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Supplemental information

Neddylation of phosphoenolpyruvate carboxykinase 1 controls glucose metabolism

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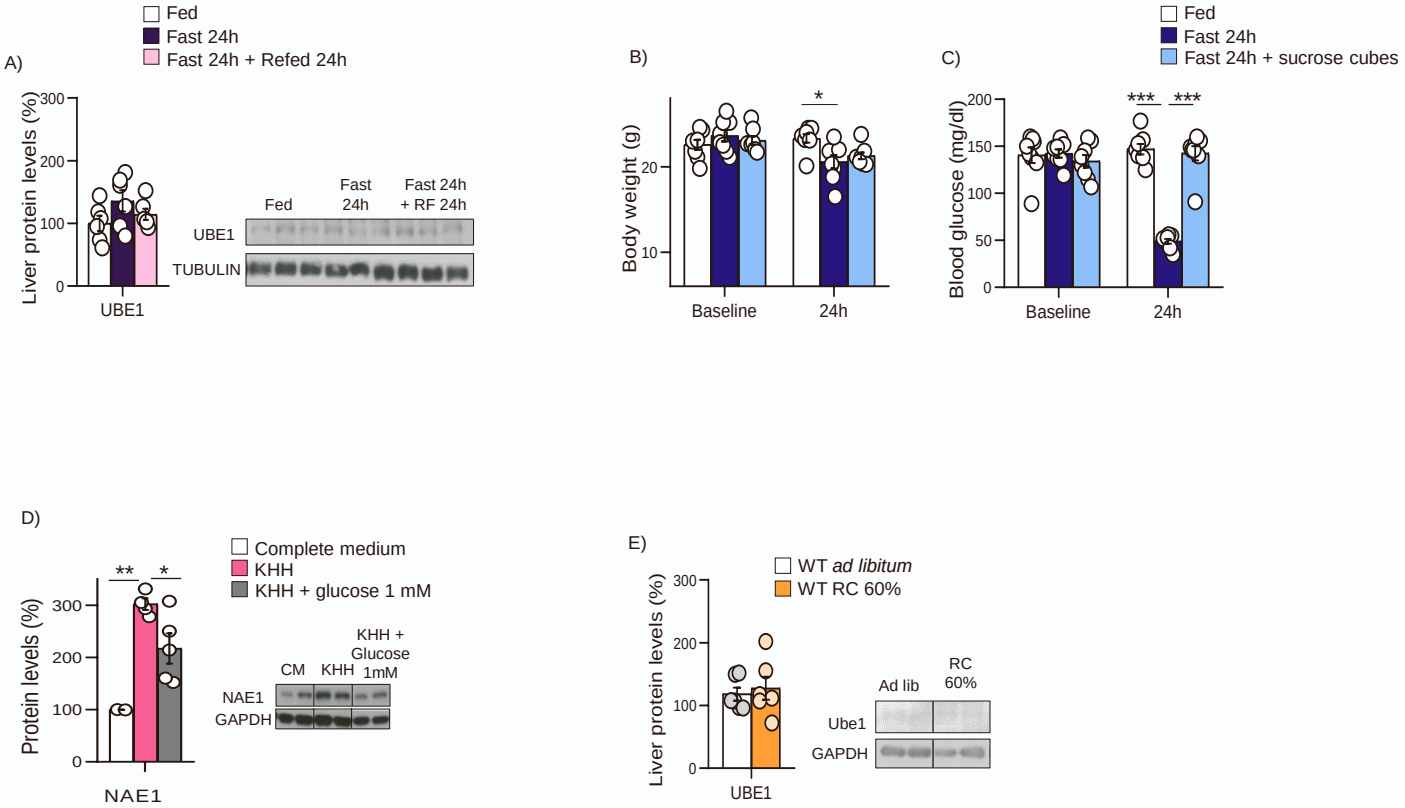

Figure S1. UBE1 is not regulated by fasting. Related to Figure 1. A) UBE1 protein levels in the liver of WT mice fed *ad libitum*, fasted for 24h, or refed for 24 h after 24 h starvation (n=6 per group). B) Body weight and C) blood glucose levels in WT mice fed *ad libitum*, fasted for 24h or fed with sugar (n=6-8 per group). D) NAE1 protein levels in THLE2 cell in complete medium, medium without nutrients (KHH) and KHH medium + glucose (n=2-6 per group). E) UBE1 protein levels in the liver of WT mice fed *ad libitum* or subjected to a 60% caloric restriction (n=6 per group). Expression of GAPDH served as a loading control, and control values were normalized to 100%. Data are presented as mean ± SEM; two-tailed unpaired *t*-test (**E**) and one-way ANOVA followed by Bonferroni *post hoc* test (**A–D**): *P<0.05, **P<0.01 and ***P<0.001.

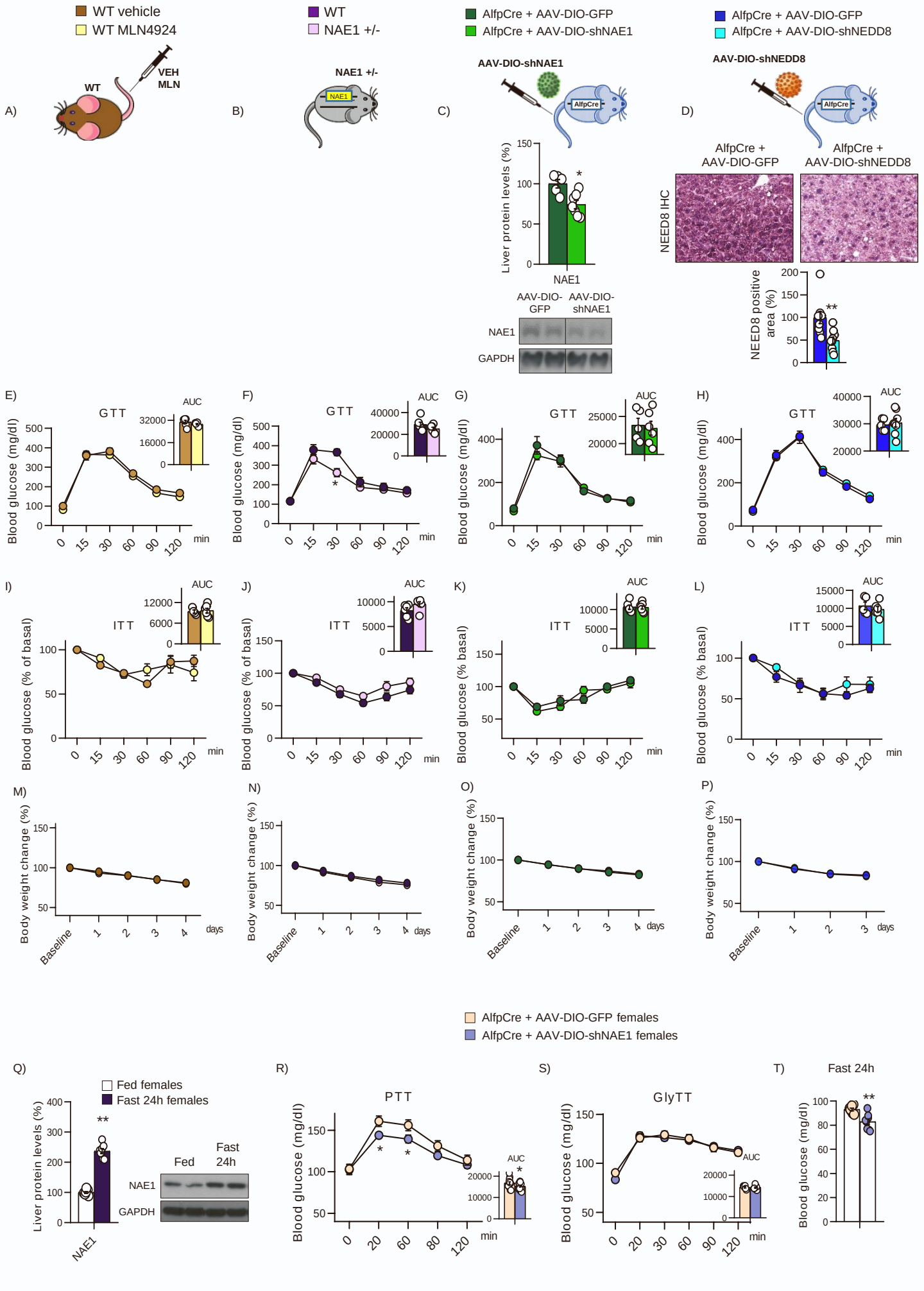
A)
WT vehicle
WT MLN4924
WT
VEH
MLN
B)
WT
NAE1 +/-
NAE1 +/-
NAE1
C)
AlfpCre + AAV-DIO-GFP
AlfpCre + AAV-DIO-shNAE1
AAV-DIO-shNAE1
AlfpCre
Liver protein levels (%)
NAE1
AAV-DIO-GFP
AAV-DIO-shNAE1
NAE1
GAPDH
D)
AlfpCre + AAV-DIO-GFP
AlfpCre + AAV-DIO-shNEDD8
AAV-DIO-shNEDD8
AlfpCre
AlfpCre + AAV-DIO-GFP
AlfpCre + AAV-DIO-shNEDD8
NEED8 IHC
NEED8 positive area (%)
E)
GTT
AUC
Blood glucose (mg/dl)
min
F)
GTT
AUC
Blood glucose (mg/dl)
min
G)
GTT
AUC
Blood glucose (mg/dl)
min
H)
GTT
AUC
Blood glucose (mg/dl)
min
I)
ITT
AUC
Blood glucose (% of basal)
min
J)
ITT
AUC
Blood glucose (% of basal)
min
K)
ITT
AUC
Blood glucose (% basal)
min
L)
ITT
AUC
Blood glucose (% basal)
min
M)
Body weight change (%)
Baseline
days
N)
Body weight change (%)
Baseline
days
O)
Body weight change (%)
Baseline
days
P)
Body weight change (%)
Baseline
days
AlfpCre + AAV-DIO-GFP females
AlfpCre + AAV-DIO-shNAE1 females
Q)
Fed females
Fast 24h females
Liver protein levels (%)
NAE1
Fed
Fast 24h
NAE1
GAPDH
R)
PTT
AUC
Blood glucose (mg/dl)
min
S)
GlyTT
AUC
Blood glucose (mg/dl)
min
T)
Fast 24h
Blood glucose (mg/dl)

Figure S2. Inhibition of hepatic neddylation does not affect glucose tolerance or insulin sensitivity. Related to Figure 2. (A), (**B**), (**C**) and (D) animal models where hepatic neddylation is inhibited (n=3-10 per group). (**C**) NAE1 protein levels in Alfp Cre+/- mice injected with AAV-DIO-GFP or AAV-dio-shNAE1 to downregulate NAE1 in the liver (n=5-7 per group). (**D**) NEDD8 IHC in the liver of Alfp Cre+/- mice injected with AAV-DIO-GFP or AAV-dio-shNEDD8 to downregulate NEDD8 in the liver (n=7-10 per group). Quantification of NEDD8 area is also shown. Glucose tolerance test (GTT) in (**E**) WT mice treated with vehicle or MLN4924, (**F**) NAE1+/- and their control littermates, (**G**) Alfp-Cre +/- mice injected with AAV-DIO expressing either GFP or shNAE1, and (**H**) Alfp-Cre +/- mice injected with AAV-DIO expressing either GFP or shNEDD8 (n=5-8 per group). Area under the curve (AUC) graphs are also shown. Insulin tolerance test (ITT) in (**I**) WT mice treated with vehicle or MLN4924, (**J**) NAE1+/- and their control littermates, (**K**) Alfp-Cre+/- mice injected with AAV-DIO expressing either GFP or shNAE1, and (**L**) Alfp-Cre+/- mice injected with AAV-DIO expressing either GFP or shNEDD8 (n=5-7 per group). AUC graphs are also shown. Body weight change during 60% caloric restriction in (**M**) WT mice treated with vehicle or MLN4929, (**N**) NAE1+/- and their control littermates, (**O**) Alfp-Cre+/- mice injected with AAV-DIO expressing either GFP or shNAE1, and (**P**) Alfp-Cre+/- mice injected with AAV-DIO expressing either GFP or shNEDD8. (**Q**) NAE1 protein levels from WT female mice fed ad libitum or subjected to fasting for 24h (n=7-10 per group). (**R**) Pyruvate Tolerance Test (PTT), (**S**) Glycerol Tolerance Test (GlyTT), and (**T**) glucose fasting levels after 24h in female Alfp-Cre+/- mice injected with AAV-DIO-GFP or AAV-DIO-shNAE1 (n=6-18 per group). Expression of GAPDH served as a loading control, and control values were normalized to 100%. Data are presented as mean ± SEM; two-tailed unpaired *t*-test: *P<0.05, **P<0.01 and ***P<0.001.

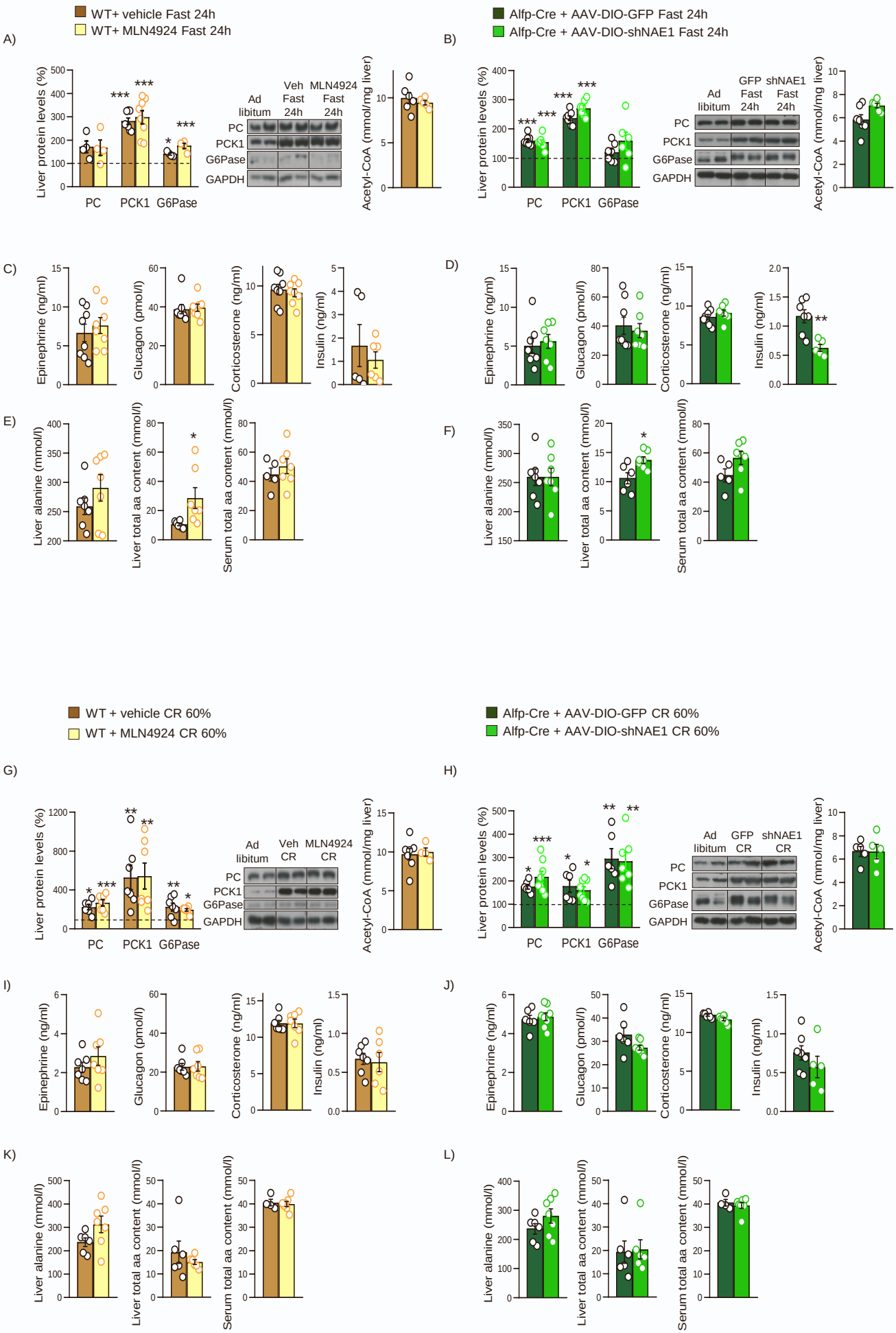
A)
WT+ vehicle Fast 24h
WT+ MLN4924 Fast 24h
Liver protein levels (%)
PC
PCK1
G6Pase
Ad libitum
Veh Fast 24h
MLN4924 Fast 24h
GAPDH
Acetyl-CoA (mmol/mg liver)
B)
Alfp-Cre + AAV-DIO-GFP Fast 24h
Alfp-Cre + AAV-DIO-shNAE1 Fast 24h
GFP Fast 24h
shNAE1 Fast 24h
C)
Epinephrine (ng/ml)
Glucagon (pmol/l)
Corticosterone (ng/ml)
Insulin (ng/ml)
D)
E)
Liver alanine (mmol/l)
Liver total aa content (mmol/l)
Serum total aa content (mmol/l)
F)
WT + vehicle CR 60%
WT + MLN4924 CR 60%
Alfp-Cre + AAV-DIO-GFP CR 60%
Alfp-Cre + AAV-DIO-shNAE1 CR 60%
G)
H)
Veh CR
MLN4924 CR
GFP CR
shNAE1 CR
I)
J)
K)
L)

Figure S3. Inhibition of hepatic neddylation does not affect circulating levels of glucose counterregulatory hormones. Related to Figure 2. Pyruvate carboxylase (PC), phosphoenolpuryvate carboxykinase 1 (PCK1) and glucose 6-phosphase (G6Pase) liver protein levels and liver acetyl-CoA levels; epinephrine, glucagon, corticosterone and insulin serum levels; and liver alanine and liver and serum amino acids content in WT mice after the administration of vehicle or MLN4924 and then (**A**, **C**, **E**) fasted for 24h, or (**G**, **I**, **K**) subjected to caloric restriction; and in Alfp Cre+/- mice injected with AAV-DIO-GFP or AAV-dio-shNAE1 subjected to (**B**, **D**, **F**) 24h fasting, and (**H**, **J**, **L**) caloric restriction. *n* = 4-8 animals per group. Expression of GAPDH served as a loading control, and control values were normalized to 100%. Spotted line indicates related to control group. Data are presented as mean ± SEM; one-way ANOVA followed by Bonferroni *post hoc* test (A, B, G and H left panels) and two-tailed unpaired *t*-test (A-L): $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

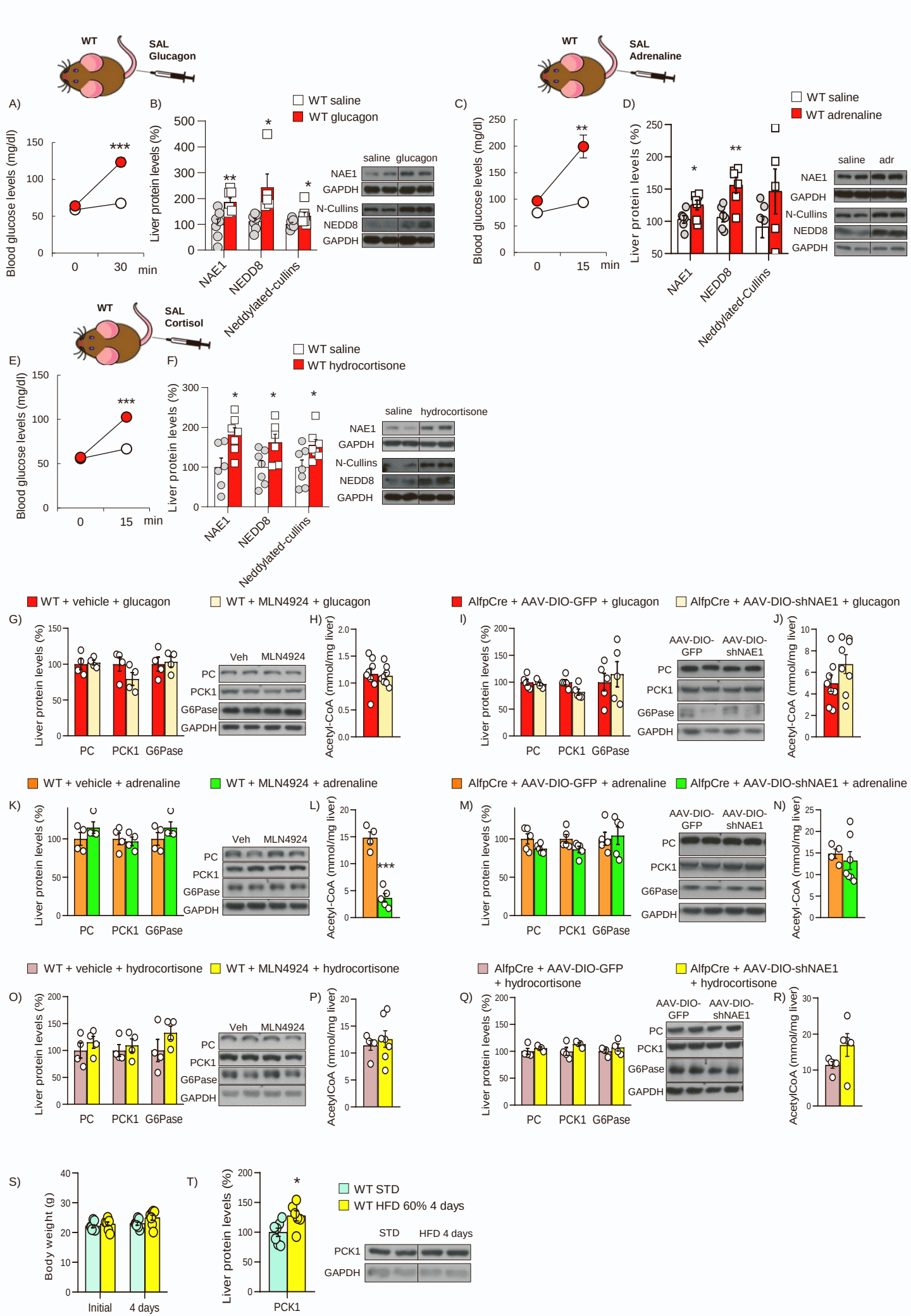
WT
SAL
Glucagon
A)
B)
WT saline
WT glucagon
Blood glucose levels (mg/dl)
Liver protein levels (%)
min
saline
glucagon
NAE1
GAPDH
N-Cullins
NEDD8
Neddylated-cullins
WT
SAL
Adrenaline
C)
D)
WT saline
WT adrenaline
saline
adr
WT
SAL
Cortisol
E)
F)
WT saline
WT hydrocortisone
saline
hydrocortisone
WT + vehicle + glucagon
WT + MLN4924 + glucagon
AlfpCre + AAV-DIO-GFP + glucagon
AlfpCre + AAV-DIO-shNAE1 + glucagon
G)
H)
I)
J)
Veh
MLN4924
AAV-DIO-GFP
AAV-DIO-shNAE1
PC
PCK1
G6Pase
GAPDH
Acetyl-CoA (mmol/mg liver)
WT + vehicle + adrenaline
WT + MLN4924 + adrenaline
AlfpCre + AAV-DIO-GFP + adrenaline
AlfpCre + AAV-DIO-shNAE1 + adrenaline
K)
L)
M)
N)
WT + vehicle + hydrocortisone
WT + MLN4924 + hydrocortisone
AlfpCre + AAV-DIO-GFP + hydrocortisone
AlfpCre + AAV-DIO-shNAE1 + hydrocortisone
O)
P)
Q)
R)
AcetylCoA (mmol/mg liver)
S)
T)
Body weight (g)
Initial
4 days
WT STD
WT HFD 60% 4 days
STD
HFD 4 days
PCK1

Figure S4. Glucose counterregulatory hormones induce neddylation in the liver. Related to Figure 2. WT mice treated with saline or glucagon (**A**) blood glucose levels; (**B**) hepatic protein levels of NAE1, NEDD8 and neddylated cullins. WT mice treated with saline or adrenaline (**C**) blood glucose levels; (**D**) hepatic protein levels of NAE1, NEDD8 and neddylated cullins. WT mice treated with saline or hydrocortisone (**E**) blood glucose levels; (**F**) hepatic protein levels of NAE1, NEDD8 and neddylated cullins. (**G**) pyruvate carboxylase (PC), phosphoenolpuryvate carboxykinase 1 (PCK1) and glucose 6-phosphase (G6Pase) liver protein levels and (**H**) acetyl-CoA serum levels in WT mice treated vehicle or MLN4924 and then with saline or glucagon (200 µg kg-1). (**I**) PC, PCK1 and G6Pase liver protein levels and (**J)** acetyl-CoA serum levels in Alfp-Cre mice injected with AAV-DIO-GFP or AAV-DIO-shNAE1 and then with saline or glucagon (200 µg kg-1). (**K**) PC, PCK1 and G6Pase liver protein levels and (**L**) acetyl-CoA serum levels in WT mice treated vehicle or MLN4924 and then with saline or adrenaline (100 µg kg-1). **(M)** PC, PCK1 and G6Pase liver protein levels and **(N)** acetyl-CoA serum levels in Alfp-Cre mice injected with AAV-DIO-GFP or AAV-DIO-shNAE1 and then with saline or adrenaline (100 µg kg-1). **(O)** PC, PCK1 and G6Pase liver protein levels and **(P)** acetyl-CoA serum levels in WT mice treated vehicle or MLN4924 and then with saline or hydrocortisone (20 mg kg-1). **(Q)** PC, PCK1 and G6Pase liver protein levels and **(R)** acetyl-CoA serum levels in Alfp-Cre mice injected with AAV-DIO-GFP or AAV-DIO-shNAE1 and then with saline or hydrocortisone (20 mg kg-1). **(S)** Body weight and (**T**) PCK1 protein levels in WT mice fed STD of HFD 60% for 4 days. *n* = 4-12 animals per group. Expression of GAPDH served as a loading control, and control values were normalized to 100%. Data are presented as mean ± SEM; two-tailed unpaired *t*-test: *P<0.05, **P<0.01, and ***P<0.001.

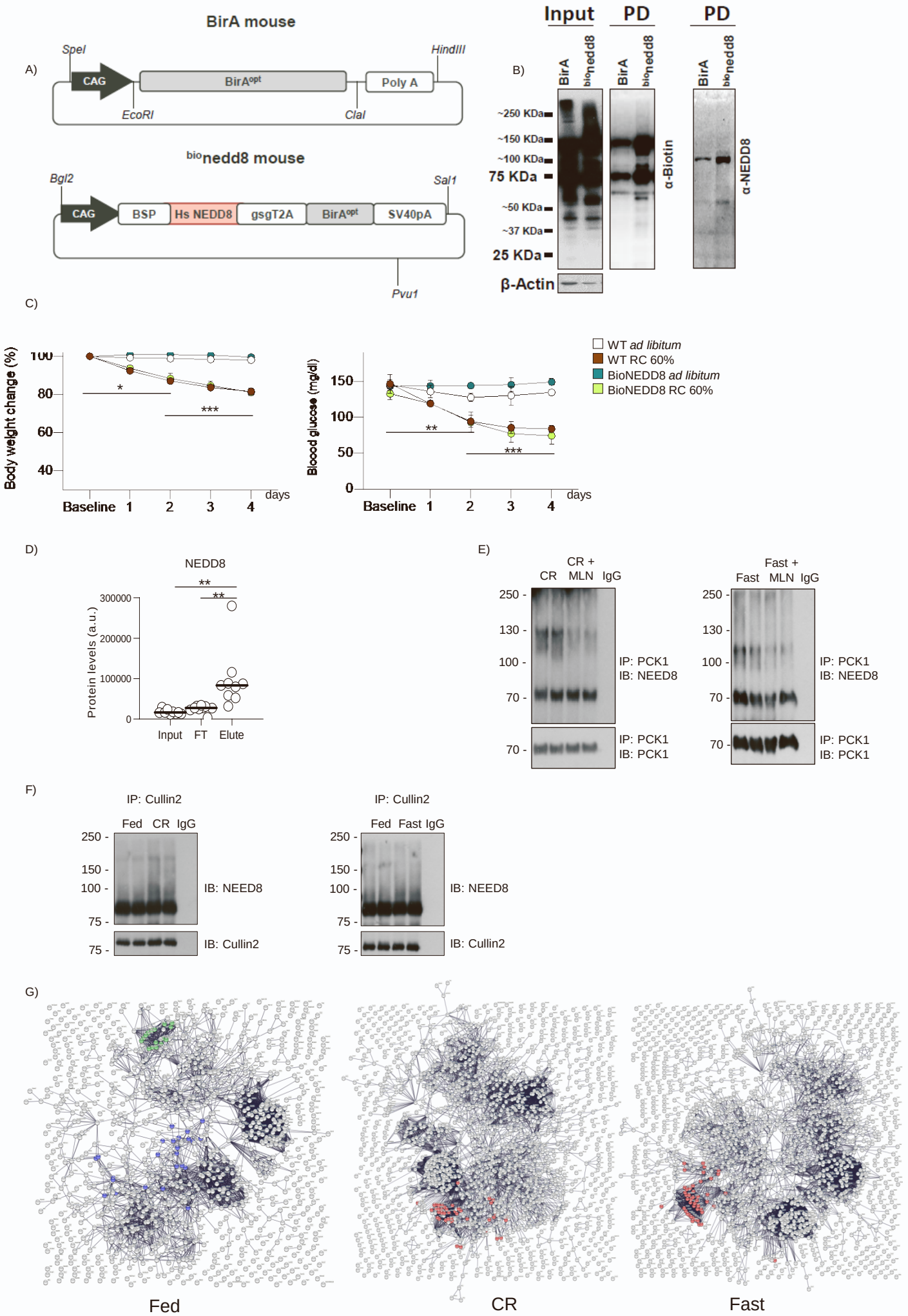
A)
BirA mouse
SpeI
HindIII
CAG
BirA^opt
Poly A
EcoRI
ClaI
bionedd8 mouse
Bgl2
Sal1
CAG
BSP
Hs NEDD8
gsgT2A
BirA^opt
SV40pA
Pvu1
B)
Input
PD
PD
BirA
bionedd8
BirA
bionedd8
BirA
bionedd8
~250 KDa
~150 KDa
~100 KDa
75 KDa
~50 KDa
~37 KDa
25 KDa
β-Actin
α-Biotin
α-NEDD8
C)
Body weight change (%)
100
80
60
40
*
***
Baseline 1 2 3 4
days
Blood glucose (mg/dl)
150
100
50
0
**
***
Baseline 1 2 3 4
days
WT ad libitum
WT RC 60%
BioNEDD8 ad libitum
BioNEDD8 RC 60%
D)
NEDD8
Protein levels (a.u.)
300000
200000
100000
0
**
**
Input
FT
Elute
E)
CR +
CR MLN IgG
250 -
130 -
100 -
70 -
IP: PCK1
IB: NEED8
70 -
IP: PCK1
IB: PCK1
Fast +
Fast MLN IgG
250 -
130 -
100 -
70 -
IP: PCK1
IB: NEED8
70 -
IP: PCK1
IB: PCK1
F)
IP: Cullin2
Fed CR IgG
250 -
150 -
100 -
75 -
IB: NEED8
75 -
IB: Cullin2
IP: Cullin2
Fed Fast IgG
250 -
150 -
100 -
75 -
IB: NEED8
75 -
IB: Cullin2
G)
Fed
CR
Fast

Figure S5. Bioneddylated mice respond normally to fasting and calorie restriction. Related to Figure 5.

(A) Characterization of the bioneddylated mice (BioNEDD8). Schematic representation of the transgenic constructs for both bioNedd8 and birA mice. Both transgenes contain the CAG promotor (CMV, ß actin, and globin), the open reading frame, and the SV40 polyadenylation signal. Restriction sites used for construct insertion and transgene excision and the PCR primers used for genotyping are indicated in materials and methods section. The control BirA mouse expresses the bacterial BirA enzyme only, while the bioNedd8 mice express a single polypeptide encoding one tagged neddylated peptide fused to BirA. In the BioNEDD8 mice the BirA enzyme mediates the biotinylation of NEDD8 with the biotin- accepting tag. Biotinylated NEDD8 is then incorporated into conjugates. **(B)** Western blot analysis of biotin and NEDD8 showing increased smear in the ^{bio}NEDD8 mice relative to the birA, both input and pull down (PD). **(C)** Body weight change and blood glucose levels in WT mice and BioNEDD8 mice fed *ad libitum* or subjected to a 60% calorie restriction for 4 days. *n* = 4-5 animals per group. **(D)** NEDD8 levels in the input, flowthrough and eluted fractions of the IP. **(E)** PCK1 and NEDD8 protein levels after the immunoprecipitation of PCK1 in WT mice after the administration of vehicle or MLN4924 and subjected to caloric restriction (CR) and a 24h-fasting. **(F)** NEDD8 and Cullin2 protein levels after the immunoprecipitation of cullin2 in WT mice subjected to caloric restriction (CR) and a 24h-fasting. **(G)** Functional association analysis for PCK1 immunoprecipitation in fed ad libitum, calorie restriction (CR) and fast conditions. Red circles show proteins related to neddylation. Data are presented as mean ± SEM; one-way ANOVA followed by Bonferroni post hoc test *P<0.05, **P<0.01, and ***P<0.001.

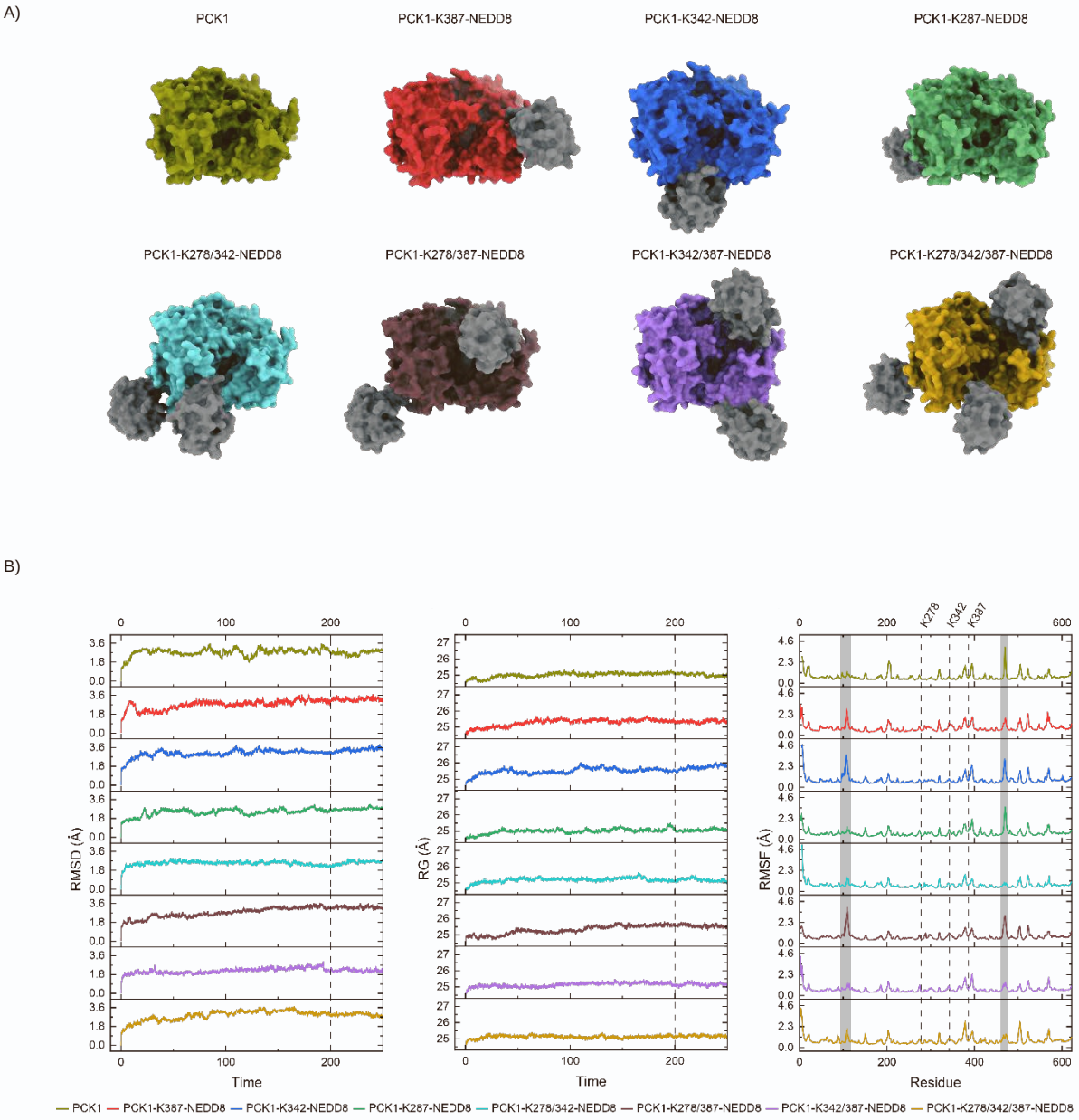

Figure S6. Molecular dynamics simulations of PCK1. Related to Figure 5. (A) Average surfaces of neddylated PCK1 after molecular dynamics simulation. The structures are aligned with respect to PKC1 module in an orientation that allows to visualize up to three NEDD8 molecules per PKC1. **(B)** Root mean square deviation (RMSD) (left); radius of gyration (RG) (middle); root mean square fluctuations (RMSF) (right) of the simulated systems with non-neddylated PCK1, mono-neddylated PCK1, bi-neddylated PCK1 and tri-neddylated PCK1. Regions corresponding to loops 95-118 and 460-470 are shadowed in gray, and the lysine residues that are modified are labelled by dashed lines.

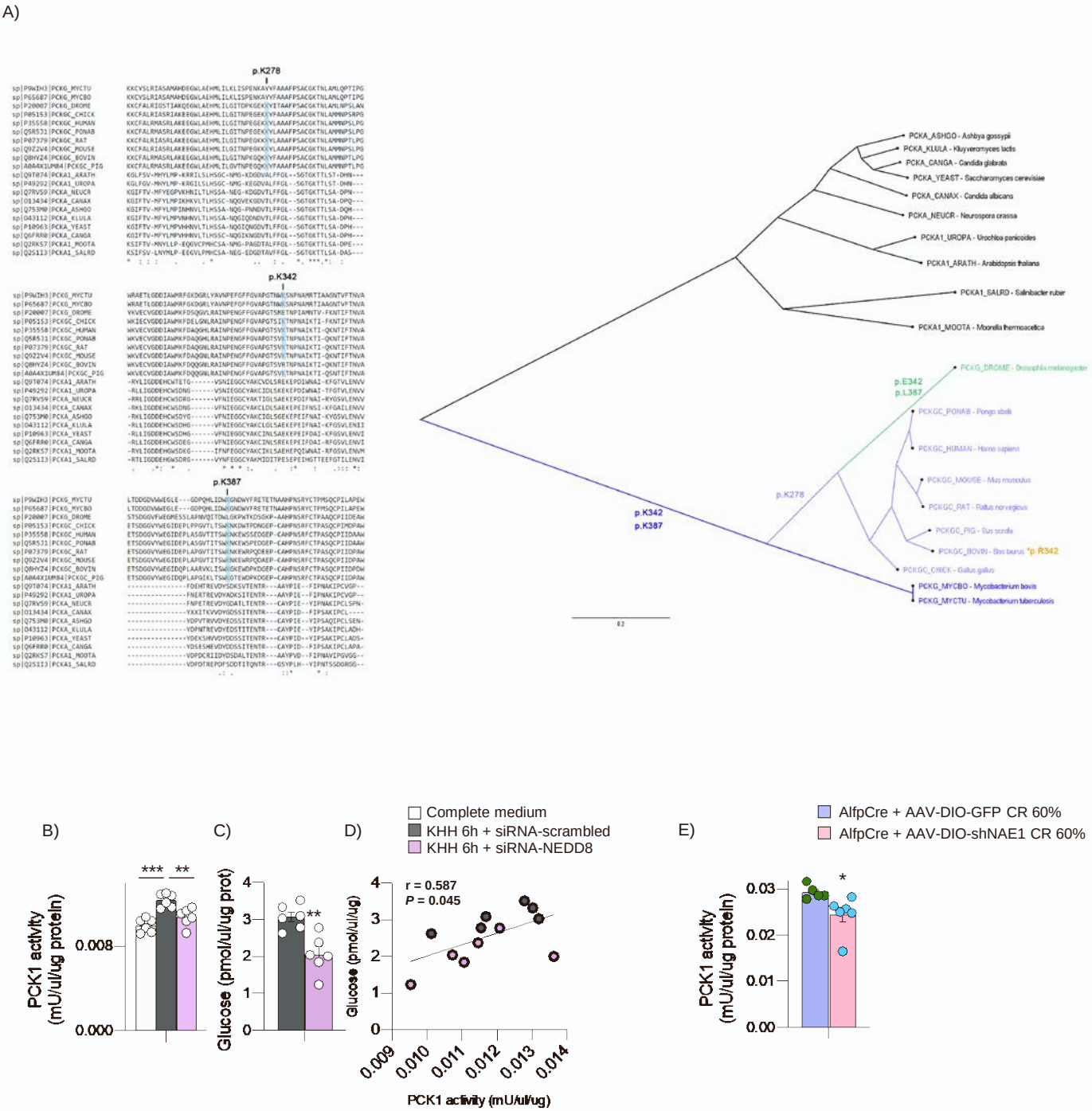

Figure S7. Phylogenesis of the three lysines of PCK1 that can be neddylated. Related to Figure 5. (A) CLUSTAL multiple sequence alignment of the regions flanking the residues K278, K342 and K387 and phylogenetic tree of PCK1 protein. The points in evolution where lysines first appear are indicated. **(B)** PCK1 activity and **(C)** glucose production in AML12 cells transfected with empty siRNA or siRNA NEDD8 and 48h later maintained in complete medium or KHH for 6h. **(D)** Correlation between glucose production and PCK1 activity in these cells is also shown. **(E)** PCK1 activity in the liver of Alfp-Cre +/- mice injected with AAV-DIO expressing either GFP or shNAE1. *n* = 5-6 per group. Data are presented as mean ± SEM; two-tailed unpaired *t*-test (**C** and **E**) or one-way ANOVA followed by Bonferroni post hoc test (**B**): *P<0.05, **P<0.01, and ***P<0.001.

Table S1. Antibodies used for western blot and immunoprecipitation. Related to STAR methods.

Protein target	Manufacter (catalog number)	Species reactivity	Dilution
NEDD8	Abcam (ab81264)	Rabbit monoclonal	1:1000
Phosphoenolpyruvate Carboxykinase 1 (PCK1)	Abcam (ab70358)	Rabbit polyclonal	1:1000
NEDD8-activating enzyme (NAE1)	Cell Signaling (14321S)	Rabbit monoclonal	1:1000
Glucose 6-Phosphatase (G6Pase)	Abcam (ab93857)	Rabbit monoclonal	1:1000
Pyruvate Carboxylase (PC)	Abcam (EPR7366)	Rabbit monoclonal	1:1000
Cullin-2	Santa Cruz (sc-166506)	Mouse monoclonal	1:1000
Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH)	Merck (CB1001)	Mouse monoclonal	1:5000
UBE1	Cell Signaling (4891)	Rabbit polyclonal	1:1000
α-Tubulin	Sigma (T5168)	Mouse monoclonal	1:5000
β -Actin	Sigma (A5316)	Mouse monoclonal	1:5000

Table S2. Anthropometric, biochemical and clinical characteristics of obese patients with normoglycemia (NG) and type 2 diabetes (T2D). Related to Figure 4.

	NG	T2D
n	30	30
Age (years, mean ± SD)	44 ± 10	50 ± 9*
Gender (F / M)	15 / 15	15 / 15
Weight (kg ± SD)	116.9 ± 25.5	117.6 ± 23.8
BMI	41.2 ± 6.6	41.9 ± 7.1
LDL (mg/dl ± SD)	118 ± 29	113 ± 38
HDL (mg/dl ± SD)	53 ± 17	45 ± 14
Triglycerides (mg/dl ± SD)	105 ± 53.6	144 ± 70*
Cholesterol (mg/dl ± SD)	191 ± 34	190 ± 43
LDL cholesterol (mg/dl ± SD)	118 ± 29	113 ± 38
HDL cholesterol (mg/dl ± SD)	53 ± 17	45 ± 14
AST (U/L ± SD)	19 ± 6	20 ± 7
ALT (U/L ± SD)	25 ± 12	26 ± 11

Glucose (mg/dl ± SD)	92 ± 6	144 ± 55***
OGTT (mg/dl ± SD)	110 ± 24	212 ± 54***
HOMA	4.32 ± 2.96	7.75 ± 5.41 **
Insulin (mU/ml)	18.6 ± 12.1	32 ± 57.1
A1c (%)	5.7 ± 0.6	8.4 ± 1.8 **

BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; AST, aspartate transaminase; ALT, alanine transaminase.