Materials and Methods

Reagents

Rapamycin and DMSO were obtained from Sigma (St Louis, MO, USA). Ghrelin peptide was purchased from Phoenix Pharmaceuticals Inc. (Burlingame, CA). Rabbit anti-phospho-S6K1 (Thr389), rabbit anti-phospho-S6 (Ser235/236), mouse anti-PCNA, mouse anti-insulin, rabbit anti-phosphor-HSL (ser563), rabbit anti-HSL, and mouse anti-β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). Mouse anti-ghrelin and mouse anti-p16 were purchased from Abcam (Cambridge, MA, USA). IRDye-conjugated affinity purified anti-rabbit, anti-mouse IgGs were purchased from Rockland (Gilbertsville, PA). Goat anti-rabbit fluoresceinisothiocyanate-conjugated IgG and goat anti-mouse Texas Red-conjugated IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). β-galactosidase antibody was from Abcam (Cambridge, MA). Trizol reagent and the reverse transcription (RT) system were from Promega Inc. (Madison, WI).

Intraperitoneal injection of rapamycin

Rapamycin ($1 mg \cdot kg^{-1} \cdot d^{-1}$) or DMSO was administered by intraperitoneal injection daily for 14 days.

Supplemental Figure Legends

Supplemental Figure 1. Regulation of ghrelin by s6k1.

Ghrelin-producing cell line, CLU122, was treated with adenovirus-s6k1 (Ad-s6k1) at various dosages for 48 hours. Ad-GFP ($15x10^6$ pfu/ml) was used as control. Cell lysate

was collected. The phosphorylation of S6 (a) and ghrelin (b) were detected by western blot. GAPDH was used as loading control.

Supplemental Figure 2. Dynamic changes of ghrelin-cre positive cells in stomach and pancreas.

ROSA-ghrelin-cre (RG) transgenic mice were generated as described in the method section and sacrificed at ages indicated. Stomach (a) and pancreas (b) were collected from *ROSA-ghrelin-cre* mice and littermates at neonatal stage (day 1-3 after birth), 1 week, 4 weeks and 8 weeks old. Animals were fixed with 4% paraformaldehyde and tissues processed for frozen sections. Co-localization of β-galactosidase (green) and ghrelin (red) was detected. Nuclei were stained with DAPI (blue). Pancreatic slides of neonatal from *ROSA-ghrelin-cre* mice and littermates were co-stained with β-galactosidase (green) and chromogranin A (red) (c) or insulin (red) (d). IgG was used as negative control. Body weight, daily food intake and random glucose were measure in ghrl-cre positive animals and wild type littermates at age of 8 weeks. After sacrifice, plasma ghrelin levels, liver and gWAT weights were measure (e). n=6 for each group.

Supplemental Figure 3. Phenotype of *mTOR-ghrl-cre* (mG) mice.

mG mice and wild-type littermates (WT) were fed with normal chow diet (NCD) or high fat diet (HFD) as indicated (**a-h**). Results were expressed as mean±SEM. n=10 for each group unless indicated otherwise. *P<0.05 vs. WT (Student's t test).

a. Representative pictures of genotyping on mG mice. Only homozygous of *mTOR* $^{flox/flox}$ mice with *ghrl-cre*$^{+}$ (shown in the red rectangle) were used as mG transgenic animals.

b. Body weights and tissue weights of mG mice and WT littermates. Tissue weights were normalized by body weights.

c-d. Circulating and hepatic triglyceride (c)/cholesterol (d) contents.

e. Intestinal genes related to lipid absorption. Jejunum was collected for mRNA extraction. qPCR was performed to measure the lipid absorption related gene expression levels, which were normalized by *β-actin*. n=7-9, *p<0.05 vs WT NCD, #p<0.05 vs. WT HFD.

f. Hepatic enzyme activities. Liver lysate was used for measuring enzyme activities, including ALT, AST and LDH, under either NCD or HFD conditions. *p<0.05 vs WT.

g. Levels of p16, PCNA and apoptosis. Frozen sections from livers of mG mice and their littermates were used for p16 and PCNA immunohistochemistry (IHC) and TUNEL staining. Red arrows indicate the positive cells.

h. Hepatic mRNA levels of senescence, proliferation and fibrosis related genes were detected by real-time quantitative PCR and normalized by *β-actin*.

i. Effects of ghrelin on proliferation and levels of fibrosis related genes in LX2 cells. LX2 cells were treated with ghrelin (10^{-8}M) for 24 hours, genes related to fibrosis were measured by qPCR and normalized by *β-actin*. MTT assay was performed to detect the proliferative effects of ghrelin on LX2 cells. Cells were treated with ghrelin at various dosages and time-course. n=6 per group.

j. Heart rate and mean blood pressure were measure in mG mice and their wild type littermates. n=11 each group.

Supplemental Figure 4. Phenotype of *TSC1-ghrl-cre* (TG) mice.

TG mice and wild-type (WT) littermates were fed with NCD or HFD for 12 weeks. Results were expressed as mean±SEM. *n*=20 for each group. *P<0.05 vs WT+NCD; #P<0.05 vs WT+HFD.

a. Representative pictures of genotyping on TG mice. Only *ghrl-cre*$^{+}$ and homozygous of *TSC1* $^{flox/flox}$ mice with *ghrl-cre*$^{+}$ were used as TG transgenic animals.

b. Weekly body weights and gross pictures of TG mice and WT littermates.

c. Weights of WAT depots were normalized by body weight (BW).

d. Cumulative food intake was measure in NCD animals for 12 weeks. Hypothalamic mRNA levels of AgRP, NPY and POMC were detected by qPCR and normalized by *β-actin*.

e. Hepatic and circulating cholesterol contents were detected by colorimetric assay.

f. mRNA levels of cholesterol-related genes were detected by real-time quantitative PCR and normalized by *β-actin*.

g. mRNA levels of lipid absorption related genes in jejunum was measure by qPCR and normalized by *β-actin*.

h. Hepatic mRNA levels of senescence, proliferation and fibrosis related genes in NCD mice were detected by real-time quantitative PCR and normalized by *β-actin*.

Supplemental Figure 5. Thermogenic phenotype of *TSC1-ghrl-cre* (TG) mice.

TG mice and wild-type (WT) littermates were fed with NCD or HFD for 12 weeks. Results were expressed as mean±SEM. *n*=20 for each group. *P<0.05 vs WT+NCD; #P<0.05 vs WT+HFD.

a. mRNA levels of lipogenic genes, adipocyte marker genes and lipid uptake related genes in eWAT were measured by qPCR and normalized by *β-actin*.

b. Protein levels of phosphor-HSL and HSL were detected in eWAT. The ratio of pHSL verses HSL was calculated by Image J software and expressed as mean±SEM in the bar graph.

c. mRNA levels of thermogenic genes in subcutaneous WAT (sWAT) were measured by qPCR and normalized by *β-actin*.

d. Animal respiratory quotient (RQ) and activities were dynamically monitored for 24 hours in metabolic cage. The average values of RQ and activities were expressed.

e. The true values of body temperature were dynamically recorded every hour during the 6-hour cold exposure.

f. Free fatty acid (FFA) profile in sWAT was analyzed. Several species with significant changes between TG and WT were shown.

g. mRNA levels of β-oxidation related genes in skeletal muscle were measured by qPCR and normalized by *α-actin*.

h. Heart rate and mean blood pressure were measure in TG mice and their wild type littermates.

Supplemental Figure 6. Effects of insulin supplement on TG phenotypes.

Pancreas slides from neonatal-aged TG and WT mice were used for ghrelin staining (**a**). IgG was used as negative control. Representative pictures of 5 animals from each group.

Pancreas slides from adult (12 weeks old) TG and WT mice were used for pS6 staining (**b**). IgG was used as negative control. Representative pictures of 6 animals from each group.

12 weeks old TG mice and wild-type (WT) littermates were supplemented with exogenous insulin (0,5U/day) for 2 weeks by subcutaneous osmotic mini-pump (c-g). Results were expressed as mean±SEM. n=7 for each group. *P<0.05 vs WT+saline; #P<0.05 vs TG+saline.

c. Pancreas sections from TG and WT mice were used for insulin staining. Plasma insulin of TG and WT mice with or without exogenous insulin supplement was measured.

d. OGTT and ITT were detected in TG and WT mice with or without exogenous insulin supplement after 16-hour overnight fasting (OGTT) or 6-hour fasting (ITT). The area under the curve was calculated.

e. mRNAs levels of lipogenesis and lipid transport related genes in liver were detected by RT-qPCR and normalized with *β-actin*.

f-g. mRNAs levels of brown- or white- markers in sWAT and BAT were detected by RT-qPCR, and normalized with *β-actin*.

Supplemental Figure 7. Effects of ghrelin supplementation on BAT and eWAT in TG mice.

Wild-type littermates (WT) and TG mice were fed with HFD for 12 weeks and implanted subcutaneously with osmotic mini-pump filled with acyl-ghrelin (11nmol/kg/day) for 2 weeks. Results were expressed as mean±SEM. *n*=10 for each group. **P*<0.05 *vs.* WT (saline), # *P* < 0.05 *vs.* TG (saline) (Student's *t* test).

a. Co-immunostaining of pmTOR (green) and ghrelin (red) in stomach was performed to show the activation of mTOR in X/A-like cells.

b. Elevation of circulating acyl-ghrelin was validated by ELISA.

c. Primary hepatocytes (HPC) were isolated and treated with ghrelin (10^{-8}M) for 12h. mRNA levels of lipogenic genes were detected by RT-qPCR and normalized with *β-actin*.

d. H&E staining of BAT.

e. mRNAs levels of brown- or white- markers in BAT were detected by RT-qPCR and normalized with *β-actin*.

f. H&E staining of eWAT.

g. mRNAs levels of brown- or white- markers in eWAT were detected by RT-qPCR, and normalized with *β-actin*.

Supplemental Figure 8. Effects of rapamycin on BAT and eWAT in TG mice.

Sixteen weeks old wild-type littermates (WT) and TG mice were intraperitoneally injected with rapamycin (1mg/kg/day) for 2 weeks.

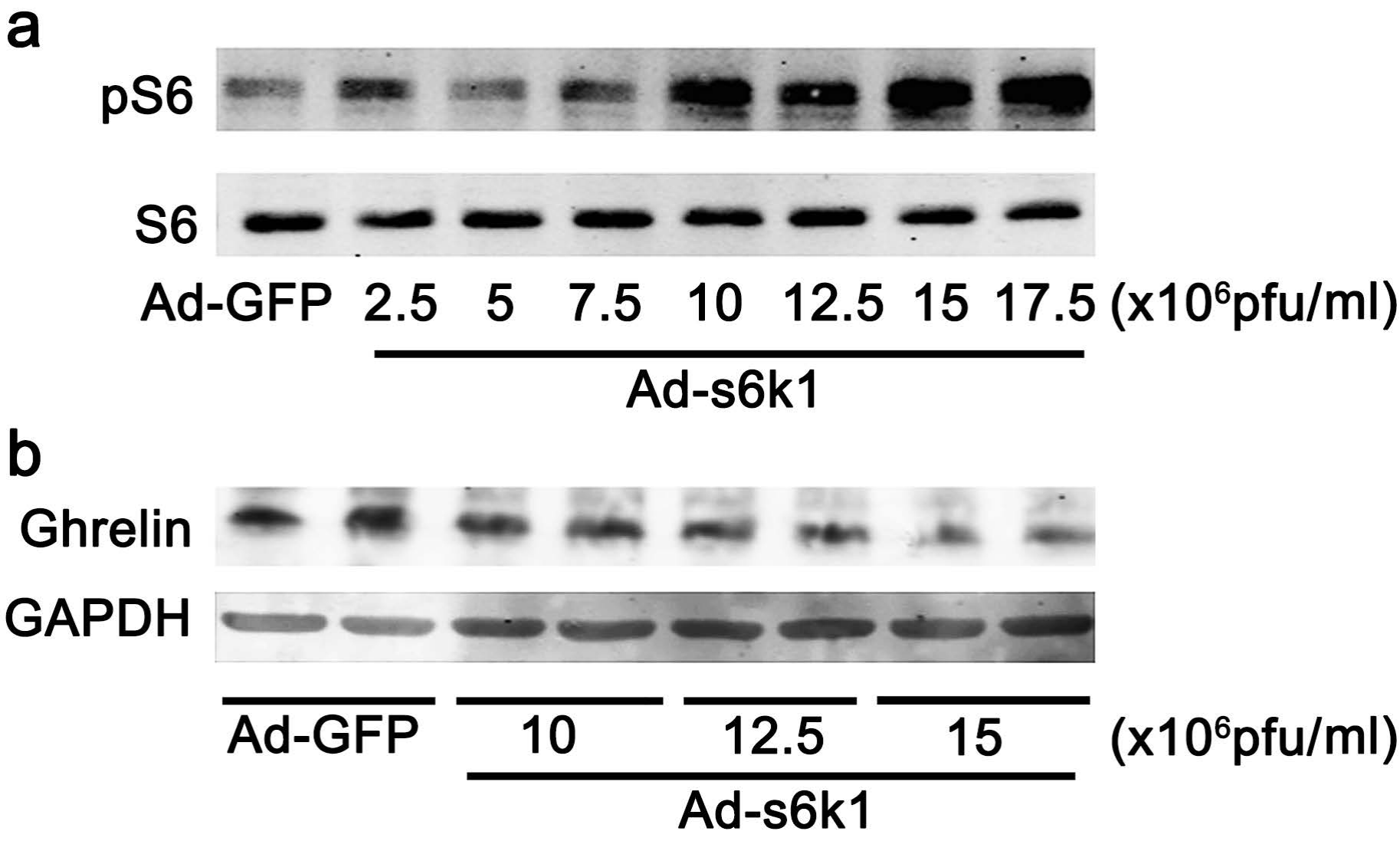
a. Co-immunostaining of pS6 (green) and ghrelin (red) in stomach was performed to validate the suppression of mTOR signaling in X/A-like cells.

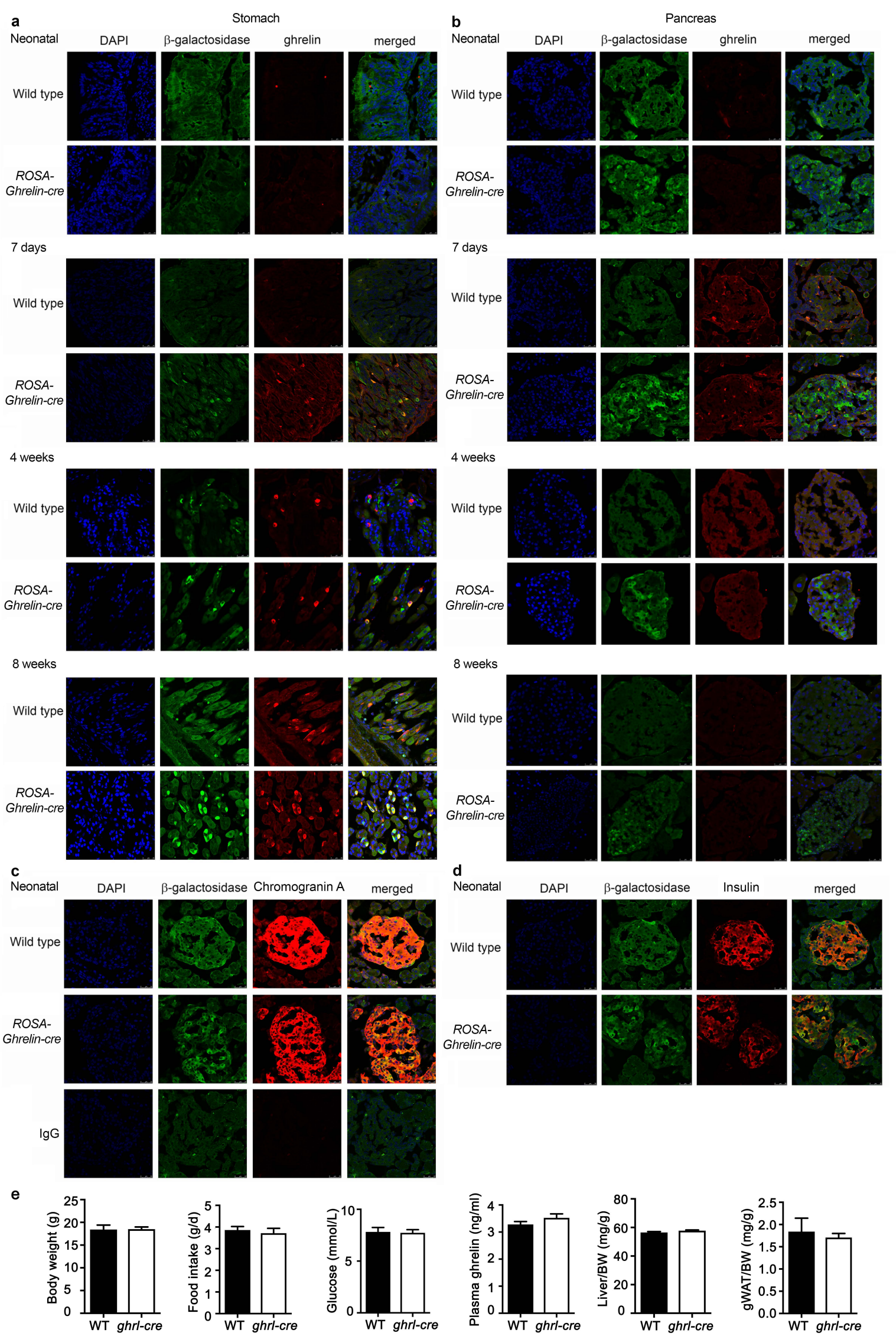
b. Brown adipose tissue (BAT) was fixed and processed for paraffin sections. Representative pictures of H&E staining were shown.

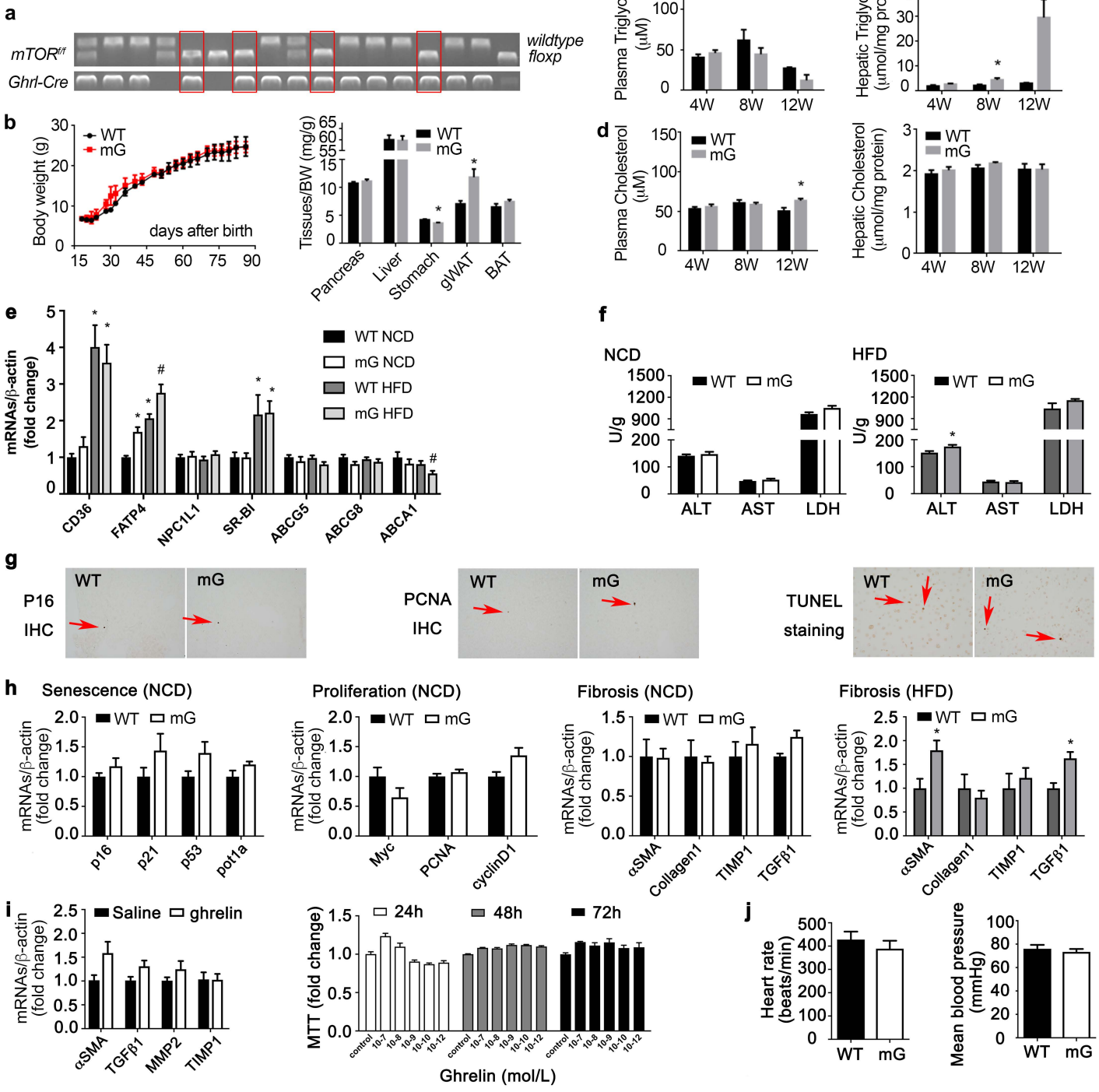
c. mRNAs levels of brown- or white- markers in BAT were measured by RT-qPCR, and normalized by *β-actin*.

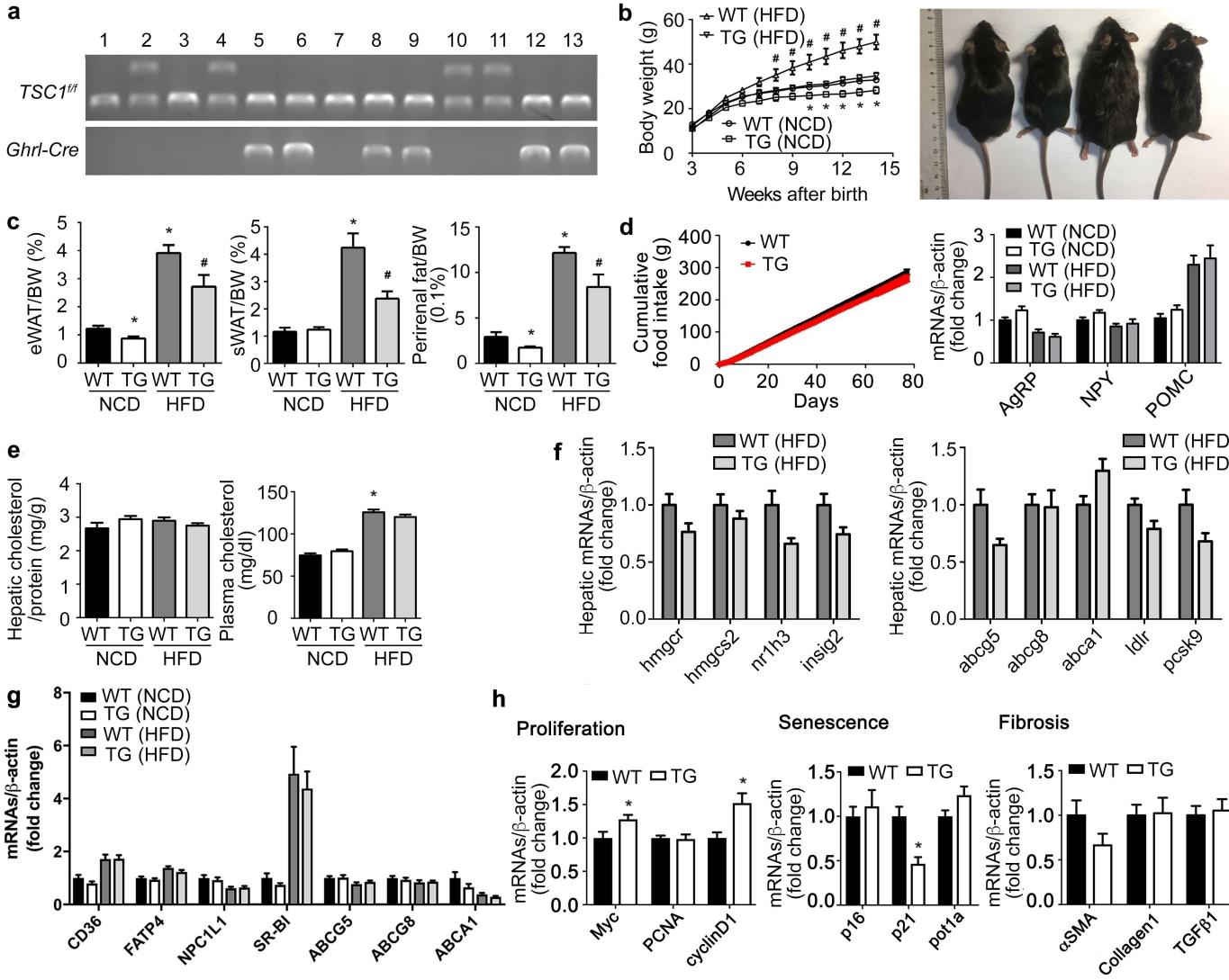
d. Representative H&E staining of epididymal WAT (eWAT).

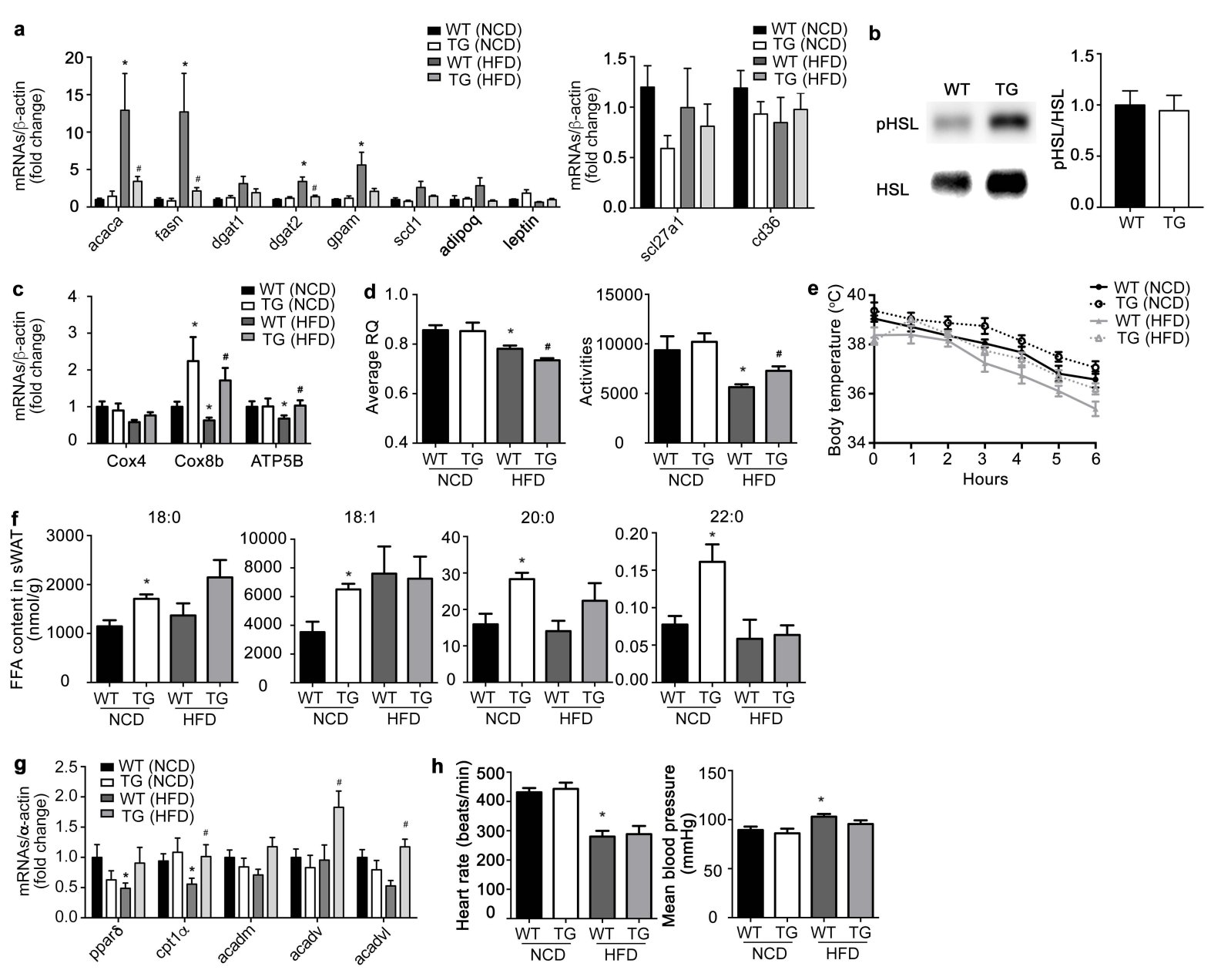
e. mRNAs levels of brown- or white- markers in eWAT were detected by RT-qPCR, and normalized by *β-actin*.

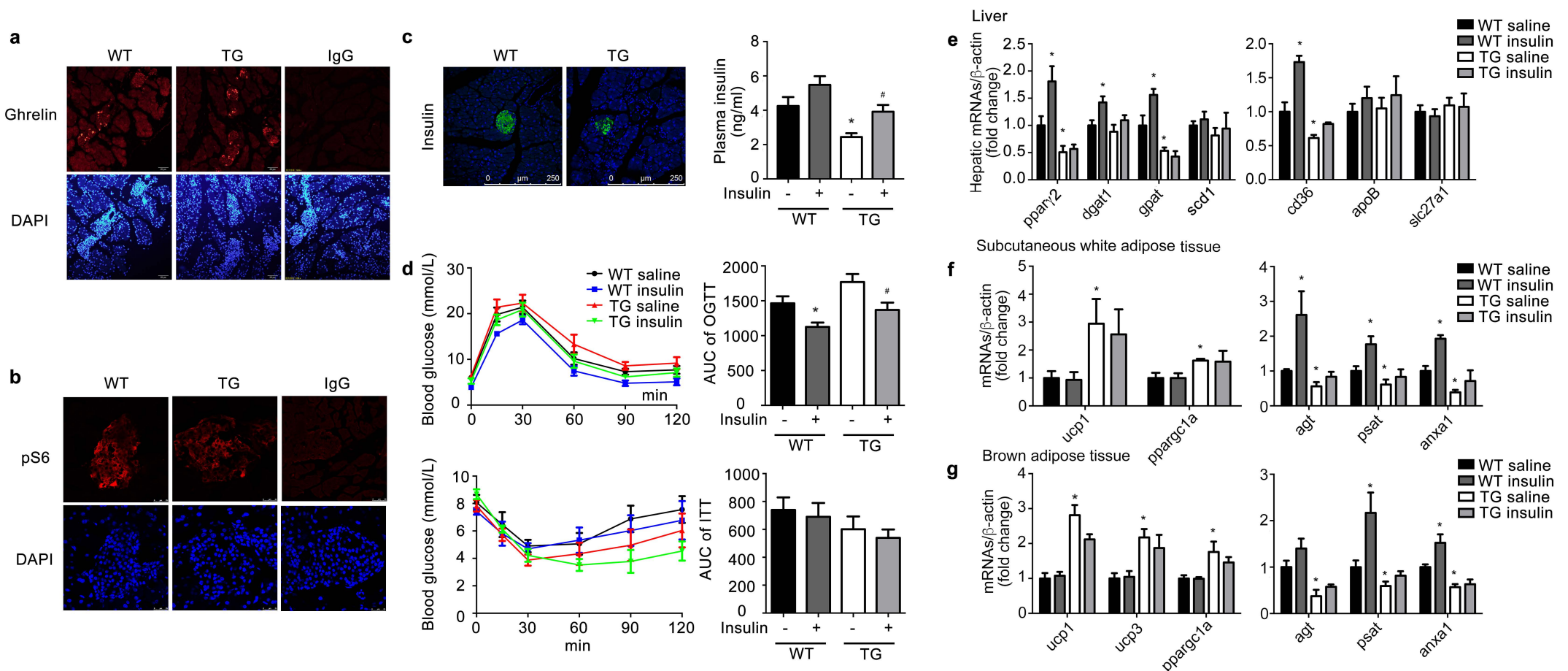

a
pS6
S6
Ad-GFP 2.5 5 7.5 10 12.5 15 17.5 (x10⁶pfu/ml)
Ad-s6k1
b
Ghrelin
GAPDH
Ad-GFP 10 12.5 15 (x10⁶pfu/ml)
Ad-s6k1

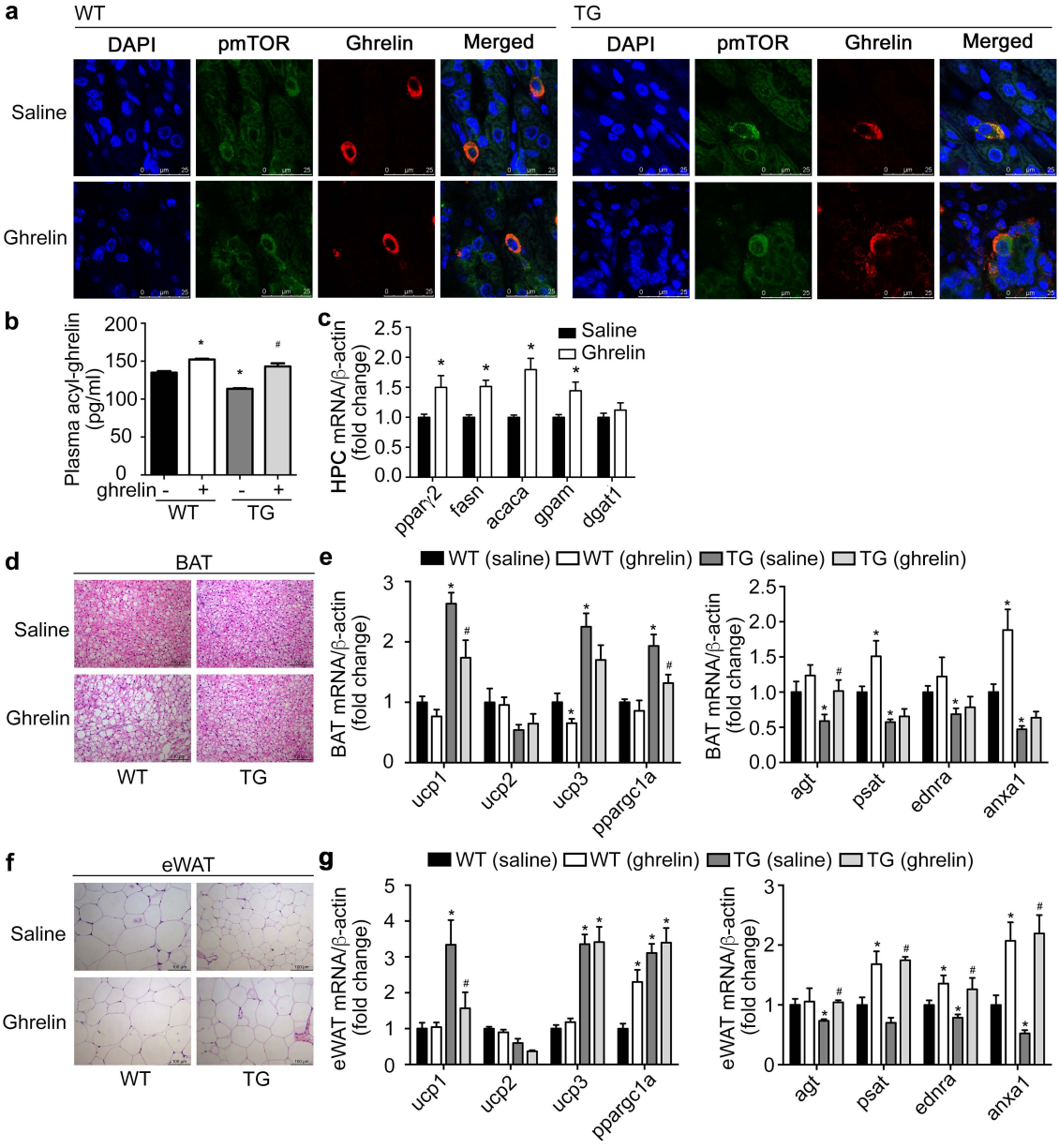

a
Stomach
Neonatal
DAPI
β-galactosidase
ghrelin
merged
Wild type
ROSA-Ghrelin-cre
7 days
4 weeks
8 weeks
b
Pancreas
c
Chromogranin A
IgG
d
Insulin
e
Body weight (g)
Food intake (g/d)
Glucose (mmol/L)
Plasma ghrelin (ng/ml)
Liver/BW (mg/g)
gWAT/BW (mg/g)
WT
ghrl-cre


a
mTOR f/f
Ghrl-Cre
wildtype
floxp
b
Body weight (g)
days after birth
WT
mG
Tissues/BW (mg/g)
Pancreas
Liver
Stomach
gWAT
BAT
c
Plasma Triglyceride (μM)
Hepatic Triglyceride (μmol/mg protein)
4W
8W
12W
d
Plasma Cholesterol (μM)
Hepatic Cholesterol (μmol/mg protein)
e
mRNAs/β-actin (fold change)
WT NCD
mG NCD
WT HFD
mG HFD
CD36
FATP4
NPC1L1
SR-BI
ABCG5
ABCG8
ABCA1
f
NCD
HFD
U/g
ALT
AST
LDH
g
P16 IHC
PCNA IHC
TUNEL staining
h
Senescence (NCD)
Proliferation (NCD)
Fibrosis (NCD)
Fibrosis (HFD)
p16
p21
p53
pot1a
Myc
PCNA
cyclinD1
αSMA
Collagen1
TIMP1
TGFβ1
i
Saline
ghrelin
MMP2
MTT (fold change)
24h
48h
72h
Ghrelin (mol/L)
j
Heart rate (beats/min)
Mean blood pressure (mmHg)


a
1 2 3 4 5 6 7 8 9 10 11 12 13
TSC1f/f
Ghrl-Cre
b
WT (HFD)
TG (HFD)
WT (NCD)
TG (NCD)
Body weight (g)
Weeks after birth
c
eWAT/BW (%)
sWAT/BW (%)
Perirenal fat/BW (0.1%)
WT TG WT TG
NCD HFD
d
WT
TG
Cumulative food intake (g)
Days
WT (NCD)
TG (NCD)
WT (HFD)
TG (HFD)
mRNAs/β-actin (fold change)
AgRP
NPY
POMC
e
Hepatic cholesterol /protein (mg/g)
Plasma cholesterol (mg/dl)
f
Hepatic mRNAs/β-actin (fold change)
hmgcr
hmgcs2
nr1h3
insig2
abcg5
abcg8
abca1
ldlr
pcsk9
g
mRNAs/β-actin (fold change)
CD36
FATP4
NPC1L1
SR-BI
ABCG5
ABCG8
ABCA1
h
Proliferation
Senescence
Fibrosis
Myc
PCNA
cyclinD1
p16
p21
pot1a
αSMA
Collagen1
TGFβ1


a
WT (NCD)
TG (NCD)
WT (HFD)
TG (HFD)
mRNAs/β-actin (fold change)
acaca
fasn
dgat1
dgat2
gpam
scd1
adipoq
leptin
scl27a1
cd36
b
WT
TG
pHSL
HSL
pHSL/HSL
c
Cox4
Cox8b
ATP5B
d
Average RQ
Activities
NCD
HFD
e
Body temperature (°C)
Hours
f
FFA content in sWAT (nmol/g)
18:0
18:1
20:0
22:0
g
mRNAs/α-actin (fold change)
pparδ
cpt1α
acadm
acadv
acadvl
h
Heart rate (beats/min)
Mean blood pressure (mmHg)


a
WT
TG
IgG
Ghrelin
DAPI
b
WT
TG
IgG
pS6
DAPI
c
WT
TG
Insulin
0 μm 250
Plasma insulin (ng/ml)
Insulin - + - +
WT
TG
d
WT saline
WT insulin
TG saline
TG insulin
Blood glucose (mmol/L)
min
AUC of OGTT
Insulin - + - +
WT
TG
Blood glucose (mmol/L)
min
AUC of ITT
Insulin - + - +
WT
TG
e
Liver
Hepatic mRNAs/β-actin (fold change)
WT saline
WT insulin
TG saline
TG insulin
pparγ2
dgat1
gpat
scd1
cd36
apoB
slc27a1
f
Subcutaneous white adipose tissue
mRNAs/β-actin (fold change)
WT saline
WT insulin
TG saline
TG insulin
ucp1
ppargc1a
agt
psat
anxa1
g
Brown adipose tissue
mRNAs/β-actin (fold change)
WT saline
WT insulin
TG saline
TG insulin
ucp1
ucp3
ppargc1a
agt
psat
anxa1


a
WT
TG
DAPI
pmTOR
Ghrelin
Merged
Saline
Ghrelin
b
Plasma acyl-ghrelin (pg/ml)
200
150
100
50
0
ghrelin - + - +
WT
TG
c
HPC mRNA/β-actin (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
Saline
Ghrelin
ppar'2
fasn
acaca
gpam
dgat1
d
BAT
Saline
Ghrelin
WT
TG
e
WT (saline)
WT (ghrelin)
TG (saline)
TG (ghrelin)
BAT mRNA/β-actin (fold change)
ucp1
ucp2
ucp3
ppargc1a
agt
psat
ednra
anxa1
f
eWAT
Saline
Ghrelin
WT
TG
g
WT (saline)
WT (ghrelin)
TG (saline)
TG (ghrelin)
eWAT mRNA/β-actin (fold change)
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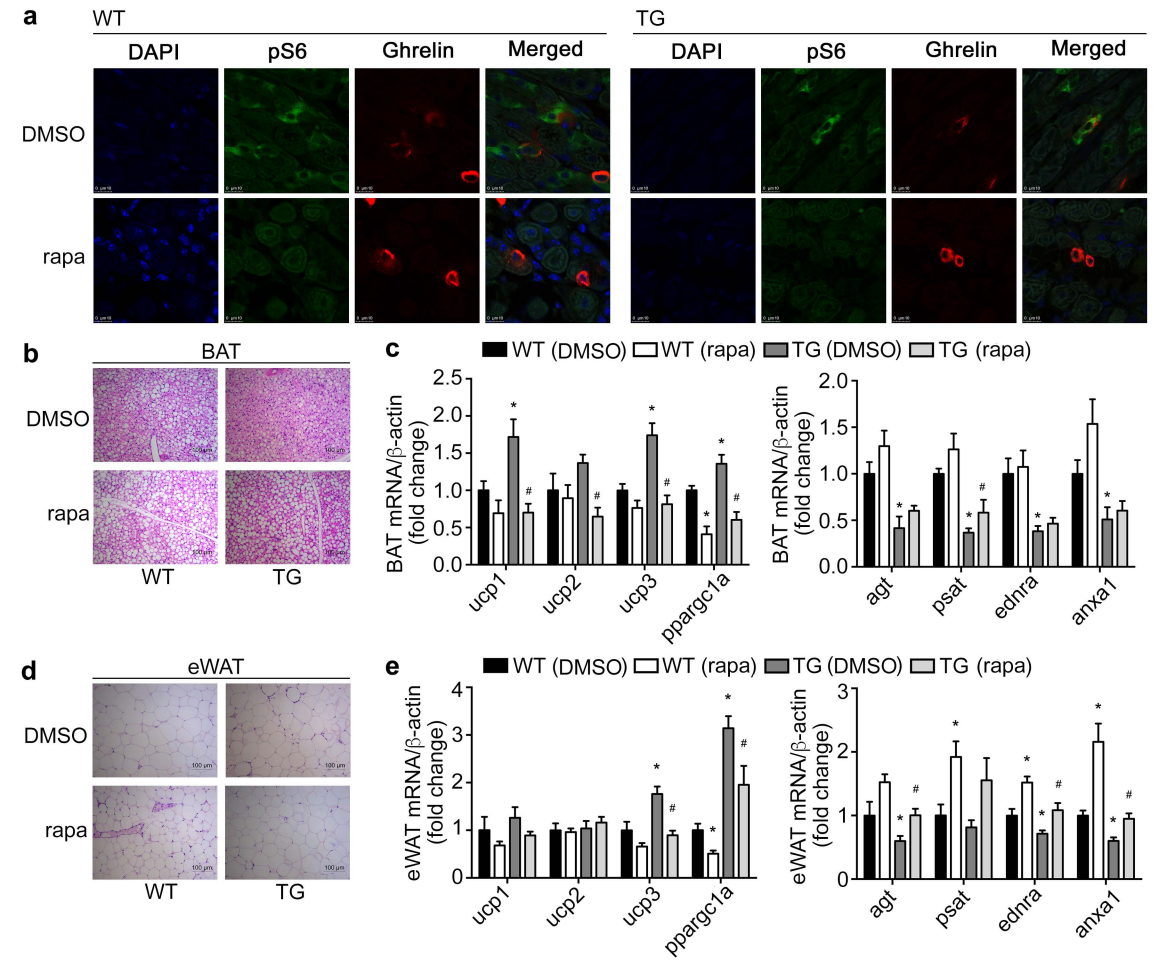
anxa1


a
WT
TG
DAPI
pS6
Ghrelin
Merged
DMSO
rapa
b
BAT
DMSO
rapa
WT
TG
c
WT (DMSO)
WT (rapa)
TG (DMSO)
TG (rapa)
BAT mRNA/β-actin (fold change)
ucp1
ucp2
ucp3
ppargc1a
agt
psat
ednra
anxa1
d
eWAT
e
eWAT mRNA/β-actin (fold change)