Supplementary Information

A branched chain amino acid metabolite drives vascular fat transport and causes insulin resistance

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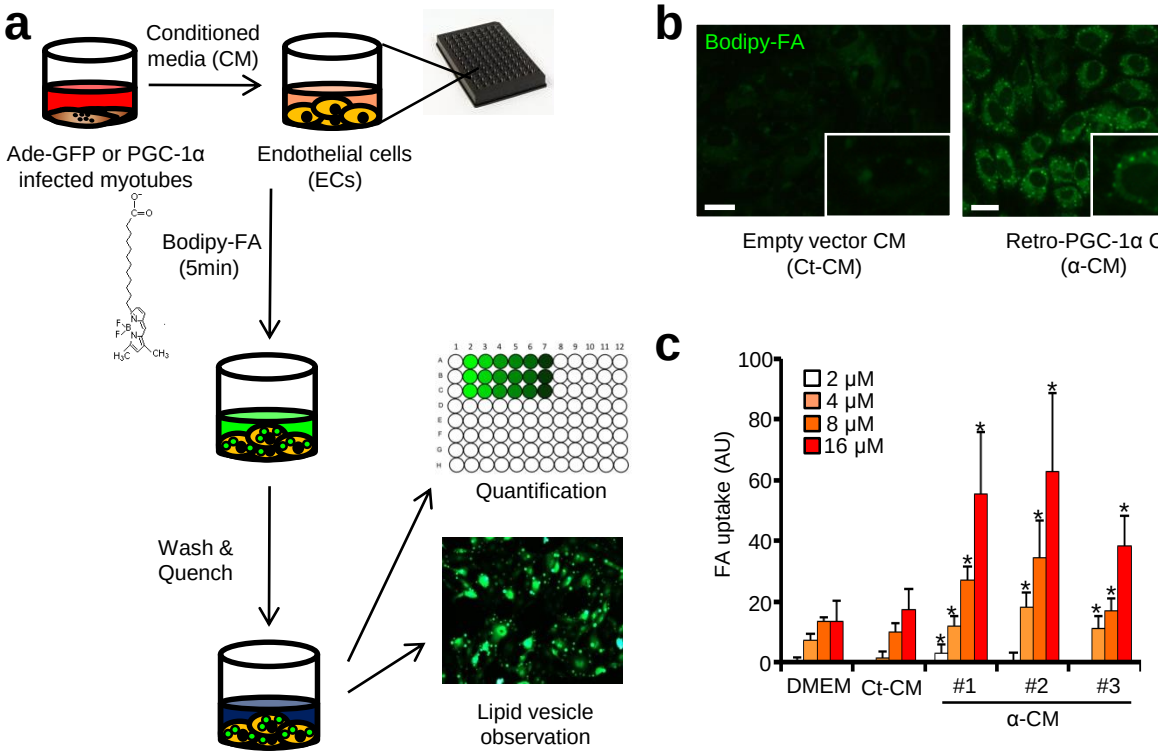
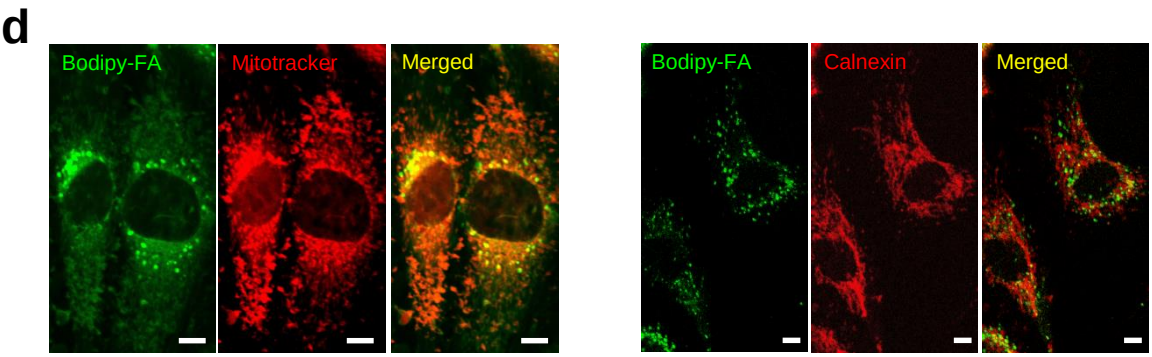
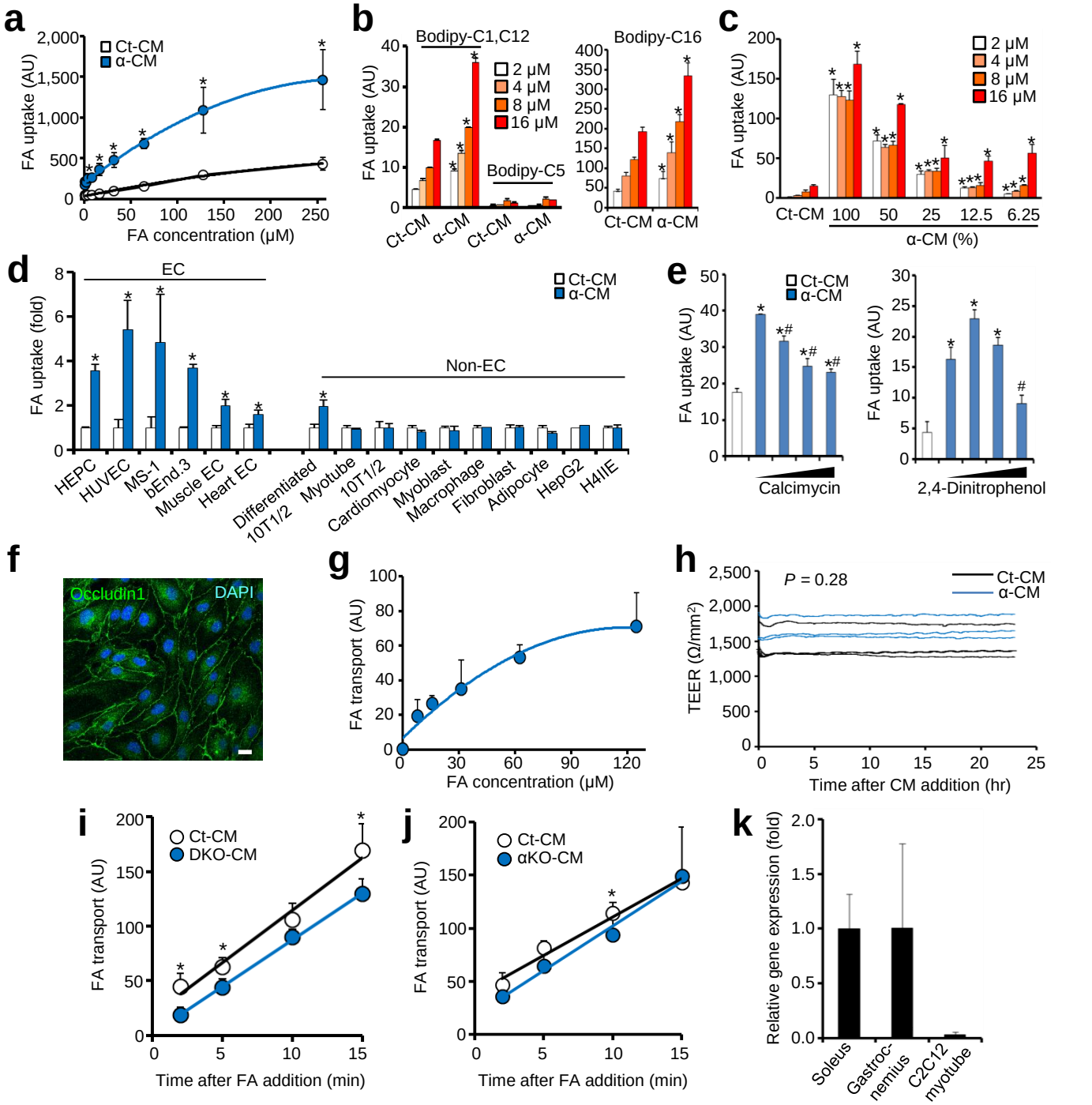
Supplementary Figs. 1-10

Supplementary Table. 1

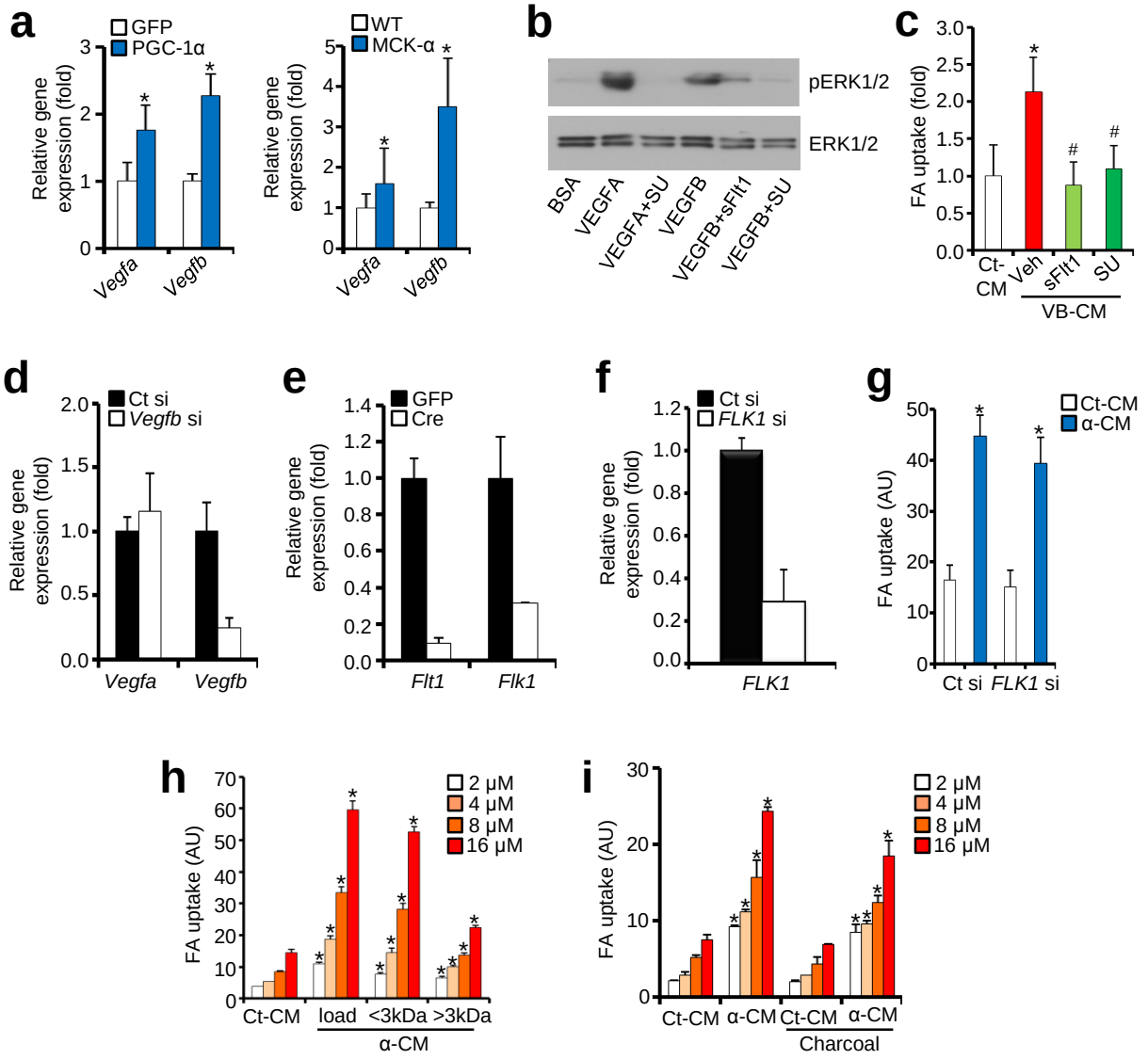




Supplementary Figure 1. PGC-1α CM induces endothelial FA uptake. (**a**) Schematic of FA uptake assay. Differentiated myotubes were infected with adenovirus expressing GFP or PGC-1α for 48 hr, and the conditioned media (CM) were harvested. Endothelial cells (ECs) in a 96 well plate were treated with CMs for 1 hr and treated with Bodipy-conjugated FA (Bodipy-FA) for 5 min. After the cells were washed and quenched, intracellular Bodipy-FA was measured by a plate reader or visualized with a fluorescence microscope. Scale bars, 25 μm. (**b**) Representative images of ECs that take up Bodipy-FA after treatment with CMs from C2C12 myotubes infected with retrovirus expressing vector control (Ct-CM) or PGC-1α (α-CM). (**c**) Quantification of Bodipy-FA (2─16 μM) uptake by ECs. Numbers (#1, #2, #3) indicate independent stable cell lines. Student's *t*-test; **P* < 0.05 *vs*. control. Data are mean ± s.d. of at least three biological replicates. (**d**) ECs with Bodipy-FA (green) after treatment of α-CM were stained with mitotracker (red, left) or immunostained with anti-Calnexin antibody (red, right) as an ER marker. Scale bars, 10 μm.



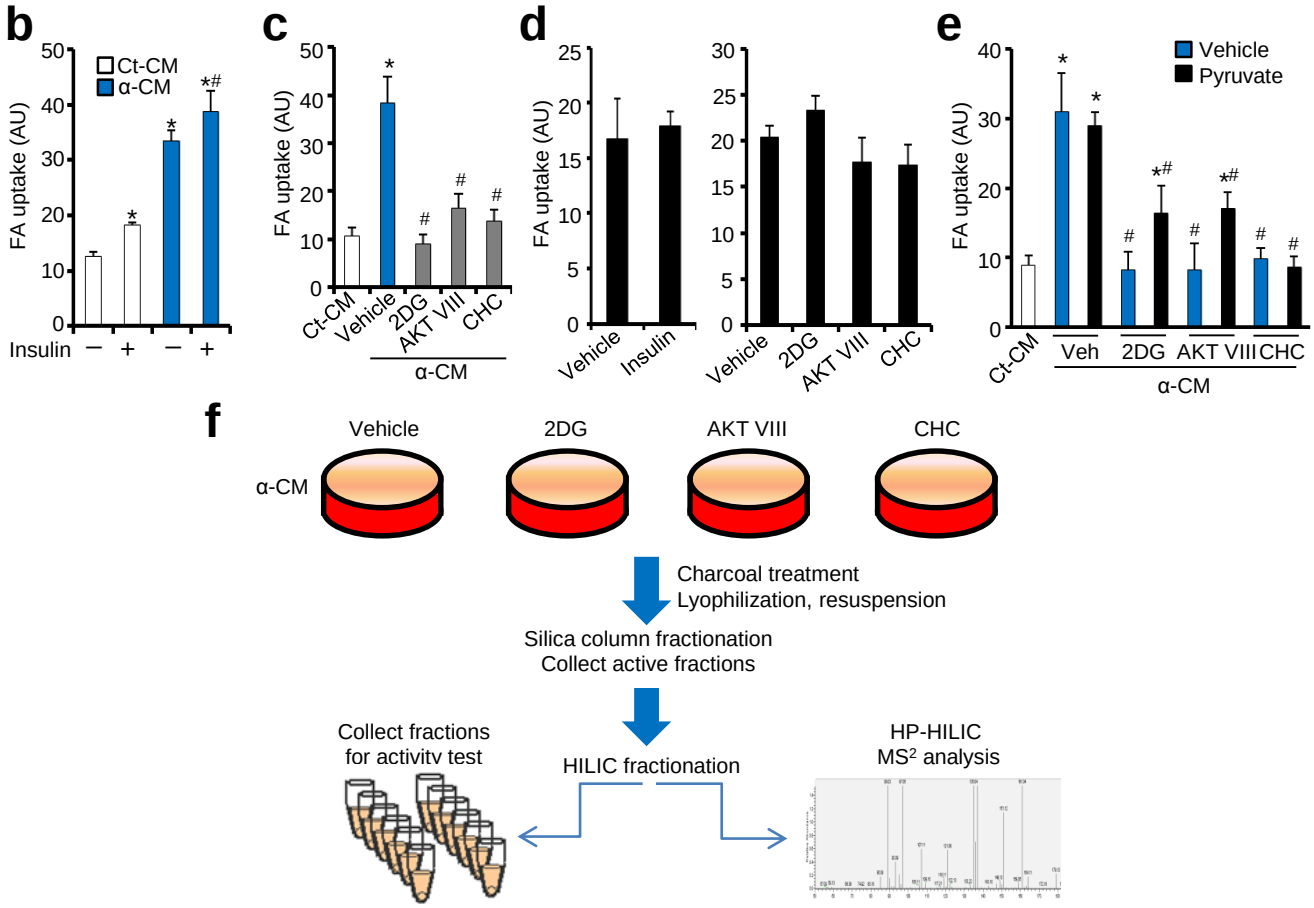
Supplementary Figure 2. Characterization of PGC-1α CM activity on endothelial FA uptake and transport. (**a**) Endothelial FA uptake with different FA concentrations at 5 min. (**b**) Endothelial FA uptake with Bodipy-conjugated long-chain FA (Bodipy-C1,C12 or Bodipy-C16) or short-chain FA (Bodipy-C5) after exposure to the indicated CM. (**c**) α-CM sequentially diluted with Ct-CM partially retains its activity. (**d**) Relative FA (2 μM) uptake by different cell types after exposure to the indicated CM for 1 hr. (**e**) FA (2 μM) uptake by HUVECs after pre-treatment with Calcimycin (left, 12.5─50 μM) or 2,4-Dinitrophenol (right, 125─500 μM) for 30 min. (**f**) Primary rat brain ECs immunostained with anti-Occludin1 antibody for tight junctions (green) and DAPI for nuclei (blue). Scale bar, 25 μm. (**g**) Endothelial FA transport (8 μM) with different FA concentrations at 5 min. (**h**) Measurement of trans-endothelial electrical resistance (TEER) of an EC monolayer after exposure to the indicated CM for 24 hr. (**i**, **j**) FA transport (8 μM) by ECs treated with CM from primary myotubes lacking both PGC-1α and PGC-1β (**i**, DKO-CM) or myotubes lacking PGC-1α alone (**j**, αKO-CM). (**k**) qPCR analysis of PGC-1α expression in the soleus, gastrocnemius muscle and C2C12 myotubes. Student's *t*-test; $^{*}P < 0.05$ *vs.* control; $^{\#}P < 0.05$ *vs.* α-CM alone. Data are mean ± s.d. of at least three biological replicates.



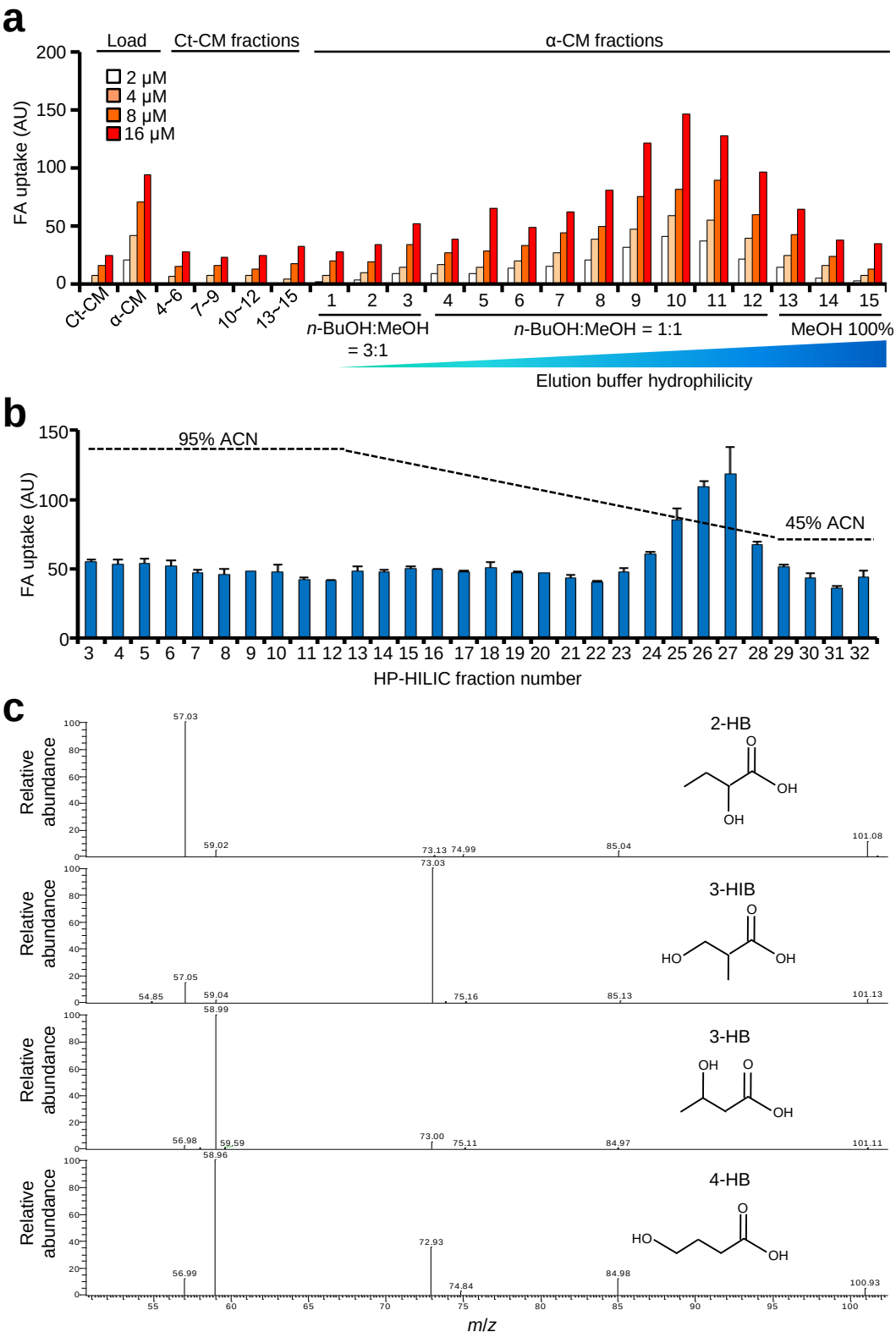
Supplementary Figure 3. The paracrine factor in PGC-1α CM is not VEGFB but small molecule(s). (**a**) qPCR analysis of C2C12 myotubes (left) expressing GFP or PGC-1α via adenovirus, or quadriceps muscle (right) from wild type or MCK-α mice. (**b**) Western blot analysis of ECs treated with recombinant VEGFA or VEGFB for 10 min with or without inhibitors (sFlt1 or SU11248). (**c**) FA uptake (2 μM) by ECs after exposure to CM from 293T cells expressing empty vector control (Ct-CM) or VEGFB (VB-CM) for 24 hr with vehicle (veh) or inhibitors. (**d**) qPCR analysis of C2C12 myotubes after transfection with control or *Vegfb* siRNA for 48 hr. (**e**) qPCR analysis of primary ECs isolated from *Flt1*$^{flox/flox}$;*Flk1*$^{flox/flox}$ mice after infection with adenovirus expressing GFP or Cre recombinase for 48 hr. (**f**) qPCR analysis of HUVECs transfected with control or *FLK1* siRNA for 48 hr. (**g**) FA uptake (2 μM) by HUVECs transfected with control or *FLK1* siRNA for 48 hr after exposure to the indicated CM for 1 hr. (**h**) Endothelial FA uptake (2─16 μM) after exposure to Ct-CM, α-CM itself (load), concentrate (>3kDa) or flow-through (<3kDa) of α-CM filtered with 3kDa membrane filter. (**i**) Endothelial FA uptake (2─16 μM) after exposure to the indicated CM with or without charcoal pre-treatment. Student's *t*-test; **P* < 0.05 *vs*. control; $^{\#}$*P* < 0.05 *vs*. α-CM alone. Two-way ANOVA for **c**. Data are mean ± s.d. of at least three biological replicates.

a

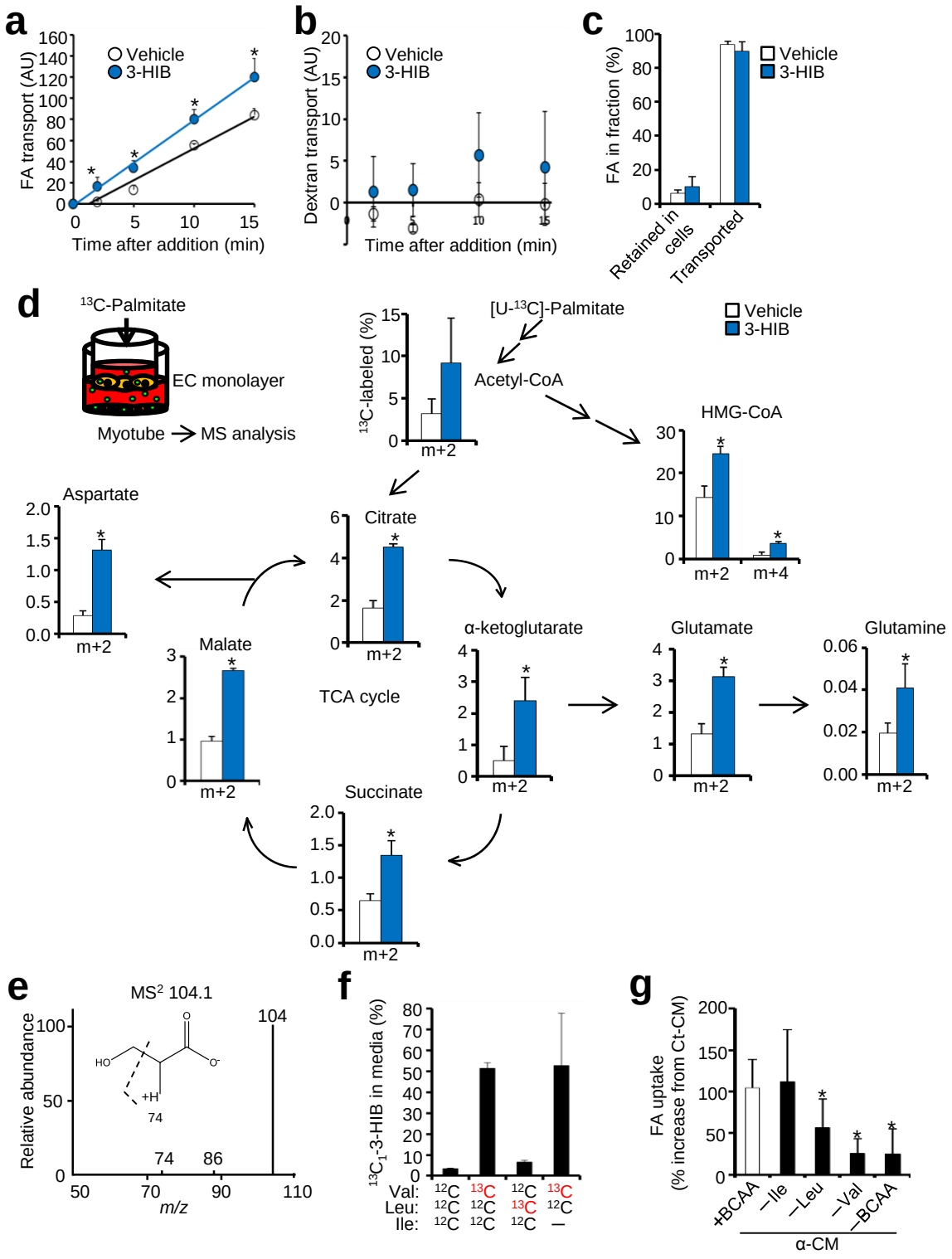
Compound	Target	α-CM activity	Compound	Target	α-CM activity
U0126	MEK1/2 ↓	—	Phenylephrine	α1 AR ↑	—
SB203580	P38 ↓	—	Ritodrine	β2 AR ↑	—
U73122	PLCγ ↓	—	Isoproterenol	β1 AR ↑	—
AKT VIII	AKT ↓	↓↓	CL316243	β3 AR ↑	—
LY294002	PI3K ↓	↓↓	XCT790	ERRα ↓	↓
Rapamycin	mTORC1 ↓	—	GW0742	PPARδ ↑	—
Torin	mTORC1,2 ↓	↓↓	Rosiglitazone	PPARγ ↑	↑
Insulin	Insulin signaling ↑	↑	Dexamethasone	Glucocorticoid ↑	—
PD98059	ERK1/2 ↓	—	2DG	Glycolysis ↓	↓↓
Compound C	AMPK ↓	↓	CHC	MCT ↓	↓↓
AICAR	AMPK ↑	—	FCCP	OXPHOS ↓	—
SP600125	JNK ↓	—	Rotenone	OXPHOS ↓	—



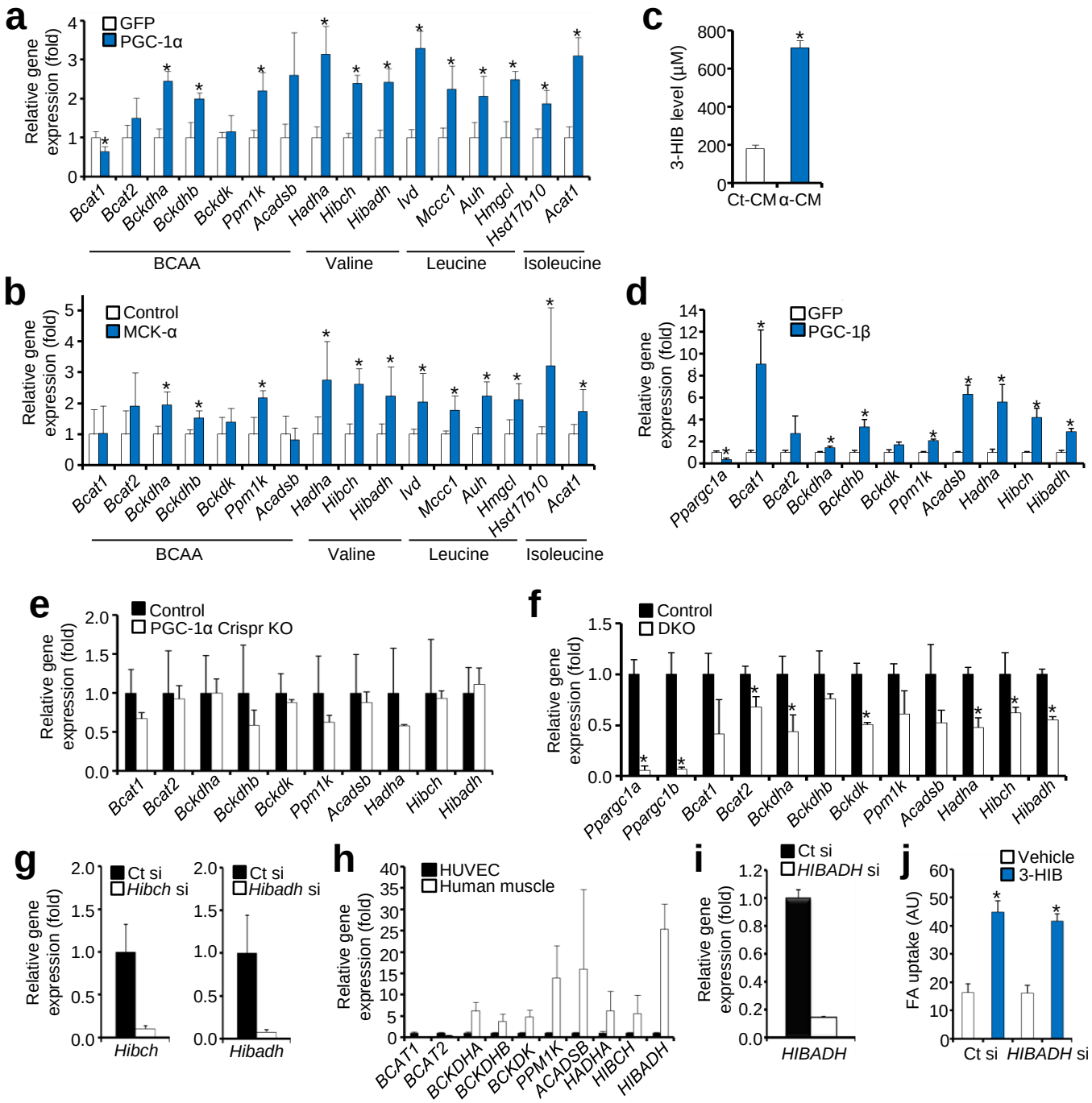
Supplementary Figure 4. Compound screen for isolation of the paracrine factor. (**a**) C2C12 myotubes expressing PGC-1α via retrovirus were treated with the indicated compound during CM generation for 48 hrs, and the activity of CM on FA uptake (2 μM) by ECs was measured. As a control, ECs were incubated with media containing the indicated compound for 1 hr to test if the compound directly affects FA uptake by ECs. The effect of compound is indicated with arrows. AR, Adrenergic receptor; MCT, monocarboxylate transporter; OXPHOS, oxidative phosphorylation. (**b**, **c**) FA uptake (2 μM) by ECs after exposure to α-CM from C2C12 myotubes treated with insulin (**b**) or the indicated inhibitor (**c**). (**d**) FA uptake (2 μM) by ECs treated with insulin (left) or the indicated inhibitor (right) for 1 hr. (**e**) FA uptake (2 μM) by ECs after exposure to α-CM from C2C12 myotubes treated with the indicated inhibitor with or without pyruvate addition (50 mM). Veh, vehicle. (**f**) α-CM treated with the indicated inhibitor were incubated with charcoal, lyophilized, and fractionated with an open silica column. Active fractions were further separated by HILIC for the activity test and HP-HILIC-MS^2 analysis. Two-way ANOVA; $^*P < 0.05$ *vs.* control; $^{\#}P < 0.05$ *vs.* α-CM alone. Data are mean ± s.d. of at least three biological replicates.



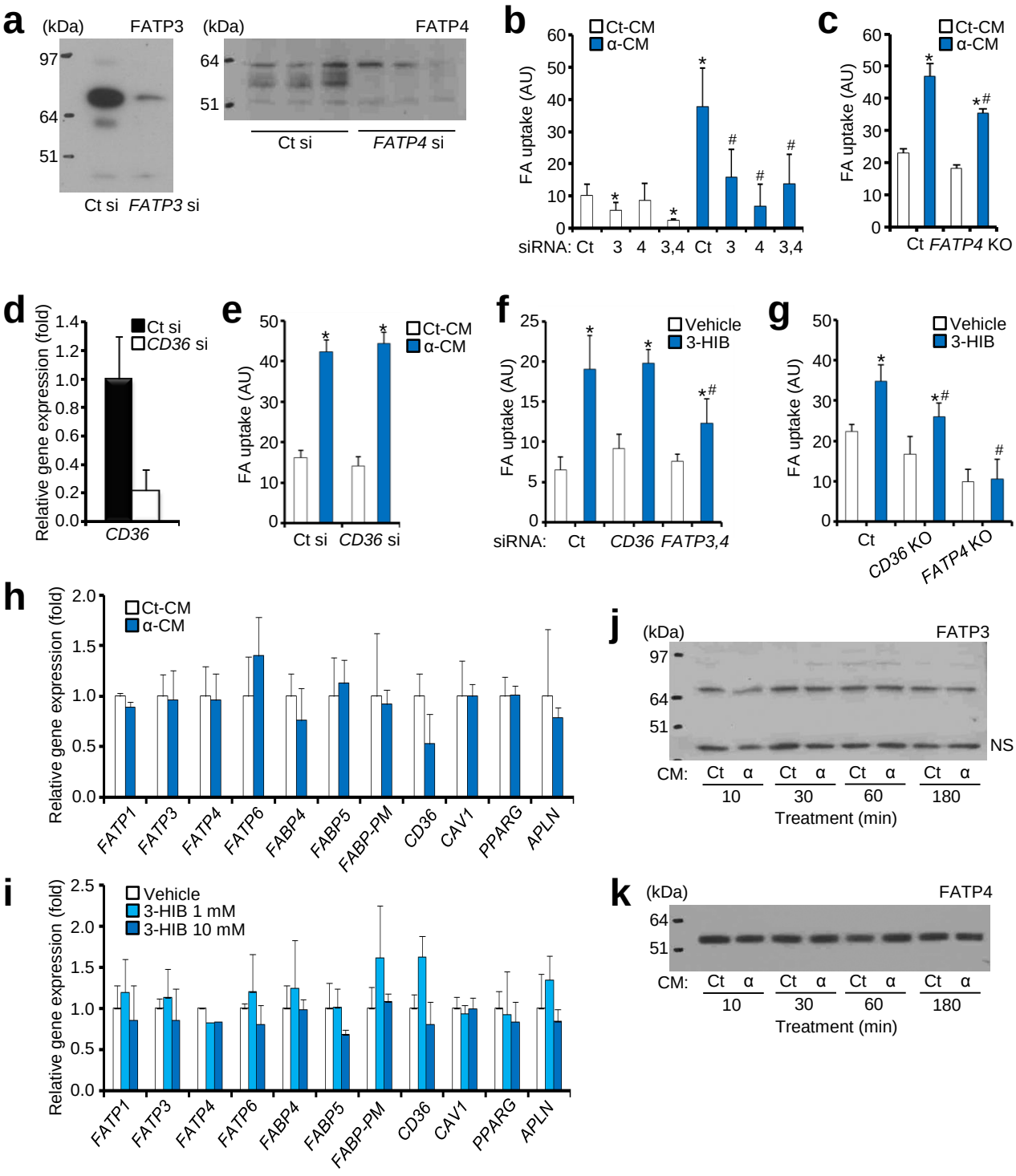
Supplementary Figure 5. Fractionation of CMs and MS2 profiles of isobaric hydroxybutyrates. (**a**) FA uptake (2─16 μM) by ECs after exposure to Ct-CM or α-CM fractions from an open silica column. (**b**) FA uptake (16 μM) by ECs after exposure to α-CM fractions from an HP-HILIC column. Detailed method is described in the Online Methods section. ACN, acetonitrile. (**c**) MS^2 fingerprints of synthetic standards of four isobaric hydroxybutyrates. Data are mean ± s.d. of at least three biological replicates.



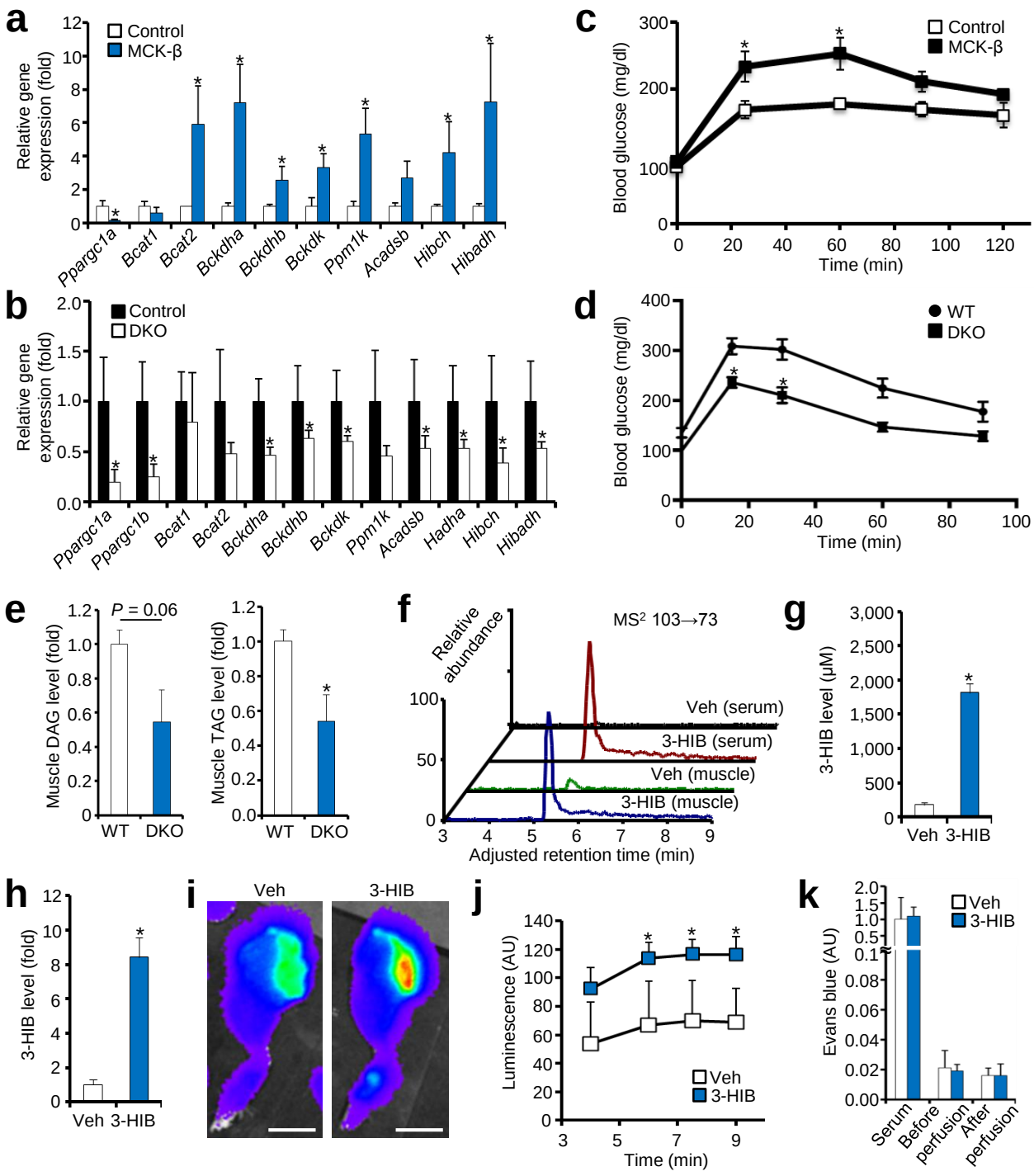
Supplementary Figure 6. 3-HIB is derived from valine and increases endothelial FA transport. (**a**, **b**) FA (**a**, 16 μM) or Dextran Texas-Red (**b**, 50 μg/mL) transport by an EC monolayer after exposure to vehicle or 3-HIB for 1 hr. (**c**) FAs retained in ECs or transported by an EC monolayer was measured. (**d**) ^{13}C-metabolic flux analysis of Acetyl-CoA, TCA intermediates and some amino acids in C2C12 myotubes after exposure to the media containing [U-^{13}C]-palmitate transported by an EC monolayer after treatment with vehicle or 3-HIB for 1 hr. M+2 or M+4 indicates two or four carbons are ^{13}C-labeled, respectively. (**e**) MS^2 spectra of $^{13}C_1$-3-HIB generated by C2C12 myotubes incubated with ^{13}C-2-Valine. (**f**) $^{13}C_1$-3-HIB production by C2C12 myotubes incubated with different combinations of ^{12}C- or ^{13}C-labeled BCAAs in the C2C12 differentiation media for 48 hr. (**g**) FA uptake (2 μM) by ECs after exposure to CMs generated with media lacking the indicated BCAA(s).*P < 0.05 *vs*. control (white bar). Data are mean ± s.d. of at least three biological replicates.



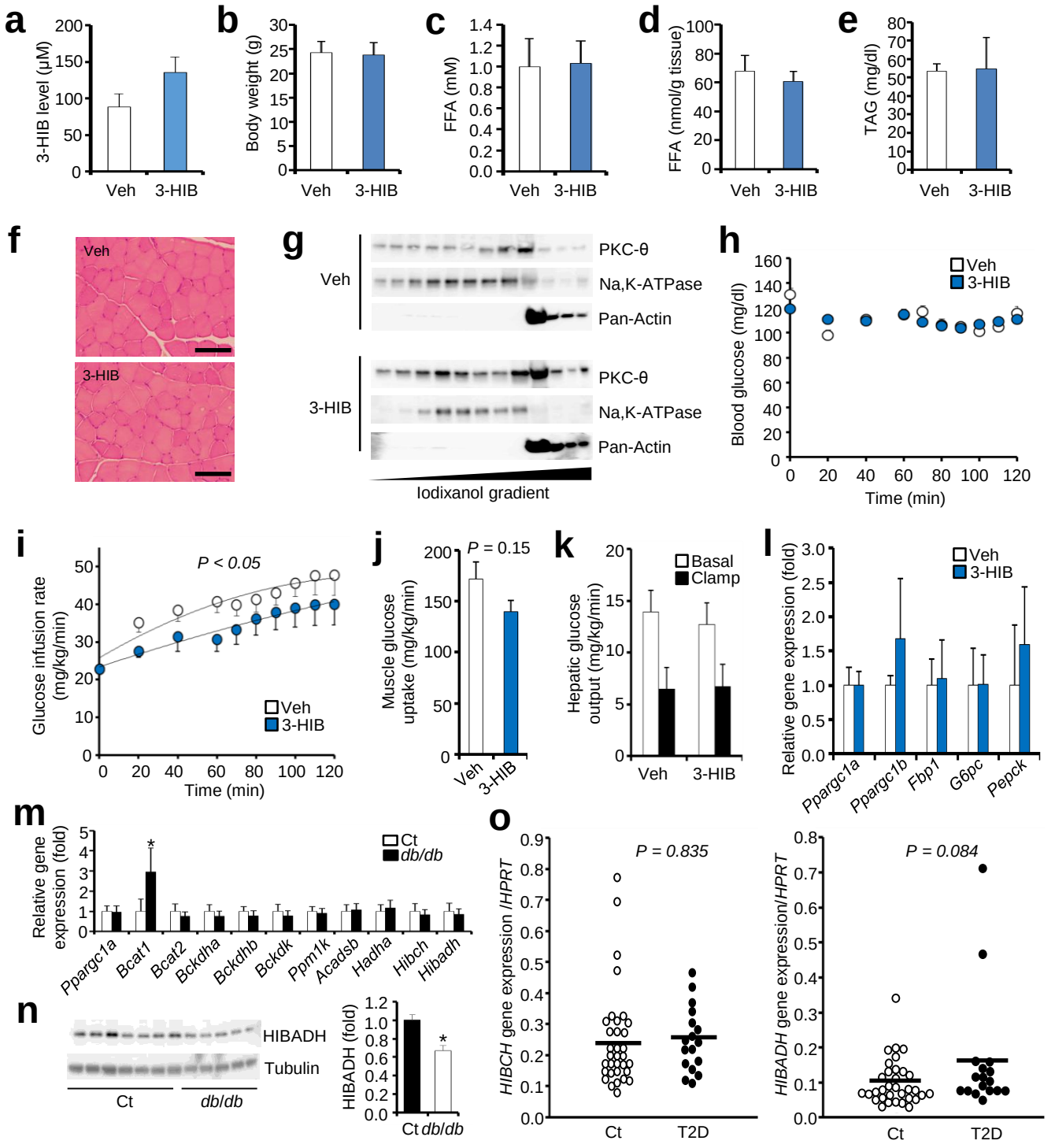
Supplementary Figure 7. PGC-1α and PGC-1β induce expression of valine catabolic enzymes in muscle. (**a**) qPCR analysis of C2C12 myotubes after transfection with adenovirus expressing GFP or PGC-1α for 48 hr. (**b**) qPCR analysis of the tibialis anterior from muscle-specific PGC-1α transgenic mice (MCK-α). (**c**) Quantification of 3-HIB in Ct-CM and α-CM generated from the C2C12 differentiation media for 48 hr. (**d**) qPCR analysis of C2C12 myotubes transfected with adenovirus expressing GFP or PGC-1β for 48 hr. (**e**) qPCR analysis of C2C12 myotubes transfected with lentivirus expressing Cas9 only (Control) or Cas9 with a PGC-1α targeting guide RNA (PGC-1α Crispr KO). The transfected cells were selected with puromycin and non-silent indel mutations of PGC-1α were confirmed by genomic DNA sequencing. (**f**) qPCR analysis of primary myotubes from muscle-specific PGC-1α and PGC-1β double knockout (DKO) mice. (**g**) qPCR analysis of C2C12 myotubes transfected with control (Ct si), *Hibch* (left) or *Hibadh* siRNA (right) for 48 hr. (**h**) qPCR analysis of HUVECs and human skeletal muscle biopsies. Note that HUVECs express much lower levels of valine catabolic genes than skeletal muscle does. (**i**) qPCR analysis of HUVECs transfected with *HIBADH* siRNA for 48 hr. (**j**) FA uptake (2 μM) by ECs treated with vehicle or 3-HIB for 1 hr after transfection with *HIBADH* siRNA for 48 hr. Student's *t*-test; $*P < 0.05$ *vs.* control. Data are mean ± s.d. of at least three biological replicates.



Supplementary Figure 8. FA transport proteins mediate endothelial FA uptake by α-CM or 3-HIB. (**a**) Western blot analysis of HUVECs transfected with control (Ct si), *FATP3* (left) or *FATP4* siRNA (right) for 48 hr. (**b**) FA uptake by HUVECs transfected with *FATP3* (3), *FATP4* (4) or both (3,4) siRNA(s) for 48 hr. (**c**) FA uptake by primary ECs isolated from control or FATP4 knockout (KO) mice. (**d**) qPCR analysis of HUVECs transfected with *CD36* siRNA for 48 hr. (**e**) FA uptake by HUVECs transfected with *CD36* siRNA for 48 hr. (**f**) FA uptake by HUVECs transfected with the indicated siRNA(s) for 48 hr. (**g**) FA uptake by primary ECs isolated from control, CD36 KO or FATP4 KO mice. Bodipy-FA (2 μM) was used for all the FA uptake experiments. (**h**, **i**) qPCR analysis of HUVECs after exposure to the indicated CM (**h**) or 3-HIB (**i**) for 1 hr. (**j**, **k**) Western blot analysis of HUVECs after exposure to the indicated CM for the indicated duration. NS, non-specific band. Student's *t*-test; $^{*}P < 0.05$ *vs.* control; $^{\#}P < 0.05$ *vs.* α-CM alone. Data are mean ± s.d. of at least three biological replicates



Supplementary Figure 9. Muscle PGC-1α/β regulates valine catabolic enzymes and acute feeding of 3-HIB increases FA uptake *in vivo*. (**a**, **b**) qPCR analysis of the quadriceps from muscle-specific PGC-1β transgenic mice (**a**, MCK-β), or muscle-specific PGC-1α and PGC-1β double knockout mice (**b**, DKO). (**c**, **d**) Glucose tolerance tests of control (**c**, $n = 6$) versus MCK-β (**c**, $n = 5$) or control (**d**, $n = 5$) versus DKO (**d**, $n = 8$). (**e**) Triglyceride (TAG) and diglyceride (DAG) levels in the quadriceps from control ($n = 4$) versus DKO ($n = 4$). (**f**–**h**) Measurements of 3-HIB levels in mice fed with vehicle (Veh) or 3-HB for 1.5 hr (**f**). 3-HIB levels in serum (**g**, $n = 2$ and 3, respectively) and muscle (**h**, $n = 4$ and 6, respectively). (**i**, **j**) Representative luminometry images of thigh muscle after removal of the skin (**i**) and quantification of luminescence in the heart (**j**, $n = 4$ per group). Scale bar, 1 cm. (**k**) Quantification of Evans blue in serum and skeletal muscle before or after PBS perfusion of the mice fed with vehicle (Veh) or 3-HIB ($n = 3$ per group). Student's *t*-test; $^*P < 0.05$ *vs.* control. Data are mean ± s.d. (**a**, **b**, **j**, **k**) or mean ±s.e. (**c**–**h**).



Supplementary Figure 10. Metabolic phenotypes of mice fed with 3-HIB for 2 weeks and 3-HIB enzyme expressions in diabetic muscle. (**a**–**f**) 3-HIB levels in muscle (**a**), body weight (**b**), levels of serum free FA (FFA) (**c**), muscle FFA (**d**), serum triglyceride (**e**), and muscle morphology (**f**) in mice provided with 3-HIB in the drinking water for 2 weeks (n = 4 per group). Scale bars, 50 μm. (**g**) Subcellular fractionation of the quadriceps from mice fed with vehicle (Veh) or 3-HIB. (**h**–**k**), Blood glucose levels (**h**), glucose infusion rate (**i**), muscle glucose uptake (**j**), and hepatic glucose output (**k**) measured by hyperinsulinemic euglycemic clamp on mice fed with vehicle (Veh) or 3-HIB (n = 8). Two-way ANOVA analysis; $P < 0.05$ *vs*. control. **l**, qPCR analysis of liver from mice fed with vehicle (Veh) or 3-HIB (n = 8). (**m**) qPCR analysis of muscle from control (Ct, n = 7) or *db*/*db* mice (n = 9). (**n**) Western blot analysis (left) and quantification (right) of HIBADH in muscle from from control (Ct, n = 7) or *db*/*db* mice (n = 5). Student's t-test; $P < 0.05$ *vs*. control. (**o**) qPCR analysis of muscle biopsies from control (Ct, n = 33) or type II diabetic patients (T2D, n = 17). for **i**. Data are mean ± s.d. (**b**–**e**, **k**–**m**) or mean ± s.e. (**a**, **h**–**j**, **n**).

Supplementary Table 1. Primers used for qPCR

gene	Primer sequence
Mouse *Bcat1* forward	CCCATCGTACCTCTTTCACCC
Mouse *Bcat1* reverse	GGGAGCGTGGGAATACGTG
Mouse *Bcat2* forward	CTCATCCTGCGCTTCCAG
Mouse *Bcat2* reverse	TCACACCCGAAACATCCAATC
Mouse *Bckdha* forward	ATCTACCGTGTCATGGACCG
Mouse *Bckdha* reverse	ATGGTGTTGAGCAGCGTCAT
Mouse *Bckdhb* forward	AGCTATTGCGGAAATCCAGTTT
Mouse *Bckdhb* reverse	ACAGTTGAAAAGATCACCTGAGC
Mouse *Bckdk* forward	ACATCAGCCACCGATACACAC
Mouse *Bckdk* reverse	GAGGCGAACTGAGGGCTTC
Mouse *Ppm1k* forward	ATGTTATCAGCGGCCTTCATTAC
Mouse *Ppm1k* reverse	GTGGAGAAGTAGCAGGCAGG
Mouse *Acadsb* forward	TGGGTCGAAGATGTGGATCAG
Mouse *Acadsb* reverse	TCGGTCTACTAAGAAGCAGGTG
Mouse *Hadha* forward	TGCATTTGCCGCAGCTTTAC
Mouse *Hadha* reverse	GTTGGCCCAGATTTCGTTCA
Mouse *Hibch* forward	GTGGAGGCGTCATAACGCTC
Mouse *Hibch* reverse	AGGAATGTGTCAGGGTCTTGT
Mouse *Hibadh* forward	GCAGCGGTGTGTTCTAGGTC
Mouse *Hibadh* reverse	ACACGTCATAGAGGATGAGTGG
Mouse *Vegfa* forward	CTGTAACGATGAAGCCCTGGAG
Mouse *Vegfa* reverse	TGGTGAGGTTTGATCCGCAT
Mouse *Vegfb* forward	GGTTGCCATACCCCACCACC
Mouse *Vegfb* reverse	CCTTGGCAATGGAGGAAGCG
Mouse *Flt1* forward	CCTGCATGATTCCTGATTGGA
Mouse *Flt1* reverse	GCCTAAGCTCACCTGCGG
Mouse *Flk1* forward	GACTTGGTTCATCAGGCTAG
Mouse *Flk1* reverse	GACGCTGTTAAGCTGCTACAC
Mouse *Ppargc1a* forward	AGCCGTGACCACTGACAACGAG
Mouse *Ppargc1a* reverse	GCTGCATGGTTCTGAGTGCTAAG
Mouse *Ppargc1b* forward	CTCCAGGCAGGTTCAACCC
Mouse *Ppargc1b* reverse	GGGCCAGAAGTTCCCTTAGG
Mouse *Fbp1* forward	TATGGTGGAAAGGGACGGGAA
Mouse *Fbp1* reverse	CCTCTGGTGATACTCAAGGATGG
Mouse *G6pc* forward	TGCAAGGGAGAACTCAGCAA
Mouse *G6pc* reverse	GGACCAAGGAAGCCACAATG
Mouse *Pepck* forward	TTGAACTGACAGACTCGCCCT
Mouse *Pepck* reverse	TGCCCATCCGAGTCATGA
Mouse *Ivd* forward	GGACGGCGAGTTTCCAGTT
Mouse *Ivd* reverse	CCACAGGCAATATCGAGTGGG
Mouse *Mccc1* forward	GGGTGATACGAACAGCCAAAA
Mouse *Mccc1* reverse	GGGTGATACGAACAGCCAAAA
Mouse *Auh* forward	TGCTCGGGATTAACAGAGCTT
Mouse *Auh* reverse	TCCACCGCTTTTGATAACATCTT
Mouse *Hmgcl* forward	GGTCTCCCCGGCTAAAGTTG
Mouse *Hmgcl* reverse	GCCAGAGCTTGACCATAGGTAT
Mouse *Hsd17b10* forward	GCTTGGTCGCGGTAGTAACTG
Mouse *Hsd17b10* reverse	TGGGGCAAATATGCAGCTTTC
Mouse *Acat1* forward	CAGGAAGTAAGATGCCTGGAAC
Mouse *Acat1* reverse	TTCACCCCCTTGGATGACATT

Supplementary Table 1. Primers used for qPCR (continued)

gene	Primer sequence
Human *FATP1* forward	TCTATGGGGTGGCTGTTCCA
Human *FATP1* reverse	TCAAACCCTCTCGCTGCA
Human *FATP3* forward	AGAGACCTTCAAACAGCAGAAAG
Human *FATP3* reverse	GTCCAGAACGTACAGTGGGT
Human *FATP4* forward	AGGCAAAGGTGCGACAGTG
Human *FATP4* reverse	CCAGTGGGTATCTGTGCCC
Human *FATP6* forward	ACAACCTCGGAAACCTTTCATC
Human *FATP* 6 reverse	CCCCTTTTTCAGAGAGGAATGG
Human *FABP4* forward	AGCACCATAACCTTAGATGGGG
Human *FABP4* reverse	CGTGGAAGTGACGCCTTTCA
Human *FABP5* forward	TTGGTTCAGCATCAGGAGTG
Human *FABP5* reverse	GATCCGAGTACAGGTGACATTG
Human *FABP-PM* forward	ATTTGGACAAGGAATACCTGCC
Human *FABP-PM* reverse	GCCACTCTTCAAGACTTCGC
Human *CD36* forward	GCCAAGGAAAATGTAACCCAGG
Human *CD36* reverse	GCCTCTGTTCCAACTGATAGTGA
Human *CAV1* forward	CATCCCGATGGCACTCATCTG
Human *CAV1* reverse	CACGGCTGATGCACTGAATCT
Human *PPARG* forward	ACCAAAGTGCAATCAAAGTGGA
Human *PPARG* reverse	AGGCTTATTGTAGAGCTGAGTCT
Human *APLN* forward	GTCTCCTCCATAGATTGGTCTGC
Human *APLN* reverse	GGAATCATCCAAACTACAGCCAG
Human *FLK1* forward	GGCCCAATAATCAGAGTGGCA
Human *FLK1* reverse	TGTCATTTCCGATCACTTTTGGA
Human *BCAT1* forward	AGCCCTGCTCTTTGTACTCTT
Human *BCAT1* reverse	CCAGGCTCTTACATACTTGGGA
Human *BCAT2* forward	CGCTCCTGTTCGTCATTCTCT
Human *BCAT2* reverse	CCCACCTAACTTGTAGTTGCC
Human *BCKDHA* forward	CTACAAGAGCATGACACTGCTT
Human *BCKDHA* reverse	CCCTCCTCACCATAGTTGGTC
Human *BCKDHB* forward	TGGAGTCTTTAGATGCACTGTTG
Human *BCKDHB* reverse	CGCAATTCCGATTCCAAATCCAA
Human *BCKDK* forward	GACTTCCCTCCGATCAAGGAC
Human *BCKDK* reverse	CTCTCACGTAGGCCCTCTG
Human *PPM1K* forward	ATAACCGCATTGATGAGCCAA
Human *PPM1K* reverse	CGCACCCCACATTTTCCAAG
Human *ACADSB* forward	GATGGCAAATGTAGACCCTACC
Human *ACADSB* reverse	AAGGCCCGGAGTATCACGA
Human *HADHA* forward	CTGCCCAAAATGGTGGGTGT
Human *HADHA* reverse	GGAGGTTTTAGTCCTGGTCCC
Human *HIBCH* forward	TGGTTCTTGCCAGAAACCTTATG
Human *HIBCH* reverse	GTAGCCACTCGAAATTGCCCA
Human *HIBADH* forward	TGCTGCCCACCAGTATCAATG
Human *HIBADH* reverse	GCAGGATCAATAGTGCTGGAATC