Supplemental Information

12-lipoxygenase regulates cold adaptation and glucose metabolism by producing the omega-3 lipid 12-HEPE from brown fat

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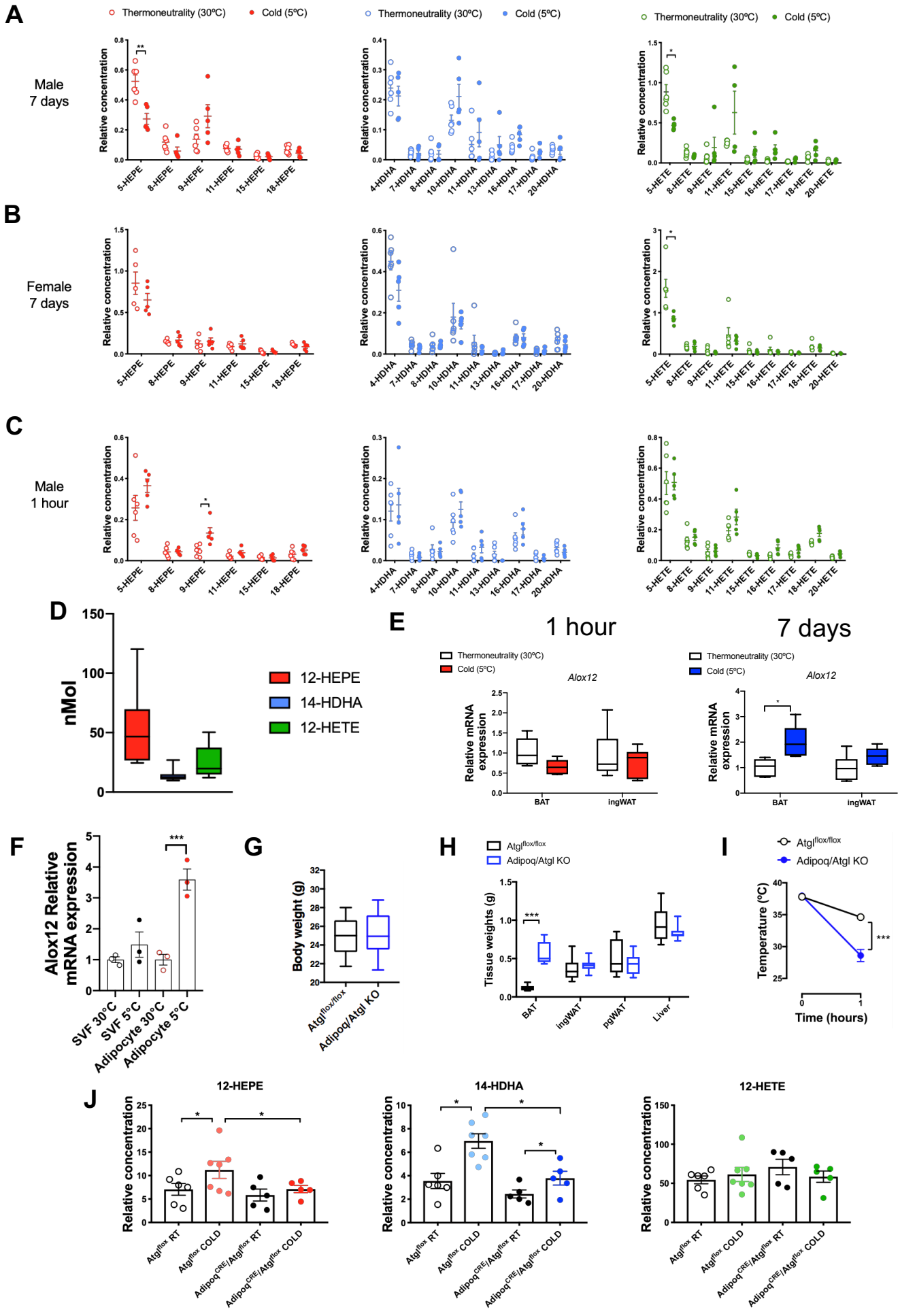

A
Male
7 days
Thermoneutrality (30ºC)
Cold (5ºC)
Relative concentration
5-HEPE
8-HEPE
9-HEPE
11-HEPE
15-HEPE
18-HEPE
4-HDHA
7-HDHA
8-HDHA
10-HDHA
11-HDHA
13-HDHA
16-HDHA
17-HDHA
20-HDHA
5-HETE
8-HETE
9-HETE
11-HETE
15-HETE
16-HETE
17-HETE
18-HETE
20-HETE
B
Female
7 days
C
Male
1 hour
D
nMol
12-HEPE
14-HDHA
12-HETE
E
1 hour
7 days
Alox12
Relative mRNA expression
BAT
ingWAT
F
Alox12 Relative mRNA expression
SVF 30°C
SVF 5°C
Adipocyte 30°C
Adipocyte 5°C
G
Body weight (g)
Atgl flox/flox
Adipoq/Atgl KO
H
Tissue weights (g)
pgWAT
Liver
I
Temperature (ºC)
Time (hours)
J
12-HEPE
14-HDHA
12-HETE
Atgl flox RT
Atgl flox COLD
Adipoq CRE/Atgl flox RT
Adipoq CRE/Atgl flox COLD

Figure S1. A coordinated 12-LOX and lipolysis-dependent release of lipid metabolites under cold exposure. *Related to Figure 1*.
A) Circulating levels of EPA, DHA and AA derived metabolites HEPEs, HDHAs and HETEs from male mice exposed to thermoneutrality or cold for 7 days (n = 5).
B) Circulating levels of the EPA, DHA and AA derived metabolites HEPEs, HDHAs and HETEs from female mice exposed to thermoneutrality or cold for 7 days (n = 5).
C) Circulating levels of the EPA, DHA and AA derived metabolites HEPEs, HDHAs and HETEs from male mice exposed to cold or room temperature for 1 hour (n = 5).
D) Quantitative values of circulating 12-HEPE, 14-HDHA and 12-HETE concentrations in mice exposed to cold or thermoneutrality for 7 days (n = 7). Targeted lipidomics was used for lipid quantification.
E) *Alox12* mRNA expression measured by qPCR in subcutaneous inguinal white adipose tissue (sqWAT) and brown adipose tissue (BAT) from C57BL6/J mice exposed to cold or thermoneutrality for 1 hour or 7 days.
F) *Alox12* mRNA expression measured by qPCR in stromal vascular fraction and adipocytes extracted from perigonadal white adipose tissue (pgWAT), ingWAT and BAT of mice housed in cold or thermoneutrality for 7 days (n= 3).
G) Body weights of $Atgl^{flox/flox}$ mice and $Adipoq^{CRE}$/Atgl KO mice (n = 10).
H) Weight of BAT, ingWAT, pgWAT and liver dissected from $Atgl^{flox/flox}$ mice and $Adipoq^{CRE}$/Atgl KO mice (n = 10).
I) Rectal temperature measured during cold tolerance test in $Atgl^{flox/flox}$ and $Adipoq^{CRE}$/Atgl KO groups (n = 5).
J) 12-HEPE, 14-HDHA and 12-HETE serum levels in $Atgl^{flox}$ and $Adipoq^{CRE}/Atgl^{flox}$ mice exposed to cold or room temperature for 1 h. (n = 5 – 6). *p<0.05, **p<0.01. N=5 per group. Data are represented as mean ± S.E.M.
All the lipid quantification data (except for Figure S1D) were detected using non-targeted lipidomics, thus relative values are shown.

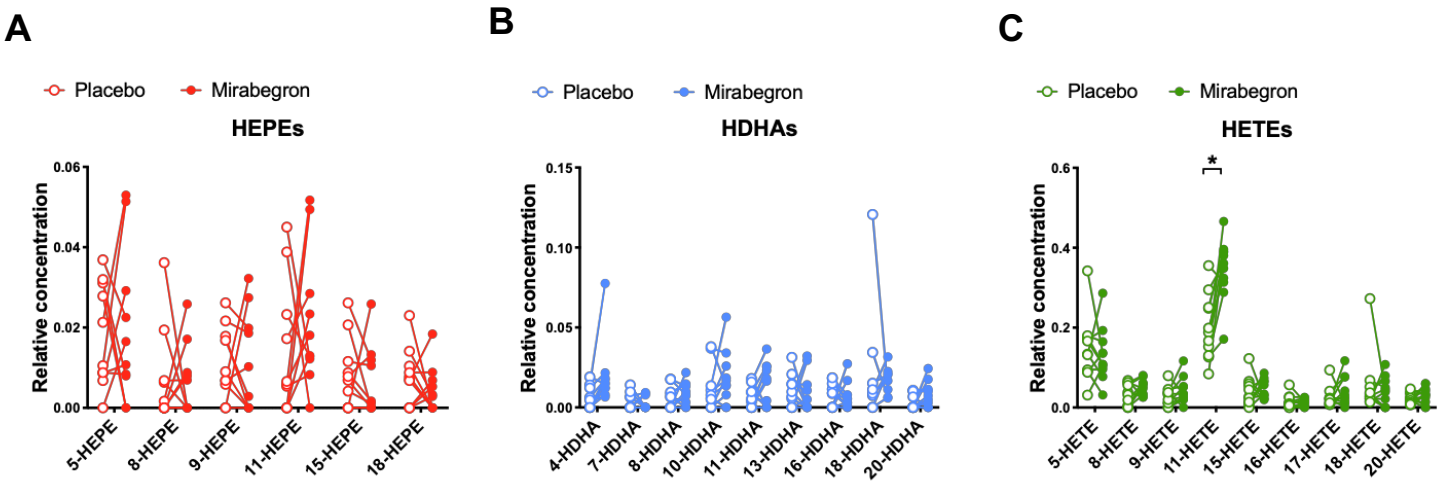


Figure S2. Effect of mirabegron treatment in the circulating levels of oxylipins. ***Related to Figure 2.***

A-C) Circulating levels of the EPA, DHA and AA derived metabolites HEPEs, HDHAs and HETEs from human subjects treated with mirabegron (200mg) or placebo. Data are represented as mean (n = 10-11). The lipid quantification data were detected using non-targeted lipidomics, thus relative values are shown.

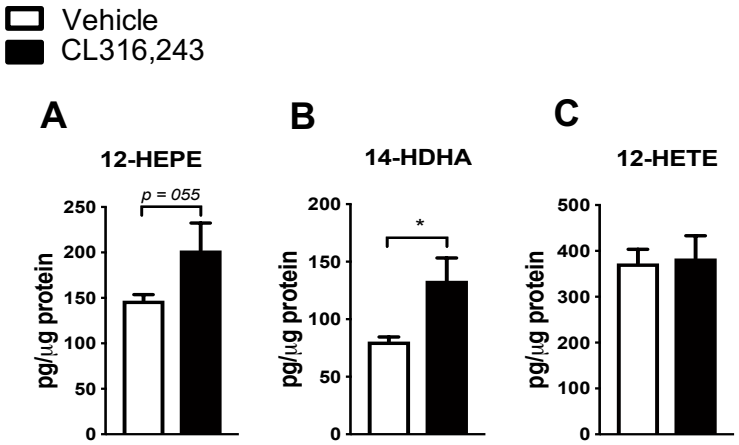


Figure S3. Production of 12-LOX metabolites from brown adipocytes in response to β3-adrenergic stimulation. *Related to Figure 3*.
A-C) 12-HEPE, 14-HDHA and 12-HETE levels in media by murine brown adipocytes treated with CL316,243. Data are represented as mean ± S.E.M (n = 5). Targeted lipidomics was used for lipid quantification.

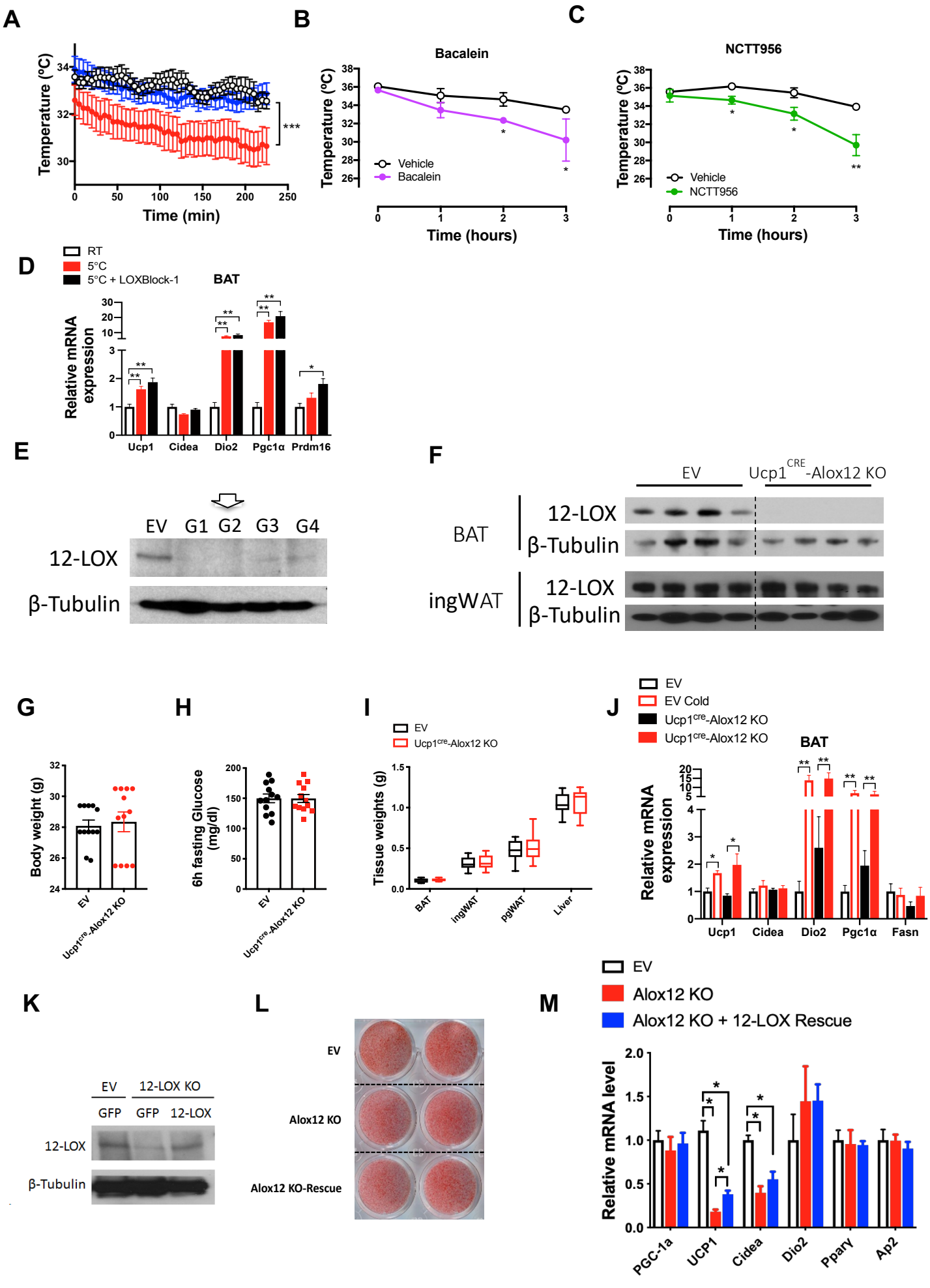

A
Temperature (ºC)
Time (min)
***
B
Bacalein
Temperature (ºC)
Vehicle
Bacalein
Time (hours)
C
NCTT956
Temperature (ºC)
Vehicle
NCTT956
Time (hours)
D
RT
5°C
5°C + LOXBlock-1
BAT
Relative mRNA expression
Ucp1
Cidea
Dio2
Pgc1α
Prdm16
E
EV
G1
G2
G3
G4
12-LOX
β-Tubulin
F
EV
Ucp1CRE-Alox12 KO
BAT
12-LOX
β-Tubulin
ingWAT
12-LOX
β-Tubulin
G
Body weight (g)
EV
Ucp1cre-Alox12 KO
H
6h fasting Glucose (mg/dl)
EV
Ucp1cre-Alox12 KO
I
EV
Ucp1cre-Alox12 KO
Tissue weights (g)
BAT
ingWAT
pgWAT
Liver
J
EV
EV Cold
Ucp1cre-Alox12 KO
Ucp1cre-Alox12 KO
BAT
Relative mRNA expression
Ucp1
Cidea
Dio2
Pgc1α
Fasn
K
EV
12-LOX KO
GFP
GFP
12-LOX
12-LOX
β-Tubulin
L
EV
Alox12 KO
Alox12 KO-Rescue
M
EV
Alox12 KO
Alox12 KO + 12-LOX Rescue
Relative mRNA level
PGC-1a
UCP1
Cidea
Dio2
Pparγ
Ap2

Figure S4. Effects of suppression of 12-LOX activity via chemical inhibition or genetic deletion in mice and in brown adipocytes. ***Related to Figure 4.***
A) Interscapular temperature measured by telemetry during cold tolerance test in C57BL6/J mice treated with vehicle or LOXBlock-1 (n = 5-6).
B) Rectal temperature measured during cold tolerance test in C57BL6/J mice treated with vehicle or Baicalein (n = 3-5).
C) Rectal temperature measured during cold tolerance test in C57BL6/J treated with vehicle or NCTT956 (n = 5).
D) mRNA expression of thermogenesis-related genes measured by qPCR in BAT from vehicle or LOXBlock-1 treated mice housed exposed to cold for 4 hours.
E) Western blotting for 12-LOX protein expression in brown adipocytes transfected with empty vector (EV), guide RNA 1 (G1), guide RNA 2 (G2), guide RNA 3 (G3) and guide RNA 4 (G4).
F) Western blotting for 12-LOX protein expression in BAT and ingWAT in EV and Ucp1^{cre}/Alox12 KO groups. (n = 6).
G) Body weights of EV mice and Ucp1^{cre}/Alox12 KO mice (n = 12).
H) 6 hour fasting glycemia in EV mice and Ucp1^{cre}/Alox12 KO mice (n = 12).
I) Weight of BAT, ingWAT, pgWAT and liver dissected from EV mice and Ucp1^{cre}/Alox12 KO mice (n = 12).
J) mRNA expression of thermogenesis-related genes measured by qPCR in BAT from EV mice and Ucp1^{cre}/Alox12 KO mice exposed to cold for 4 hours or kept at room temperature (n = 6).
K) Western blotting for 12-LOX protein expression in EV brown adipocytes transfected with GFP and 12-LOX KO brown adipocytes transfected with GFP or with *Alox12* cDNA.
L) Oil red-O staining in EV brown adipocytes transfected with GFP and 12-LOX KO brown adipocytes transfected with GFP or with *Alox12* cDNA.
M) mRNA expression of thermogenesis- and adipogenesis-related genes measured by qPCR in EV brown adipocytes transfected with GFP and 12-LOX KO brown adipocytes transfected with GFP or with *Alox12* cDNA.
*P<0.05, **P<0.01. Data are represented as mean ± S.E.M.

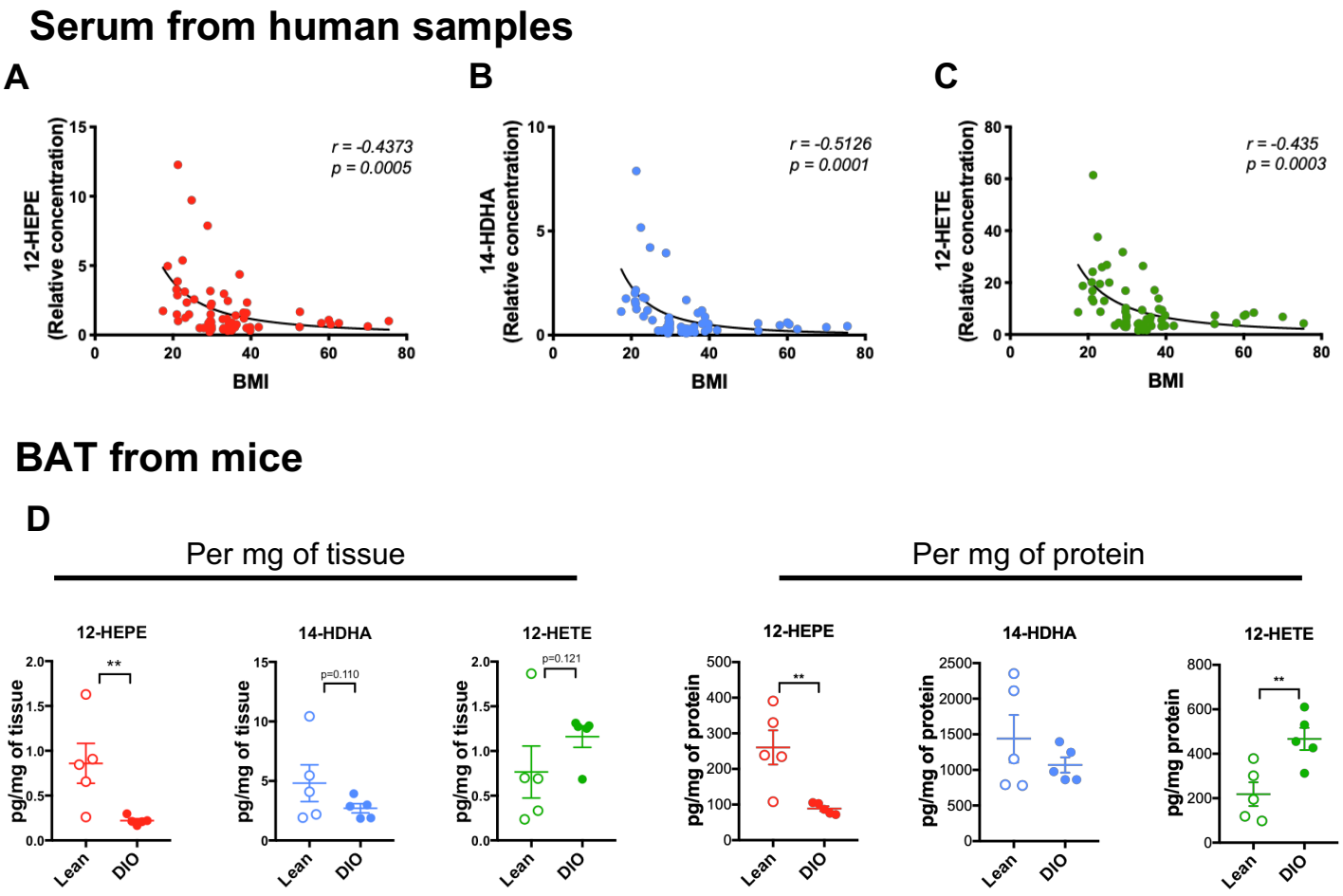


Figure S5. Obesity reduces circulating levels of 12-LOX products in humans and impairs biosynthesis of 12-LOX metabolites in mouse BAT. *Related to Figure 5*.

A-C) Spearman correlation between circulating 12-LOX metabolite levels and body mass index (BMI) in lean (BMI<25), overweight (BMI>25, <30) and obese (BMI>30) subjects (n = 60).

D) Lipid concentration in BAT from lean (Chow) and DIO (HFD) mice normalized to tissue mass and protein content (n = 5-7). Targeted lipidomics was used for lipid quantification.

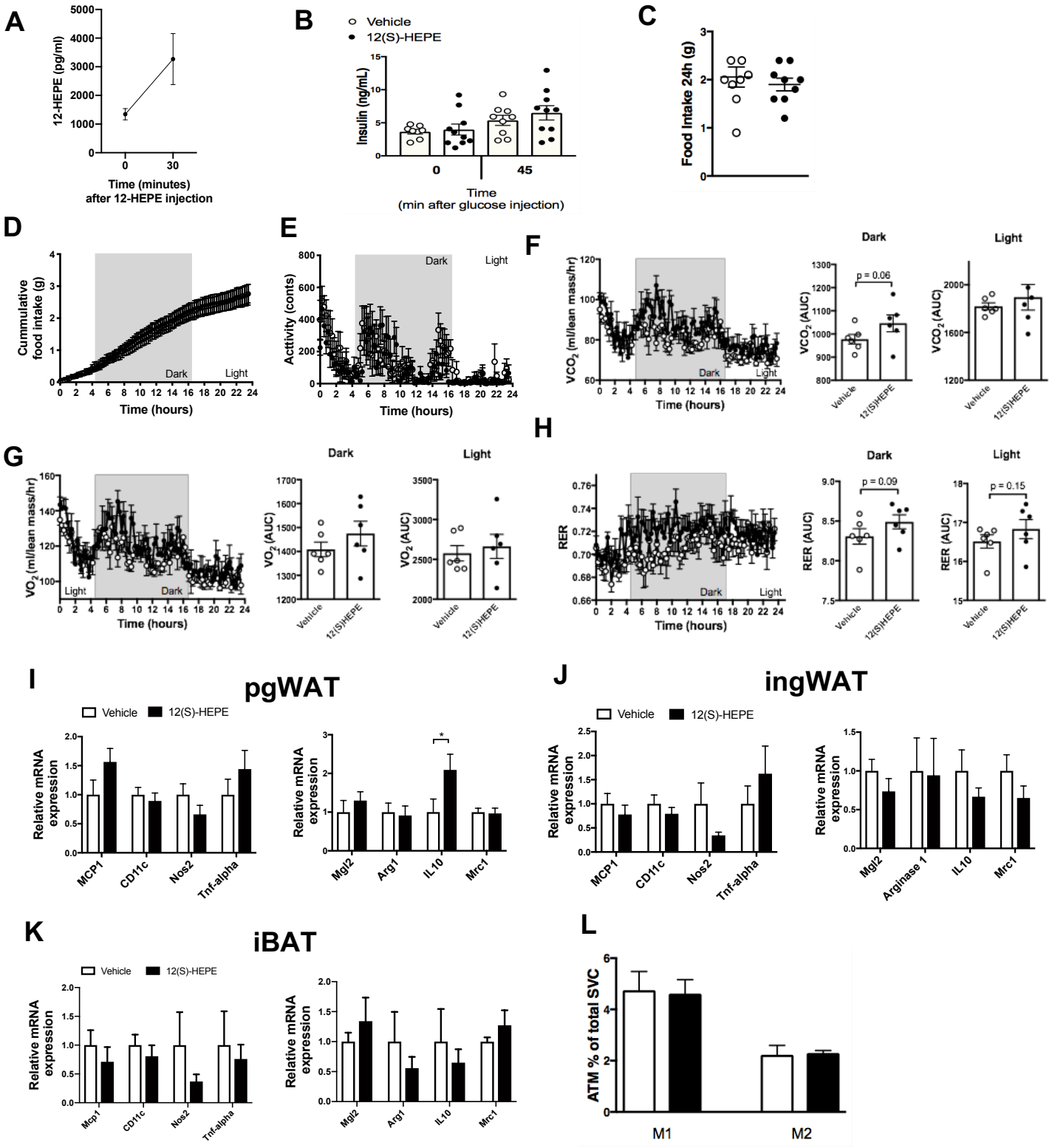
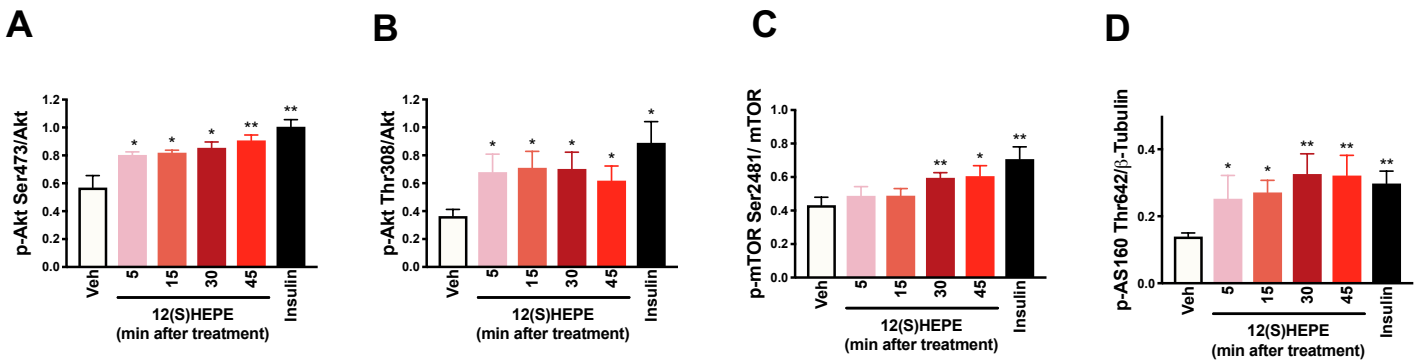
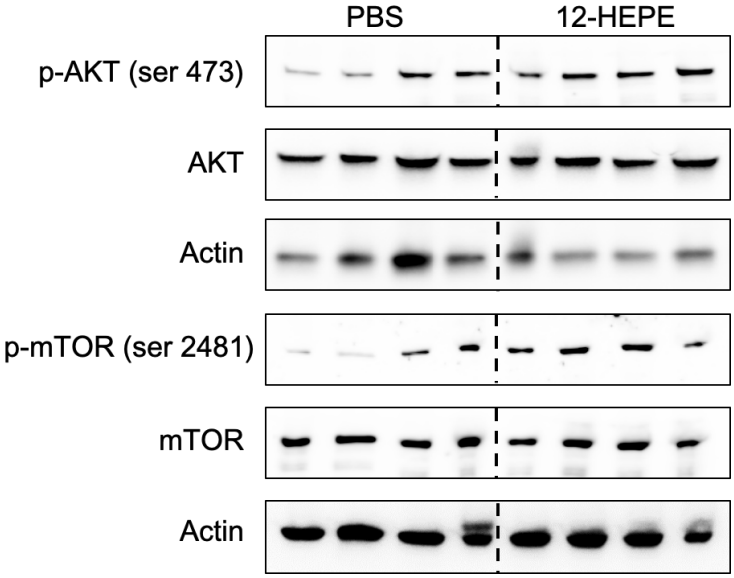
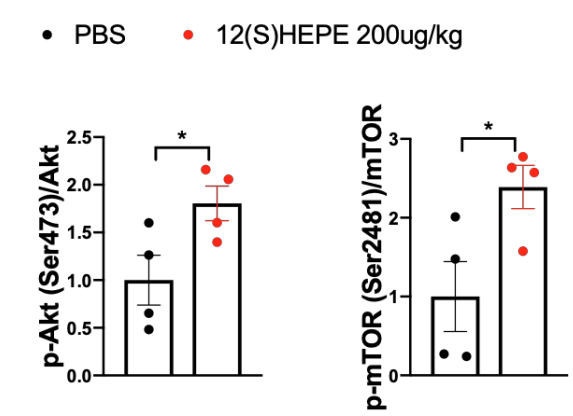

A
12-HEPE (pg/ml)
Time (minutes)
after 12-HEPE injection
B
Vehicle
12(S)-HEPE
Insulin (ng/mL)
Time
(min after glucose injection)
C
Food Intake 24h (g)
D
Cummulative
food intake (g)
Dark
Light
Time (hours)
E
Acttivity (conts)
Dark
Light
Time (hours)
F
Dark
Light
p = 0.06
Vehicle
12(S)HEPE
Time (hours)
G
Light
Dark
Time (hours)
H
RER
p = 0.09
p = 0.15
I
pgWAT
Vehicle
12(S)-HEPE
Relative mRNA
expression
MCP1
CD11c
Nos2
Tnf-alpha
Mgl2
Arg1
IL10
Mrc1
J
ingWAT
Arginase 1
K
iBAT
Mcp1
L
ATM % of total SVC
M1
M2

Figure S6. Effects of 12(S)-HEPE treatment in DIO mice. *Related to Figure 6*.
A) Plasma levels of 12-HEPE, before and 30 minutes after a 200µg/kg i.p. injection of 12(S)-HEPE in C57BL6/J mice.
B) Glucose-stimulated insulin release measured at time 0 and 45 min after dextrose injection (2g/kg) in DIO mice treated chronically with 12(S)-HEPE or Vehicle.
C) Food intake of DIO mice treated chronically with 12(S)-HEPE or Vehicle, measured in individual cages.
D) Cumulative food intake of DIO mice treated chronically with 12(S)-HEPE or Vehicle,
monitored during 24 hours by CLAMS (n = 6).
E) Spontaneous activity of DIO mice treated chronically with 12(S)-HEPE or Vehicle, monitored during 24 hours by CLAMS (n = 6).
F-H) Volume of carbon dioxide consumption (VCO_2), volume of oxygen consumption (VO_2), and respiratory exchange ratio (RER) of DIO mice chronically treated with 12(S)-HEPE or Vehicle, monitored during 24 hours by CLAMS (n = 6).
I-K) Relative mRNA expression of anti-inflamatory and pro-inflammatory genes measured qPCR in pgWAT (H), ingWAT (I) and BAT (J) from DIO mice chronically treated with 12(S)-HEPE or Vehicle.
L) Flow cytometry analysis of M1 and M2 macrophage infiltration in the stromal vascular fraction (SVF) of pgWAT from mice treated with 12(S)-HEPE or vehicle. M1 macrophages were identified as F4/80+/CD11c+/CD206- and M2 were identified as F4/80+/CD11c-/CD206+ cells (n = 8).


A
p-Akt Ser473/Akt
B
p-Akt Thr308/Akt
C
p-mTOR Ser2481/ mTOR
D
p-AS160 Thr642/β-Tubulin
Veh
5
15
30
45
Insulin
12(S)HEPE
(min after treatment)


E
PBS
12-HEPE
p-AKT (ser 473)
AKT
Actin
p-mTOR (ser 2481)
mTOR
Actin


F
PBS
12(S)HEPE 200ug/kg
p-Akt (Ser473)/Akt
p-mTOR (Ser2481)/mTOR

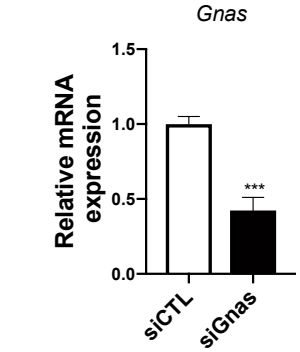

G
Gnas
Relative mRNA expression
siCTL
siGnas

Figure S7. 12(S)-HEPE stimulates the AKT/mTORC2 pathway in brown adipocytes *in vitro* and *in vivo*. *Related to Figure 7*.
A) Quantification of Akt phosphorylated at Ser473 protein expression detected by western blotting of samples from murine brown adipocytes treated with 12(S)-HEPE or vehicle normalized to total Akt protein expression (n = 5).
B) Quantification of Akt phosphorylated at Th308 protein expression detected by western blotting of samples from murine brown adipocytes treated with 12(S)-HEPE or vehicle normalized to total Akt protein expression (n = 5).
C) Quantification of mTORC2 phosphorylated at Ser2481 protein expression detected by western blotting of samples from murine brown adipocytes treated with 12(S)-HEPE or vehicle normalized to total mTor protein expression (n = 6).
D) Quantification of AS160 phosphorylated at Th642protein expression detected by western blotting of samples from murine brown adipocytes treated with 12(S)-HEPE or vehicle normalized to β-Tubulin protein expression (n = 5).
E) Western blot images of phospho-AKT (Ser473), Akt, phospho-mTORC2 (Ser2481), mTOR and actin in BAT from 12(S)-HEPE or PBS-injected mice (i.v., 10min).
F) Quantification of protein expression of phospho-AKT (Ser473) normalized by total Akt or actin, and phospho-mTORC2 (Ser2481) normalized by total mTOR or actin in BAT from 12(S)-HEPE or PBS-injected mice. n*P<0.05, **P<0.01, ***P<0.001, in comparison to vehicle treated cells. Data are represented as mean ± S.E.M.
G) *Gnas* mRNA expression in murine brown adipocytes transfected with *Gnas* siRNA or control siRNA.

Supplemental Table 1: List of primers and sequences. *Related to Star Methods*.

Gene name	Forward Primer	Reverse Primer
Alox12	TCCCTCAACCTAGTGCGTTTG	GTTGCAGCTCCAGTTTCGC
Gnas	CAGAGCCTCCATTGGGGTC	GCTTCTCGCTCAACTGGGG
Glut1	CAGTTCGGCTATAACACTGGTG	GCCCCCGACAGAGAAGATG
Glut4	GTGACTGGAACACTGGTCCTA	CCAGCCACGTTGCATTGTAG
Fasn	GGCTCTATGGATTACCCAAGC	CCAGTGTTCGTTCCTCGG
Scd1	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
Chrebp-beta	TCTGCAGATCGCGTGGAG	CTTGTCCCGGCATAGCAAC
Chrebp-alpha	CGACACTCACCCACCTCTTC	TTGTTCAGCCGGATCTTGTC
Srebp1c	GCAGCCACCATCTAGCCTG	CAGCAGTGAGTCTGCCTTGAT
Ucp1	CTGCCAGGACAGTACCCAAG	TCAGCTGTTCAAAGCACACA
Acl	GCCAGCGGGAGCACATC	CTTTGCAGGTGCCACTTCATC
Ppargc1a	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCCTGTTTTC
Dio2	CAGTGTGGTGCACTGCTCCAATC	TGAACCAAAGTTGACCACCAG
Cpt1b	CGAGGATTCTCTGGAACTGC	GGTCGCTTCTTCAAGGTCTG
Cidea	ATCACAACTGGCCTGGTTACG	TACTACCCGGTGTCCATTTCT
Mcp1	AGGTCCCTGTCATGCTTCTG	TCATTGGGATCATCTTGCTG
Nos2	TAGTTTCCAGAAGCAGAATGTGACC	CCAAGACTCTAAATCGGATCTCTC
Cd11c	CAGAACTTCCCAACTGCACA	TCTCTGAAGCTGGCTCATCA
Tnfa	CCACCACGCTCTTCTGTCT	GCTCCTCCACTTGGTGGTTT
Mgl2	GGAAGCCAAGACTTCACACA	CTCTTCCCGCTCCAAGTTCT
Arginase1	AGACCACAGTCTGGCAGTTG	CCACCCAAATGACACATAGG
Il10	AGTGGAGCAGGTGAAGAGTG	CACTGCAGGTGTTTTAGCTTT
Mrc1	TGATTACGAGCAGTGGAAGC	GTTCACCGTAAGCCCAATTT