Additional File

Including Supplementary Tables S1-3, Supplementary figure legends and Supplementary Figures S1-3

Ammonia-lowering activities and carbamoyl phosphate synthetase 1 (Cps1) induction mechanism of a natural flavonoid

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Running title: Ammonia control and urea cycle regulation by a flavonoid

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Supplementary Table S1: Primers for construction of reporter constructs.

Name	**Sequence**
pF	5'-GGCCCCGGGAGATCTGGAGATACACAGTAAATT-3'
pR	5'-GGCCCATGGCAGCTCCTCCTTTCCTTAGCCCCT-3'
P-C/EBPmutF	5'-GAGAAGGTGCCACTTGTTATATTATGATTTGTATGACATGTCCATT-3'
P-C/EBPmutR	5'-AATGGACATGTCATACAAATCATAATATAACAAGTGGCACCTTCTC-3'
dF	5'-GGCCCCGGGCAGTTTCCGAGAATCTGAAACACA-3'
dR	5'-GGCCCATGGTTTGAAAACAGCAAATTCATCAGC-3'

Supplementary Table S2: Primers for real-time qPCR analysis.

Target genes	**Forward primer**	**Reverse primer**
GAPDH	5'-CAAGGTCATCCATGACAACTTTG-3'	5'-GGCCATCCACAGTCTTCTGG-3'
Cps1	5'-CACCAATTTCCAGGTGACCA-3'	5'-TACTGCTTTAGGCGGCCTTT-3'
Otc	5'-AGGGTCACACTTCTGTGGTTC-3'	5'-CAGAGAGCCATAGCATGTACTG-3'
Ass1	5'-ACACCTCCTGCATCCTCGT-3'	5'-GCTCACATCCTCAATGAACACCT-3'
Asl	5'-CTATGACCGGCATCTGTGGAA-3'	5'-AGCAACCTTGTCCAACCCTTG-3'
Arg1	5'-TTGGGTGGATGCTCACACTG-3'	5'-GTACACGATGTCTTTGGCAGA-3'
Cebpa	5'-CAAGAACAGCAACGAGTACCG-3'	5'-GTCACTGGTCAACTCCAGCAC-3'
Cebpb	5'- ACCGGGTTTCGGGACTTGA -3'	5'- GTTGCGTAGTCCCGTGTCCA -3'

Supplementary Table S3: Mass spectrometry data table for the 164KD band shown in the Supplemental Figure 1B.

Accession	Description	Score	Coverage	Unique Peptides	PSMs	MW [kDa]
124248512	carbamoyl-phosphate synthetase [ammonia], mitochondrial precursor [Mus musculus]	13654.31	66.47	106	778	164.5
124486747	glycogen debranching enzyme [Mus musculus]	1273.25	36.88	54	85	174.2
146219837	eukaryotic translation initiation factor 3 subunit A [Mus musculus]	870.20	35.04	49	69	161.8
19527028	vigilin [Mus musculus]	1147.90	38.17	45	78	141.7
93102409	fatty acid synthase [Mus musculus]	614.67	13.50	29	31	272.3
110347469	alpha-2-macroglobulin precursor [Mus musculus]	631.93	23.68	27	47	165.7
114205420	aldehyde oxidase 3 [Mus musculus]	862.84	21.95	25	37	146.8
77682555	xanthine dehydrogenase/oxidase [Mus musculus]	704.11	19.70	25	39	146.5
254553372	isoleucine--tRNA ligase, cytoplasmic [Mus musculus]	342.94	22.98	25	28	144.2

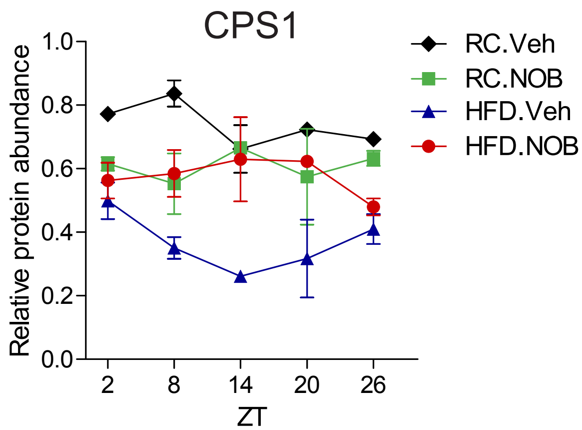
Supplementary figure legends:

Supplementary Figure S1. NOB modulates CPS1 expression. (A) Quantification of CPS1 protein levels from three independent experiments including the blot shown in Fig. 2A. RC, regular chow; HFD, high-fat diet; Veh, vehicle; NOB, Nobiletin. Two-way ANOVA with Bonferroni *post-hoc* tests shows significant statistical differences between HFD.Veh and other three groups (p<0.0001). Furthermore, one-way ANOVA with Bonferroni tests shows significant difference (p<0.05) between ZT time points in RC.Veh but not in other groups. **(B)** Coomassie blue staining of CPS1. Mouse liver protein lysates were separated on SDS-PAGE gel and stained with Coomassie blue. The predominant 164KD band was validated by mass spectrometry as CPS1 (Table S3). **(C)** Control microscopy images for Figure 2B using rabbit IgG.

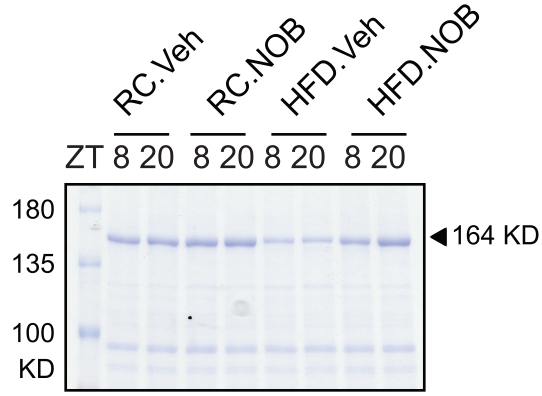
Supplementary Figure S2. NOB rescued C/EBP protein circadian expression in the liver from HFD fed mice. (A) Quantification of C/EBPα p42 protein levels from three independent experiments including the blot shown in Figure 3B. Two-way ANOVA with Bonferroni *post-hoc* tests shows significant difference between RC.Veh and HFD.Veh (p<0.05), indicating diet effect on the C/EBPα p42 expression level. Importantly, RC.NOB was not significantly different from HFD.NOB, suggesting NOB reversed the reducing effect of HFD on the C/EBPα p42 expression. **(B)** Quantification of C/EBPα p30 protein levels from three independent experiments including the blot shown in Figure 3B. Two-way ANOVA with Bonferroni *post-hoc* tests shows significant difference between RC.Veh and HFD.Veh (p<0.01), but RC.NOB and HFD.NOB was not significantly different, again suggesting NOB reversed the reducing effect of HFD on the p30 expression. **(C)** Quantification of C/EBPβ protein levels from three independent experiments including the blot shown in Figure 3B. Two-way ANOVA with Bonferroni *post-hoc* tests shows significant difference between RC.Veh and HFD.Veh (p<0.0001), but RC.NOB and HFD.NOB was not significantly different.

Supplementary Figure S3. NOB restored CPS1 and C/EBP protein levels in a clock-dependent manner. **(A)** Quantification of CPS1 protein levels under constant darkness (DD) conditions from three independent experiments including the blot shown in Figure 6A. Two-way ANOVA with Bonferroni *post-hoc* tests shows significant statistical difference between WT and Clk ($Clock^{\Delta 19/\Delta 19}$) ($p$<0.0001). **(B)** Quantification of C/EBPα p42 protein levels under constant darkness (DD) conditions from three independent experiments including the blot shown in Figure 6A. Two-way ANOVA with Bonferroni *post-hoc* tests shows significant statistical differences between WT and Clk (p<0.05). **(C)** Quantification of C/EBPα p30 protein levels under constant darkness (DD) conditions from three independent experiments including the blot shown in Figure 6A. Two-way ANOVA with Bonferroni *post-hoc* tests shows significant statistical difference between WT and Clk (p<0.01). **(D)** Quantification of C/EBPβ protein levels under constant darkness (DD) conditions from three independent experiments including the blot shown in Figure 6A. Two-way ANOVA with Bonferroni *post-hoc* tests shows significant statistical difference between WT and Clk (p<0.05).

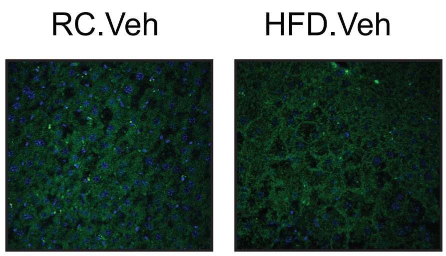
A


CPS1
Relative protein abundance
1.0
0.8
0.6
0.4
0.2
0.0
2
8
14
20
26
ZT
RC.Veh
RC.NOB
HFD.Veh
HFD.NOB

B

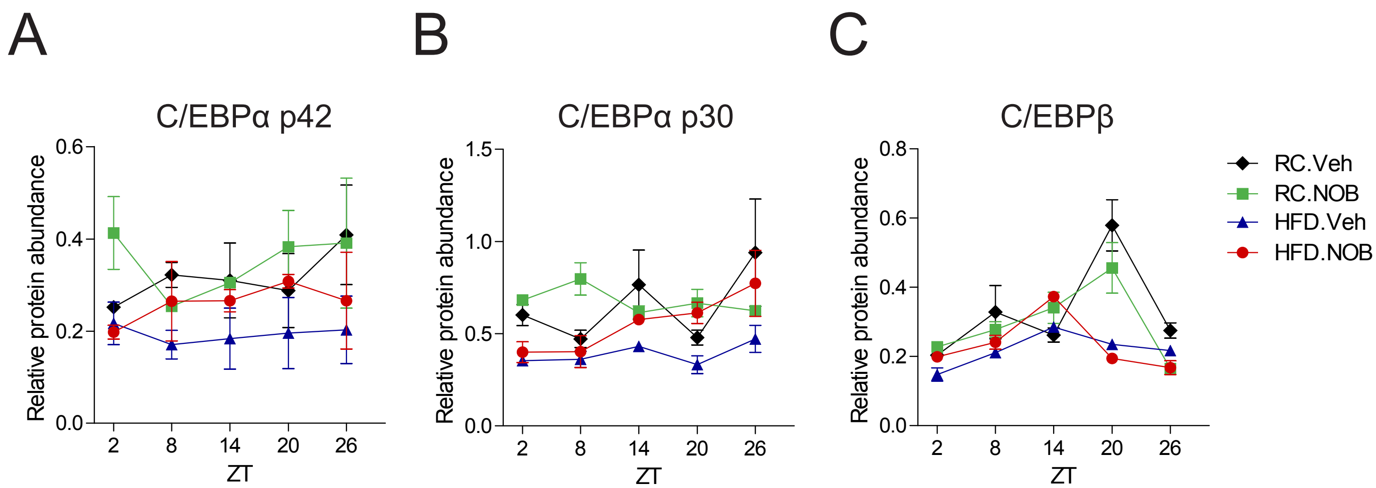

RC.Veh
RC.NOB
HFD.Veh
HFD.NOB
ZT 8 20 8 20 8 20 8 20
180
135
100
KD
164 KD

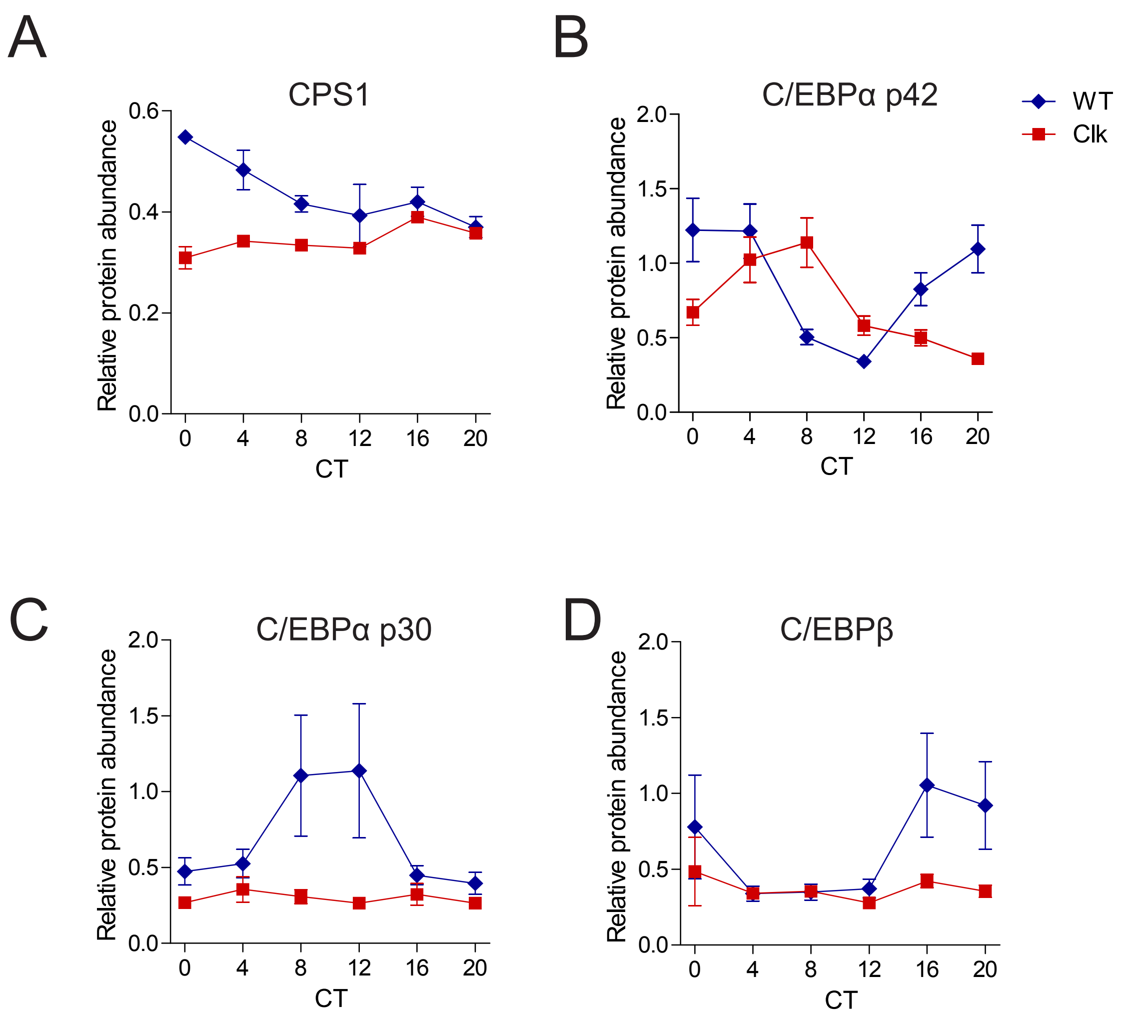
C


RC.Veh
HFD.Veh

ZT 2

Supplementary Figure S2




A
CPS1
Relative protein abundance
0.0
0.2
0.4
0.6
0
4
8
12
16
20
CT
B
C/EBPα p42
WT
Clk
Relative protein abundance
0.0
0.5
1.0
1.5
2.0
CT
C
C/EBPα p30
Relative protein abundance
CT
D
C/EBPβ
Relative protein abundance
CT