Supplementary Information

A non-enzymatic function of neuraminidase 1 restrains hepatic glucagon response in mice

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Supplementary Methods

Liver-specific transfection in mice

We employed a TBG promoter-driven expression system to achieve hepatocyte-specific overexpression of NEU1, mice were injected with 80 μL of AAV8-*Neu1* plasmid virus suspension (virus titer > 10^{13}, Hanbio Biotechnology, China) blended with 120 μL saline. Control mice were injected with AAV8-NC. Liver-specific knockdown of *Gcn5* in mice were created using a AAV8- *Gcn5* shRNA, while mice for control were injected with AAV8-NC shRNA. Four weeks later, mice were randomly divided into different groups. The shRNA oligo used in our experiments are as follows:
NC shRNA: TTCTCCGAACGTGTCACGT;
Gcn5 shRNA: GAAGCCUUCUACGGUCCAUUU.

Glucagon, oral glucose and pyruvate tolerance tests (GTT, OGTT and PTT)

For glucagon, oral glucose, and pyruvate tolerance tests, mice were free access to water but without food for 14 h. The fasting duration was from 5:00 PM to 7:00 AM the following day. Next, mice were administered with glucagon (2 mg/kg) or pyruvate (1.5 g/kg or 2 g/kg) by intraperitoneal injection. In the case of oral glucose tolerance test, fasted mice were orally administered with glucose (2.5 g/kg) by gavage. Tail-blood glucose was measured at 0, 15, 30, 60, 90, and 120 min after challenge using the OneTouch VerioVue blood glucose meters (Lifescan, America).

Cell Transfection

For *Neu1*, *Gcn5* and *Sam68* knockdown in cells, primary hepatocytes were transfected with siRNA using Lipofectamine® 2000 transfection reagent (ThermoFisher, America). NC siRNA was used as a normal control. For *Neu1*, *Sam68* and *Ppargc1a* overexpression, primary hepatocytes/HEK-293T cells were transfected with plasmids according to the manufacturer's protocol. Empty vector (pcDNA3.1) was used as a control. Briefly, the culture medium was changed to Opti-MEM prior to transfection, and the mixture of 2.5 μL/mL Lipofectamine® 2000 and 1 μg/mL of each siRNA or plasmid pool were added to the cells. Media containing 10% FBS was added to the cells after 4-6 h incubation at 37 ℃. Plasmids were bought from the Public Protein/Plasmid Library in China. The siRNA oligos used are as follows:

NC siRNA: UUCUCCGAACGUGUCACGUTT
ACGUGACACGUUCGGAGAATT;
Gcn5 siRNA: GAAGCCUUCUACGGUCCAUUU
AAAUGGACCGUAGAAGGCUUC;
Neu1 siRNA: CCAAGGCUGAGAACGACUUTT
AAGUCGUUCUCAGCCUUGGTT;
Sam68-1 siRNA: ACCCACAACAGACAAGUAAUU
AAUUACUUGUCUGUUGUGGGU;
Sam68-2 siRNA: CCAGAAACAUACGAAGAUUAU
AUAAUCUUCGUAUGUUUCUGG;
Sam68-3 siRNA: CGAGGAGAAUUAUUUGGAUUU
AAAUCCAAAUAAUUCUCCUCG.

Hepatic glucose production

Primary hepatocytes were maintained in the DMEM with 10% FBS. Then the cells were washed with PBS and cultured in Krebs-Ringer HEPES (KRH) buffer for 2 h. Next, the cells were washed again and cultured in KRH buffer supplemented with 10 mM pyruvate, 100 nM glucagon for 6 h. The supernatant of cells was then collected for glucose analysis using the Glucose Assay Kit (Jiancheng bioengineering, China), and normalized by total cellular protein content.

Flow cytometry

Primary hepatocytes were isolated from C57BL/6J mice and cultured overnight to allow for cell attachment. Cells were then transfected with wild-type NEU1 plasmid or its enzyme active site mutant (catalytically inactive) plasmid. At 24 h post-transfection, cells were detached with trypsin without EDTA for 90 s, collected as a cell suspension, and centrifuged at 300 × g for 5 min. The supernatant was discarded, and the cell pellet was collected. The cell pellet was washed three times with PBS, followed by fixation with 4% paraformaldehyde for 15 min at room temperature. After fixation, cells were washed once with PBS and blocked with 5% BSA blocking solution for 30 min at room temperature. Following blocking, cells were washed once with PBS and incubated with Fluorescein-conjugated SNA (Sambucus Nigra lectin) for 30 min at room temperature in the dark. Cells were then washed twice with PBS by centrifugation. Finally, cells were resuspended in PBS and analyzed by flow cytometry. Fluorescence signals were detected through the FL1 channel (FITC, excitation wavelength 488 nm) on a Celesta cytometer (BD Biosciences, America). Samples were analyzed using FlowJo software (TreeStar, America).

Immunoprecipitation

Liver or cell samples were lysed in 500 μL IP Lysis Buffer (Thermo Scientific, America), centrifuged at 12,000g for 10 min at 4 ℃ and collected the supernatants. After measuring the protein concentrations, a 400 μg protein was transferred and incubated with IgG or primary antibodies overnight at 4 ℃. Then, 30 μL protein A/G magnetic agarose beads (MCE, China) were added and incubated with the lysates for another 6 h. Subsequently, the beads were washed 5 times with the PBS followed by the addition of 40 μL 1 × SDS-PAGE loading buffer (Beyotime, China) and Western blotting analysis.

Luciferase reporter assay

HEK-293T cells were transfected *Gcn5* luciferase reporter constructs plasmid (1 μg/mL). Renilla Luciferase plasmid (0.1 μg/mL) was used as an internal control. After 24 h transfection, cell lysates were used for luciferase assays using a 96-well luminometer with a luciferase substrate system (Promega, America).

Biochemical assays

Serum glucagon was measured by using the Mouse Glucagon ELISA Kit (CUSABIO, China) according to the manufacturer's instructions. Serum ALT, AST, and ALP levels

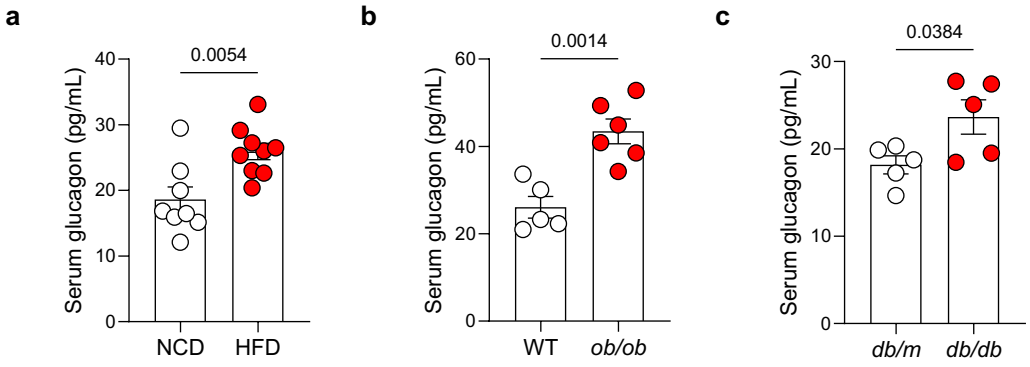
were measured using commercial kits (Jiancheng bioengineering, China). Reaction products were measured with microplate reader (SpectraMax iD5, Molecular Devices, America).

ChIP assays

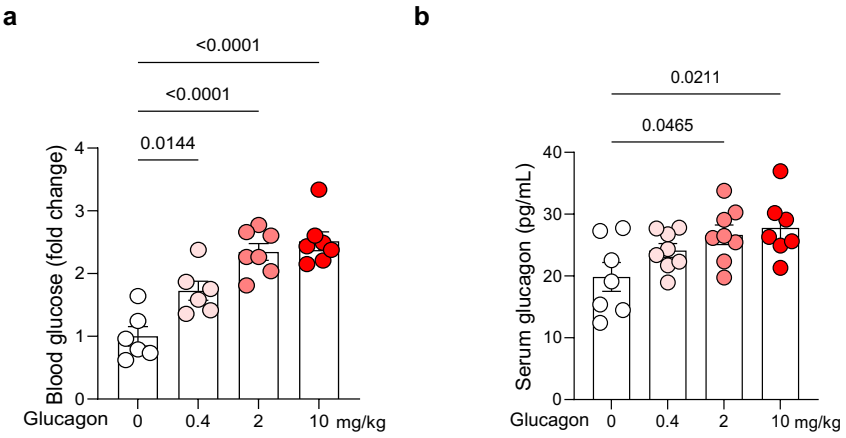
Chromatin immunoprecipitation (ChIP) assays were performed using a SimpleChIP® Plus Enzymatic Chromatin IP Kit (Cell Signaling Technology, America) according to the manufacturer's instructions. Briefly, chromatin in primary hepatocytes was cross-linked in 1% formaldehyde and subsequently lysed using 1% SDS lysis buffer. Chromatin was fragmented by SFX250 Ultrasonic Cell Disruption System (Branson, America). Soluble chromatin was immunoprecipitated with an SAM68 antibody (Proteintech, China). The de-cross-linked samples were incubated with RNase A and proteinase K. DNA was purified using a Phenol/Chloroform extraction method. The following primers were used during qRT-PCR detection:

Site A: Forward primer TGCAGCTTTTGTCCAGAACC
Reverse primer CCGTTGCTCAATCCCATCAG;

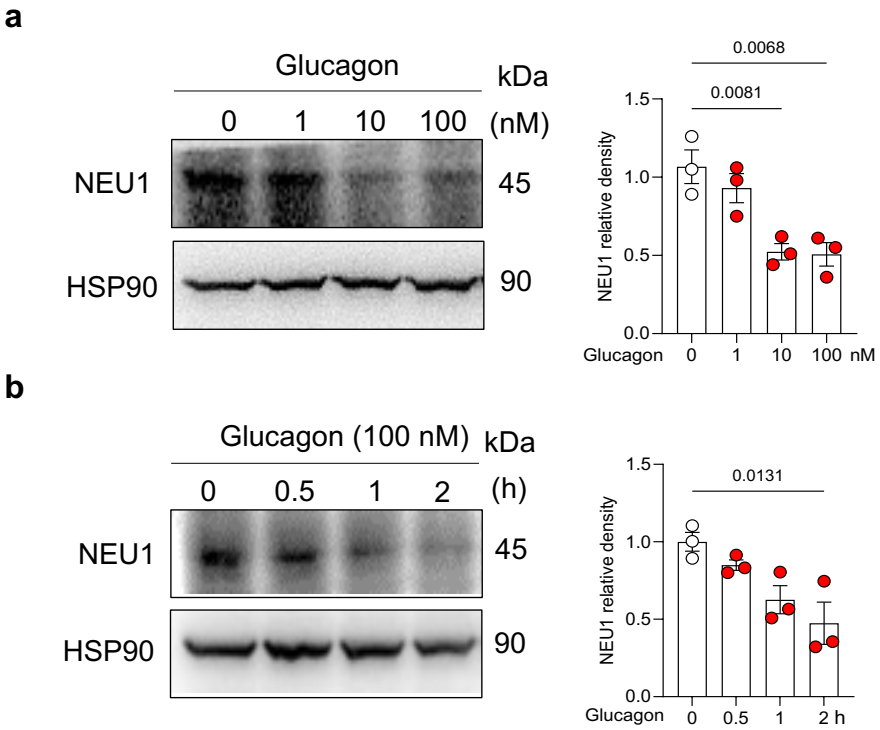
Site B: Forward primer CCCACTCTTCCCATCCAGAA
Reverse primer GGTTCTGGACAAAAGCTGCA.



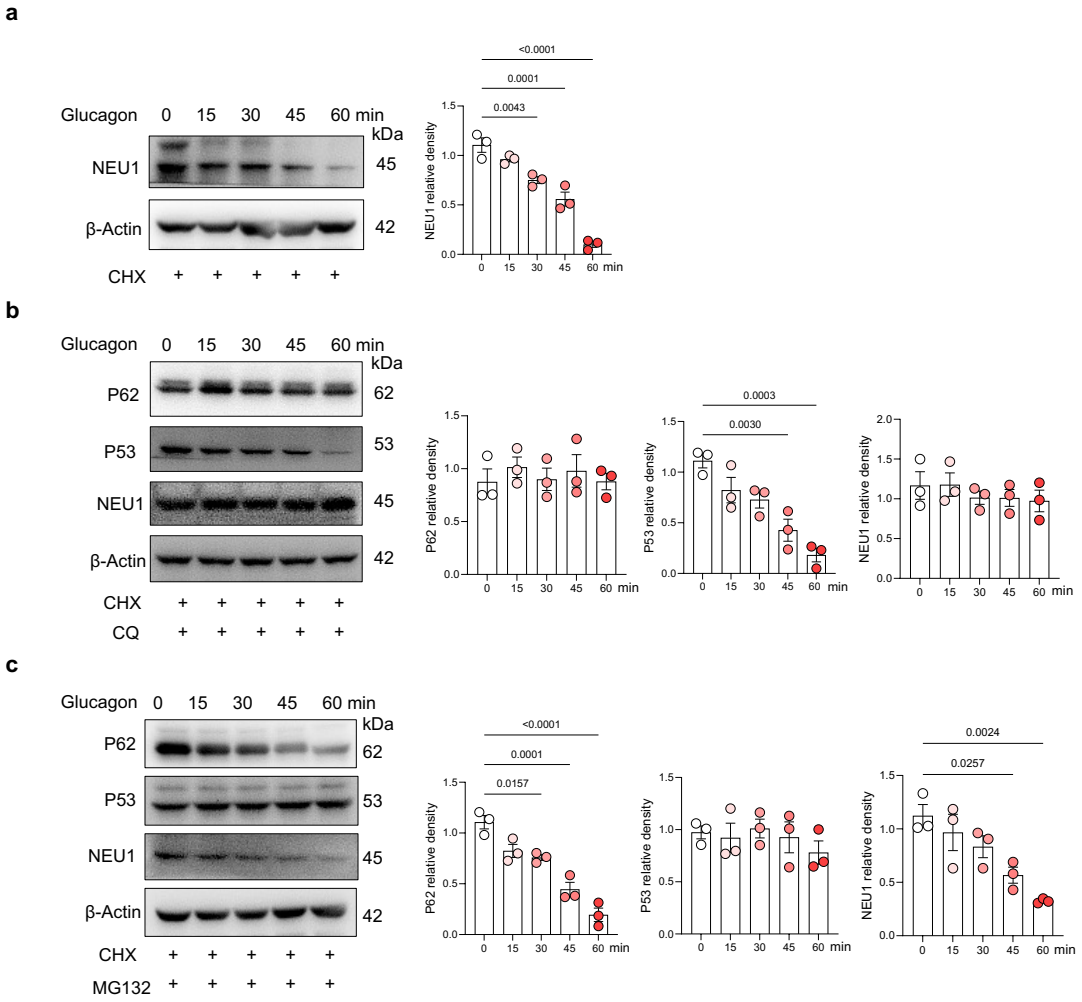
Supplementary Figure 1. Serum glucagon levels in HFD, *ob*/*ob* and *db*/*db* mice. a-c Serum glucagon levels in (**a**) HFD mice (n = 9), (**b**) *ob/ob* mice (n = 6) and (**c**) *db/db* mice (n = 5). NCD normal chow diet, HFD high-fat diet, WT wild type. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test (**a-c**). Source data are provided as a Source Data file.



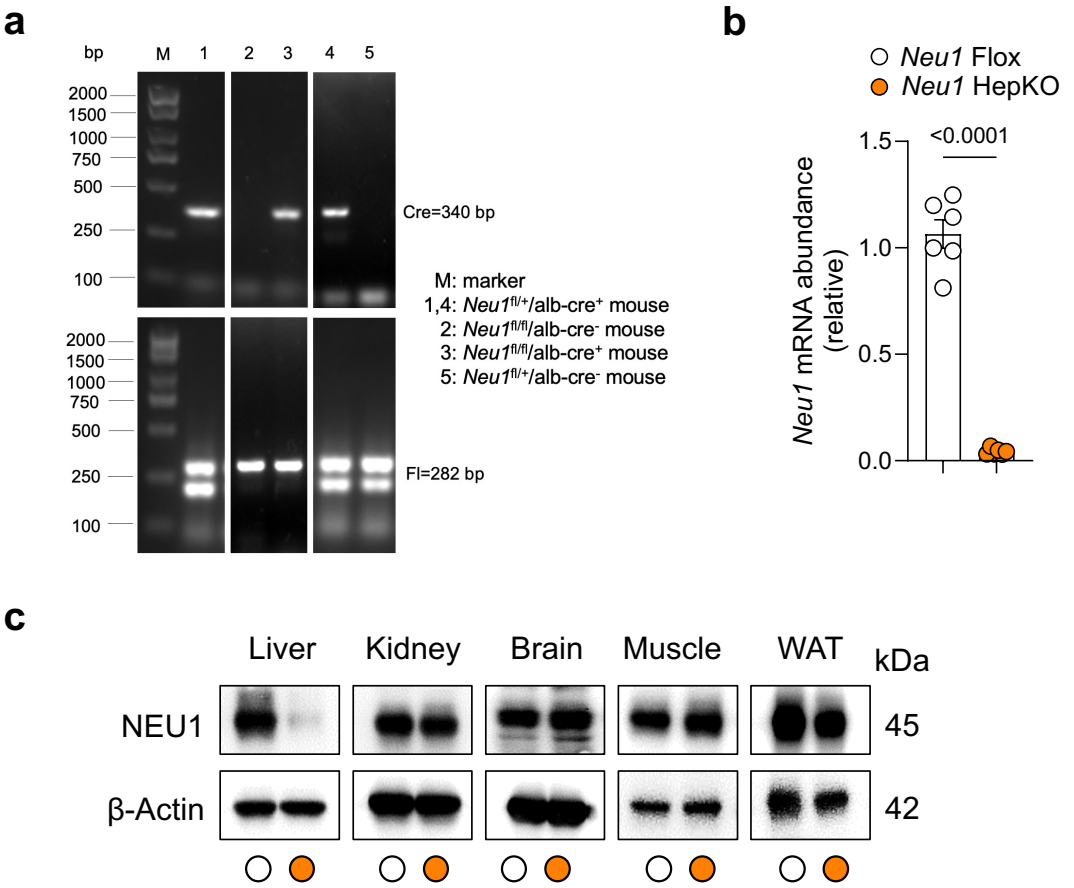
Supplementary Figure 2. Blood glucose and serum glucagon levels in glucagon-challenged mice. a-b Blood glucose (**a**, n = 6, 6, 7, 7) and serum glucagon (**b**, n = 7, 8, 8, 7) levels in glucagon-challenged mice (0, 0.4, 2, and 10 mg/kg). Bars represent mean ± SEM values. Significant differences are analyzed using a one-way ANOVA with multiple comparisons (**a-b**). Source data are provided as a Source Data file.



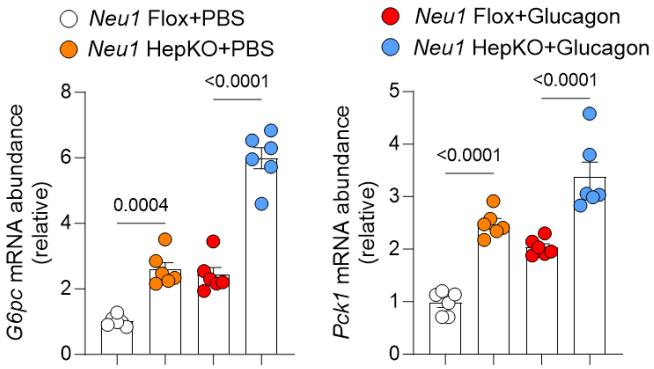
Supplementary Figure 3. Glucagon impaired the protein level of NEU1 in primary hepatocytes. a Protein levels of NEU1 in primary hepatocytes treated with glucagon (0, 1, 10, and 100 nM) for 1 h (n = 3). **b** Protein levels of NEU1 in primary hepatocytes treated with 100 nM glucagon (0, 0.5, 1, and 2 h, n = 3). Bars represent mean ± SEM values. Significant differences are analyzed using a one-way ANOVA with multiple comparisons (**a-b**). Source data are provided as a Source Data file.



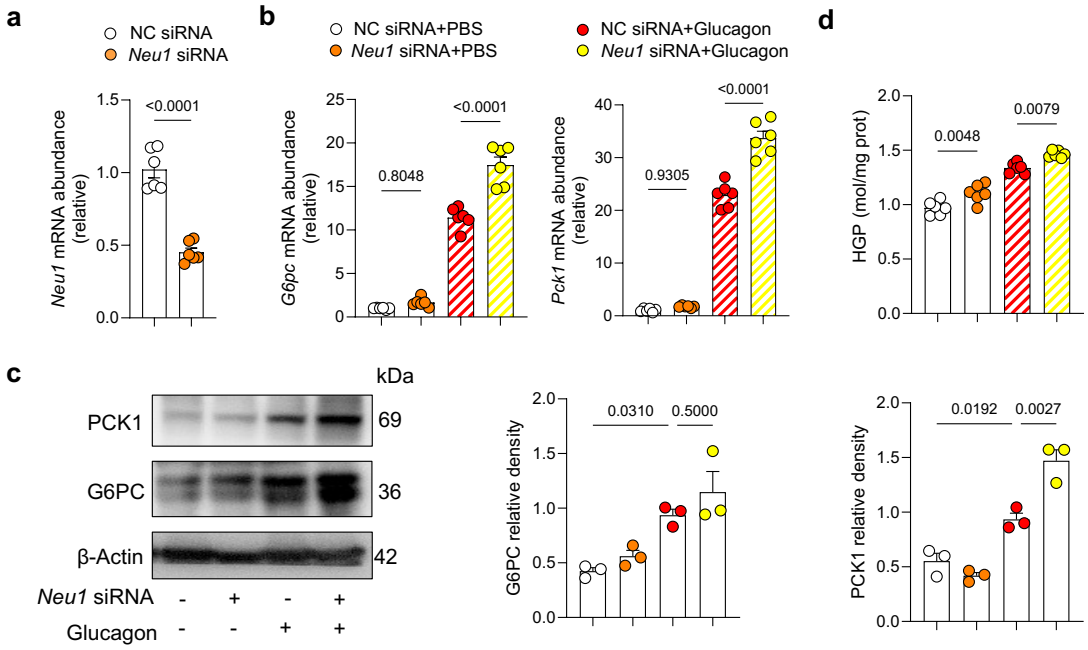
Supplementary Figure 4. Glucagon impaired NEU1 protein via lysosomal degradation pathway. a NEU1 protein levels in glucagon-stimulated (100 nM, 0, 15, 30, 45, and 60 min) primary hepatocytes treated with CHX (20 μM) (n = 3). **b** NEU1 protein levels in glucagon-stimulated (100 nM, 0, 15, 30, 45, and 60 min) primary hepatocytes treated with CHX (20 μM) and CQ (20 μM) (n = 3). **c** NEU1 protein levels in glucagon-stimulated (100 nM, 0, 15, 30, 45 and 60 min) primary hepatocytes treated with CHX (20 μM) and MG132 (20 μM) (n = 3). P53 and P62 were used as positive controls for the ubiquitin-proteasome pathway and the autophagy pathway, respectively. CHX cycloheximide, CQ chloroquine. Bars represent mean ± SEM values. Significant differences are analyzed using a one-way ANOVA with multiple comparisons (**a-c**). Source data are provided as a Source Data file.



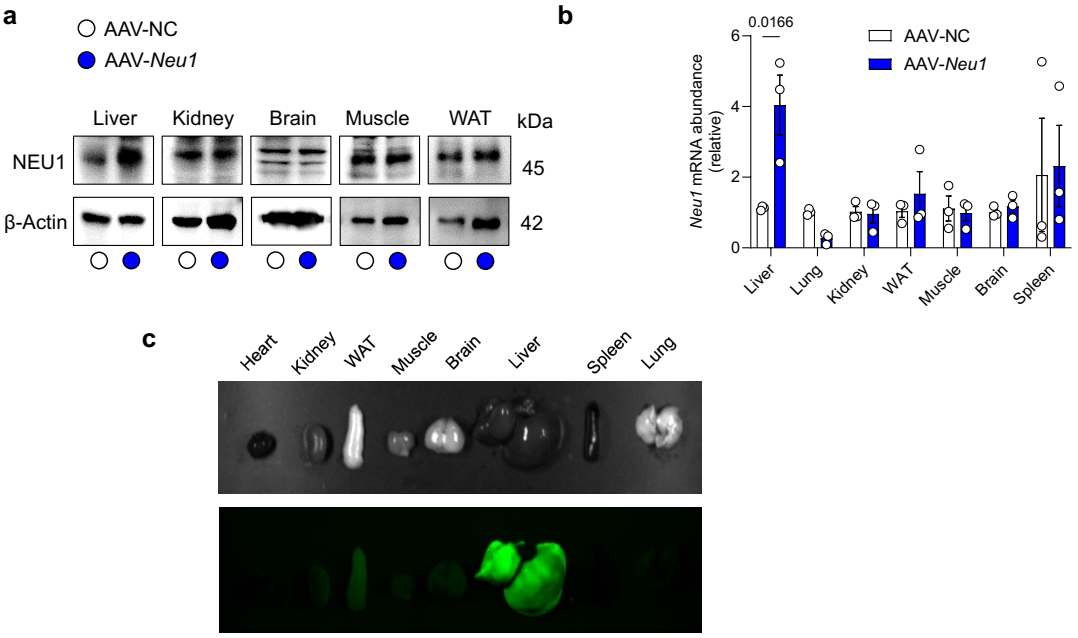
Supplementary Figure 5. The confirmation of NEU1 expression in the liver of *Neu1* HepKO mice. a PCR genotyping of *Neu1* Flox mice or *Neu1* HepKO mice**. b** Relative mRNA levels of *Neu1* in the liver of *Neu1* HepKO mice (n = 6). **c** Western blot analysis of NEU1 protein levels in the liver, kidney, brain, muscle, and WAT of *Neu1* Flox mice or *Neu1* HepKO mice. M marker, WAT white adipose tissue. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test (**b**). Source data are provided as a Source Data file.



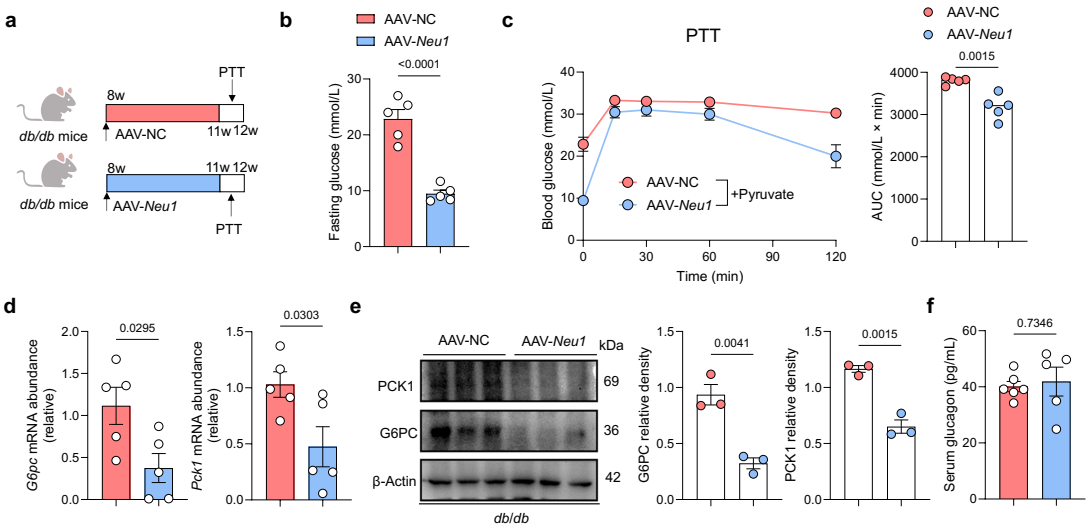
Supplementary Figure 6. Hepatic *Neu1* knockout promoted hepatic glucagon response. Relative mRNA levels of *G6pc* and *Pck1* in primary hepatocytes from *Neu1* HepKO or *Neu1* Flox mice with 100 nM glucagon stimulation for 1 h (n = 6). PBS phosphate buffer solution. Bars represent mean ± SEM values. Significant differences are analyzed using a one-way ANOVA with multiple comparisons. Source data are provided as a Source Data file.



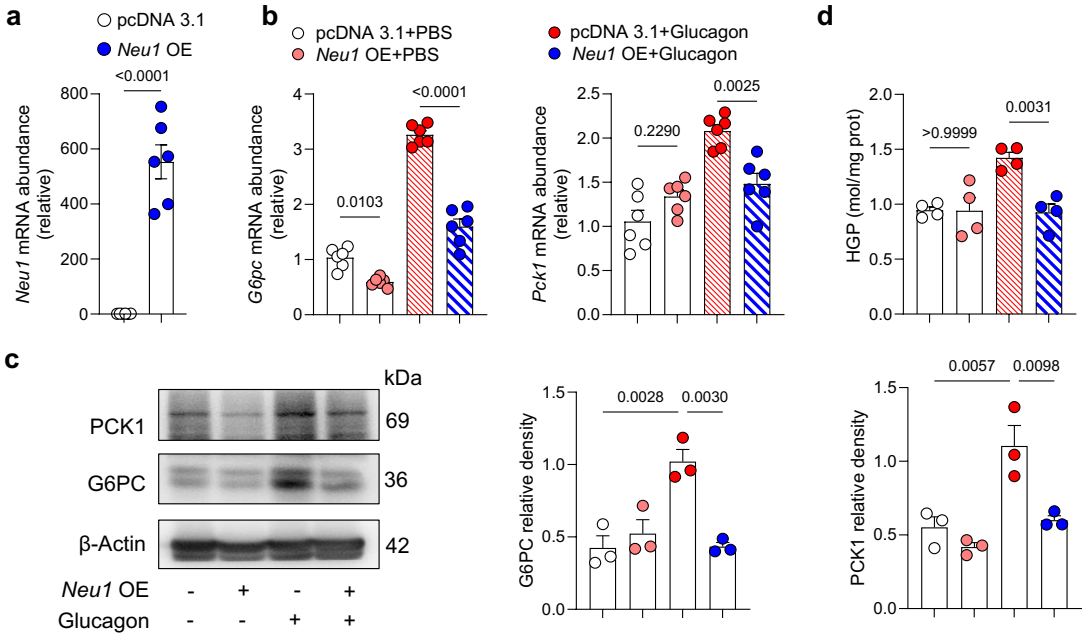
Supplementary Figure 7. *Neu1* knockdown promoted hepatic glucagon response in primary hepatocytes. a mRNA levels of *Neu1* in primary hepatocytes transfected with NC siRNA or *Neu1* siRNA. **b** mRNA levels of *Pck1* and *G6pc* in primary hepatocytes transfected with NC siRNA or *Neu1* siRNA and then stimulated with glucagon (100 nM, 1 h) (n = 6). **c** Protein levels of PCK1 and G6PC under the same treatment conditions as in (**b**) (n = 3). **d** HGP under the same treatment conditions as in (**b**) (n = 6). HGP hepatic glucose production, NC normal control, PBS phosphate buffer solution. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test (**a**), and a one-way ANOVA with multiple comparisons (**b-d**). Source data are provided as a Source Data file.



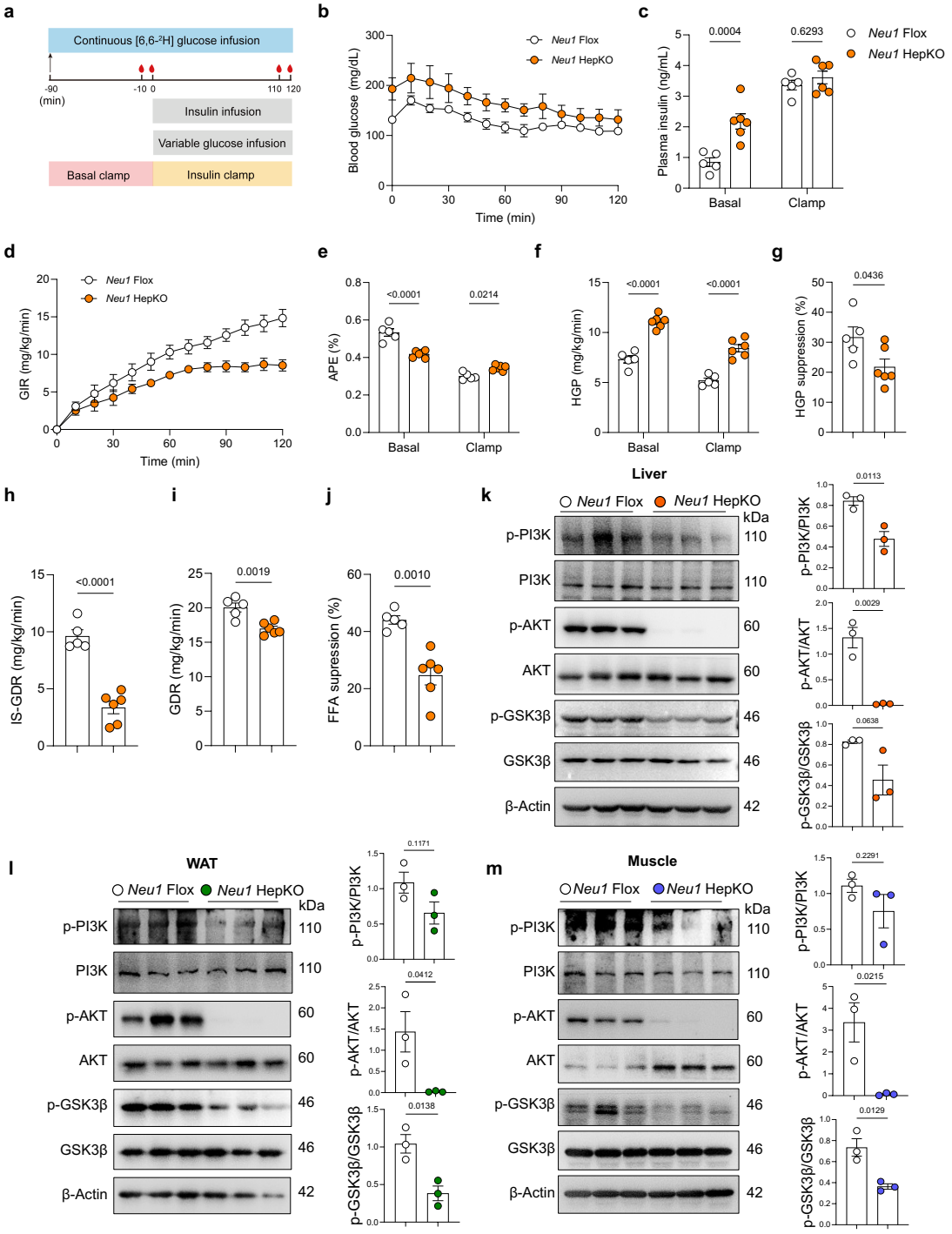
Supplementary Figure 8. The confirmation of NEU1 expression in the liver of AAV-*Neu1* injected mice. a Protein levels of NEU1 in the liver, kidney, brain, muscle, and WAT from mice injected with AAV8-*Neu1* or AAV8-NC. **b** mRNA levels of *Neu1* in the liver, lung, kidney, WAT, muscle, brain, and spleen of mice injected with AAV8-*Neu1* or AAV8-NC (n = 3). **c** Fluorescence image of green fluorescent protein expression in the heart, kidney, WAT, muscle, brain, liver, spleen, and lung of mice injected with AAV8-*Neu1*. AAV adeno-associated virus, NC normal control, WAT white adipose tissue. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test (**b**). Source data are provided as a Source Data file.



Supplementary Figure 9. *Neu1* overexpression inhibited hepatic glucagon response in *db*/*db* mice. a The scheme for *db/db* mice. 8-week-old *db/db* mice injected with AAV8-NC or AAV8-*Neu1* (**b-f**). **b** Fasting blood glucose levels (n = 5). **c** PTT (2 g/kg). AUC is indicated on the right (n = 5). **d-e** Hepatic (**d**) mRNA (n = 5) and (**e**) protein levels (n = 3) of G6PC and PCK1. **f** Serum glucagon levels (AAV-NC group: n = 6; AAV- *Neu1* group: n = 5). AAV adeno-associated virus, AUC area under the curve, NC normal control, PTT pyruvate tolerance test. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test (**b-f**). Source data are provided as a Source Data file.

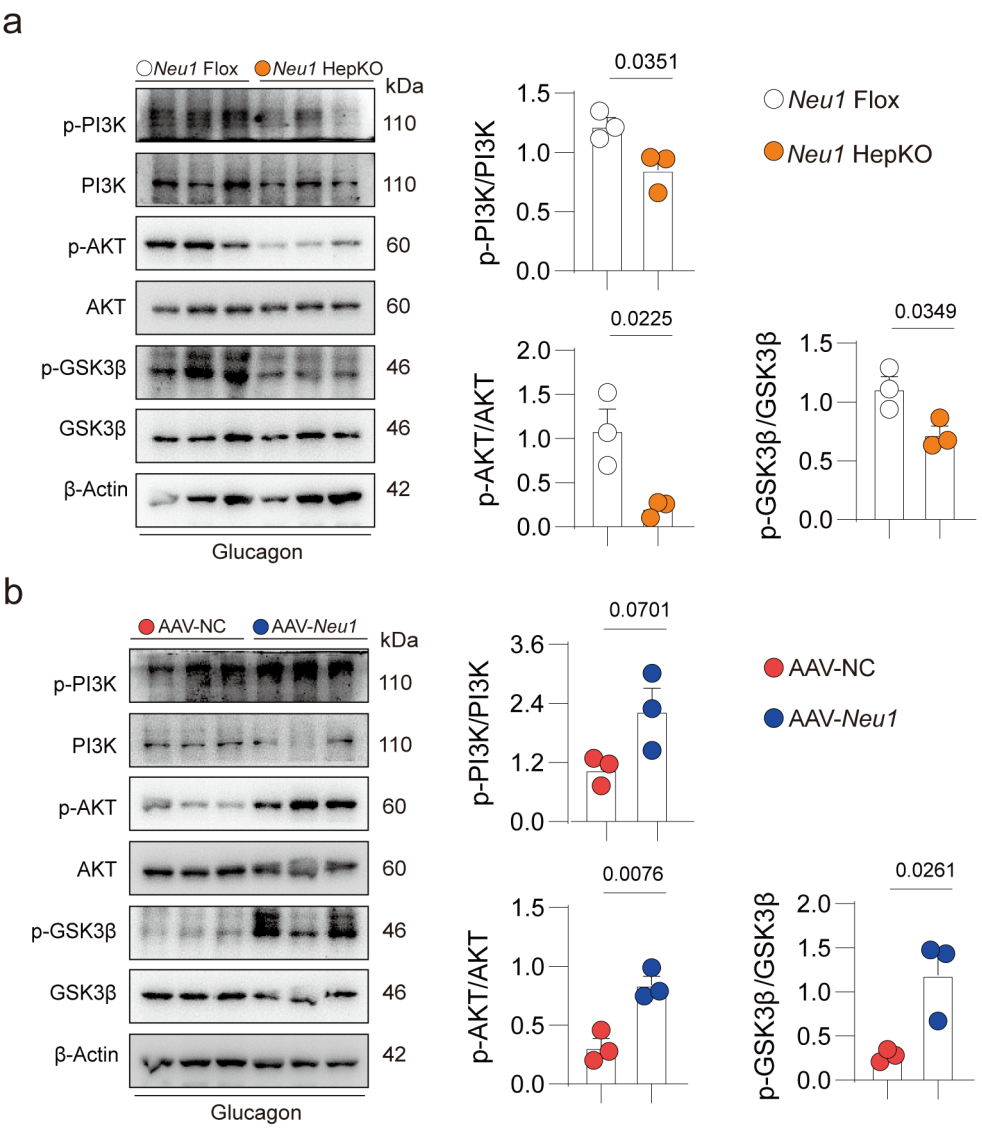


Supplementary Figure 10. *Neu1* inhibited hepatic glucagon response in primary hepatocytes. a mRNA levels of *Neu1* in primary hepatocytes transfected with pcDNA3.1 or *Neu1* plasmid (n = 6). **b** mRNA levels of *Pck1* and *G6pc* in primary hepatocytes transfected with pcDNA3.1 or *Neu1* plasmid and then stimulated with glucagon (100 nM, 1 h) (n = 6). **c** Protein levels of PCK1 and G6PC under the same treatment conditions as in (**b**) (n = 3). **d** HGP under the same treatment conditions as in (**b**) (n = 4). HGP hepatic glucose production, NC normal control, OE overexpression, PBS phosphate buffer solution. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test (**a**), and a one-way ANOVA with multiple comparisons (**b-d**). Source data are provided as a Source Data file.

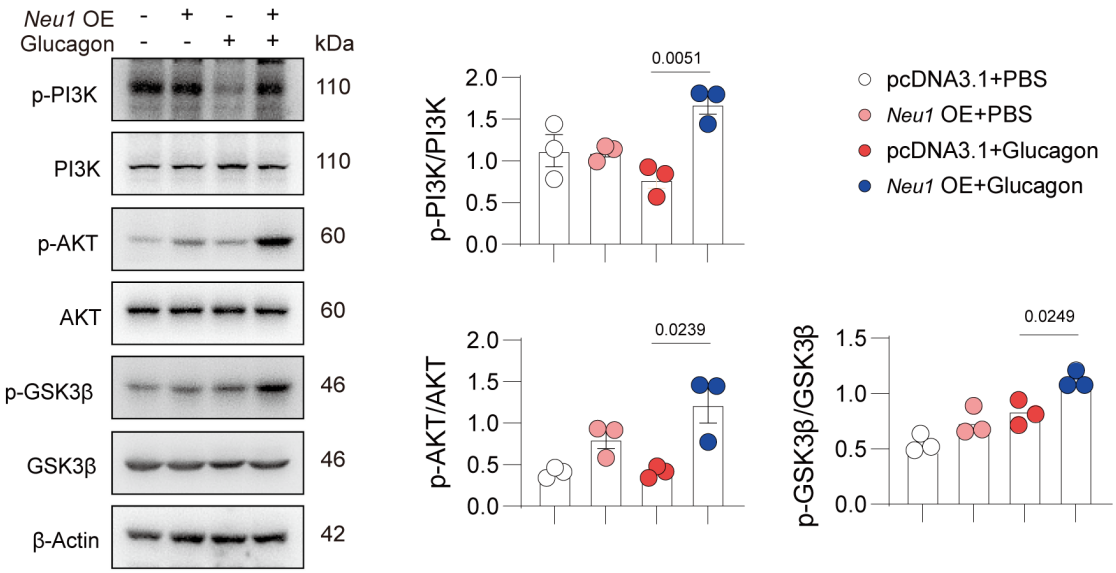


Supplementary Figure 11. Evaluation of insulin sensitivity by hyperinsulinemic-euglycemic clamps using [6,6-^{2}H] glucose in *Neu1* HepKO mice. a The scheme for hyperinsulinemic-euglycemic clamp assays. **b** Blood glucose levels measured during clamp studies (*Neu1* Flox group: n = 5; *Neu1* HepKO group: n = 6). **c** Plasma insulin levels under basal and clamp conditions (*Neu1* Flox group: n = 5; *Neu1* HepKO group: n = 6). **d** GIR measured during clamp studies (*Neu1* Flox group: n = 5; *Neu1* HepKO group: n = 6). **e-f** APE (**e**) and HGP (**f**) under basal and clamp conditions (*Neu1* Flox group: n = 5; *Neu1* HepKO group: n = 6). **g-i** HGP suppression (**g**), GDR (**h**), and IS-GDR (**i**) under clamp conditions (*Neu1* Flox group: n = 5; *Neu1* HepKO group: n = 6).

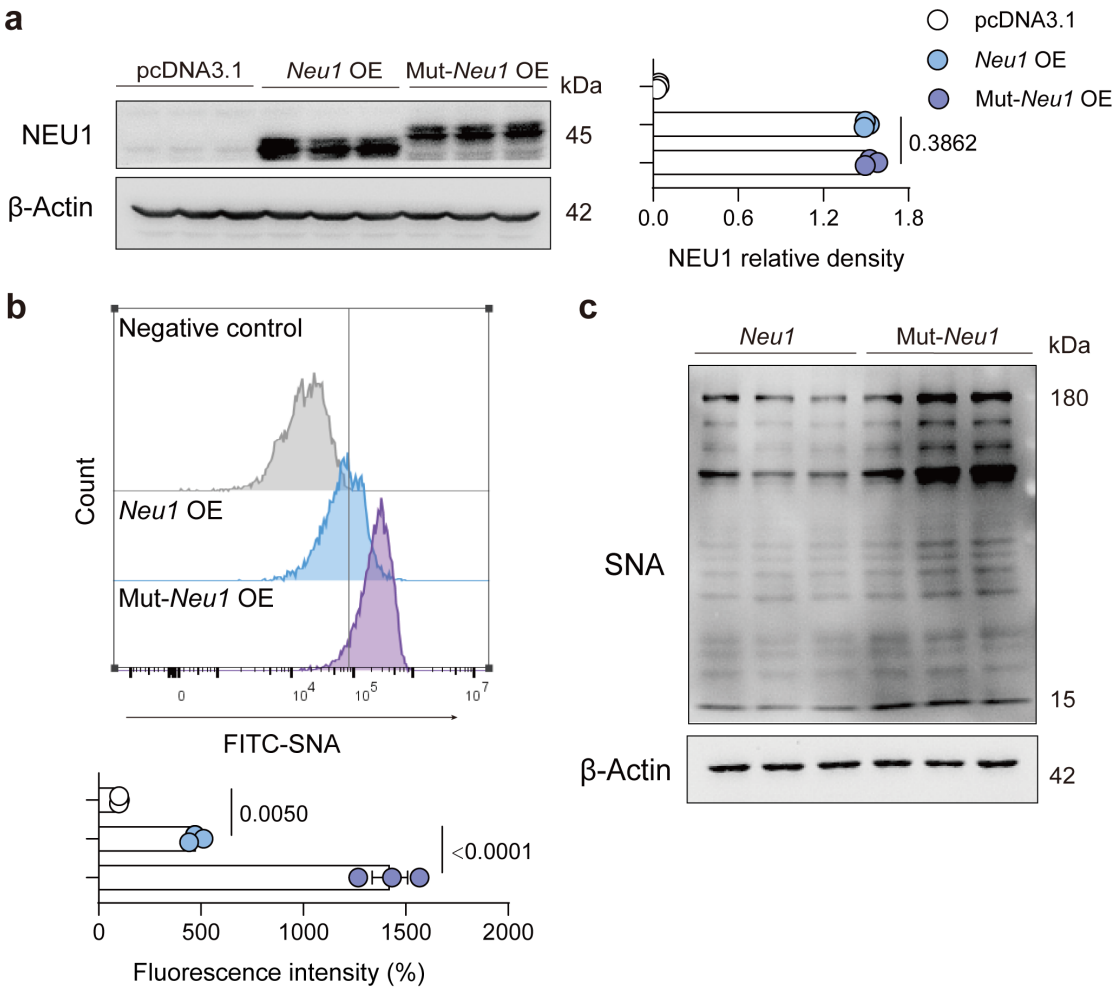
j FFA suppression (*Neu1* Flox group: n = 5; *Neu1* HepKO group: n = 6). **k-m** Protein levels of p-PI3K, p-AKT, p-GSK3β in liver (**k**), WAT (**l**), and muscle (**m**) from *Neu1* HepKO mice (n = 3). GTT glucagon tolerance test, AUC area under the curve, GIR glucose infusion rate, APE Atom percent excess, HGP hepatic glucose production, GDR glucose disposal rate, IS-GDR insulin-stimulated GDR. FFA Free fatty acid, WAT white adipose tissue. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test (**b-m**). Source data are provided as a Source Data file.



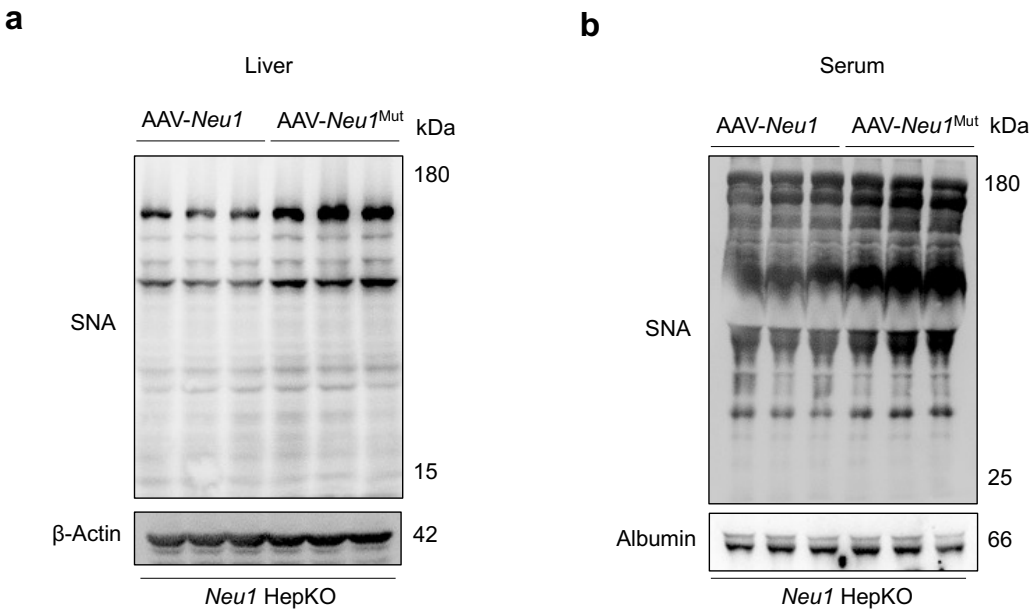
Supplementary Figure 12. NEU1 is involved in the insulin signaling pathway in glucagon-stimulated mice. a Hepatic protein levels of p-PI3K, p-AKT and p-GSK3β in glucagon-challenged *Neu1* HepKO mice (2 mg/kg, n = 3). **b** Hepatic protein expression of p-PI3K, p-AKT, and p-GSK3β in Neu1-overexpressing mice following glucagon treatment. (2 mg/kg, n = 3). AAV adeno-associated virus, NC normal control. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test (**a-b**). Source data are provided as a Source Data file.



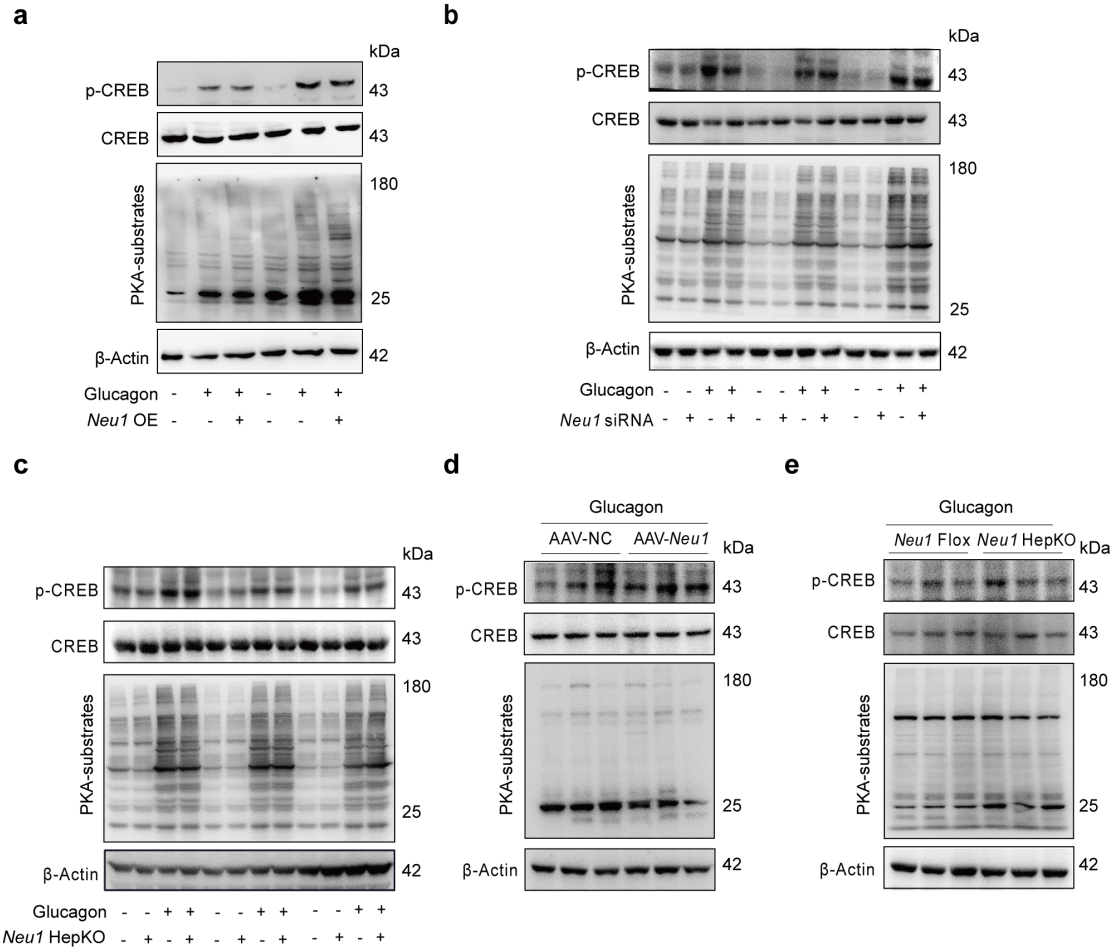
Supplementary Figure 13. Overexpression of *Neu1* enhanced insulin signaling pathway in hepatocytes under glucagon stimulation. Protein levels of p-PI3K, p-AKT and p-GSK3β in *Neu1*-overexpressed hepatocytes with 100 nM glucagon stimulation for 1 h (n = 3). PBS phosphate buffer solution, OE overexpression. Bars represent mean ± SEM values. Significant differences are analyzed using a one-way ANOVA with multiple comparisons. Source data are provided as a Source Data file.



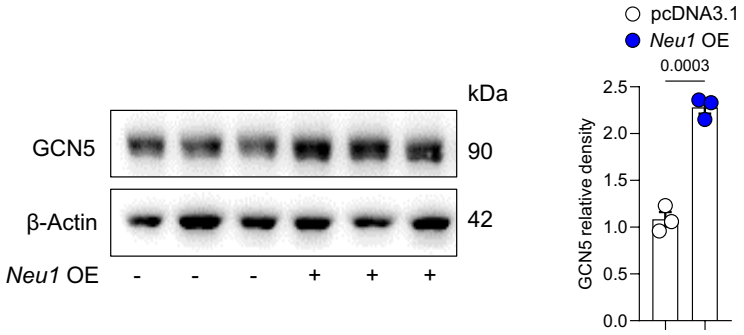
Supplementary Figure 14. Enzyme-active mutant plasmid was successfully constructed. a Protein levels of NEU1 in hepatocytes transfected with *Neu1* or Mut-*Neu1* plasmids (n = 3). **b-c** Flow cytometry analysis of FITC-SNA (**b**) and SNA-lectin blotting (**c**) in hepatocytes transfected with *Neu1* plasmid or Mut-*Neu1* plasmid (n = 3). Mut mutation, OE overexpression, SNA sambucus nigra agglutinin. Bars represent mean ± SEM values. Significant differences are analyzed using a one-way ANOVA with multiple comparisons (**a-b**). Source data are provided as a Source Data file.



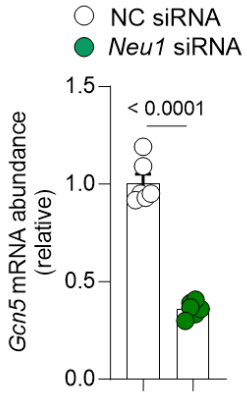
Supplementary Figure 15. The NEU1 enzyme-active mutant successfully caused elevated SNA levels in the liver and serum of mice. a-b SNA-lectin blotting of (**a**) liver and (**b**) serum from *Neu1* HepKO mice injected with AAV-*Neu1* or AAV-*Neu1*^{Mut} (n=3). AAV adeno-associated virus, Mut mutation, SNA sambucus nigra lectin. Source data are provided as a Source Data file.



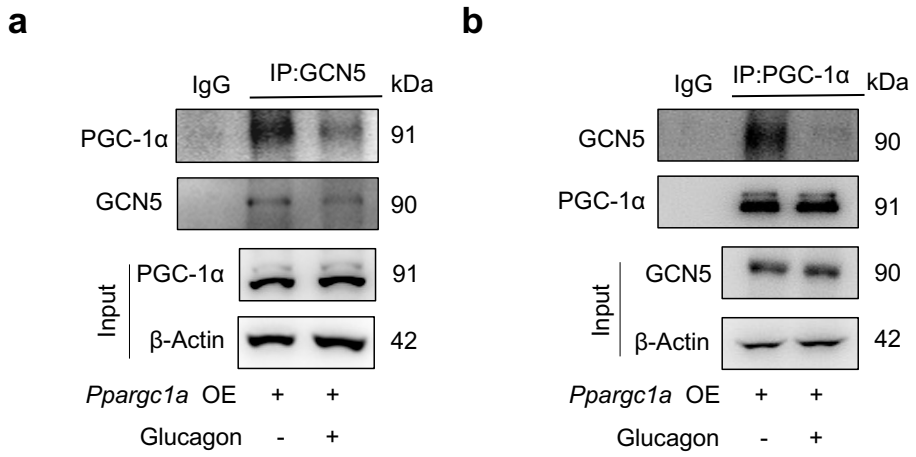
Supplementary Figure 16. NEU1 inhibited hepatic glucagon response independent of PKA/CREB pathway. a-c Western blotting analysis of PKA-substrates, CREB and p-CREB expression in *Neu1*-overexpresed/knockdown/knockout primary hepatocytes treated with glucagon (100 nM, 1 h). **d** Western blotting analysis of PKA-substrates, CREB and p-CREB expression in AAV-NC or AAV-*Neu1* mice treated with glucagon (2 mg/kg, 1 h, n = 3). **e** Western blotting analysis of PKA-substrates, CREB and p-CREB expression in *Neu1* Flox or *Neu1* HepKO mice treated with glucagon (2 mg/kg, 1 h, n = 3). AAV adeno-associated virus, NC normal control, OE overexpression. Source data are provided as a Source Data file.



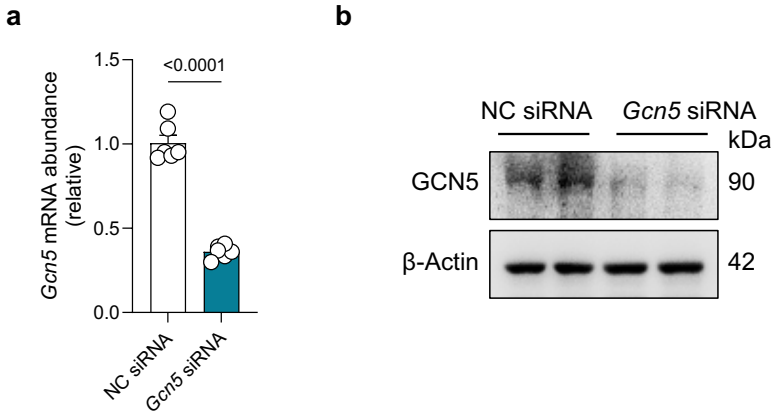
Supplementary Figure 17. NEU1 increased the protein expression of GCN5. Protein levels of GCN5 in *Neu1*-overexpressed primary hepatocytes (n = 3). OE overexpression. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test. Source data are provided as a Source Data file.



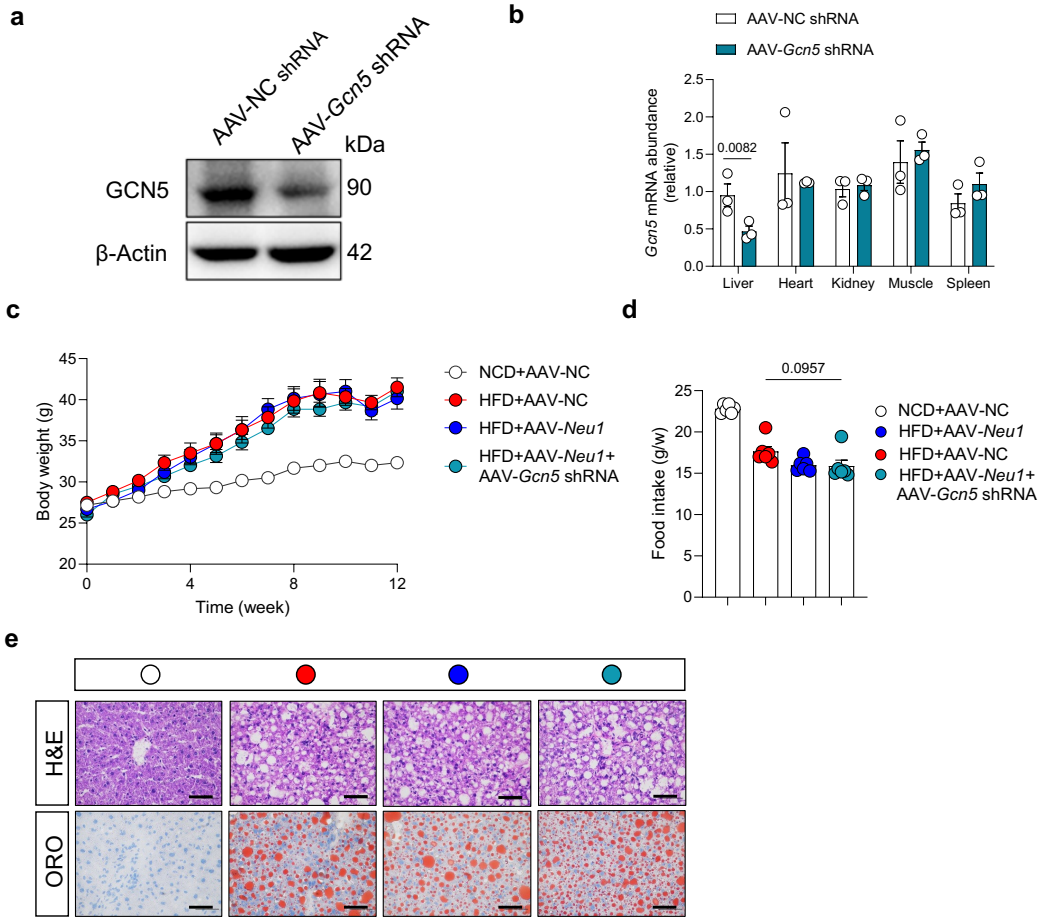
Supplementary Figure 18. Knockdown of NEU1 downregulated the expression of *Gcn5*. mRNA levels of *Gcn5* in *Neu1*-knockdown primary hepatocytes (n = 6). NC normal control. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test. Source data are provided as a Source Data file.



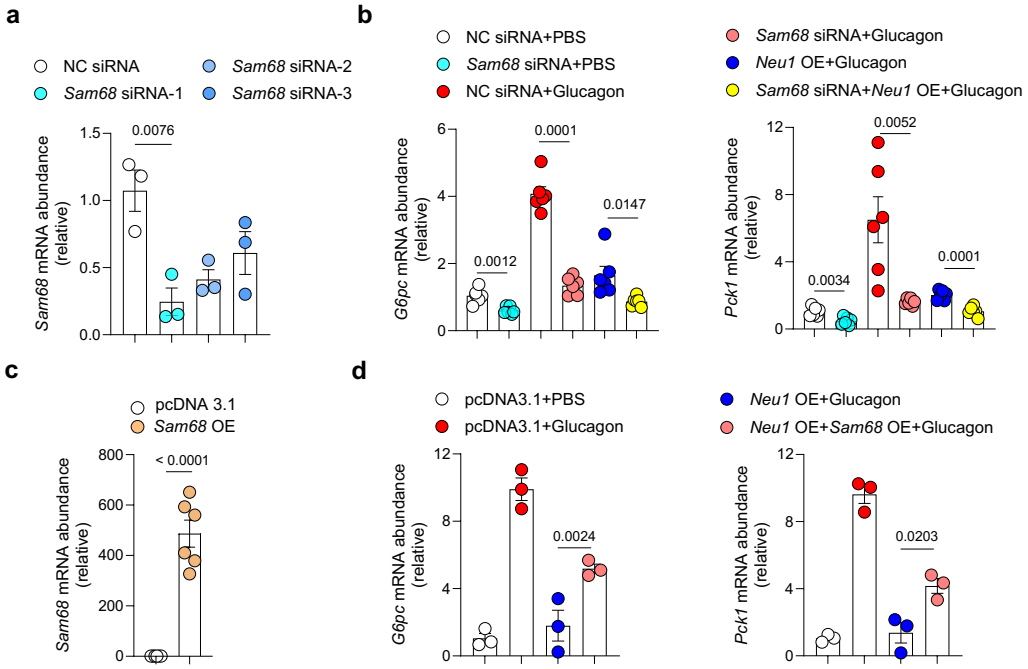
Supplementary Figure 19. Glucagon suppressed the interaction between PGC-1α and GCN5. a-b Immunoprecipitation analysis of interaction of GCN5 with PGC-1α in *Ppargc1a*-overexpressed hepatocytes with or without 100 nM glucagon stimulation for 1 h. OE overexpression, IgG immunoglobulin G. Source data are provided as a Source Data file.



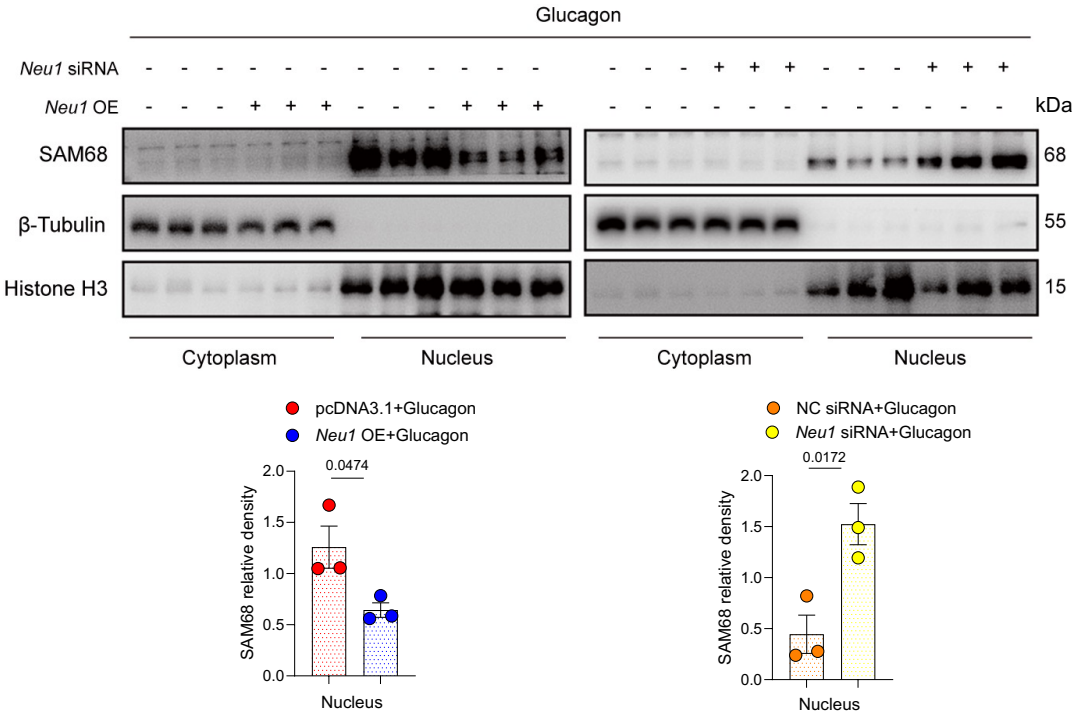
Supplementary Figure 20. GCN5 is knockdown in hepatocytes via *Gcn5* siRNA. **a** mRNA levels of *Gcn5* in hepatocytes transfected with NC siRNA or *Gcn5* siRNA (n = 6). **b** Western blot analysis of GCN5 in hepatocytes transfected with NC siRNA or *Gcn5* siRNA. NC normal control. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test (**a**). Source data are provided as a Source Data file.



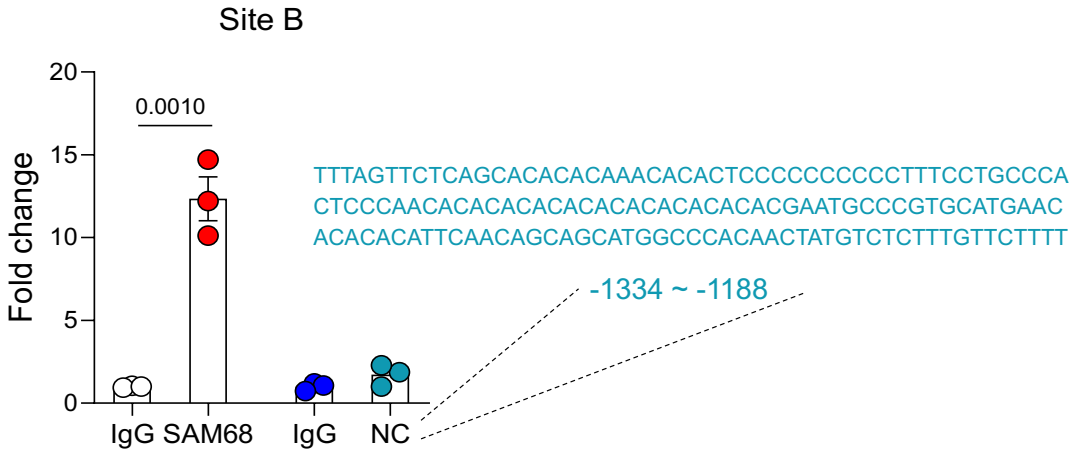
Supplementary Figure 21. Hepatic *Gcn5*-shRNA had no significant influences in body weight, food intake and lipid accumulation in HFD mice injected with AAV-*Neu1*. a-e 8-week-old mice injected with AAV-NC, AAV-*Neu1* or AAV-*Neu1*+ AAV-*Gcn5* shRNA were fed with NCD or HFD for 12 weeks. **a** Western blot analysis of GCN5 of AAV-NC shRNA or AAV-*Gcn5* shRNA mice. **b** mRNA levels of *Gcn5* in the liver, heart, kidney, muscle, and spleen of mice injected with AAV-*Gcn5* shRNA or AAV-NC shRNA (n = 3). **c-d** Body weight (**c**) and food intake (**d**) (n = 6). **e** Representative images of H&E and ORO-stained liver sections. Scale bar, 50 μm. AAV adeno-associated virus, HFD high-fat diet, NC normal control, NCD normal chow diet, H&E hematoxylin and eosin staining, ORO oil red o staining. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test (**b**), and a one-way ANOVA with multiple comparisons (**c-d**). Source data are provided as a Source Data file.



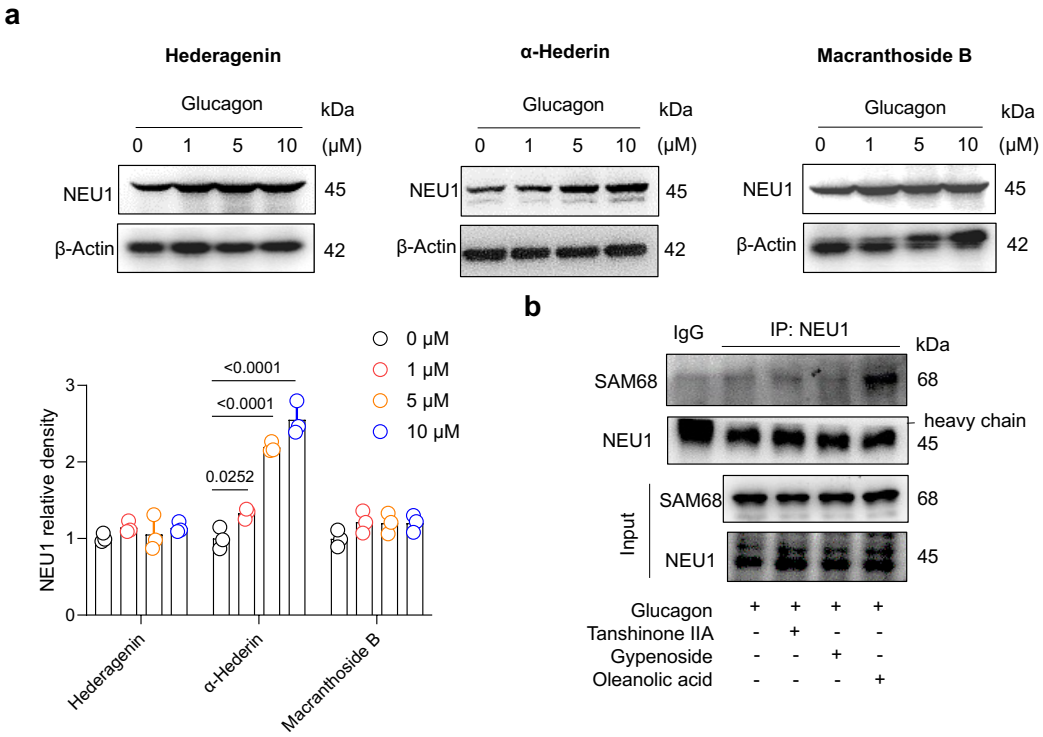
Supplementary Figure 22. NEU1 interacted with SAM68 to inhibit glucagon response. a mRNA levels of *Sam68* in primary hepatocytes transfected with different siRNA sequences (n = 3). **b** mRNA levels of *G6pc* and *Pck1* in *Neu1*-overexpressed, *Sam68*-knockdown primary hepatocytes stimulated with 100 nM glucagon (1 h, n = 6).**c** mRNA levels of *Sam68* in hepatocytes after transfected with *Sam68* plasmid (n = 6). **d** mRNA levels of *G6pc* and *Pck1* in *Neu1*-overexpressed, *Sam68*-overexpressed primary hepatocytes stimulated with 100 nM glucagon (1 h, n = 3). PBS phosphate buffer solution, OE overexpression, NC normal control. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test (**c**), and a one-way ANOVA with multiple comparisons (**a-b**, **d**). Source data are provided as a Source Data file.



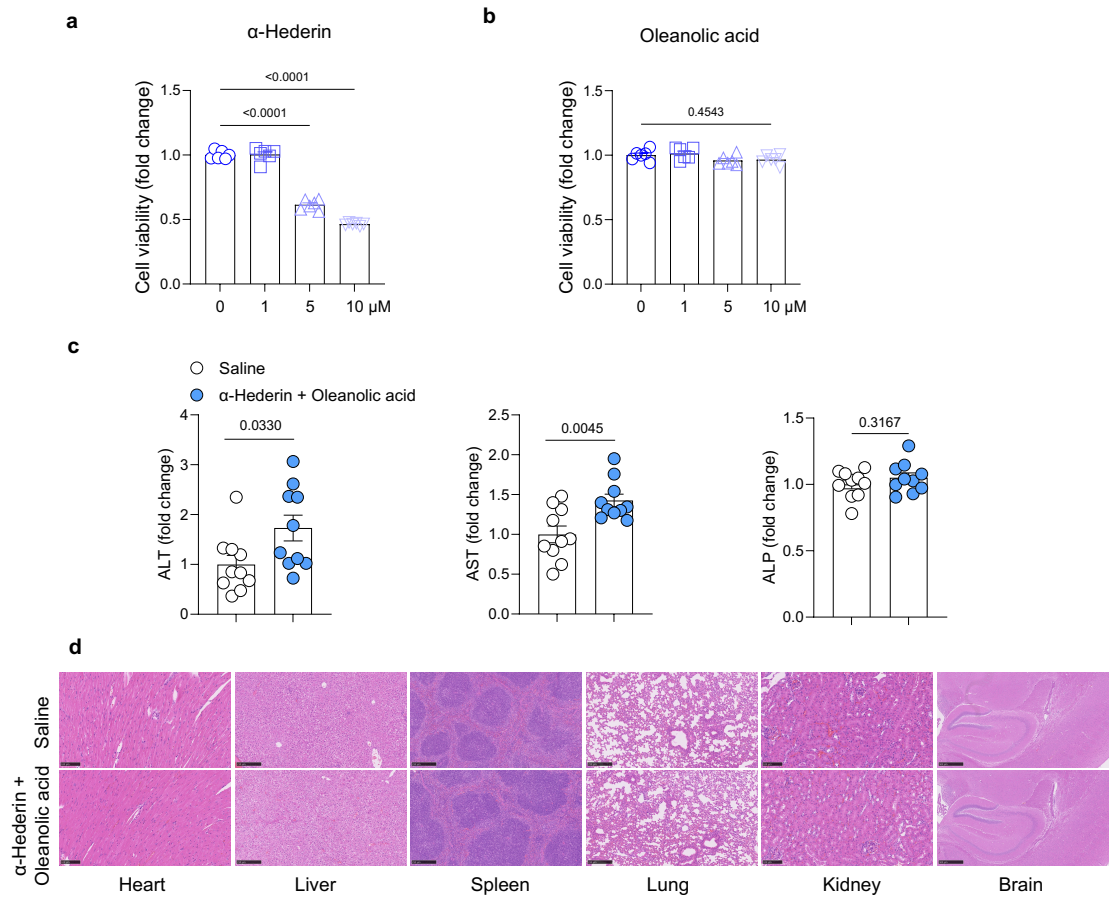
Supplementary Figure 23. NEU1 impaired the nuclear accumulation of SAM68. SAM68 protein levels in cytoplasmic and nuclear fractions from *Neu1*-overexpressed or -knockdown primary hepatocytes treated with glucagon (100 nM, 1 h; n = 3). OE overexpression, NC normal control. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test. Source data are provided as a Source Data file.



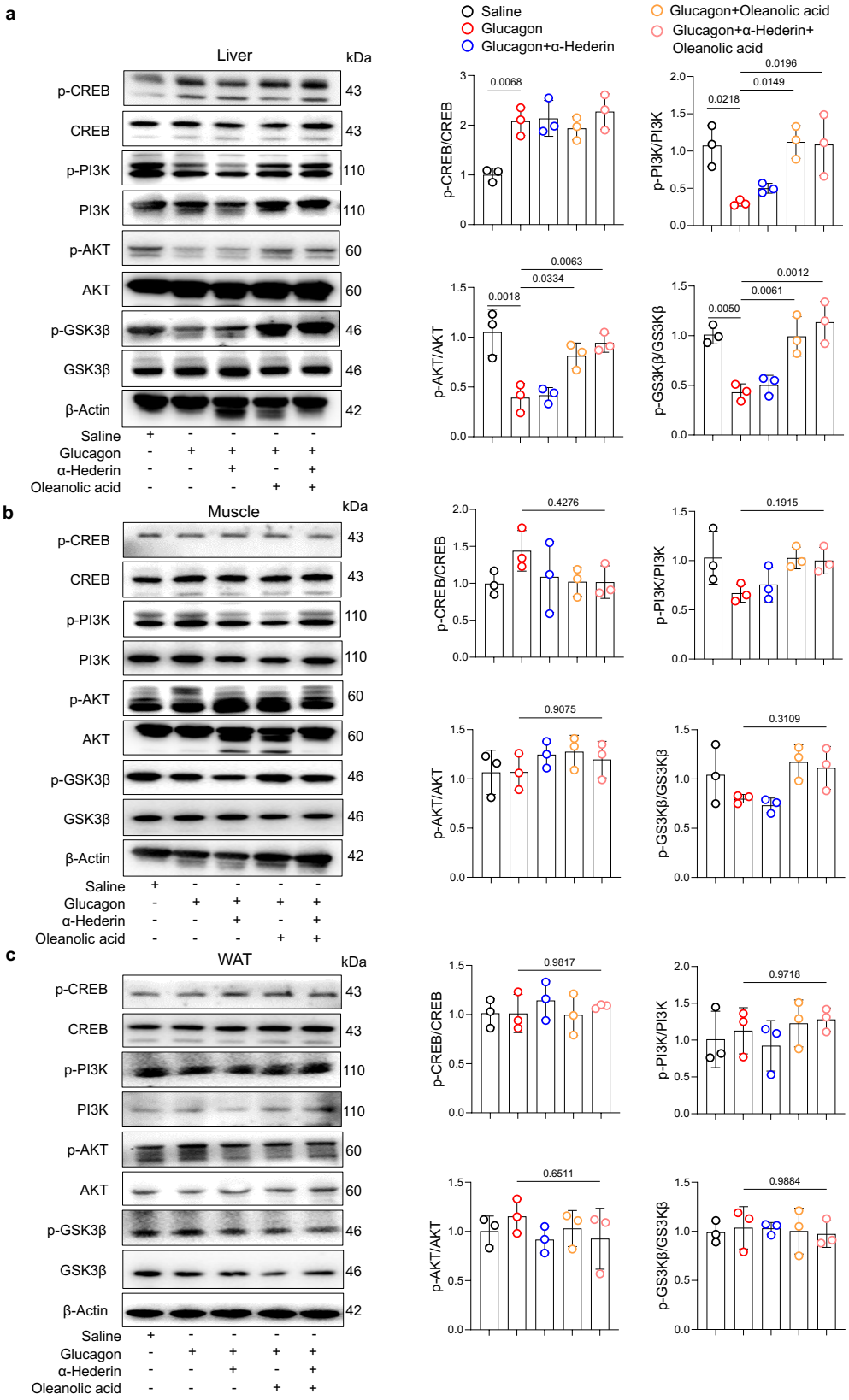
Supplementary Figure 24. SAM68 bound to the promoter region of *Gcn5*. ChIP analysis using a SAM68-specific antibody to detect SAM68 occupancy at the promoter site B of *Gcn5* in primary hepatocytes (n = 3). ChIP Chromatin Immunoprecipitation, NC negative control. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test. Source data are provided as a Source Data file.



Supplementary Figure 25. α-Hederin upregulated NEU1 and oleanolic acid enhances NEU1-SAM68 interaction in hepatocytes. a NEU1 protein levels in glucagon-stimulated (100 nM, 1 h) hepatocytes treated with candidate compounds (n = 3). **b** Immunoprecipitation analysis of interaction of NEU1 with SAM68 in hepatocytes with 100 nM glucagon stimulation for 1 h after treating with candidate compounds. Bars represent mean ± SEM values. Significant differences are analyzed using a one-way ANOVA with multiple comparisons (**a**). Source data are provided as a Source Data file.



Supplementary Figure 26. The toxicity of α-hederin and oleanolic acid has been evaluated both *in vitro* and *in vivo*. a-b Cell viability of hepatocytes treated with α-hederin or oleanolic acid (n = 6). **c** Serum ALT, AST, and ALP levels of mice (n = 10). **d** H&E staining of tissue sections following treatment with α-hederin and oleanolic acid (200 mg/kg each). Scale bar, 500 μm (brain), 250 μm (liver, spleen, lung), and 100 μm (heart, kidney). ALT Alanine Aminotransferase, AST Aspartate Aminotransferase, ALP, Alkaline Phosphatase, H&E hematoxylin and eosin staining. Bars represent mean ± SEM values. Significant differences are analyzed using two-tailed *t*-test (**c**), and a one-way ANOVA with multiple comparisons (**a-b**). Source data are provided as a Source Data file.



Supplementary Figure 27. Oleanolic acid affects the insulin signaling pathway in mouse liver. a-c Protein levels of p-CREB, p-PI3K, p-AKT, p-GSK3β in (**a**) liver, (**b**) muscle, and (**c**) WAT from C57BL/6J mice treated with 2 mg/kg glucagon ± 1 mg/kg α-hederin or/and 1 mg/kg oleanolic acid (n = 3). WAT white adipose tissue. Bars

represent mean ± SEM values. Significant differences are analyzed using a one-way ANOVA with multiple comparisons (**a-c**). Source data are provided as a Source Data file.

Supplementary Table 1. Clinical and metabolic characteristics in this study.

Subject characteristics	ND (n = 10)	T2D (n = 10)	*P* value
Gender (F/M)	1/9	4/6	-
Mean age (y)	60.40 ± 2.65	65.70 ± 3.24	0.22
BMI (kg/m^2)	24.35 ± 1.67	24.21 ± 0.88	0.94
FPG (mmol/L)	4.73 ± 0.17	9.82 ± 0.70	1.35E-6
AST (U/L)	47.62 ± 10.33	41.79 ± 8.55	0.67
ALT (U/L)	44.94 ± 12.63	58.15 ± 25.19	0.64

The patients were seen in the same medical centre (Nanjing Drum Tower Hospital, China). ND: Non-diabetes. T2D: Type 2 diabetes. BMI: Body mass index. FPG: Fasting plasma glucose. AST: Aspartate aminotransferase. ALT: Alanine aminotransferase. Data are represented as means ± SEM.

Supplementary Table 2. Primers for RT-qPCR.

Gene	Orientation	Oligonucleotide Sequence (5'-3')
Neu1 (mice)	Forward	CCTACGAGCTTCCAGATGGC
	Reverse	CAGGGTCGAAGGTCACATCC
Pck1 (mice)	Forward	TGGCATCCCCGAATATGATGA
	Reverse	GGGCGAGTCTGTCAGTTCAAT
G6pc (mice)	Forward	CGACTCGCTATCTCCAAGTGA
	Reverse	GTTGAACCAGTCTCCGACCA
Ppargc1a (mice)	Forward	ATACCGCAAAGAGCACGAGAAG
	Reverse	CTCAAGAGCAGCGAAAGCGTCACAG
Gcn5 (mice)	Forward	GAAGAGGACCCTCATCCTCA
	Reverse	GTGAAGCCTGACTCCCAGAT
Sirt1 (mice)	Forward	GCTGACGACTTCGACGACG
	Reverse	TCGGTCAACAGGAGGTTGTCT
Sirt2 (mice)	Forward	GCCTGGGTTCCCAAAAGGAG
	Reverse	GAGCGGAAGTCAGGGATACC
Sirt3 (mice)	Forward	ATCCCGGACTTCAGATCCCC
	Reverse	CAACATGAAAAAGGGCTTGGG
Sirt4 (mice)	Forward	CAGATGTCGTTTTCTTCG
	Reverse	CCAGAGTATACCTGCAAGG
Sirt5 (mice)	Forward	CTCCGGGCCGATTCATTTCC
	Reverse	GCGTTCGCAAAACACTTCCG
Sirt6 (mice)	Forward	ATGTCGGTGAATTATGCAGCA
	Reverse	GCTGGAGGACTGCCACATTA
Actb (mice)	Forward	AGTGTGACGTTGACATCCGTA
	Reverse	GCCAGAGCAGTAATCTCCTTCT

Supplementary Table 3. Antibodies for immunoblotting.

Antibody	Company	Catalog #
NEU1	Santa Cruz	sc-166824
NEU2	Santa Cruz	sc-100570
NEU3	Proteintech	27879-1-AP
NEU4	Proteintech	12995-1-AP
β-Actin	Santa Cruz	sc-517582
PKA Substrate Antibody	Cell Signaling Technology	9621S
p-CREB (Ser133)	Cell Signaling Technology	9198S
CREB	Cell Signaling Technology	9197S
Acetylated-Lysine Antibody	Cell Signaling Technology	9441S
PGC-1α	Proteintech	66369-1-Ig
HSP90	Beyotime	AF0192
GCN5	Cell Signaling Technology	3305S
SAM68	Proteintech	10222-1-AP
Anti-Mouse IgG	Beyotime	A0216
Protein A/G Magnetic Beads	MCE	HY-K0202
PI3K	HUABIO	ET1609-3O
p-PI3K	HUABIO	HA721672
GSK-3β	HUABIO	ET1607-71
Phospho-GSK-3β (Ser9) (5B3)	Cell Signaling Technology	9323S
P53	Santa Cruz	sc-71818
P62	HUABIO	HA721171
G6PC	Abcam	AB319053
PCK1 (D12F5)	Cell Signaling Technology	12940S
Sambucus Nigra Lectin (SNA, EBL)	Vector Laboratories	B-1305
p-AKT (Ser473)	Cell Signaling Technology	4060S
AKT (C67E7)	Cell Signaling Technology	4691S
HRP-conjugated Streptavidin	Proteintech	SA00001-0
Sambucus Nigra Lectin (SNA, EBL), Fluorescein	Vector Laboratories	FL-1301
Insulin Receptor B(4B8)	Cell Signaling Technology	3025T
Anti-Ubiquitin Antibody	Cell Signaling Technology	58395S
Anti-HA-Tag (C29F4)	Cell Signaling Technology	3724T
Anti-anti DDDDK-Tag	ABclonal	AE005
Histone H3(1B1B2)	Cell Signaling Technology	14269S
β-Tubulin	Proteintech	10094-1-AP