Supplementary Information Titles

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Article Title:	An inhibitor of the protein kinases TBK1/IKKε improves obesity-related metabolic dysfunctions
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Supplementary Figure 2	Amlexanox increases energy expenditure with no effect on respiratory quotient or total activity
Supplementary Figure 3	Amlexanox does not improve insulin sensitivity in ND fed mice
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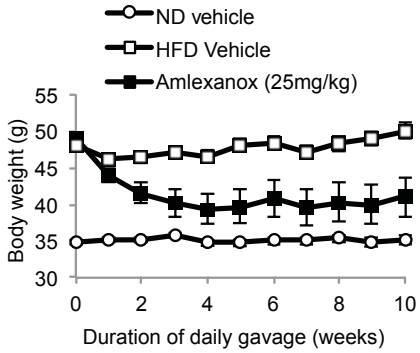
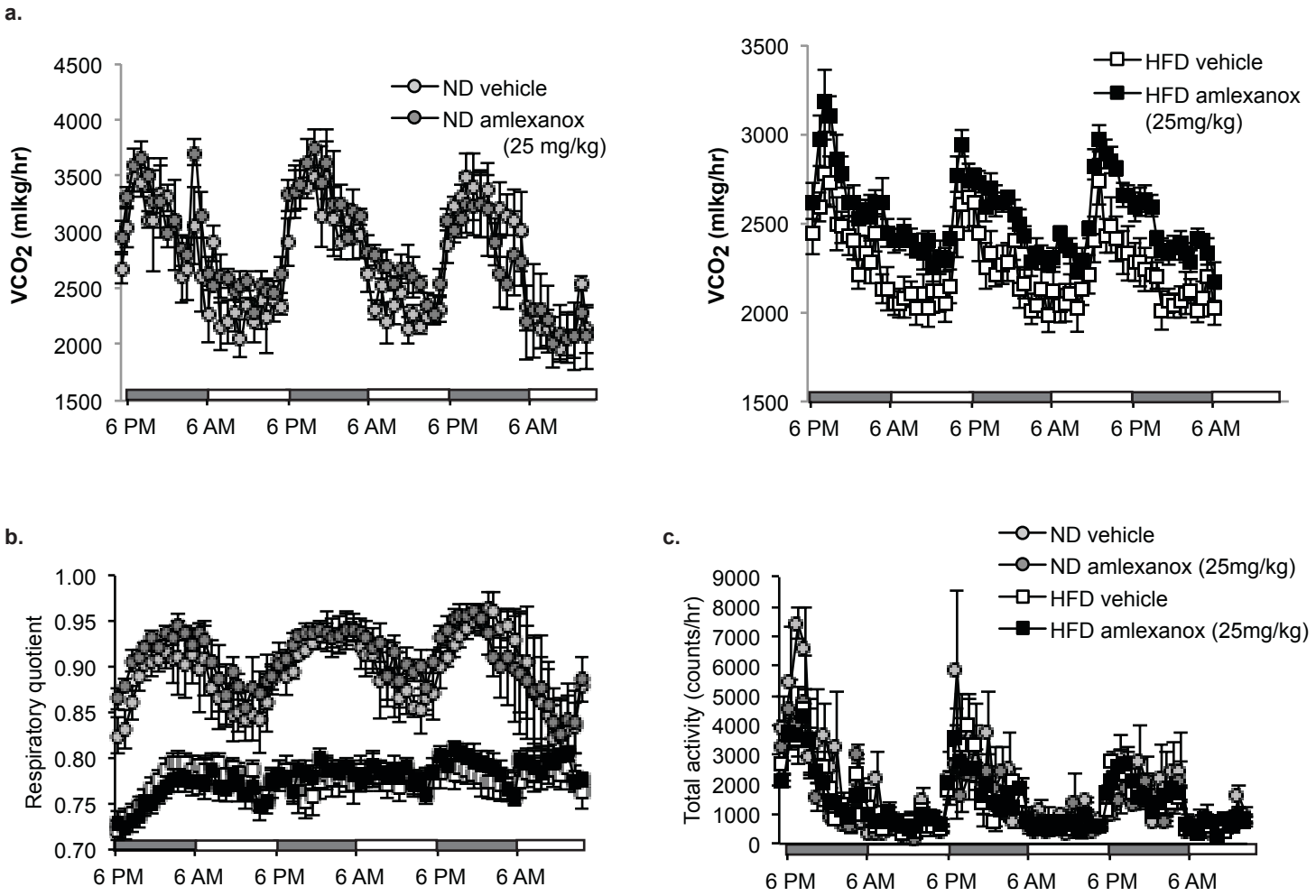
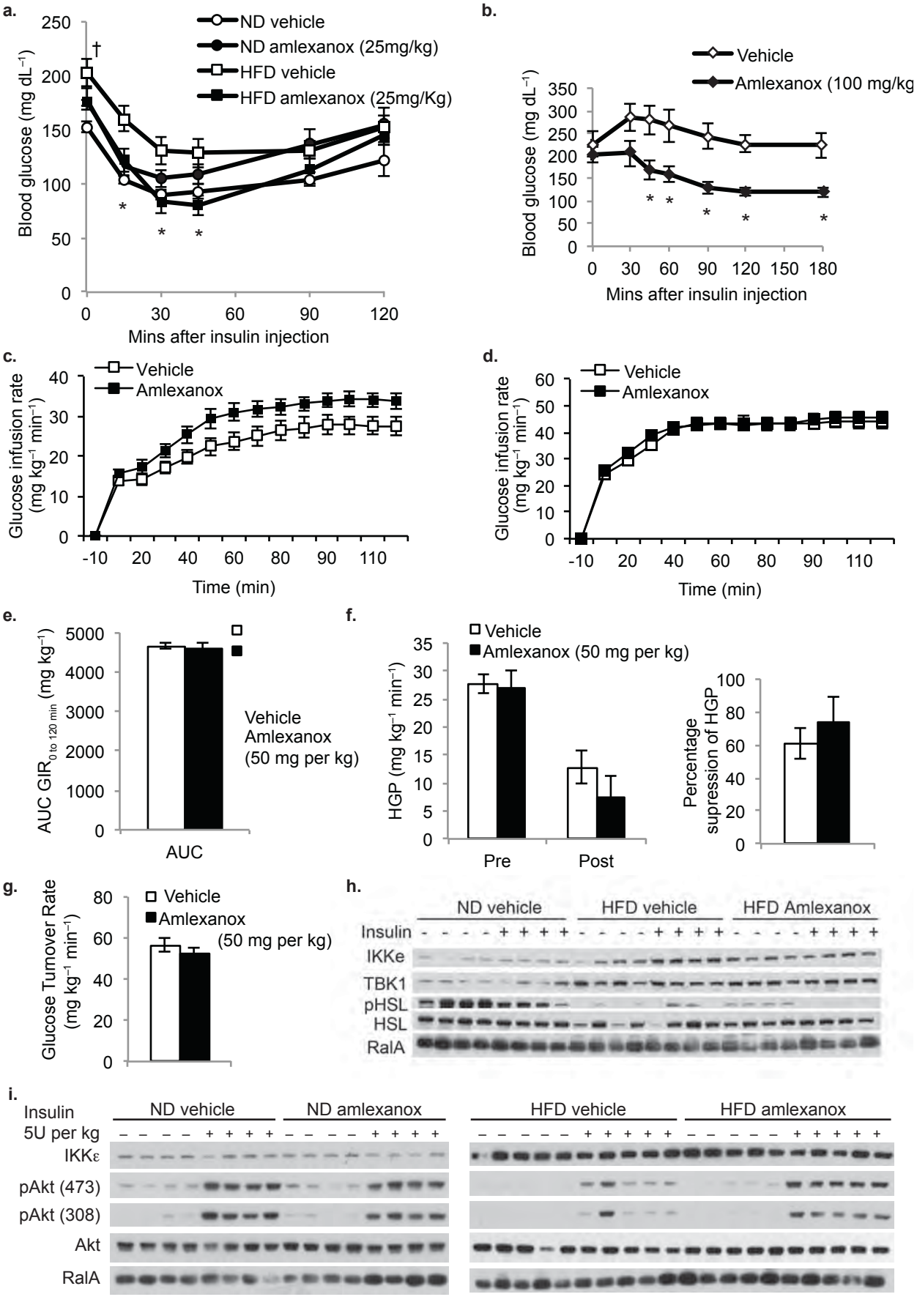


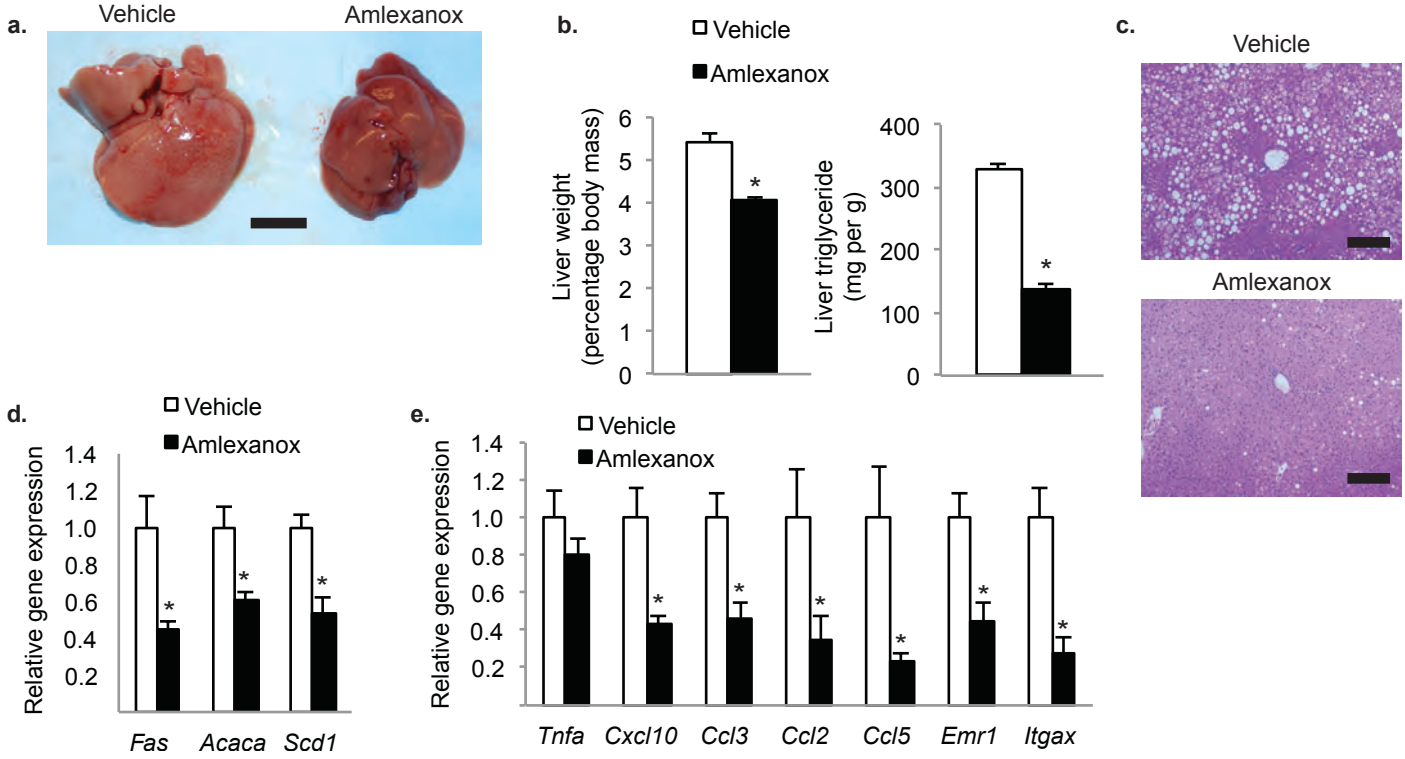
Supplementary Figure 1: Weight loss with amlexanox treatment. Body weight of treatment group: amlexanox (black squares) or vehicle control (white squares). Mice maintained on ND and gavaged with vehicle control are also shown (white circles). (*n*=7 per group).



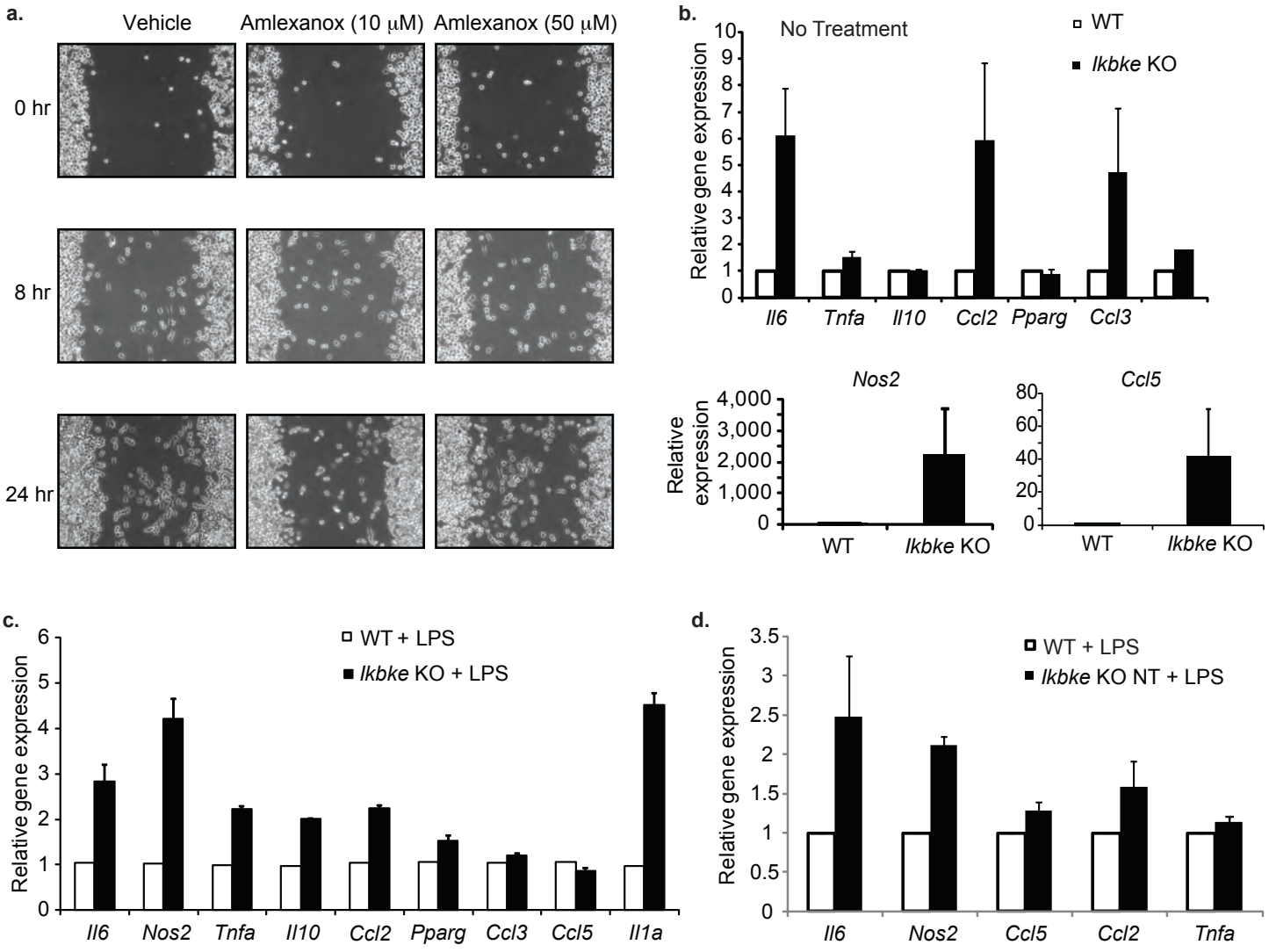
Supplementary Figure 2: Amlexanox increases energy expenditure with no effect on respiratory quotient or total activity. (**a**) Carbon dioxide production (VCO_2) in mice on ND (left panel – light grey circles = vehicle, dark grey circles = 25 mg per kg amlexanox) and HFD (right panel – white squares = vehicle, black squares = 25 mg per kg amlexanox). HFD amlexanox treated values are significantly higher than HFD vehicle values during all three light and dark cycles, p-value < 0.05. (**b**) Respiratory quotient ($RQ = VCO_2 / VO_2$) analyzed in metabolic cages. (**c**) Total activity for all groups. (*n*=4 for ND groups, *n*=8 for HFD groups).

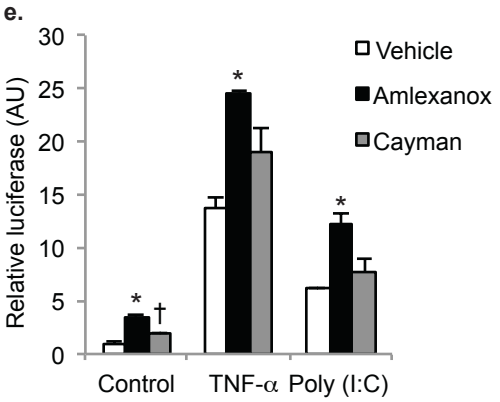

a.
ND vehicle
ND amlexanox (25mg/kg)
HFD vehicle
HFD amlexanox (25mg/Kg)
Blood glucose (mg dL−1)
Mins after insulin injection
b.
Vehicle
Amlexanox (100 mg/kg
Blood glucose (mg dL−1)
Mins after insulin injection
c.
Vehicle
Amlexanox
Glucose infusion rate (mg kg−1 min−1)
Time (min)
d.
Vehicle
Amlexanox
Glucose infusion rate (mg kg−1 min−1)
Time (min)
e.
AUC GIR0 to 120 min (mg kg−1)
Vehicle
Amlexanox
(50 mg per kg)
AUC
f.
Vehicle
Amlexanox (50 mg per kg)
HGP (mg kg−1 min−1)
Pre
Post
Percentage supression of HGP
g.
Vehicle
Amlexanox
(50 mg per kg)
Glucose Turnover Rate (mg kg−1 min−1)
h.
ND vehicle
HFD vehicle
HFD Amlexanox
Insulin
IKKe
TBK1
pHSL
HSL
RalA
i.
Insulin
5U per kg
ND vehicle
ND amlexanox
HFD vehicle
HFD amlexanox
IKKε
pAkt (473)
pAkt (308)
Akt
RalA

Supplementary Figure 3: Amlexanox does not improve insulin sensitivity in ND fed mice. (**a**) Insulin tolerance test of treatment group presented as absolute blood glucose values. ND vehicle control (white circles), HFD vehicle control (white squares) HFD 25 mg per kg amlexanox (black squares). (*n*=8 per group). (**b**) Insulin tolerance test ob/ob mice gavaged with 100 mg per kg amlexanox (black diamonds) or vehicle control (white diamonds). (*n*=8 per group) presented as absolute blood glucose values. * *P* value < 0.05 HFD vehicle control versus HFD amlexanox treated, or ob/ob vehicle control versus ob/ob amlexanox treated. † *P* value <0.05 ND vehicle control versus ND amlexanox treated. Glucose infusion rate during hyperinsulinemic euglycemic clamp for mice on (**c**) HFD or (**d**) ND. (**e**–**g**) Clamp data for ND fed mice (*n*=6 per group). (**e**) Area under the curve for glucose infusion rate during the clamp. (**f**) Hepatic glucose production and percent suppression of hepatic glucose production. (**g**) Glucose turnover rate during the clamp. (**c**–**g**) vehicle-treated shown in white, amlexanox-treated shown in black. Phosphorylation of hormone sensitive lipase (HSL) at serine 563 (**h**) and Akt at Ser308 and Ser473 (**i**) in epididymal adipose tissue of mice treated with amlexanox. Mice were fasted twelve hours then injected with 2.5 U per kg insulin control 20 min before sacrifice (**h**) or 5U per kg insulin 15 min before sacrifice (**i**), saline injection for control mice.

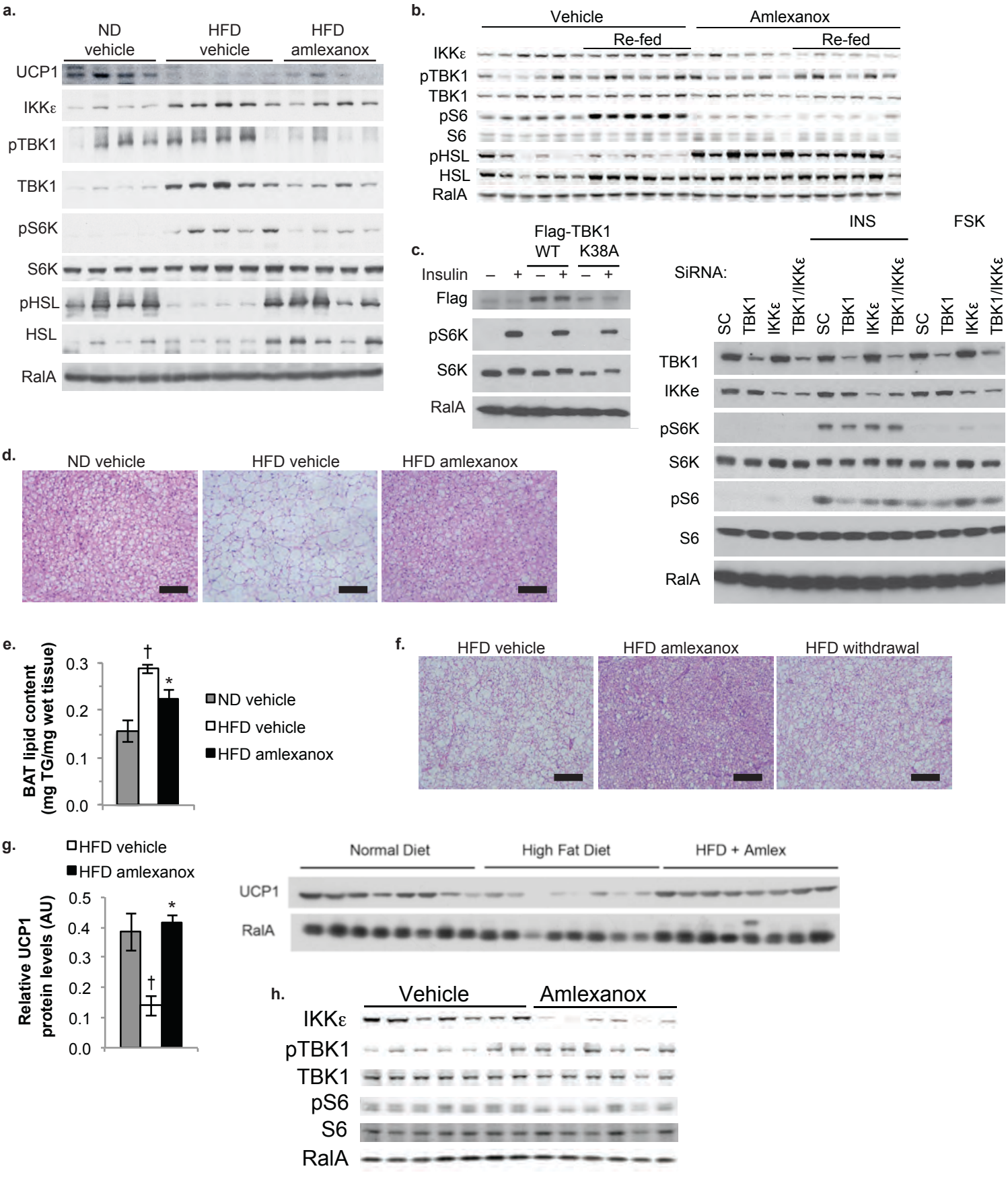


Supplementary Figure 4: Amlexanox treatment reverses hepatic steatosis in ob/ob mice. (**a**) Macroscopic pictures of ob/ob livers; left: vehicle control; right: 100 mg per kg amlexanox (scale bar = 1 cm) (**b**) Relative liver weight (left panel) and triglyceride (TG) content (right panel) of ob/ob mice gavaged with vehicle (white bars) or 100 mg per kg amlexanox (black bars) (*n*=6 per group). (**c**) Representative images of H and E-stained liver sections from ob/ob mice; top: vehicle control; bottom: 100 mg per kg amlexanox (scale bar = 2 mm). Expression of lipid metabolism genes (**d**) and inflammatory genes (**e**) in livers from ob/ob mice gavaged with vehicle control (white bars) or 100 mg per kg amlexanox (black bars) (*n*=6 per group). * *P* value < 0.05 HFD vehicle control versus HFD amlexanox treated or ob/ob vehicle versus amlexanox treated mice.

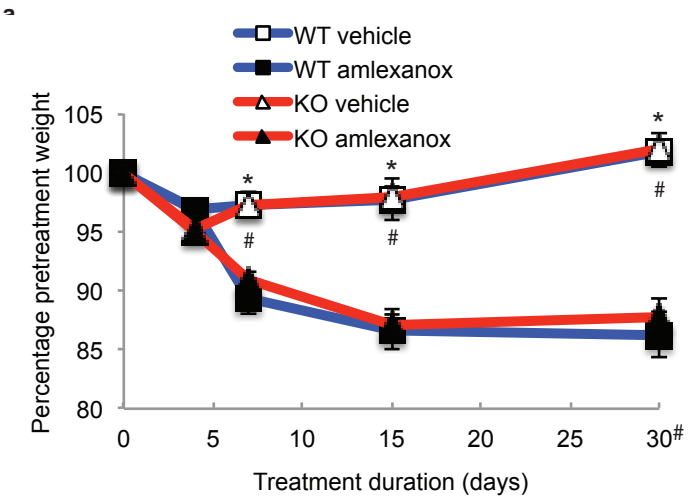


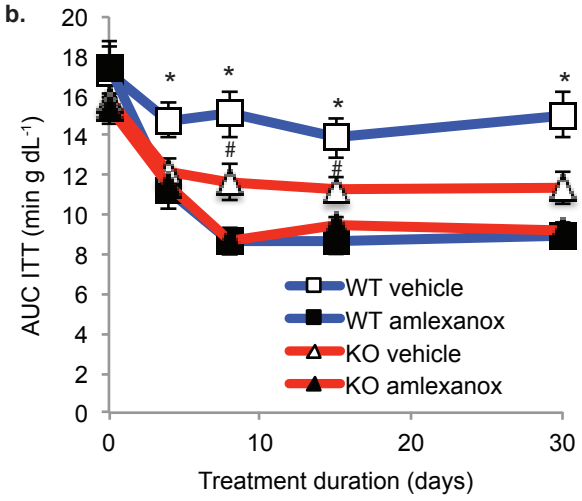


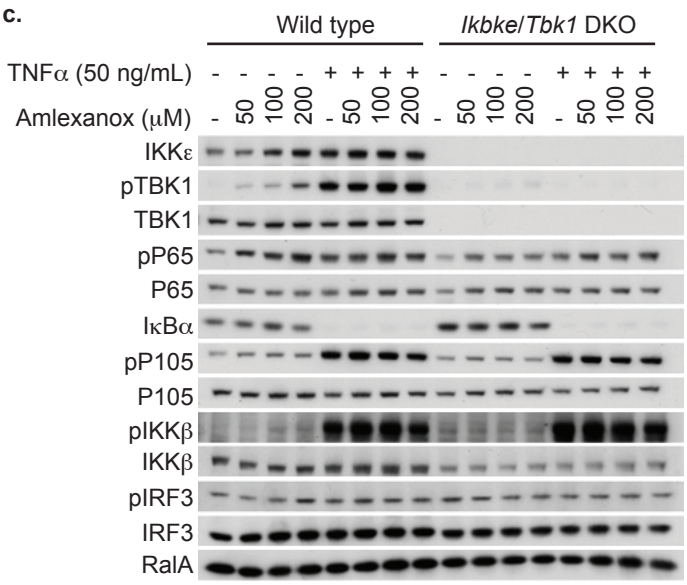
Supplementary Figure 5: Inflammatory control by IKKε. (**a**) Migration of RAW264.7 macrophages treated with amlexanox or vehicle control was tested in a 24hr *in vitro* wound-healing assay. (**b**) Relative expression of cytokines in BMDM from WT (white bars) and *Ikbke* KO (black bars) mice, normalize to WT expression (n=2 per group). (**c**) LPS simulated cytokine expression in BMDM from WT (white bars) and *Ikbke* KO (black bars) mice. Graphs are a representation of one experiment with at least two replicated experiments (n=2 per group). (**d**) Peritoneal macrophages from WT (white bars) and *Ikbke* KO (black bars) treated with LPS. Normalized to WT with LPS values. (n=2 per group). Results are a summary of at least three experiments. (**e**) Promoter reporter assay of NF-κB activity in 3T3-L1 cells stimulated with TNF-α or poly (I:C) after pretreatment with amlexanox (black bars), cayman (grey bars) or vehicle control (white bars). Results were replicated in multiple experiments. * P value < 0.05 vehicle versus amlexanox, † P value < 0.05 vehicle versus cayman.

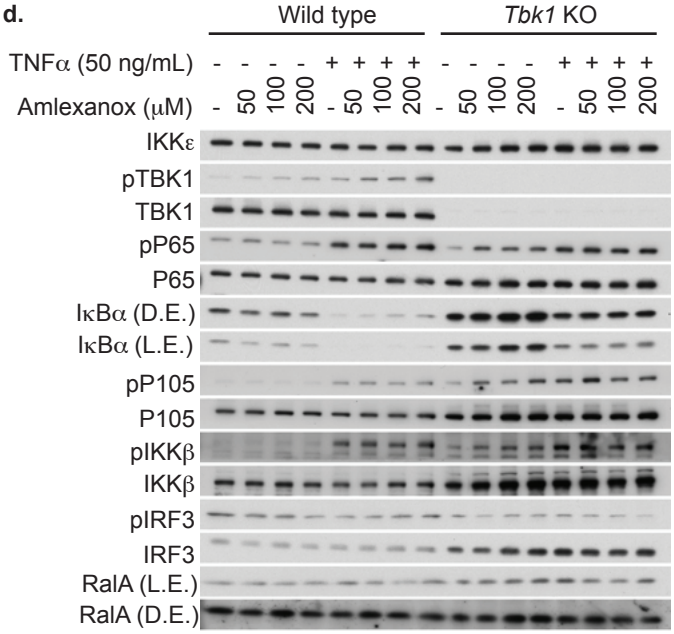

a.
ND vehicle
HFD vehicle
HFD amlexanox
UCP1
IKKε
pTBK1
TBK1
pS6K
S6K
pHSL
HSL
RalA
b.
Vehicle
Amlexanox
Re-fed
Re-fed
IKKε
pTBK1
TBK1
pS6
S6
pHSL
HSL
RalA
c.
Flag-TBK1
WT
K38A
Insulin – + – + – +
Flag
pS6K
S6K
RalA
INS
FSK
SiRNA:
SC
TBK1
IKKε
TBK1/IKKε
TBK1
IKKe
pS6K
S6K
pS6
S6
RalA
d.
ND vehicle
HFD vehicle
HFD amlexanox
e.
BAT lipid content (mg TG/mg wet tissue)
0.3
0.2
0.1
0.0
†
*
ND vehicle
HFD vehicle
HFD amlexanox
f.
HFD vehicle
HFD amlexanox
HFD withdrawal
g.
HFD vehicle
HFD amlexanox
Relative UCP1 protein levels (AU)
0.5
0.4
0.3
0.2
0.1
0.0
*
†
Normal Diet
High Fat Diet
HFD + Amlex
UCP1
RalA
h.
Vehicle
Amlexanox
IKKε
pTBK1
TBK1
pS6
S6
RalA

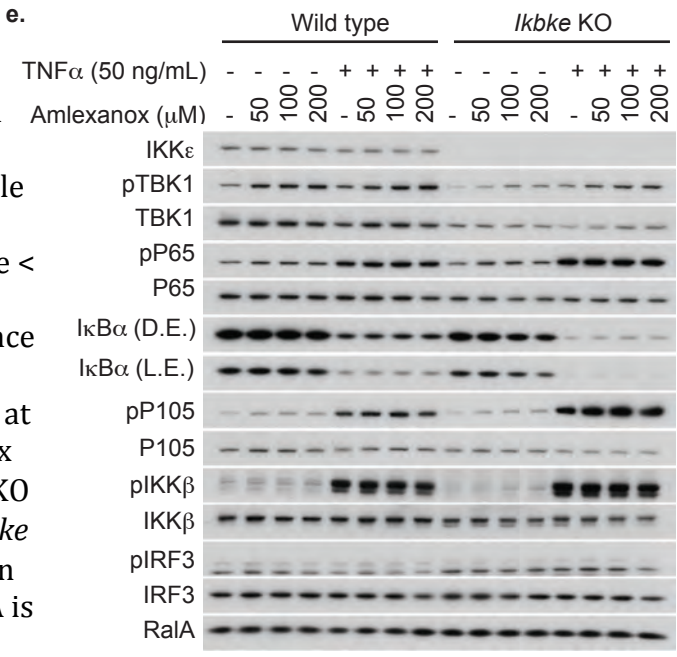
Supplementary Figure 6: Metabolic regulation by IKKε and TBK1 in adipocytes and adipose tissue. (**a**) Western blot analysis of WAT protein levels of UCP1, noncanonical IKKs, and phosphorylation of S6K at threonine 389 and HSL at serine 562. RalA is a loading control. (**b**) Western blot quantified in Figure 6d. (**c**) Left panel: Effect of TBK1 overexpression on insulin stimulated S6K phosphorylation at threonine 389. RalA is a loading control. Right Panel: Effect of IKKε/TBK1 knockdown on S6K and S6 phosphorylation at serine 235/236. RalA is a control. (**d**) Representative images of H and E-stained sections of BAT from mice in treatment group; left panel: ND vehicle control; middle panel: HFD vehicle control; right panel: HFD 25 mg per kg amlexanox (scale bars = 2 mm). (**e**) BAT triglyceride content in vehicle treated ND (grey bars) and HFD (white bars) and amlexanox treated HFD (black bars) mice (*n*=8 per group). (**f**) Representative images of H and E-stained BAT sections from withdrawal group; left: high fat diet vehicle control, middle: high fat diet gavaged daily with 25 mg per kg amlexanox; right: amlexanox withdrawal for ten weeks after eight weeks treatment (scale bars = 2 mm). (**g**) UCP-1 protein levels normalized to RalA in BAT of vehicle and amlexanox treated mice. Left panel: quantification ND (grey bars) and HFD (white bars) and amlexanox treated HFD (black bars) mice (*n*=8 per group). Right panel: western blot. (**h**) BAT protein levels of noncanonical IKKs, and phosphorylation of S6 at serine 235/236 quantified in Figure 6g.











Supplementary Figure 7: Effects of amlexanox treatment on IKKε knockout mice and MEFs. (**a**) Percentage body pretreatment body weight and (**b**) area under the ITT curve of WT (squares) and *Ikbke* KO (triangles) mice treated with amlexanox (black) or vehicle control (white) (n=12 per group). * P value < 0.05 amlexanox versus vehicle control in WT mice, # † P value < 0.05 amlexanox versus vehicle control in KO mice. (**c**–**e**) Amlexanox treatment of MEFs in the presence and absence of TNF-α. (**c**) Amlexanox stimulated induction of TBK1 phosphorylation at serine 172 and P65 phosphorylation at serine 536 in wildtype but not DKO MEFs. (**d**) Amlexanox stimulated IKKβ phosphorylation at serine 177 in *Tbk1* KO MEFs. D) Amlexanox stimulated IκBα degradation in *Ikbke* KO MEFs. No effects of amlexanox or TNFα treatments on IRF3 phosphorylation at serine 396 were observed. RalA is a loading control.

Supplementary Table 1: RT-PCR primer sequences

Gene	Forward primer	Reverse primer
Ikbke	5'-ACAAGGCCCGAAACAAGAAAT-3'	5'-ACTGCGAATAGCTTCACGATG-3'
Tbk1	5'-ACTGGTGATCTCTATGCTGTCA-3'	5'-TTCTGGAAGTCCATACGCATTG-3'
Fasn	5'-GGAGGTGGTGATAGCCGGTAT-3'	5'-TGGGTAATCCATAGAGCCCAG-3'
Me1	5'-GTCGTGCATCTCTCACAGAAG-3'	5'-TGAGGGCAGTTGGTTTTATCTTT-3'
Acaca	5'-TAATGGGCTGCTTCTGTGACTC-3'	5'-CTCAATATCGCCATCAGTCTTG-3'
Scd1	5'-GCTGGAGTACGTCTGGAGGAA-3'	5'-TCCCGAAGAGGCAGGTGTAG-3'
Ppara	5'-AACATCGAGTGTCGAATATGTGG-3'	5'-AGCCGAATAGTTCGCCGAAAG-3'
Pparg	5'-TCGCTGATGCACTGCCTATG-3'	5'-GAGAGGTCCACAGAGCTGATT-3'
Cd36	5'-ATGGGCTGTGATCGGAACTG-3'	5'-GTCTTCCCAATAAGCATGTCTCC-3'
Fabp4	5'-GGGGCCAGGCTTCTATTCC-3'	5'-GGAGCTGGGTTAGGTATGGG-3'
Lpl	5'-TGTGTCTTCAGGGGTCCTTAG-3'	5'-GGGAGTTTGGCTCCAGAGTTT-3'
G6pc	5'-CGACTCGCTATCTCCAAGTGA-3'	5'-GTTGAACCAGTCTCCGACCA-3'
Pck1	5'-CTGCATAACGGTCTGGACTTC-3'	5'-CAGCAACTGCCCGTACTCC-3'
Pklr	5'-TCAAGGCAGGGATGAACATTG-3'	5'-CACGGGTCTGTAGCTGAGTG-3'
Gck	5'-ATGGCTGTGGATACTACAAGGA-3'	5'-TTCAGGCCACGGTCCATCT-3'
Tnfa	5'-ACGGCATGGATCTCAAAGAC-3'	5'-AGATAGCAAATCGGCTGACG-3'
Cxcl10	5'-CCAAGTGCTGCCGTCATTTTC-3'	5'-GGCTCGCAGGGATGATTTCAA-3'
Ccl3	5'-TTCTCTGTACCATGACACTCTGC-3'	5'-CGTGGAATCTTCCGGCTGTAG-3'
Ccl3	5'-TTAAAAACCTGGATCGGAACCAA-3'	5'-GCATTAGCTTCAGATTTACGGGT-3'
Cybb	5'-TTGGGTCAGCACTGGCTCTG-3'	5'-TGGCGGTGTGCAGTGCTATC-3'
Ccl5	5'-GCTGCTTTGCCTACCTCTCC-3'	5'-TCGAGTGACAAACACGACTGC-3'
Emr1	5'-CTGGGATCCTACAGCTGCTC-3'	5'-AGGAGCCTGGTACATTGGTG-3'
Itgax	5'-CTGGATAGCCTTTCTTCTGCTG-3'	5'-GCACACTGTGTCCGAACTCA-3'
Il1a	5'-TGTTCCTGAACTCAACTGTG-3'	5'-AGACAGGCTTGTGCTCTG-3'
Il6	5'-TAGTCCTTCCTACCCCAATTTCC-3'	5'-TTGGTCCTTAGCCACTCCTTC-3'
Il10	5'-GCTCTTACTGACTGGCATGAG-3'	5'-CGCAGCTCTAGGAGCATGTG-3'
Nos2	5'-CCAAGCCCTCACCTACTTCC-3'	5'-CTCTGAGGGCTGACACAAGG-3'
Slc2a4	5'-GTGACTGGAACACTGGTCCTA-3'	5'-CCAGCCACGTTGCATTGTAG-3'
Prdm16	5'-CCACCAGACTTCGAGCTACG-3'	5'-ACACCTCTGTATCCGTCAGCA-3'
Ppargc1a	5'-CCACTTCAATCCACCCAGAAAG-3'	5'-TATGGAGTGACATAGAGTGTGCT-3'
Ppargc1b	5'-TCCTGTAAAAGCCCGGAGTAT-3'	5'-GCTCTGGTAGGGGCAGTGA-3'
Cidea	5'-TGACATTCATGGGATTGCAGAC-3'	5'-GGCCAGTTGTGATGACTAAGAC-3'
Ucp1	5'-AGGCTTCCAGTACCATTAGGT-3'	5'-CTGAGTGAGGCAAAGCTGATTT-3'
Elovl3	5'-TTCTCACGCGGGTTAAAAATGG-3'	5'-GAGCAACAGATAGACGACCAC-3'
Cox7a1	5'-GCTCTGGTCCGGTCTTTTAGC-3'	5'-GTACTGGGAGGTCATTGTCGG-3'
Cox7a2	5'-GCTGGCCCTTCGTCAGATT-3'	5'-GGCATCCCATTATCCTCCTGAA-3'
Cox8b	5'-AGTGGGCTAAGACCCATCCTG-3'	5'-TGTGGGGATCTCAGCCATAGT-3'

Supplementary methods

Tissue Specific Insulin signaling. After a 16 hour fast we injected saline control or insulin (2.5 U per kg or 5 U per kg) into the peritoneal cavity, and collected tissues 15–20 min latter.

IKKε knockout mice. IKKε knockout breeding pairs were generously provided by Dr. Tom Maniatis. These mice were housed and fed under conditions identical to wild type mice. Insulin tolerance tests in these mice were performed using a dose of 1.2 U per kg insulin.

Wound healing assay: RAW cells were plated in 6-well dishes to almost confluency. A "wound" was created by scraping the plate with a pipette tip and monitored for 24 hours and photographed at the indicated times. Cells were treated with the indicated concentrations of amlexanox or MRT at time 0.

Bone marrow-derived macrophages. Bone marrow derived macrophages (BMDM) from wildtype and IKKε knockout mice were harvested after high-fat diet. Bone marrow was extracted from the femur and tibia with PBS. Bone marrow was pooled from at least 3 mice. Cells were plated in the presence of 20% L929 cell conditioned media and DMEM with 10% heat inactivated FBS. After overnight culture, nonadherent cells were collected and plated for experiments. Cells were used on day six or seven post culture. BMDM cells were treated *ex vivo* with 10ng ml^{-1} LPS for 24 hours.

Peritoneal Macrophages. Thioglycollate-elicited peritoneal macrophages (PM) were harvested from wildtype and IKKε knockout mice three days after 2 mL intraperitoneal injection of 4% Brewer's thioglycollate (Sigma) into mice. PM cells were plated in DMEM containing 10% heat inactivated FBS for three hours at which time media was changed and cells were treated with 10 ng ml^{-1} LPS.

Overexpression and gene knockdown. 3T3-L1 adipocytes were routinely transfected with plasmids and siRNA by electroporation as described previously [2]. Stealth(tm) duplex siRNA was obtained from Invitrogen, using 5'-AGAACAGUGUAUAAACUCCCACAGG-3' and 5'-CCUGUGGGAGUUUAUACACUGUUCU-3' against mouse TBK1, 5'-UGGACAUGCUGAACAGAGUUAGAGG-3' and 5'-CCUCUAACUCUGUUCAGCAUGUCCA-3' against mouse IKKε. Primers were designed using the Block-IT™ program from Invitrogen, with the scrambled primers used as a control. TBK1 expressing plasmids were kindly provided by Dr. Tom Maniatis [3].

Promoter reporter assay. 3T3-L1 cells were electroporated with 10 μg pBIX, which contains a luciferase reporter driven by a 6 x NFKB response element, and 0.1 μg pRL-SV40 expressing renilla as a normalizing control. 24 hours after transfection cells were pretreated with 50 μM amlexanox or 2 μM cayman for one hour, then TNFα (10ng ml^{-1}) or poly (I:C) (100 μg ml^{-1}) were added for an additional 7 hours. Cells were harvested in passive lysis buffer (Promega). Samples were subjected to two freeze-thaw cycles and then cleared by centrifugation at 14,000 × *g* for 1 min. The supernatant was assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

References

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2. Inoue, A., S.Y. Sawata, and K. Taira, *Molecular design and delivery of siRNA.* Journal of drug targeting, 2006. **14**(7): p. 448-55.
3. Sharma, S., et al., *Triggering the interferon antiviral response through an IKK-related pathway.* Science, 2003. **300**(5622): p. 1148-51.