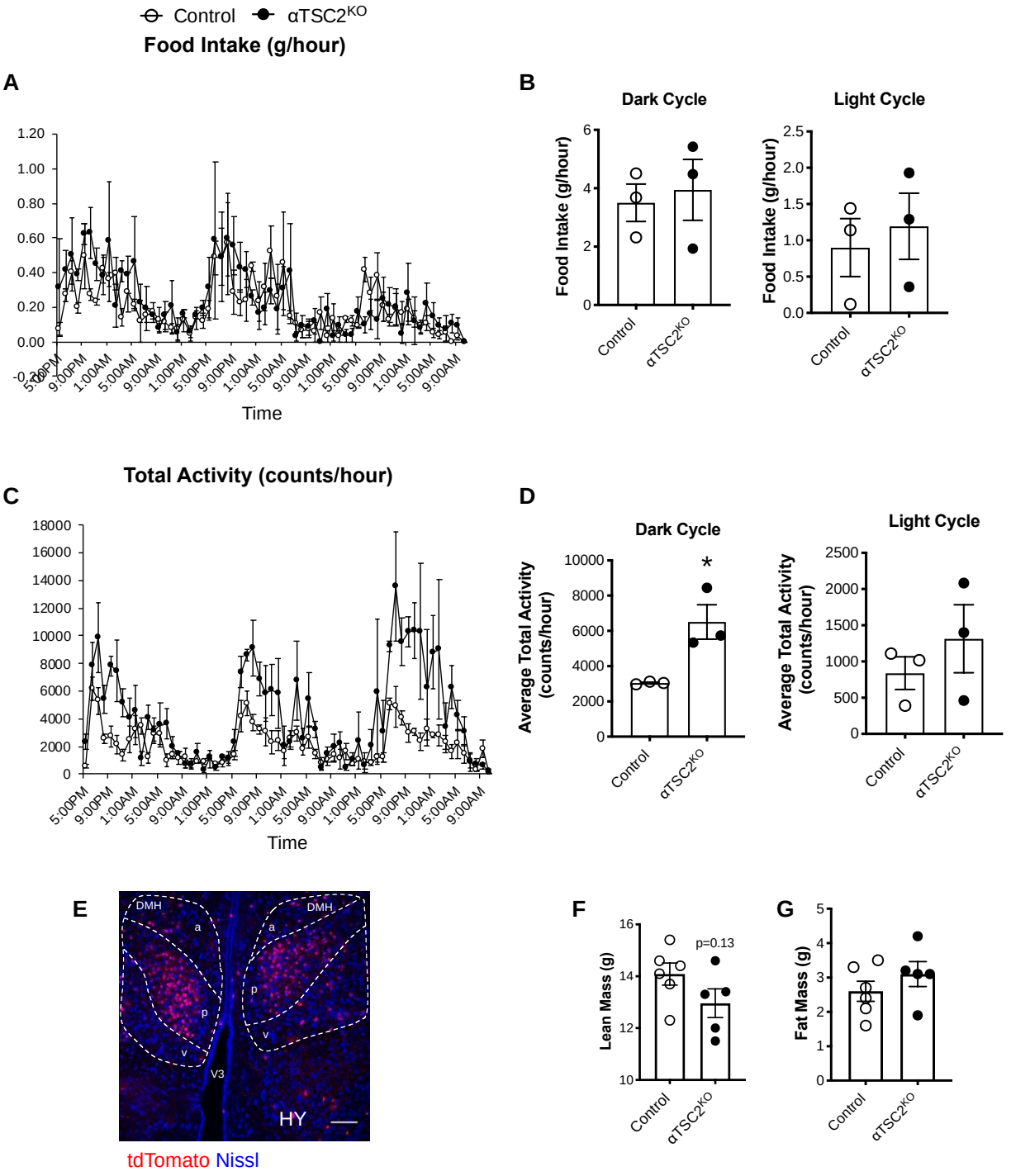
FC=Flow Cytometry

WB=Western Blot

Supplemental Table 2. Primer Sequences

Gene	Forward	Reverse
GCGR	ACGGTACAGCCAGAAGAT	CTACCAGCAACCAGCAATAG
PEPCK	ATCATCTTTGGTGGCCGTAG	ATCTTGCCCTTGTGTTCT
G6Pase	CCGGTGTTTGAACGTCATCT	CAATGCCTGACAAGACTCCA
GCK	CTGTTAGCAGGATGGCAGCTT	TTTCCTGGAGAGATGCTGTGG
FAS	AGAAGCCATGTGGGGAAGATT	AGCAGGGACAGGACAAGACAA
SDS	CACTGGCCTCGCTGGTTGTCATT	GTGGCCAGGGCAGCAGCAGAT
GPT1	CTTTGAAAGCAGTGCAGCGT	AAATCAGGCCTACACCCAGC
PC	ATCCAGCGGCGGCACCAGA	GCGGGAATTGACCTCGATGAAGTA
GOT1	TCTGACCGTGGTCGGAAAAG	TTTGGTGGCGTGAACTACGA
CRTC2	GGTGGTTCTCTGCCCAATGT	AGTCAGAGCTTGTCCTTCGC
18S	GCAATTATTCCCCATGAACG	GGGACTTAATCAACGCAAGC

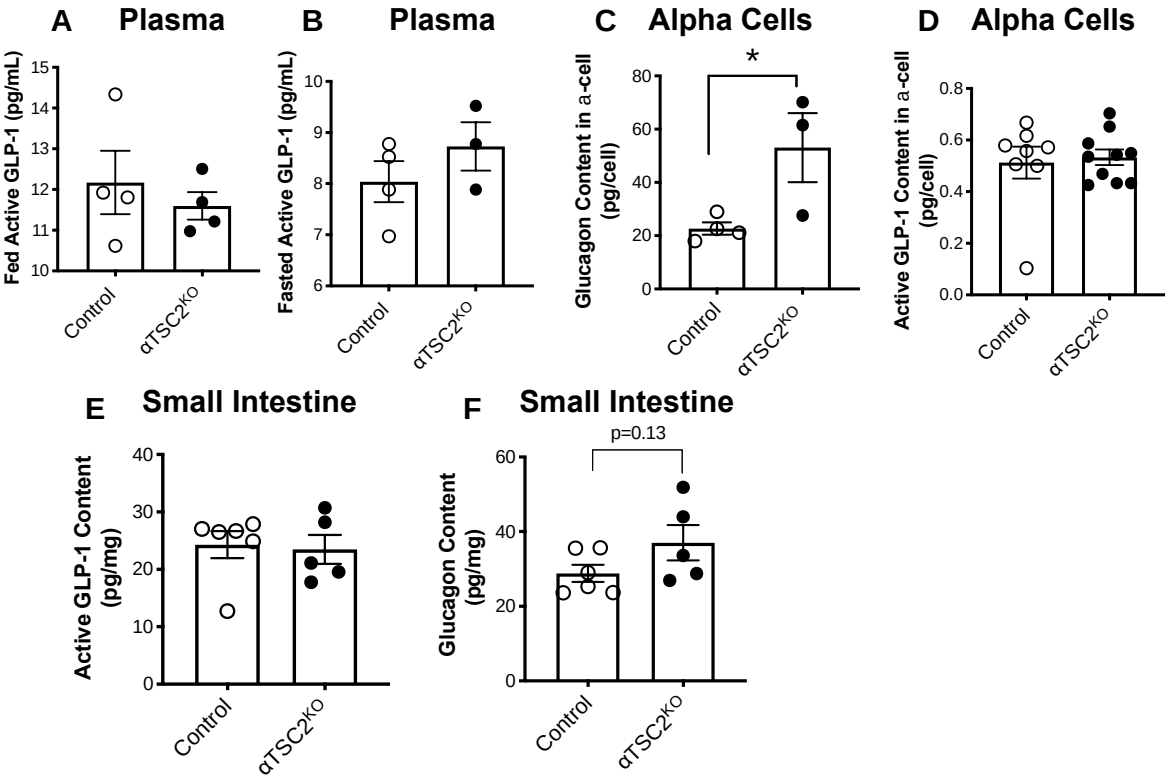
Supplemental Figure 1

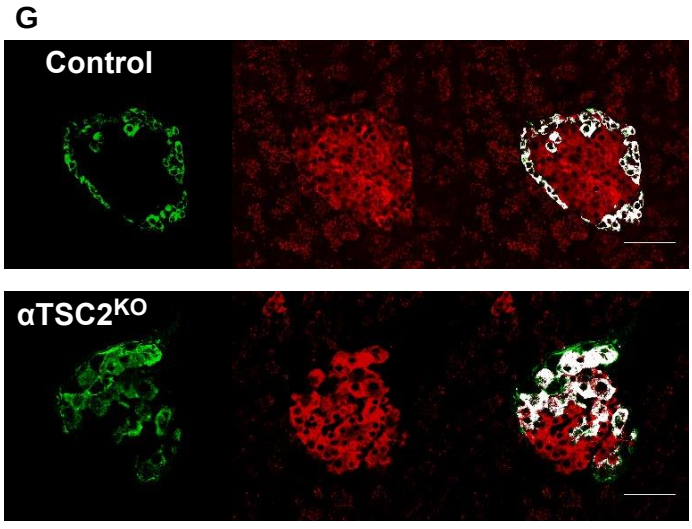


HY – Hypothalamus
DMH – Dorsomedial Nucleus of Hypothalamus (a-anterior, p-posterior, v-ventral)
V3 – Third Ventricle

Supplemental Figure 1. αTSC2^{KO} mice have lower body weight associated with increase in activity levels. A and B. Food intake (grams/hour) for 3 days in 2-month old male control and αTSC2^{KO} mice (n=3). **C and D.** Locomotor activity levels (counts/hour) in for 3 days in 2-month old male control and αTSC2^{KO} mice (n=3). **E.** Immunofluorescent images (scale=50 μm) of *Glucagon-Cre* recombination in the DMH region of the hypothalamus using a reporter transgenic mouse *CAG-tdTomato*. **F.** Lean and **G**. Fat mass in in 2-month old male control and αTSC2^{KO} mice (n=5-6). Data are shown as means ± S.E.M. *p<0.05; (Student's 2-tailed *t* test).

Supplemental Figure 2





Supplemental Figure 2. Glucagon, but not GLP-1 content, is increased in αTSC2^{KO} mice. **A.** Fed active GLP-1 in plasma (n=4) and **B.** Fasted active GLP-1 levels in plasma (n=3-4). **C.** Glucagon content per α-cell isolated by FACS (100 cells) from Glucagon-Cre$^{CAG\text{-}tdTomato}$ control and αTSC2$^{KO;\ CAG\text{-}tdTomato}$ mice (n=3-4). **D.** Active GLP-1 content per α-cell isolated by FACS (100 cells) from Glucagon-Cre$^{CAG\text{-}tdTomato}$ control and αTSC2$^{KO;\ CAG\text{-}tdTomato}$ mice (n=8-10). **E.** Active GLP-1 content and **F.** Glucagon content from whole small intestine in control and αTSC2$^{KO;\ CAG\text{-}tdTomato}$ mice (n=5-6). **G.** Immunostaining images (scale 50 μm) of Glucagon (green) PC2 (red) and Glucagon/PC2 co-localization (white) in alpha-cells (n=4). Data are shown as means ± S.E.M. *p<0.05; (Student's 2-tailed *t* test).