Supplementary Data

Hepatic sialic acid synthesis modulates glucose homeostasis in both liver and skeletal muscle

Jun Peng[1*]
Liming Yu[1,8]
Linzhang Huang[1.9]
Vivian A. Paschoal[2]
Haiyan Chu[1,10]
Camila O. de Souza[2]
Joseph V. Varre[3]
Da Young Oh[2]
Jennifer J. Kohler[4]
Xue Xiao[5]
Lin Xu[5]
William L. Holland[3]
Philip W. Shaul[1,7*]
Chieko Mineo[1,6,7*]

[1]Center for Pulmonary and Vascular Biology, Dept. of Pediatrics,
[2]Dept. of Internal Medicine, [4]Dept. of Biochemistry,
[5]Peter O'Donnell Jr. School of Public Health, [6]Dept. of Cell Biology,
University of Texas Southwestern Medical Center,
5323 Harry Hines Blvd. Dallas, TX, 75390, USA.

[3]Dept. of Nutrition & Integrative Physiology,
University of Utah College of Health,
250 1850 E, Salt Lake City, UT, 84112, USA.

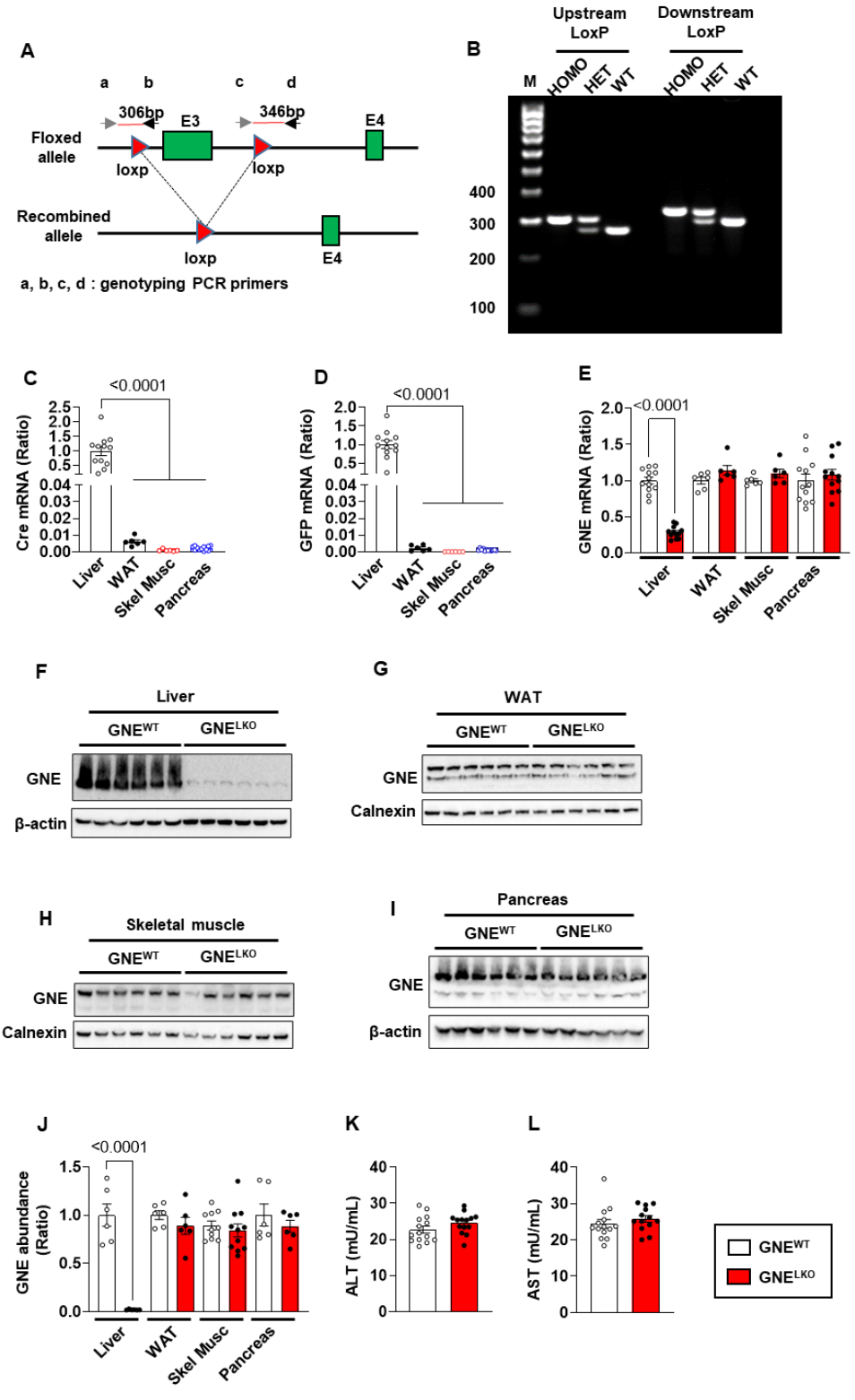
[7]Authors contributed equally

[8]Current address: Dept. of Pharmacology, UT Health San Antonio, 7703 Floyd Curl Drive, MC7764, San Antonio, TX, 78229, USA.

[9]Current address: Shanghai Key Laboratory of Metabolic Remodeling and Health, Fudan University, Shanghai 200433, China.

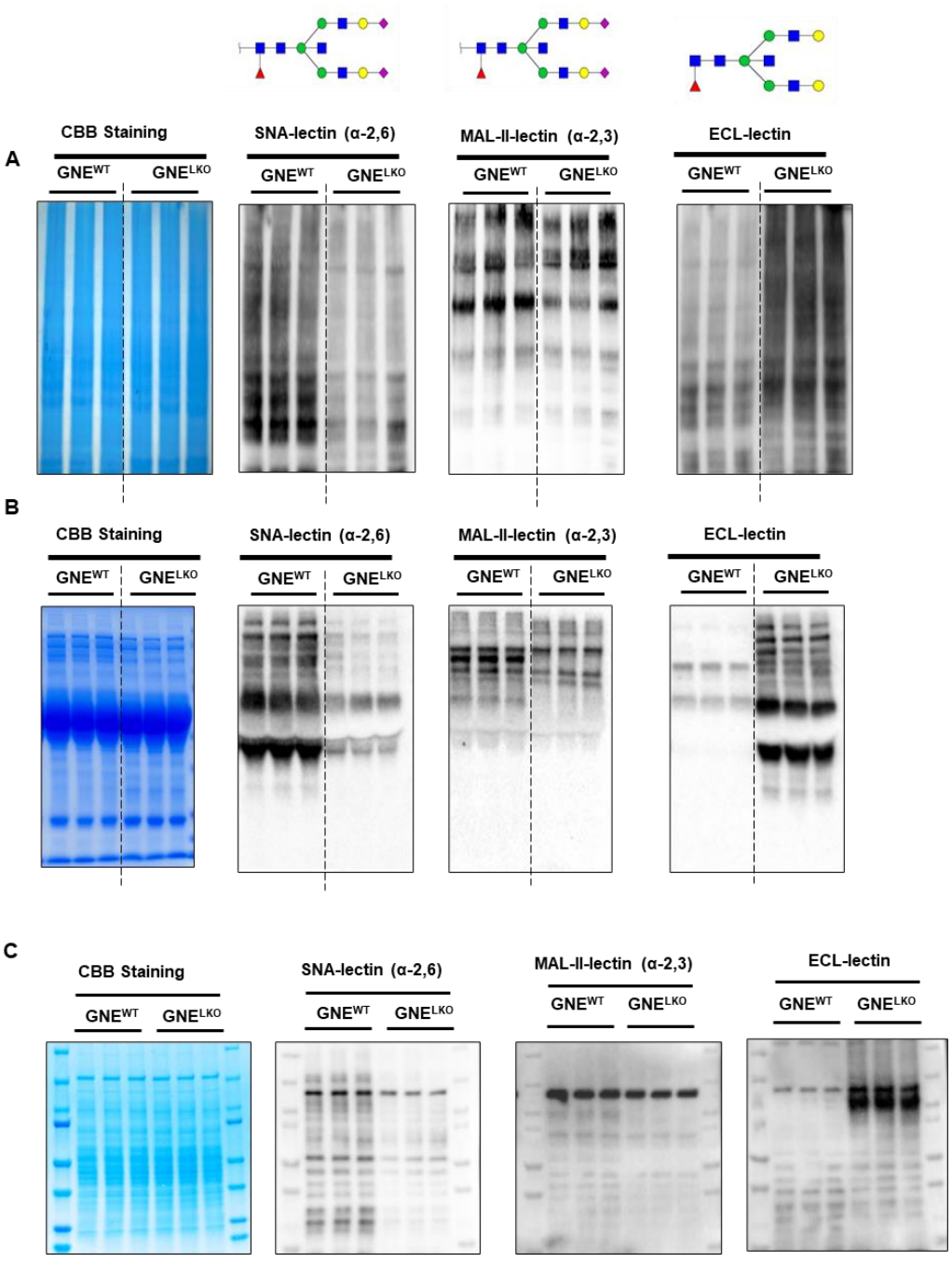
[10]Current address: Shanghai Zymogen Biotechnology Co., LTD., Shanghai 200131, China

[*]Corresponding authors.



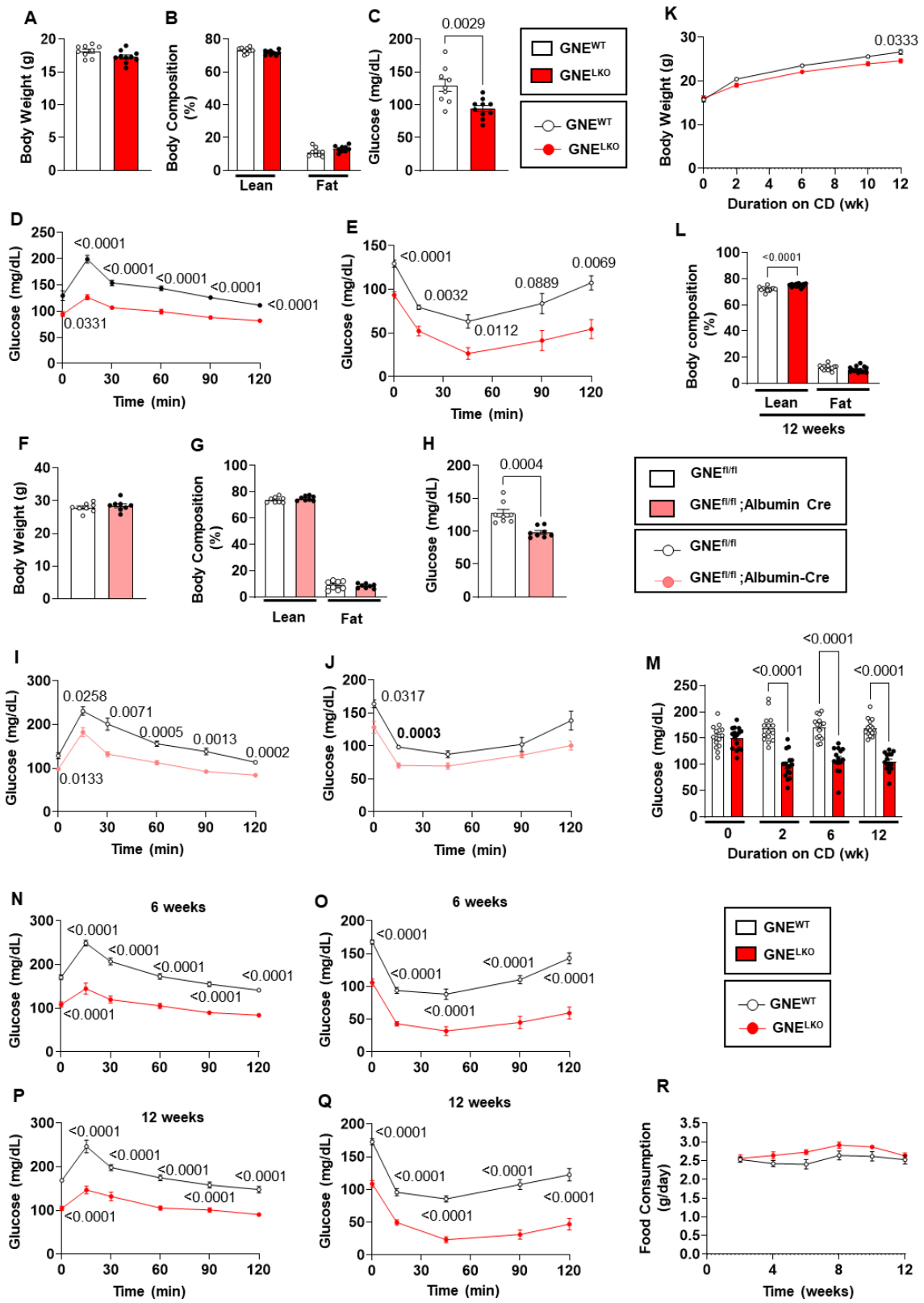
Supplementary Figure S1. Inducible deletion of GNE selectively in hepatocytes in mice.

(**A**) Floxed GNE mice ($GNE^{fl/fl}$) were generated by inserting LoxP sites within intron 2 and 3 of the mouse GNE gene allowing Cre-recombinase-mediated excision of exon 3.
(**B**) PCR genotyping of wild-type mice (WT) and mice heterozygous or homozygous for the floxed allele (HET, HOMO).
(**C-E**) In $GNE^{fl/fl}$ mice injected with AAV8-GFP or AAV8-Cre, mRNA expression for Cre recombinase (**C**) or GFP (**D**) was detected by qRT-PCR in the liver, but not in the white adipose tissues (WAT), skeletal muscle (Skel Musc) or pancreas. In $GNE^{fl/fl}$ mice injected with AAV8-Cre, GNE mRNA expression was decreased in the liver, but not in the other tissues (**E**). N=12/group for liver and pancreas, and N=6/group for WAT and Skel Musc.
(**F-J**) GNE protein expression was assessed by immunoblot using tissue lysates from $GNE^{fl/fl}$ mice injected with control AAV8-GFP or AAV8-Cre. GNE was selectively deleted in the liver (**F**), with no decrease in WAT (**G**), Skel Musc (**H**) or pancreas (**I**). Quantitation of GNE protein expression (**J**). N=6/group for liver, WAT, pancreas and N=12/group for Skel Musc.
(**K, L**) Plasma ALT (**K**) and AST levels (**L**) were assessed after 2 weeks of AAV injection. N=13-15/group.
Data are represented as mean±SEM. Significance was determined by one-way ANOVA with Tukey's multiple comparison (C, D), unpaired two tailed Student's t test with Welch's correction (Liver in E and in J), and by unpaired two-tailed Student's t test (WAT, Skel Musc, Pancreas in E and J, K, L).



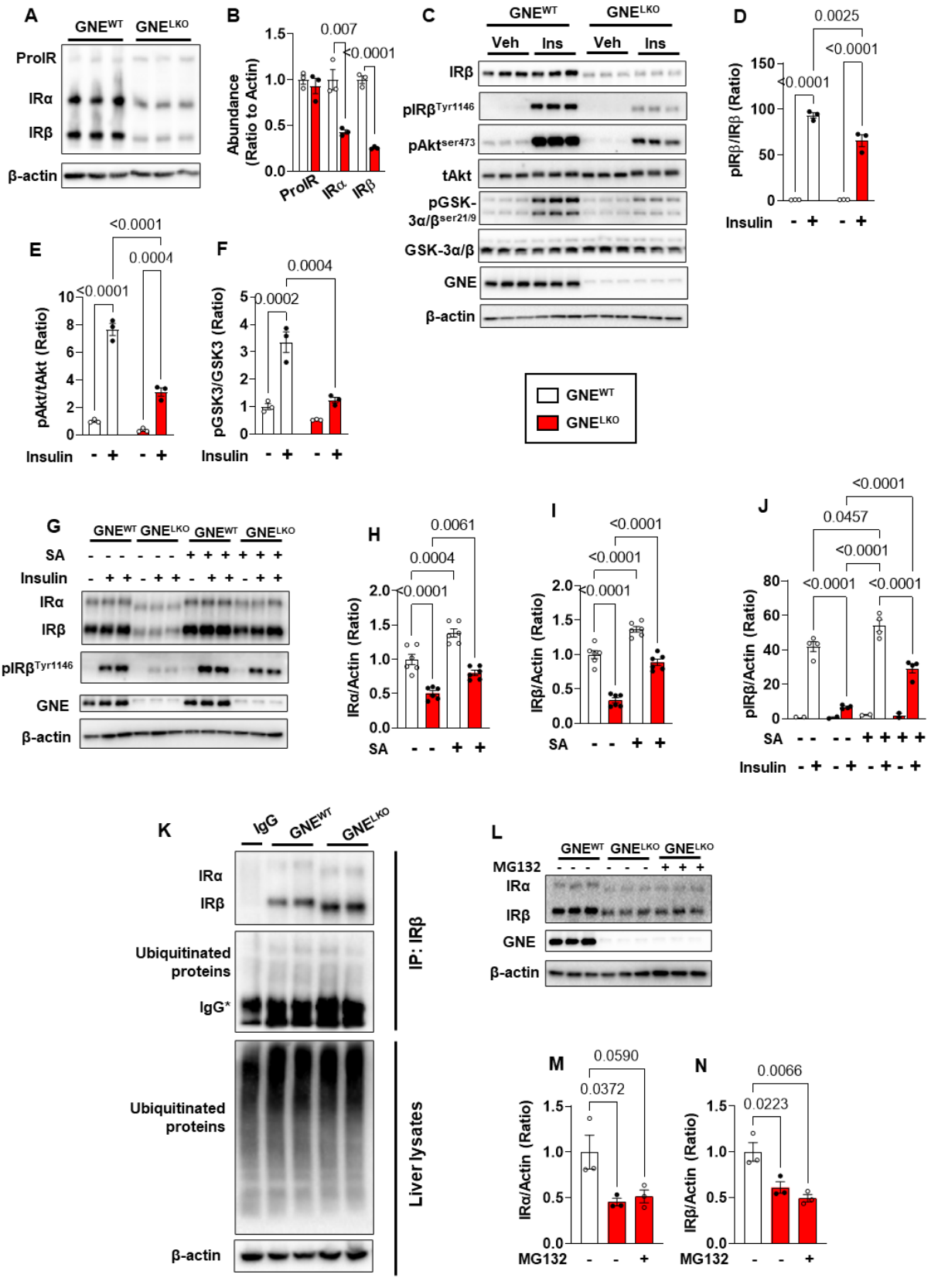
Supplementary Figure S2. Changes in protein sialylation in liver, plasma and primary mouse hepatocytes by hepatic GNE deletion.
(**A, B**) Male $GNE^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age and fed control diet for 2 weeks. Liver lysate (**A**) and plasma (**B**) were obtained, Coomassie brilliant blue staining was performed (CBB), terminal sialylation was assessed using SNA-lectin and MAL-II-lectin, and terminal galactosylation was assessed by ECL-lectin blotting. Three samples are shown per group.

(**C**) Male $GNE^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age and fed control diet for 2 weeks. Primary hepatocytes were isolated and cultured for 24h, and protein sialylation and galactosylation were assessed by SNA-lectin, MAL-II-lectin and ECL-lectin blotting. Three samples are shown per group.



Supplementary Figure S3. Effect of hepatocyte-specific deletion of GNE on glucose metabolism in control diet fed mice.

(**A-E)** Female $GNE^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age, and 2 weeks post-AAV injection, body weights (**A**, N=9,10/group) and lean or fat mass (**B**, N=9,10/group) were assessed. (**C**) Plasma glucose concentrations after 6h fast. N=9,10/group. (**D**) Glucose tolerance tests (1g/kg glucose) after 6h fast. N=9,10/group. (**E**) Insulin tolerance tests (0.5U/kg insulin) after 6h fast. N=9,10/group.
(**F-J**) $GNE^{fl/fl}$ mice were crossed with mice expressing Albumin-Cre recombinase. Male $GNE^{fl/fl}$ mice and $GNE^{fl/fl}$;Albumin Cre mice were fed control diet. Body weights (**F**, N=8/group) and lean or fat mass (**G**, N=8/group) were assessed at 15 weeks of age. (**H**) Plasma glucose concentrations after 6h fast. N=8/group. (**I**) Glucose tolerance tests (1g/kg glucose) after 6h fast. N=8/group. (**J**) Insulin tolerance tests (0.5U/kg insulin) after 6h fast. N=8/group.
(**K-R**) Male $GNE^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age, and fed control diet for an additional 0-12 weeks. Body weights were monitored (K, N=15-16/group). (**L**) Body composition was assessed by NMR at 12 week post AAV8 injections (N=13-15/group). (**M**) Plasma glucose concentrations after 6h fast. N=15-16/group. (**N, P**) Glucose tolerance tests (1g/kg glucose) after 6h fast, 6 weeks (**N**) or 12 weeks (**P**) post AAV8 injections. N=15-16/group. (**O, Q**) Insulin tolerance tests (0.5U/kg insulin) after 6h fast, 6 weeks (**O**) or 12 weeks (**Q**) post AAV8 injections N=15-16/group. (**R**) Food consumption was measured every 2 weeks (N=6, 5/group).
Data are represented as mean±SEM. Significance was determined by unpaired two-tailed Student's t test (A-C, F-H, L) or two-way repeated measures ANOVA with Sidak's multiple comparison test (D, E, I-K, M-R).


A
GNEWT GNELKO
ProIR
IRα
IRβ
β-actin
B
Abundance (Ratio to Actin)
0.007
<0.0001
ProIR
IRα
IRβ
C
GNEWT GNELKO
Veh Ins Veh Ins
IRβ
pIRβTyr1146
pAktser473
tAkt
pGSK-3α/βser21/9
GSK-3α/β
GNE
β-actin
D
pIRβ/IRβ (Ratio)
0.0025
<0.0001
<0.0001
Insulin - + - +
E
pAkt/tAkt (Ratio)
<0.0001
0.0004
<0.0001
Insulin - + - +
F
pGSK3/GSK3 (Ratio)
0.0004
0.0002
Insulin - + - +
GNEWT
GNELKO
G
GNEWT GNELKO GNEWT GNELKO
SA - - - - - - + + + + + +
Insulin - + + - + + - + + - + +
IRα
IRβ
pIRβTyr1146
GNE
β-actin
H
IRα/Actin (Ratio)
0.0061
0.0004
<0.0001
SA - - + +
I
IRβ/Actin (Ratio)
<0.0001
<0.0001
<0.0001
SA - - + +
J
pIRβ/Actin (Ratio)
<0.0001
0.0457
<0.0001
<0.0001
<0.0001
SA - - - - + + + +
Insulin - + - + - + - +
K
IgG GNEWT GNELKO
IRα
IRβ
Ubiquitinated proteins
IgG*
IP: IRβ
Ubiquitinated proteins
β-actin
Liver lysates
L
GNEWT GNELKO GNELKO
MG132 - - - - - - + + +
IRα
IRβ
GNE
β-actin
M
IRα/Actin (Ratio)
0.0590
0.0372
MG132 - - +
N
IRβ/Actin (Ratio)
0.0066
0.0223
MG132 - - +

Supplementary Figure S4. Effect of hepatocyte-specific deletion of GNE on insulin receptor processing.

(**A-B**) Male $GNE^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age and fed control diet for 2 weeks. Primary hepatocytes were isolated, and the abundance of insulin receptors were assessed by immunoblot (**A**). Quantitation of immunoblots by densitometry is shown in **B** (N=3/group).

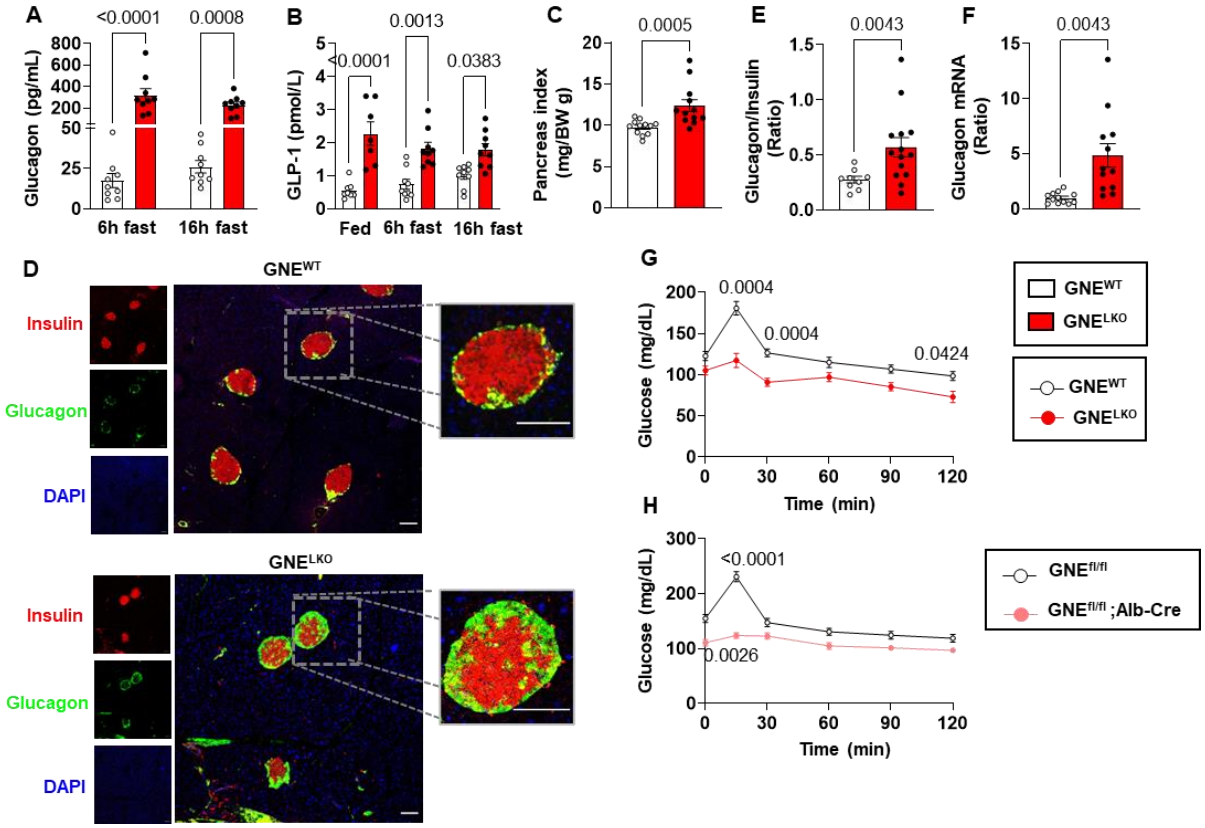
(**C-F**) Primary hepatocytes were isolated and cultured for 24h, treated with insulin (100nM) for 15min, and IRβ-Tyr1146, Akt-Ser473 and GSK3α/β-Ser21/9 phosphorylation was assessed by immunoblot. (**C**, 3 samples/group shown). Quantitation of immunoblots by densitometry is shown in **D-F** (N=3/group).

(**G-J**) Primary hepatocytes were isolated and pretreated with or without 5 mM sialic acid for 3 days, then treated with insulin (100nM) for 15 min, and IRα and IRβ expression was assessed by immunoblot (**G**, 3 samples/group shown). Quantitation of immunoblots by densitometry is shown in **H, I** (N=6/group). IRβ-Tyr1146 phosphorylation was also assessed by immunoblot (2 samples/group shown). Quantitation of immunoblots by densitometry is shown in **J** (N=4/group).

(**K**) Male $GNE^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age and fed control diet for 2 weeks, IRβ was immunoprecipitated from liver lysate, and IR ubiquitination was evaluated (2 samples/group shown). Upper panel is IR immunoblot.

(**L-N**) Primary hepatocytes were isolated and cultured for 24h, treated with vehicle or the proteosome inhibitor MG132 (10μM for 18h), and IR abundance was assessed by immunoblot (**L**, 3 samples/group shown). Quantitation of immunoblots by densitometry is shown in **M, N**.

Data are represented as mean±SEM. Significance was determined by unpaired two tailed Student's t test (B), two-way ANOVA with Tukey's multiple comparison test (D-F, J), or one-way ANOVA with Tukey's multiple comparison (H-I, M-N).



Supplementary Figure S5. Effects of hepatic GNE deletion on glucagon production and action.

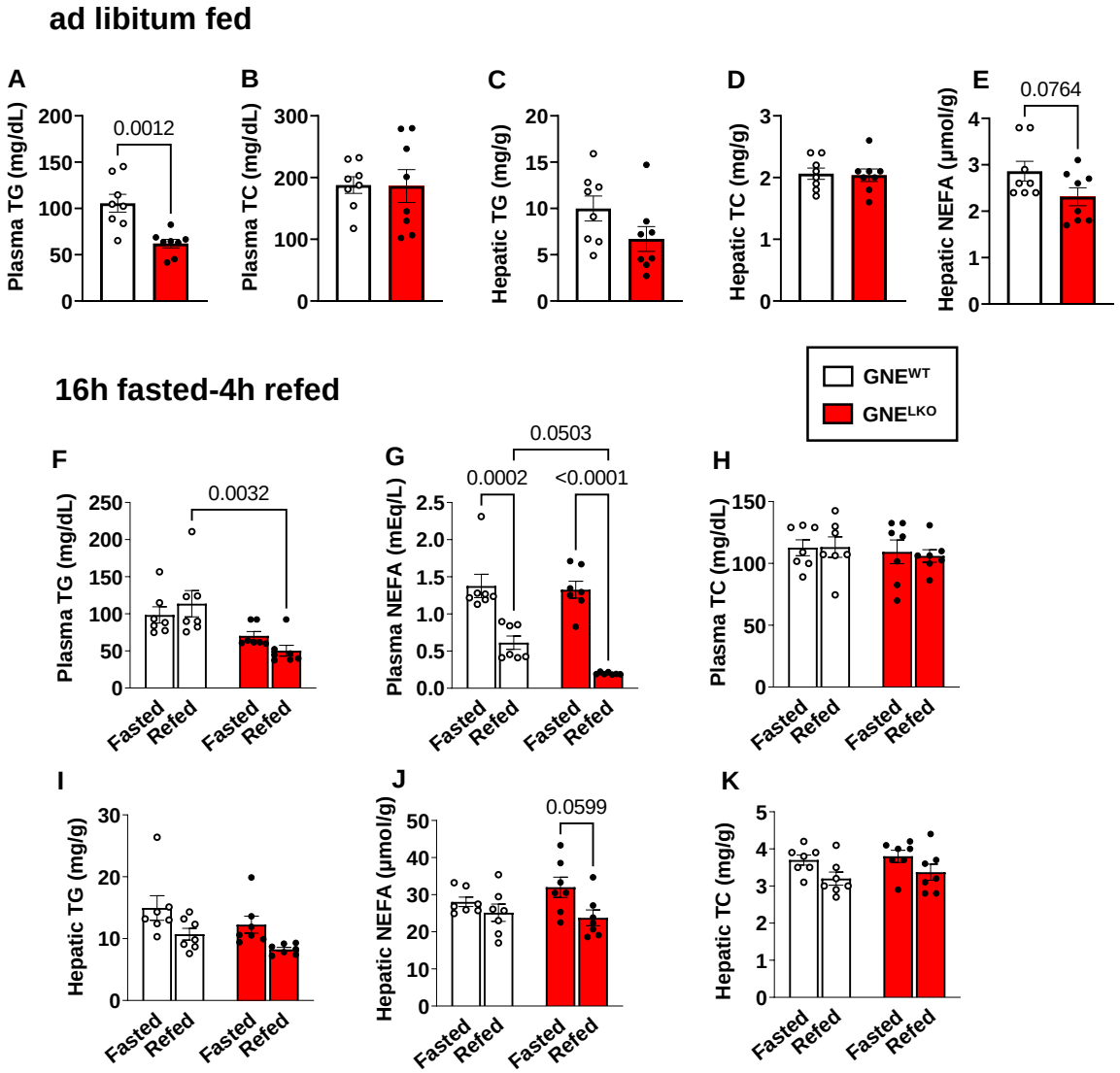
(**A,B**) Male GNE$^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age and fed control diet for 2 weeks, and plasma glucagon and GLP-1 levels were measured without fasting or after a 6h or 16 fast. N=7-9/group.

(**C-F**) Pancreatic glucagon production was assessed. (**C**) Pancreatic hyperplasia was assessed by calculating pancreas weight/body weight. N=12/group. (**D, E**) Immunofluorescence images of pancreas, showing insulin-producing β cells in red and glucagon-producing α cells in green (**D**, Scale bar=100 μm). Ratio of glucagon to insulin positive area was calculated (**E**, N=9,14/group). (**F**) Glucagon mRNA expression in pancreas was determined by qRT-PCR (N=12/group).

(**G**) Female GNE$^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age and fed control diet for 2 week, and glucagon tolerance tests were performed after 6h fast. N=9/group.

(**H**) Male GNE$^{fl/fl}$ mice or GNE$^{fl/fl}$;Albumin Cre mice were fed control diet for 10 weeks, and glucagon tolerance tests were performed after 6h fast. N=8/group.

Data are represented as mean±SEM. Significance was determined by two-way ANOVA with Tukey's multiple comparison test (A, B), unpaired two-tailed Mann-Whitney test (C, E), Student's t test with Welch's correction (F), or two-way repeated measures ANOVA with Sidak's multiple comparison test (G, H),

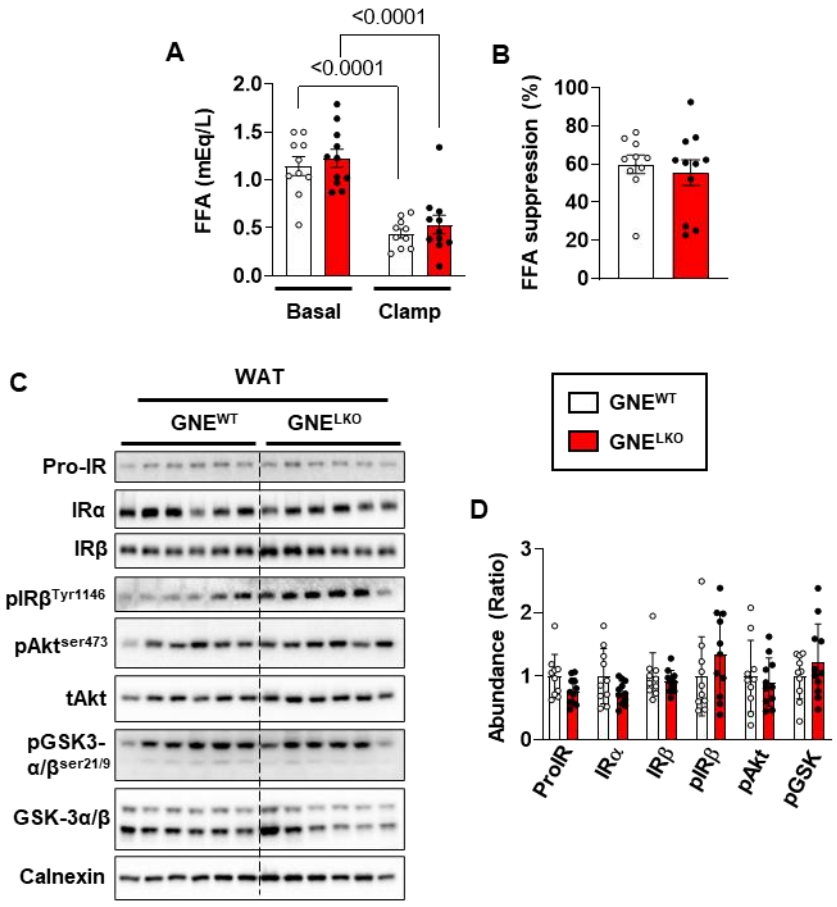


Supplementary Figure S6. Effect of hepatic GNE deletion on plasma and liver lipids

(A-E) Male GNE$^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age and fed control diet for 2 weeks, and levels of triglyceride (TG), total cholesterol (TC) and free fatty acid (NEFA) in plasma and liver were measured in ad libitum fed state. N=9/group.

(F-K) Male GNE$^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age and fed control diet for 2 weeks. Mice were fasted for 16h or fasted for 16h followed by refeeding for 4h, the plasma and livers were harvested, and levels of triglyceride (TG), total cholesterol (TC) and free fatty acid (NEFA) in plasma and liver were measured. N=7/group.

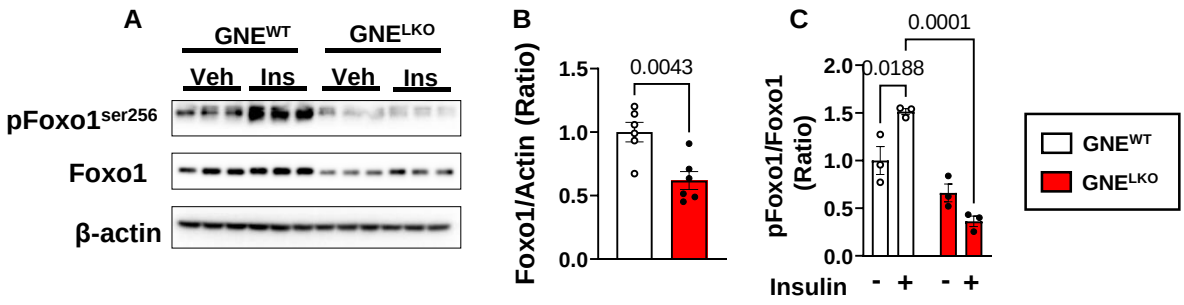
Data are represented as mean±SEM. Significance was determined by unpaired two tailed Student's t test (A-E) and two-way ANOVA with Tukey's multiple comparison analysis (F-K).



Supplementary Figure S7. Effects of hepatic GNE deletion on insulin suppression of lipolysis and insulin signaling in WAT.

(**A,B**) Male GNE$^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age and fed control diet for 2-4 weeks, and plasma levels of free fatty acids (FFA) were measured before and after a hyperinsulinemic-euglycemic clamp. N=10,11/group. (**C,D**) After the clamp, inguinal WAT was isolated, and the expression of IR precursor (Pro-IR), IRα and IRβ, and IRβ-Tyr1146, Akt-Ser473 and GSK3α/β-Ser21/9 phosphorylation were assessed by immunoblot (**C**). Quantitation by densitometry is shown in **D** (N=10,11/group).

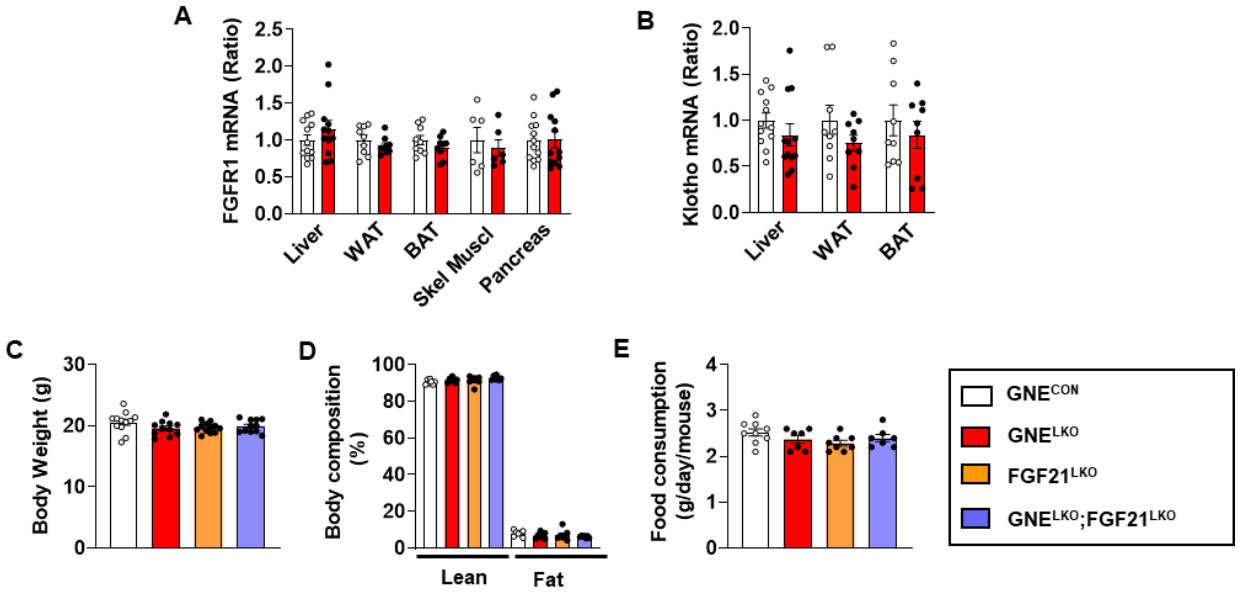
Data are represented as means±SEM. Significance was determined by two-way ANOVA with Tukey's multiple comparison test (A), or unpaired two tailed Student's t test with or without Welch's correction or unpaired two tailed Mann-Whitney test (B, D).



Supplementary Figure S8. Effects of hepatic GNE deletion on FoxO1 expression and phosphorylation.

(**A-C**) Male GNE$^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age and fed control diet for 2 weeks, and the abundance of Fox01 phosphorylation at Ser256, total FoxO1 and β-actin were assessed by immunoblot (A). Quantitaion by densitometry is shown in B, C. N=6/group).

Data are represented as means±SEM. Significance was determined by unpaired two tailed Student's t test (B) or two-way ANOVA with Tukey's multiple comparison test (C).



Supplementary Figure S9. Studies of liver GNE and FGF21.

(**A,B**) Male GNE$^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age and fed control diet for 2 weeks, and the expression of FGFR1 (**A**), and Klotho (**B**) was evaluated in various tissues by qRT-PCR. N=12/group for liver and pancreas, N=9/group for WAT and BAT, and N=6/group for skeletal muscle.

(**C-E**) Male GNE$^{fl/fl}$, FGF21$^{fl/fl}$ or GNE$^{fl/fl}$;FGF$^{fl/fl}$ mice were injected with control AAV8-GFP or AAV8-Cre at 5 weeks of age and fed control diet for 2 weeks, and body weight (**C**, N=11-12/group), lean and fat mass (**D**, N=7/group) and food consumption (**E**, N=7-9/group) were measured.

Data are represented as means±SEM. Significance was determined by unpaired two tailed Student's t test with or without Welch's correction or unpaired two tailed Mann-Whitney test (A, B) or one-way ANOVA with Tukey's multiple comparison (C-E).