

Gut bacteria enriched in obese mice produce leucic acid to counter obesity through HCAR2

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Abstract

Gut microbiota modulates a wide range of physiological processes in the host. However, whether host genes shape microbial communities to affect host physiology remains poorly explored. Using *Aida*^{-/-} mice that develop obesity due to increased intestinal fat absorption, we identify two *Clostridium* strains enriched in their fat-depleted gut environment. We demonstrate that the growth of these strains is suppressed by oleate both *in vivo* and *in vitro*, mirroring their selective expansion. Paradoxically, these strains protect against diet-induced obesity. Integrated metabolomics and ¹³C-tracing reveal that these strains biosynthesise leucic acid, whose administration reduces adiposity and enhances lean mass and exercise capacity by suppressing lipid synthesis and promoting catabolism. By combining computational prediction with transcriptomic and human genetic evidence, we identify HCAR2 as a candidate receptor for leucic acid and confirm this by demonstrating direct binding and showing that HCAR2 is required for metabolic effects of leucic acid *in vitro* and in adipose tissue *in vivo*. We thus establish a homeostatic host-microbiome feedback loop wherein a host gene-defined metabolic niche selects for microbes that produce a signalling metabolite, which in turn activates a physiologically relevant receptor to suppress obesity.

Main

Coevolution between hosts and their resident microbes has fostered a mutual dependence¹⁻³, in which the physiology in the host affects microbial communities, and vice versa⁴⁻⁷. Environmental perturbations, such as diet⁸, antibiotic exposure⁹ and cold stress¹⁰, can elicit changes in this symbiosis and trigger a wide range of physiological changes in the host, including alterations in lipid metabolism, immune function and energy balance. In addition, emerging evidence shows that host genetics may have a critical impact on the microbiome composition¹¹⁻¹⁵. For example, genome-wide association studies (GWAS) have linked the *ABO* blood group and *FUT2* polymorphisms with luminal acetylgalactosamine (GalNAc) dynamics, which enrich *Faecalibacterium prausnitzii*¹⁶⁻¹⁹. Similarly, intestinal HIF-2α regulates lactate levels to influence *Bacteroides vulgatus* and *Ruminococcus torques*²⁰. These studies have emphasised luminal metabolites as central mediators of the crosstalk between host genetic background and gut microbes. However, mechanistic links connecting host genetic background with resulting phenotypes and microbial composition/functions remain poorly defined.

We previously established *Aida* as an anti-thrifty gene that limits intestinal lipid absorption and enhances energy expenditure to counter fat-induced obesity^{21,22}. We hypothesised that fat depletion from the chyme resulting from *Aida* deletion would create an environment that favours specifically adapted bacterial taxa. Here, through systematic culturing of *Aida*^{-/-} cecal content, we isolated two hitherto unidentified anaerobes, designated lipid-depletion-enriched (LDE)-1 (an *Oscillospira* strain) and LDE-2 (a tentative new genus within *Lachnospiraceae*), both exhibiting oleate-sensitive growth suppression. Intriguingly, these strains function as feedback metabolic regulators, as administering either strain attenuated diet-induced obesity, suggesting a host-elicited microbial adaptation to counteract lipid

dysregulation in *Aida*^{-/-} mice. Metabolomic analysis revealed that both strains biosynthesise leucic acid (α-hydroxyisocaproic acid or 2-hydroxy-4-methylpentanoic acid) via conserved 2-hydroxyisocaproate dehydrogenases (HicDHs), which are responsible for the anti-obesity effects. Mechanistically, we identified the membrane receptor hydroxycarboxylic acid receptor 2 (HCAR2) as the receptor for leucic acid, which signals to suppress adipose lipid anabolism and promote catabolism. These findings define a feedback host-microbe-metabolite loop, in which host metabolic defects resulting from a genetic alteration reshape the gut microbiome, leading to the production of specific metabolites that rebalance host metabolism.

Results

AIDA depletion enriches specific gut bacterial species linked to adiposity

To determine whether *Aida* depletion alters gut microbiota composition in the presumably altered chyme, we performed 16S rRNA gene sequencing on cecal contents from high-fat diet (HFD)-fed WT and *Aida*^{-/-} mice (Supplementary Table 1). The overall bacterial composition at the phylum level did not change significantly in *Aida*^{-/-} mice (Extended Data Fig. 1a). We also determined the richness and evenness of the bacterial species and found no significant differences (Extended Data Fig. 1b-f). We then assessed the beta diversity of operational taxonomic units (OTUs) using principal coordinate analysis (PCoA). Again, the *Aida*^{-/-} samples exhibited a similar bacterial profile to that of the WT group (Fig. 1a). These data indicate that the deletion of *Aida* does not systematically disturb gut microbiota.

However, we observed that 6 out of the 271 OTUs identified in the microbiota profiling exhibited significant abundance changes in *Aida*^{-/-} mice (Fig. 1b). OTU68 (*Anaerotruncus colihominis*), OTU209 (unclassified member of the *Lachnospiraceae* family), and OTU228 (unclassified member of the genus of *Clostridium*) were enriched, while OTU21 (*Holdemanella massiliensis*), OTU59 (*Adlercreutzia mucosicola*), and OTU141 (unclassified members of the genus of *Ruminiclostridium*) were decreased (Fig. 1b and Extended Data Fig. 1g). Strikingly, analysis of metagenomic data from 355 normal-weight and 631 obese human fecal samples (Supplementary Table 2) revealed that OTU68 (*A. colihominis*) was significantly less abundant in obese compared to normal-weight individuals (Fig. 1c) and inversely correlated with body mass index (BMI) in obesity (Fig. 1c). Species phylogenetically related to other *Aida*-related OTUs, including *Holdemanella filiformis* for OTU21 and *Adlercreutzia equolifaciens* for OTU59, showed no BMI association despite being mildly reduced in obesity (Extended Data Fig. 1h,i). In mice, OTU209 abundance in cecal contents strongly correlated with fat mass in *Aida*^{-/-} background (Pearson $r = 0.9369$, $P = .0059$) (Fig. 1d; Extended Data Fig. 1j). These data suggest that OTU68 and OTU209 are associated with the development of obesity.

To isolate bacterial strains linked to *Aida*-associated metabolic phenotypes, we cultured cecal content from HFD-fed *Aida*^{-/-} mice under stringent anaerobic conditions (0% O₂), targeting the obligate anaerobes OTU68 and OTU209. Using nine distinct media, we obtained 254 isolates, resolving 30 taxonomically

characterised species and more than 20 novel species based on 16S rRNA sequence identity thresholds (Extended Data Fig. 1k; Supplementary Table 3). Among these, strains LDE-1, which represents OTU68, and LDE-2, which represents OTU209, were prioritised for further functional studies. Scaffolded genome sequencing revealed that LDE-1 represented a new strain of *A. colihominis* (family *Oscillospiraceae*; Supplementary note 1), while LDE-2 represented a putative new genus within the family *Lachnospiraceae* (Supplementary note 2). Whole-genome phylogeny corroborated these classifications, confirming the novelty of LDE-2 at the genus level.

To define how *Aida* depletion reshapes the luminal lipid microenvironment to favour these strains, we quantified cecal fatty acid profiles via GC-MS. This revealed a significant reduction in oleate (C18:1) and palmitate (C18:0) in *Aida*^{-/-} mice compared to WT controls (Fig. 1e). In contrast, laurate (C12:0), myristate (C14:0), stearate (C18:0), arachidate (C20:0), and arachidonate (C20:4) did not show significant change, indicating selective depletion of specific luminal LCFAs. Strikingly, *in vitro* growth assays demonstrated that oleate potently inhibited the growth of LDE-1 and LDE-2 at concentrations as low as 10 μ M. As a comparison, *Bacteroides uniformis*, which did not show changes in abundance in the gut, remained resistant to oleate (Fig. 1f). In comparison, palmitate, stearate, and myristate, as well as short-chain (acetate, propionate, and butyrate) and medium-chain fatty acids (caprylate and decanoate), exhibited no suppressive effect (Fig. 1g-j). Critically, *in vivo* validation via gavage of oleate-rich olive oil recapitulated these findings, markedly reducing the abundance of OTU68 and OTU209 in *Aida*^{-/-} mice (Fig. 1k). These data demonstrated that accelerated intestinal lipid absorption in *Aida*^{-/-} mice depletes luminal LCFAs, particularly oleate, thereby alleviating the growth suppression of lipid-sensitive anaerobes and promoting their enrichment.

LDE-1 and LDE-2 ameliorate diet-induced obesity and metabolic dysfunction in mice

We next investigated whether the obesity-associated enrichment of LDE-1 and LDE-2 reflects a maladaptive bystander effect or a protective feedback response. To this end, we administered these strains to HFD-fed WT mice with prior subsection to antibiotic (ABX)-treatment (Fig. 2a). Strikingly, monocolonization with either strain robustly attenuated body weight gain, with LDE-1- and LDE-2-treated mice exhibiting a 10% reduction relative to controls at 8 weeks post-gavage (Fig. 2b). In contrast, the probiotic *Escherichia coli* Nissle 1917 (EcN) showed no significant anti-obesity effect (Extended Data Fig. 2a), underscoring the specificity of LDE-1 and LDE-2. At 5 weeks post-gavage, LDE-1 and LDE-2 administration markedly reduced gonadal white adipose tissue (gWAT) and inguinal WAT (iWAT) mass compared to controls (Fig. 2c,d). Histological analysis confirmed marked reductions in adipocyte hypertrophy in both gWAT and iWAT (Fig. 2e-h and Extended Data Fig. 2b-e). Notably, neither strain induced muscle wasting. LDE-2 even increased soleus muscle mass by 18% (Fig. 2i,j). Treadmill testing revealed a significant increase in running distance and prolongation of time to exhaustion in LDE-2-treated mice (Fig. 2k,l), while both strains improved grip strength (Fig. 2m). Importantly, there were no substantial changes in serum alanine transaminase (ALT) or blood urea nitrogen (BUN) levels (Fig. 2n,o), suggesting these strains did not cause liver or renal damage. In addition, food intake among the groups was similar (Fig. 2p), indicating that the observed anti-obesity effects are not caused by a consequence

of bacterial gavage-induced anorexia. These results demonstrated that LDE-1 and LDE-2 uncouple obesity resistance from muscle wasting, a critical limitation of conventional weight-loss therapies.

We further explored the long-term effects of LDE-2, which appeared to exert more beneficial effects than LDE-1, in mice maintained on HFD for 16 weeks following the last gavage. Notably, LDE-2 sustained 30% lower body weight gain (Extended Data Fig. 2f) and improved fasting glycemia (Fig. 2q). Hepatic steatosis was attenuated, with 22% reduced liver weight and steatosis scores reduced to 0 (Fig. 2r-t). A mild increase in rectus femoris muscle mass further underscored its musculoskeletal benefits (Extended Data Fig. 2g).

Leucic acid is increased by LDE-1 and LDE-2 strains

To identify factors mediating the metabolic effects of LDE-1 and LDE-2, we performed untargeted metabolomics on pre- and post-culture media using ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS). Focusing on metabolites commonly upregulated by both strains, we identified six metabolites that were upregulated in both LDE-1 and LDE-2 cultures, including leucic acid, butyric acid, N-methyltryptamine, thymine, 4-hydroxy-3-methoxycinnamaldehyde and Tyr-Pro (Fig. 3a-c; Supplementary Table 4,5).

To validate the *in vivo* enrichment of these metabolites, we analysed cecal contents of HFD-fed mice using gas chromatography-mass spectrometry (GC-MS). After testing trimethylsilyl (TMS), *tert*-butyldimethylsilyl (TBDMS), and sequential (TMS followed by trifluoroacetyl) derivatization approaches, we selected TMS based on peak resolution, sensitivity, and reproducibility for leucic acid, the most markedly changed metabolite, while maintaining compatibility with the detection of a broad spectrum of other metabolites (Extended Data Fig. 3a). Indeed, *Aida*^{-/-} mice exhibited higher leucic acid abundance compared to WT controls (Extended Data Fig. 3b,c), confirming that AIDA depletion drives accumulation of this microbially-derived metabolite. Critically, structurally related hydroxy acids (2-hydroxybutyric acid, 3-hydroxybutyric acid, and 2-hydroxy-3-methylbutyric acid) and leucine, the precursor for leucic acid, were unchanged in *Aida*^{-/-} mice (Extended Data Fig. 3d-g). To assess non-hydroxylated SCFAs, we used derivatisation-independent GC-MS, and found that butyric acid together with propionic acid, pentanoic acid, isobutyric acid, 2-methylbutanoic acid, and isovaleric acid, remained unchanged or even decreased (Extended Data Fig. 3h-m), underscoring the specificity of leucic acid induction among SCFA family. In addition, N-methyltryptamine, thymine, 4-hydroxy-3-methoxycinnamaldehyde, and Tyr-Pro were not detectable using either approach, consistent with their much lower confidence score compared to leucic acid and butyric acid in the untargeted LC-MS data (Supplementary Table 4,5). These data establish that AIDA-deficient mice enrich LDE-1 and LDE-2 that produce leucic acid.

Leucic acid phenocopies the beneficial effects of LDE-1 and LDE-2 in mice

To determine whether leucic acid mediates the anti-obesity effects of LDE-1 and LDE-2, we supplemented drinking water with 50 mM leucic acid, a dose in line with prior studies of branched-chain amino acid derivatives and short-chain fatty acids²³⁻²⁷. Leucic acid robustly attenuated HFD-induced

weight gain in both WT and *Aida*^{-/-} mice (Extended Data Fig. 4a). Strikingly, WT mice receiving leucic acid maintained body weights comparable to those of normal chow diet (NCD)-fed controls, achieving a 17% reduction in adiposity by week 12 (Fig. 3d). Consistent with the impact observed in LDE-1- or LDE-2-gavaged mice, gWAT and iWAT mass decreased by 46% and 50%, respectively (Fig. 3e,f; Extended Data Fig. 4b-e). Like administration of LDE-1 or LDE-2, leucic acid treatment did not cause muscle wasting (Extended Data Fig. 4f,g), accompanied by larger myofiber cross-sectional areas in the quadriceps (Extended Data Fig. 4h,i), longer distance to exhaustion on the treadmill, and greater grip strength (Fig. 3g,h). The enhanced grip strength was also observed in *Aida*^{-/-} mice administered leucic acid, although their exercise endurance was not significantly changed (Extended Data Fig. 4j-l). Notably, leucic acid did not elevate levels of ALT or BUN in WT or *Aida*^{-/-} mice, indicating no hepatorenal toxicity (Extended Data Fig. 5a-d). However, unlike LDE-2, leucic acid failed to improve fasting glycemia or reduce hepatic steatosis (Extended Data Fig. 5e,f), suggesting additional mediators in LDE-2 contribute to long-term metabolic benefits.

To assess whether leucic acid has an impact on systemic energy metabolism to reduce adiposity and confer other benefits, WT mice were subjected to metabolic cage analysis. The leucic acid-treated mice exhibited a pronounced reduction in respiratory quotient (RQ) (Extended Data Fig. 5g), indicative of preferential lipid oxidation over catabolism of carbohydrate or amino acid. Leucic acid also induced an increase in basal rectal temperature (Fig. 3i), suggesting elevated heat production. These metabolic alterations occurred independently of changes in food intake, locomotor activity (Extended Data Fig. 5h,i), or faecal lipid excretion (Extended Data Fig. 5j,k), excluding malabsorption or anorexia as confounding factors.

Bacterial HicDHs drive leucic acid production and are phylogenetically restricted in human gut clades

To identify the enzymes responsible for leucic acid production, we performed Conserved Domain (CD) Searches²⁸ in strains LDE-1 and LDE-2. For reference, genes in these newly sequenced genomes were assigned arbitrary numeric identifiers during annotation. Both encode a branched-chain-amino-acid aminotransferase (BCAT) homolog for converting L-leucine to 2-ketoisocaproic acid (KIC) (Extended Data Fig. 6a,b). We identified six putative 2-hydroxyacid dehydrogenases (HicDHs) capable of reducing KIC to leucic acid (Supplementary Table 6,7). Phylogenetic analysis separated these into two distinct clusters. The proteins encoded by *GM000059* and *GM003503* in LDE-1, and *GM002896*, *GM003422*, and *GM003150* in LDE-2, formed a clade with various known D-HicDHs related proteins. The protein encoded by *GM003311* in LDE-1 grouped with L-HicDH family proteins (Extended Data Fig. 6c). AlphaFold-predicted structures confirmed conservation, with low root mean square deviation (RMSD) values (0.928-1.907 Å) to reference enzymes (Extended Data Fig. 7a), and key nicotinamide adenine dinucleotide (NAD⁺) co-substrate binding residues were strictly conserved (Extended Data Fig. 7b-g). Functional validation in *E. coli* revealed that only three HicDHs, *GM003311* (LDE-1), *GM003150* (LDE-2), and *GM003422* (LDE-2), produced deuterated leucic acid from L-leucine-5,5,5-D₃ (Extended Data Fig. 8a), despite being expressed at similar levels (Extended Data Fig. 8b). Enantiomer-specific triple quadrupole

GC-MS analysis confirmed strict stereochemical partitioning. LDE-1 produced >98% L-leucic acid, while LDE-2 produced >98% D-leucic acid (Extended Data Fig. 8c,d), consistent with phylogenetic predictions.

We assessed the human relevance of this pathway by probing the Unified Human Gastrointestinal Genome (UHGG) database²⁹, and identified 27,237 putative homologs ($\geq 50\%$ similarity, $\geq 80\%$ coverage) across 288 species. A high-confidence subset (344 homologs, $\geq 70\%$ similarity) was enriched within 13 species from the *Lachnospiraceae* and *Ruminococcaceae* families, matching the lineages of LDE-2 and LDE-1, respectively (Extended Data Fig. 8e,f and Supplementary Table 8). This indicates leucic acid production is a specialised trait enriched in specific gut clades.

Genomic analysis confirmed that this reductive pathway dominates in both strains, as we found no homologs for alternative routes such as leucine 2,3-aminomutase (LAM) or the branched-chain 2-keto acid dehydrogenase (BCKDH) complex (Extended Data Fig. 6a). This absence correlates with our observation of unchanged isobutyric acid and isovaleric acid levels (the end products of those pathways) in bacterial culture media (Supplementary Table 4,5) and the reduced levels of these acids in the ceca of *Aida*^{-/-} mice (Fig. 3l,n). These findings establish HicDH-mediated reductive catabolism as the dominant leucine metabolic pathway in both strains, explaining their selective leucic acid production and metabolic effects.

Leucic acid suppresses lipid storage through metabolic rewiring and enantioselective catabolic activation

To elucidate the metabolic mechanism of leucic acid, we performed integrated proteomic and transcriptomic profiling of gWAT from leucic acid-treated WT mice. Multi-omics analysis identified 40 significantly downregulated candidates ($|\log_2(\text{fold change})| > 0.5$, $P < 0.05$) enriched in lipid anabolic pathways, including *triglyceride biosynthesis* (Reactome: R-MMU-75109), *fatty acid biosynthesis* (GO:0006633) and *lipid storage* (GO:0019915) (Fig. 4a and Supplementary Table 9,10). Critically, leucic acid suppressed key enzymes catalysing triglyceride synthesis (DGAT2, MOGAT2) and fatty acid synthesis (ACSS2, FASN, SCD2 and ELOVL6) (Fig. 4b,c and Extended Data Fig. 9a). These findings were recapitulated in *Aida*^{-/-} gWAT transcriptomes (Extended Data Fig. 9b-d and Supplementary Table 11) and validated by immunoblotting in both genotypes (Fig. 4d,e), confirming AIDA-independent suppression of lipid storage.

In iWAT, leucic acid administration upregulated 14 genes in both WT and *Aida*^{-/-} iWAT, enriched for pathways linked to muscle-like metabolic activation, such as *muscle contraction* (Reactome: R-MMU-397014, GO:0006936), *myofibril* (GO:0030016) and *sarcomere* (GO:0030017) (Extended Data Fig. 9e and Supplementary Table 12,13), indicative of adipose tissue beiging³⁰. Strikingly, Gene Set Enrichment Analysis (GSEA) of *Aida*^{-/-} iWAT demonstrated amplified thermogenic activation, with robust induction of *thermogenesis* (NES = 2.7437, adjusted $P < .0001$) and *fatty acid degradation* (NES = 2.3071, adjusted $P < .0001$) (Fig. 4f and Supplementary Table 14). These transcriptomic changes were biochemically validated by an increase in UCP1 protein abundance in both genotypes, correlating with the leucic acid-

induced elevation of basal core body temperature (Fig. 3i), alongside a coordinated downregulation of lipid anabolism proteins DGAT2 and FASN via immunoblotting (Fig. 4g,h).

We chose 50 μ M, which was within the supplemented range *in vivo* (Extended Data Fig. 10a-g), to investigate direct cellular mechanisms. At this dose, leucic acid enantiomers significantly reduced lipid accumulation in oleate-challenged MEFs, with both enantiomers decreasing lipid stores by $\sim$ 60% in WT and $\sim$ 70% in *Aida*^{-/-} cells (Fig. 5a,b). Notably, this potent lipid-lowering effect occurred despite the 1.9-fold higher lipid content in *Aida*^{-/-} MEFs, which is consistent with AIDA being able to reduce lipid absorption²¹ and suggests an AIDA-independent activity of leucic acid.

We next investigated the catabolism of fatty acids using [U-¹³C]oleic acid tracing. In beige-like adipocytes, treatment with D-leucic acid significantly enhanced isotopic enrichment in α -ketoglutarate (36%) and citrate (47%), while L-leucic acid showed a weaker increase in α -ketoglutarate labelling (23%) and no citrate effect (Fig. 5c). This enantioselectivity was also found in *Aida*^{-/-} MEFs. Both forms increased labelled α -ketoglutarate (D: 14%; L: 16%) and malate (D: 34%; L: 33%), but only D-leucic acid dramatically elevated proline (53%), a derivative of α -ketoglutarate (Extended Data Fig. 11a-d). Critically, unchanged total metabolite pools (Extended Data Fig. 11e-h) excluded biosynthesis alterations, instead implicating enhanced fatty acid-derived acetyl-CoA flux into the TCA cycle.

Functional validation via oxygen consumption assays revealed that D-leucic acid markedly elevated respiration rate in WT and *Aida*^{-/-} MEFs, while L-leucic acid induced subtler increases (Fig. 5d). Collectively, leucic acid exerts AIDA-independent suppression of lipid storage across adipose depots and genotypes, while exhibiting enantiomer-selective amplification of lipid catabolism in thermogenic adipocytes and *Aida*^{-/-} cells, with a mechanistic divergence between gWAT and iWAT at physiologically relevant concentrations (Fig. 5e).

HCAR2 mediates leucic acid-induced suppression of lipid accumulation

To identify the molecular target through which leucic acid regulates lipid metabolism, we performed integrative target prediction for L- and D-enantiomers using complementary computational approaches, TargetNet employing deep learning on 3D features³¹ and SwissTargetPrediction utilising similarity-based machine learning³² (Supplementary Table 15). Intersection analysis revealed 13 consensus targets (Fig. 6a), mitigating the single-platform biases common in endogenous metabolite studies, with both enantiomers exhibiting full compliance with Lipinski's Rule of Five³³ (Extended Data Fig. 12a), indicating favourable drug-likeness.

To prioritise these candidates based on human genetic evidence, we screened the GWAS Catalog³⁴, which uncovered 44 obesity-associated intergenic or regulatory region SNPs near *HCAR2* (*Hydroxycarboxylic Acid Receptor 2*). In contrast, only one SNP was found near *GRM2* (*Glutamate Metabotropic Receptor 2*), and no significant associations were observed for other candidates (Fig. 6b and Supplementary Table 16). Transcriptomic analysis of adipose tissue from leucic acid-treated mice

identified *Hcar2* and *Car4* (*Carbonic Anhydrase 4*) as the sole genes that were significantly differentially expressed across both absolute abundance change and fold-change metrics (Fig. 6c). This convergence of genetic association and transcriptional responsiveness prioritised HCAR2 for mechanistic validation (Fig. 6d).

Molecular docking suggested potential binding of HCAR2 for both D-leucic acid ($\Delta G \approx -5.2$ kcal/mol) and L-leucic acid ($\Delta G \approx -5.3$ kcal/mol) (Fig. 6e), with binding affinities comparable to established ligands niacin ($\Delta G \approx -5.9$ kcal/mol) and β -hydroxybutyrate ($\Delta G \approx -4.8$ kcal/mol) calculated under identical parameters (Extended Data Fig. 12b). We experimentally validated receptor engagement using ^{19}F -NMR ligand-detected binding assay^{35,36}. Incubation of 4-fluoro-leucic acid with HCAR2-expressing cells induced a pronounced chemical shift ($\Delta\delta = 0.08$ ppm), indicative of ligand-receptor engagement (Fig. 6f). By contrast, no shift occurred in HCAR1-expressing cells, confirming the specificity of HCAR2.

The requirement of HCAR2 to mediate the role of leucic acid was further investigated through loss-of-function experiments. Leucic acid enantiomers reduced lipid accumulation in beige-like adipocytes and oleate-loaded MEFs expressing control shRNA, but this effect was abolished in *Hcar2*-knockdown cells (Fig. 6g,h and Extended Data Fig. 12c-f). Similarly, *Hcar2* knockdown eliminated leucic acid-stimulated oxygen consumption (Fig. 6i). For *in vivo* validation, we knocked down *Hcar2* via AAV in one side of the iWAT, using the contralateral side injected with control shRNA as an internal control (Fig. 6j and Extended Data Fig. 12g). While *Hcar2* knockdown alone did not alter baseline iWAT mass, leucic acid administration induced significant mass reduction and pronounced browning in control shRNA-treated iWAT. Crucially, the same treatment resulted in significantly attenuated fat mass reduction and diminished browning in contralateral *Hcar2*-knockdown tissue relative to control tissues within the same animals (Fig. 6k,l). Collectively, these findings establish HCAR2 as the cognate receptor for leucic acid in mediating the suppression of lipid storage (Extended Data Fig. 12h).

Discussion

Our study reveals that the textural changes of chyme, caused by depletion of luminal fat as a result of AIDA deficiency, can critically influence microbial growth and metabolite production, which in turn plays a significant role in regulating host physiology. Specifically, the absence of AIDA in mice resulted in the selective enrichment of oleate-suppressed *Clostridia* strains LDE-1 and LDE-2. Paradoxically, these strains exert anti-obesity effects, as we found that gavage with LDE-1 or LDE-2 ameliorates obesity in mice fed a high-fat diet, forming a host-directed adaptive loop where microbial expansion counteracts host metabolic dysfunction. Mechanistically, we identified leucic acid as a key bioactive metabolite produced by HicDHs in these strains. Leucic acid counters obesity and enhances exercise performance. Moreover, we show that leucic acid suppresses lipid storage via divergent regulation of lipid anabolism and catabolism across adipose depots mediated by HCAR2.

The selective enrichment of these beneficial strains is likely driven by a specific ecological niche, as varied abilities of the host to absorb different nutrients can alter the contents of residual nutrients in the

chyme. Our analyses *in vitro* on the two strains LDE-1 and LDE-2 showed high sensitivity of the LDE strains to oleate, consistent with their enrichment in the fat-depleted gut of *Aida*^{-/-} mice. Thus, AIDA deficiency, by promoting lipid absorption through ERAD-mediated degradation of re-esterification enzymes (GPAT3, MOGAT2 and DGAT2)²¹, creates a lipid-depleted luminal environment that favours the growth of oleate-sensitive bacteria. This model is further supported by the selective reduction of oleate and palmitate in the *Aida*^{-/-} cecum, likely reflecting the preference of intestinal triglyceride synthesis enzymes for these acyl-CoAs^{37,38}. Although dietary fatty acids generally exhibit antimicrobial properties, the particular sensitivity of LDE-1 and LDE-2 to oleate, a trait shared by bacteria like *Lactobacillus iners*³⁹, coupled with their resistance to SCFAs or MCFAs that bypass intestinal re-esterification, defines a unique ecological niche.

Leucic acid has been previously identified as a microbial metabolite that shows beneficial effects on metabolic dysfunction-associated steatohepatitis (MASH)⁴⁰. We have found that leucic acid production by LDE-1 and LDE-2 represents a previously unrecognised mode of host-microbe crosstalk emerging from the changed luminal environment resulting from the genetic mutation of the host. The lipid-depleted conditions in *Aida*^{-/-} gut create an ecological niche analogous to time-restricted feeding⁴⁰. Our data show that leucic acid exerts striking metabolic benefits across both WT and *Aida*^{-/-} genetic backgrounds, including reduction of adiposity and enhancement of exercise capacity. These effects stem from the combined roles of suppression of lipid synthesis in gWAT and promotion of lipid catabolism and thermogenesis in iWAT.

The physiochemical properties of leucic acid salts facilitate HCAR2 engagement through efficient aqueous diffusion enabled by their hydrophilicity (TPSA 57-60 Å², logP -0.8567 to 0.478). This mechanism gains human relevance from GWAS linking intergenic variants near the *HCAR2* locus to obesity traits. Although physiological levels of leucic acid are modest in humans, these genetic associations likely represent conserved signalling through endogenous HCAR2 ligands such as β-hydroxybutyrate⁴¹ and niacin⁴², positioning this pathway as an evolutionarily conserved regulator of energy homeostasis. Taken together, we have presented an exemplary mode of host-microbe relationship, in which the host physiological state can influence the community of gut microbes that exert feedback controls on the host.

Methods

Animals and ethics

All experiments utilised male C57BL/6J mice. Homozygous *Aida*^{-/-} mice and wild-type (WT) littermate controls were generated by crossing heterozygous *Aida*^{-/-} parents as described previously²¹. WT mice administered LDE-1, LDE-2, or leucic acid were sourced from the Specific Pathogen-Free (SPF) facility at the Laboratory Animal Center of Xiamen University (Xiamen, China). For metabolic studies, 8-week-old male mice were fed a high-fat diet (HFD; D12492, Research Diets, New Brunswick, NJ, USA; 60% kcal

from fat, primarily lard and soybean oil) ad libitum and housed under controlled conditions (temperature: 22-24 °C; humidity: 55-60%; light/dark cycle: 12 h, lights on at 8:00 AM; enrichment: nesting materials replaced weekly). All animal procedures were approved by the Xiamen University institutional animal care and use committee (Approval No. XMULAC20220188).

Cell culture

Beige-like adipocytes were derived from 3T3-L1 preadipocytes (ATCC CL-173) via a beige protocol. Cells were maintained in an induction medium (DMEM/F12 1:1 medium with 10% FBS containing 5 µg/mL insulin (BS901, Biosharp, Hefei, China), 1 nM 3,3',5-triiodo-L-thyronine (T3) (T-2877, Sigma-Aldrich, Taufkirchen, Germany), 125 µM indomethacin (S1723, Sigma-Aldrich), 0.5 mM isobutylmethylxanthine (IBMX) (I5879, Sigma-Aldrich) and 1 µM dexamethasone (DEX) (D4902, Sigma-Aldrich)) for 2 days. Cells were switched to maintenance medium (DMEM/F12 1:1 medium with 10% FBS supplemented with 5 µg/mL insulin and 1 nM T3) for 6 days, with medium refreshed every 48 h. Differentiated beige-like adipocytes were confirmed by multilocular lipid droplet formation. MEFs were maintained in DMEM with 10% FBS.

Analysis of human faecal metagenomic data

Publicly available metagenomic datasets (accession codes: CNP0003084, PRJEB12123)⁴³ were analysed, comprising 355 normal-weight (BMI 18.5-24.9) and 631 obese (BMI > 30) individuals. Taxon abundances were expressed as relative abundances, calculated by dividing the read count of each taxon by the total read count across all taxa. Zero values were substituted with the detection limit (1×10^{-6}), and $\log_{10}$ transformation was applied. Covariates (sampling batch, sex, age) were assessed but showed no significant association with taxon abundances, thus, no adjustments were made.

Gut microbiota analysis by 16S rRNA sequencing

Cecal content samples were collected from 6 homozygous *Aida*^{-/-} mice and 6 WT littermates after 4 months of HFD feeding. Genomic DNA was extracted using the MagBeads Fecal DNA Kit (116570400, MP Biomedicals, Santa Ana, CA, USA). The V3-V4 hypervariable regions of bacterial 16S rRNA genes were amplified and analysed using an Illumina HiSeq2500 platform and sequenced on an Illumina HiSeq2500 platform (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China). Sequences with $\geq 97\%$ similarity were assigned to the same operational taxonomic units (OTUs). Microbial alpha diversity indexes were obtained from the free online platform of the Majorbio Cloud Platform (www.majorbio.com). The 16S rRNA sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) GenBank database under BioProject accession number PRJNA1311729.

Quantitative PCR (qPCR)

DNA from cecal content was extracted as above. Specific primers were designed for the amplification of fragments within 16S rRNA genes of OTU68 and OTU209, as well as universal primers targeting the 16S rRNA gene for normalization purposes⁴⁴. The specificity of these primers was validated through in silico analysis and confirmed experimentally using known positive controls. OTU68: 187 bp product (F: 5'-GCGTCCGATTAGCCAGTTGG-3'; R: 5'-GCCTGCCTCTACTTCACTCAAG-3'). OTU209: 227 bp product (F: 5'-GCGTGGGTGAAGAAGCGCC-3'; R: 5'-TCTTGGAACTGCGTGGCTG-3'). Universal 16S: 177 bp product (F: 5'-ACTCCTACGGGAGGCAGCAGT-3'; R: 5'-ATTACCGCGGCTGCTGGC-3'). The qPCR was performed in triplicate using ChamQ SYBR qPCR Master Mix (Q312, Vazyme Biotech, Nanjing, China). Primer specificity was confirmed by Sanger sequencing of amplicons. Relative abundances were calculated using the $2^{-\Delta\Delta C_t}$ method normalised to universal 16S rRNA.

Isolation of AIDA depletion-related bacterial strains

Culture media preparation

Nine agar plate formulations were used for anaerobic bacterial isolation: modified Gifu anaerobic medium (mGAM) and yeast casitone fatty acids broth (YCFA), with or without 1 g/L taurocholic acid sodium salt, respectively. mGAM was made by supplementing GAM with 10% bovine rumen fluid. Modified chopped meat medium (CMM), TSA medium (with 5% defibrinated sheep blood), rumen bacteria medium (TPY), modified reinforced clostridial medium (RCM) and modified PYG medium were also used. Media were autoclaved (121 °C, 20 min), and 5% sheep blood was added post-sterilisation. Plates were pre-reduced in an anaerobic workstation (D500G, Gene Science, Wilmington, DE, USA; atmosphere: 85% N₂, 5% CO₂, 10% H₂) for at least 24 h before use. Strict anaerobic conditions of liquid media were generated by using an intelligent anaerobic culture preparation system (DLQ120-B, Beijing Ai Si Pu Technology Co., Ltd., Beijing, China).

Cecal sample processing and anaerobic cultivation

Cecal contents from 6-month HFD-fed *Aida*^{-/-} mice were divided into two aliquots. Ethanol-treated fraction: Resuspended in 1 mL sterile PBS, incubated in 70% ethanol (4 h, 25 °C), and filtered (0.22 µm) to remove host cells and debris. Sequential 10-fold dilutions (10⁻³ to 10⁻⁶) were plated (100 µL/plate). Untreated fraction: Directly diluted in PBS and plated. The plates were incubated at 37 °C in the anaerobic chamber (D500G, GeneScience). Plates were incubated at 37 °C under anaerobic conditions for up to 30 days. Colonies were monitored at 2, 4, 6, 8, 10, 15, 20, and 30 days, with six morphologically distinct colonies per plate isolated and subcultured for 2-7 days. Colony morphology and spatial distribution were documented to avoid redundancy.

Bacterial identification via 16S rRNA gene sequencing

Single colonies were lysed in 3 µL of NaOH/SDS buffer (0.8% NaOH, 1% SDS), diluted in 67 µL ddH₂O, boiled (95 °C, 30 min), and centrifuged (5000 g, 5 min). Supernatants were used as templates for PCR amplification of near-full-length 16S rRNA genes using universal primers⁴⁵: 27F: 5'-

AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTTACGACTT-3'. PCR products (~1,500 bp) were Sanger-sequenced (Sangon Biotech Co., Ltd., Shanghai, China). Sequences were analysed using NCBI BLAST against a custom database of *Aida*-related OTUs, the Nucleotide Collection (nr/nt) and the 16S rRNA database (Bacteria and Archaea). Taxonomic assignments followed established thresholds with $\geq 98.7\%$ identity to reference strains as the same species.

LDE-1 and LDE-2 were deposited under the Budapest Treaty at the China General Microbiological Culture Center (CGMCC), Beijing, China. The assigned deposit numbers are CGMCC No.46618 for LDE-1 and CGMCC No.46619 for LDE-2. These strains are available for research purposes from CGMCC under the terms of the Material Transfer Agreement.

Negative stain electron microscopy

Bacterial isolates were resuspended in 20 μ L of 0.1 M phosphate buffer. A portion of this suspension was carefully applied onto a carbon/formvar-coated grid and allowed to adhere for 15 s, blotted off and stained with 1% uranyl acetate for 15 s. Grids were air-dried for 3 h at 25 °C and imaged on a Hitachi HT-7800 transmission electron microscope (HT-7800, Hitachi, Tokyo, Japan). Images were captured at magnifications ranging from 4,000 $\times$ to 10,000 $\times$ at 80 kV.

Bacterial genome sequencing and assembly

DNA extraction and library preparation

Genomic DNA was extracted from 1 g of pure LDE-1 or LDE-2 cultures and subjected to bacteria draftmap analysis by Novogene Co., Ltd. (Beijing, China). DNA was randomly sheared into short fragments, end-repaired, A-tailed, and ligated with an Illumina adapter. The DNA fragments with adapters were size-selected, PCR amplified and purified. The library was checked with Qubit and real-time PCR for quantification, and a bioanalyzer for size distribution detection.

Sequencing and quality control

Paired-end sequencing (2 $\times$ 150 bp) was performed on an Illumina NovaSeq 6000 platform (Novogene Co., Ltd., Beijing, China). The reads with > 40% low-quality bases (mass value ≤ 20), with > 10% unrecognised nucleotides (N), or adapter contamination (15 bp overlap, < 3 mismatches).

Genome Assembly and Annotation

The clean data were assembled with SOAPdenovo, SPAdes, and ABySS software. Contigs from all assemblies were integrated with CISA software and polished with GapCloser software to fill gaps. Final scaffolds (> 500 bp) were annotated using GeneMarkS. Functional annotation involved performing a whole-genome Blast search against GO and KEGG databases. Genome assemblies were deposited in NCBI GenBank under BioProject accession PRJNA1311729.

Strain classification and phylogenetic analysis

Taxonomic assignment of the strains was performed using a dual approach: 16s rRNA sequence analysis and the Genome Taxonomy Database Toolkit (GTDB-Tk). Full-length 16S rRNA sequences, extracted from whole-genome assemblies, were first queried against the GenBank/ENA/DDBJ and EzBioCloud databases (<https://ezbiocloud.net/>) to identify closest relatives. Sequence alignment was executed using ClustalX (v. 1.838), and phylogenetic trees were constructed using the neighbour-joining algorithm in MEGA11⁴⁶ with bootstrap support based on 1,000 replicates. For higher-resolution classification, draft genome assemblies were analysed with GTDB-Tk (v. 1.7.0) via the KBase platform (<https://narrative.kbase.us/>). This workflow leverages 120 conserved bacterial marker genes from the Genome Taxonomy Database (GTDB, v. R06-RS202), which integrates curated RefSeq and Genbank reference genomes. Strains were taxonomically assigned by placement into a domain-specific reference phylogeny, refined using relative evolutionary divergence (RED) thresholds and pairwise average nucleotide identity (ANI) values relative to GTDB reference genomes^{47,48}. All GTDB-Tk analyses were finalised on 2024-05-08.

Bacterial growth inhibition assays by fatty acids

Fatty acid solution preparation

Sodium salt solutions were prepared according to chain length. Acetate (S818277, Macklin Biochemical Technology, Shanghai, China), propionate (S817368, Macklin Biochemical Technology), and butyrate (S817488, Macklin Biochemical Technology) were dissolved in ddH₂O to prepare 100 mM stocks, 0.22 µm-filtered, and assayed at 5 mM. Caprylate (S6096, Macklin Biochemical Technology), decanoate (629-25-4, MedChemExpress, Shanghai, China), and myristate (822-12-8, MedChemExpress) were dissolved in 2% DMSO in ddH₂O to prepare 10 mM stocks, sonicated or vortexed, 0.22 µm nylon-filtered (84311ES03, Yeasen Biotechnology, Shanghai, China), and tested at 100 µM. Palmitate (S161420, Aladdin Scientific, Shanghai, China), stearate (822-16-2, Aladdin Scientific), and oleate (S817542, Macklin Biochemical Technology) were added into mGAM media, autoclaved to dissolve, tested at 10 µM or 100 µM. All solutions were validated precipitate-free. NaCl controls (matched to experimental concentrations) were prepared equivalently.

Bacterial growth analysis

LDE-1, LDE-2 and *Bacteroides uniformis* were inoculated in 96-well plates (1 mL per well) or jars (40 mL per jar) containing fatty acid working solutions or controls. LDE-1 was inoculated at a density of OD₆₀₀ = 0.6 and LDE-2 at a density of OD₆₀₀ = 0.15 to mGAM media. Cultures were normalised to an identical starting OD₆₀₀. Media pH was verified to be pH 7.0 ± 0.2 pre-inoculation. Plates or jars were maintained in an anaerobic chamber (D500G, GeneScience) throughout incubation. Growth was monitored at 24 h intervals by measuring OD₆₀₀. Background absorbance was corrected by subtracting cell-free supernatant OD₆₀₀ from total readings. Post-assay culture purity was confirmed via 16S rRNA sequencing.

Bacterial preparation and oral administration

Two-month-old male mice were randomised into experimental groups based on body weight and housed under SPF conditions. Mice were fed HFD for 3-5 days, followed by broad-spectrum antibiotic (ABX) treatment (1 g/L ampicillin, 0.5 g/L vancomycin, 1 g/L neomycin, 1 g/L metronidazole in drinking water) for 5-7 days to deplete gut microbiota.

The LDE-1 culture was inoculated into fresh mGAM and cultured anaerobically (85% N₂, 5% CO₂, 10% H₂) at 37 °C for 48 h. After incubation, the culture was centrifuged (10,000 *g*, 8 min). The supernatant was discarded, and the bacterial pellet was washed with sterile PBS. The bacteria were then resuspended in fresh mGAM to achieve OD₆₀₀ = 1.3. The control group received the same volume of fresh mGAM administered by oral gavage, once every three days for ten administrations. The preparation of LDE-2 was similar to that of LDE-1, with the following modifications. The culture was inoculated at a 1:25 dilution, incubated for 60 h, centrifuged at 6,000 × *g* for 5 min, and resuspended to achieve OD₆₀₀ = 0.6. The control group received the same volume of sterile PBS administered by oral gavage, once daily for 14 days. The *Escherichia coli* Nissle 1917 (EcN) culture was inoculated at a 1:50 dilution into fresh LB liquid medium and incubated in a shaker at 37 °C for 18-22 h. The culture was then centrifuged at 6,000 × *g* for 5 min. The supernatant was discarded, and the bacterial pellet was washed with sterile PBS. The bacteria were resuspended in sterile PBS to achieve an OD₆₀₀ = 1.3. The control group received the same volume of sterile PBS administered by oral gavage, once daily for 10 days. After the last gavage, the mice that received LDE-1 were kept for an additional 10 days, while those that received LDE-2 were observed for 16 weeks before sacrifice to minimise any acute effects of gavage. The mice were fasted for 6 h (8:30 a.m. to 2:30 p.m.) before sacrifice.

Administration of leucic acid in mice

Two-month-old male mice were divided into two comparable groups. HFD feeding started at least 2 days before the leucic acid administration. DL-leucic acid (S161215, Aladdin Scientific, Shanghai, China) was solubilised in the drinking water to make a final concentration of 50 mM leucic acid (pH = 2.5). The drinking water was acidified using HCl (ADW) to the same pH as the control mice. The drinking water was replaced with distilled water containing 50 mM leucic acid or ADW every 5 days. The mice were fasted for 6 h (8:30 a.m. to 2:30 p.m.) before sacrifice.

Biochemical analysis

Blood and faecal parameters were measured in mice after a 6-h fast (8:30 a.m. to 2:30 p.m.). Blood glucose was quantified by a glucose meter (OneTouch UltraVue, Johnson & Johnson, New Brunswick, NJ, USA). Commercial kits were utilised to determine the levels of triglyceride (TAG, A110-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), non-esterified fatty acids (NEFA, A042-1-1, Nanjing Jiancheng), alanine aminotransferase (ALT, C009-2-1, Nanjing Jiancheng) and blood urea nitrogen (BUN, C013-2-1, Nanjing Jiancheng) in accordance with the manufacturer's protocols.

Measurement of basal core body temperature

Following one week of leucic acid or ADW administration to mice acclimated to HFD for 5 days, basal core body temperature was measured. Using a calibrated rectal probe, temperatures were recorded between 14:30 and 15:30 after a 4-h fast. Two independent measurements were taken per mouse at 48 h intervals, ensuring consistent probe insertion depth of 1.5 cm and a dwell time of 30 seconds.

Mass-spectrometry-based metabolomics for bacterial secreted metabolites

Sample preparation

Untargeted metabolomics was performed on culture media from LDE-1 and LDE-2 strains (Metware Biotechnology Inc., Wuhan, China). Strains were grown in modified GAM medium to the exponential phase. Pre- and post-culture media were collected. A 150 μ L extraction solution (acetonitrile: methanol = 1:4, v:v) containing 1 μ g/mL of 2-chlorophenylalanine (internal standard) was added into a 50 μ L sample. Then the sample was vortexed (3 min), centrifuged (12,000 g, 10 min, 4 °C). Supernatant was collected and incubated at -20 °C (30 min), recentrifuged (12,000 g, 3 min, 4 °C). 120 μ L supernatant was aliquoted for LC-MS.

LC-MS analysis

LC-MS analysis was performed using UHPLC (Shimadzu Nexera LC-30A, Kyoto, Japan) and MS (SCIEX TripleTOF 6600+, Foster City, CA, USA) in positive/negative ionisation modes. Waters ACQUITY Premier HSS T3 Column (1.8 μ m, 2.1 mm $\times$ 100 mm) using 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B in the following gradient: 5 to 20% in 2 min, increased to 60% in the following 3 min, increased to 99% in 1 min and held for 1.5 min, then come back to 5% mobile phase B within 0.1 min, held for 2.4 min. The analytical conditions were as follows: column temperature, 40 °C; flow rate, 0.4 mL/min; injection volume, 4 μ L. Mass spectrometry data acquisition was operated using information-dependent acquisition (IDA) mode using Analyst TF software v. 1.7.1 (SCIEX, Concord, Canada). The source parameters (ion source Gas 1/Gas 2: 50 psi; curtain gas: 25 psi; temperature: 550 °C; ion spray voltage floating: 5000 V or -4000 V; decluttering potential: $\pm$ 60 V). TOF-MS scan parameters (mass range: 50-1000 Da; accumulation time: 200 ms; and dynamic background subtract: on). Product ion scan parameters (mass range: 25-1000 Da; accumulation time: 40 ms; collision energy: $\pm$ 30; collision energy spread: 15; resolution: UNIT; charge state: 1 to 1; intensity: 100 cps; exclude isotopes within 4 Da; mass tolerance: 50 ppm; maximum number of candidate ions to monitor per cycle: 18).

Data Processing

Raw data were converted to mzXML format by ProteoWizard software. Peak extraction, alignment, and retention time correction were performed in XCMS program. Peak areas normalised using support vector regression (SVR). Low-abundance peaks (< 50% detection rate per group) were excluded. Metabolic identification information was obtained by searching in-house, public, and AI-predicted databases and

MetDNA web server. The metabolomics data have been deposited to MetaboLights⁴⁹ repository with the study identifier MTBLS12947.

Determination of leucic acid concentration by GC-MS

The concentrations of leucic acid in mouse faeces, serum, and tissues were measured using GC-MS. To optimise this determination, different derivatisation approaches and different capillary columns were compared.

Sample preparation involved homogenising approximately 50 mg of tissue or cecal content in a 20-fold volume of 80% (v:v) methanol aqueous solution containing 10 µg/mL 2-hydroxyoctanoic acid as an internal standard. The homogenate was centrifuged at 18,000 × *g* and 4 °C for 15 min. A 200 µL aliquot of the supernatant was dried using a vacuum centrifugal concentrator. The residue was reconstituted in 50 µL pyridine (270407, Merck, Darmstadt, Germany) via sonication. Derivatisation was then performed using one of three methods. The first method involved derivatisation with 50 µL N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, M85387, Meryer, Shanghai, China) at 37 °C for 1 h to form trimethylsilyl (TMS) derivatives. The second method involved sequential derivatisation, first with 50 µL MSTFA at 37 °C for 1 h, followed by 50 µL N-Methyl-bis(trifluoroacetamide) (MBTFA, N814244, Macklin, Shanghai, China) at 37 °C for 1 h to form TMS and trifluoroacetyl (TFA) derivatives. The third method used 50 µL N-*tert*-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA, M-108, Merck, Darmstadt, Germany) at 55 °C for 1 h to form *tert*-butyldimethylsilyl (TBDMS) derivatives. All derivatisation reactions occurred in a heating block. Following derivatisation, samples were centrifuged at 18,000 × *g* for 5 min, and the supernatant was transferred to GC vials for analysis.

For the comparison of low-polarity (HP-5MS) and mid-polarity (DB-1701) capillary columns, TMS-derivatised samples prepared as described were analysed. Both GC-MS methods employed a 1 mL/min helium carrier gas flow rate, a 1 µL injection volume, a 10:1 split ratio, and a 250 °C injector temperature. However, the temperature programs were adjusted to account for the different column characteristics and ensure appropriate elution of the leucic acid di-TMS derivative. The HP-5MS method used an initial hold at 70 °C for 2 min, ramped at 7 °C per min to 140 °C, then ramped at 30 °C per min to 310 °C with a final hold for 9 min. The DB-1701 method used an initial hold at 80 °C for 2 min, ramped at 5 °C per min to 140 °C, then ramped at 10 °C per min to 200 °C, followed by a ramp at 30 °C per min to 290 °C with a final hold for 9 min. Detection was performed in combined scan/selected ion monitoring (SIM) mode. The SIM ions were set at *m/z* 103, 159, 233, and 261, with a solvent delay of 7 min.

Indirect calorimetry

Mice were acclimated to individual housing in metabolic chambers (CAB-16-1-EU, Sable Systems International, North Las Vegas, NV, USA) for 3 days to minimise stress from environmental changes. Following acclimation, oxygen consumption (VO₂), carbon dioxide production (VCO₂), and respiratory exchange ratio (RER) were measured over 48 h at 5-min intervals using high-resolution sensors. Food

intake was estimated daily per cage and normalised to individual mouse intake by dividing total cage consumption by the number of mice.

Body composition analysis

Fat and lean mass were quantified in conscious mice using a 3-in-1 EchoMRI Composition Analyzer (100H, Echo Medical Systems, Houston, TX, USA). Triplicate measurements per mouse were performed and averaged to ensure precision.

Muscle performance assessments

Grip strength test

Forelimb grip strength was measured using a digital force meter (47200, Ugo Basile, Gemonio, Italy). The maximum grip strength was measured for each mouse, with claws of forelimbs held against the device. Each mouse was tested three times to ensure reliable data collection.

Treadmill exhaustion test

A motorised treadmill (47300, Ugo Basile, Gemonio, Italy) was used to assess endurance. Mice were fasted for 1 h prior to an acclimatisation phase on the treadmill two days before the formal test (5° incline, 5 m/min speed). The formal test mirrored the acclimatisation protocol but with a higher speed limit (initial speed = 5 m/min, increased by 1 m/min until reaching 25 m/min and held constant). Exhaustion was defined as the mouse remaining on the electrified rear of the treadmill (> 5 s) despite mild stimulation. Total distance and time to exhaustion were recorded.

Structure prediction and alignment for bacterial HicDHs

Putative 2-hydroxyisocaproate dehydrogenase (HicDH) structures from LDE-1 and LDE-2 were modelled using AlphaFold2⁵⁰. Predicted structures were aligned to experimentally resolved templates (PDB IDs: 1DXY [*Lactobacillus casei* D-2-HicDH]⁵¹, 1HYH [*Weissella Confusa* L-2-HicDH]^{52,53} in PyMOL (v. 3.1.6.1, Schrödinger, New York, NY, USA). Root-mean-square deviation (RMSD) values were calculated by PyMOL using the entire protein structure.

Phylogenetic analysis of putative HicDHs

A neighbour-joining⁵⁵ tree was constructed in MEGA11⁴⁶. Each of the protein sequences of the six putative HicDHs from LDE-1 and LDE-2 was used for BLAST of 3 proteins with top total scores, which belong to 3 different genera within NCBI *refseq_protein*. Three well-characterised HicDHs, including L-2-HicDH of *Weissella confusa*, D-2-HicDH of *Lactobacillus casei*, and LDHD of *Mus musculus*, were additionally included. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches⁵⁵. The evolutionary distances were

computed using Poisson correction^{56,57} distances with complete deletion of gaps/missing data (279 positions retained).

Cloning of putative 2-hydroxyacid dehydrogenases

Putative 2-hydroxyacid dehydrogenases were identified through in silico screening of predicted proteins encoded by the draft genomes of strains LDE-1 and LDE-2. A batch conserved domain (CD)-Search against the NCBI CDD database (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>)²⁸ revealed six candidates annotated as 2-hydroxyisocaproate dehydrogenase (HicDH), HicDH-like proteins, or 2-hydroxyacid dehydrogenases. Coding sequences for these candidates were codon-optimised for heterologous expression in *Escherichia coli* using ExpOptimizer (NovoPro, <https://www.novoprolabs.com/tools/codon-optimization>), chemically synthesised (Tsingke Biotech, Beijing, China), and cloned into the expression vector pGEX-4T-1 for downstream functional characterisation.

Determination of the enantiomeric excess of leucic acid

Enantiomeric excess analysis was performed with modifications to a previously described method⁵⁸. Strains were cultured in mGAM medium and 4-Methyl-2-oxovaleric acid (1 mM, M104530, Aladdin Scientific, Shanghai, China), adjusted to pH 7.0 with NaOH, was added 12 h prior to sampling. Leucic acid was analysed as methyl esters by a trimethylsilyldiazomethane methylation approach. The methylation procedure was as follows. Leucic acid was extracted three times with diethyl ether after acidification with 0.2 M HCl. Pooled ether layers were dried under nitrogen gas. Residues were reconstituted in 90 μ L methanol:ether (2:7, v:v), cooled to 4 °C, and reacted with 10 μ L trimethylsilyldiazomethane (T140538, Aladdin Scientific) for 3 h at 25 °C. The resulting leucic acid methyl esters, along with a commercial racemic mixture standard (D9509, TargetMol Chemicals, Shanghai, China), were quantified using a CP-Chirasil-Dex CB column (25 m $\times$ 0.25 mm $\times$ 0.25 μ m) on an GC-MS system (Agilent 8890B GC system and 7000D MSD system, Agilent Technologies, Santa Clara, CA, USA) using helium at 1 mL/min (constant flow). Temperature program was as follows: 80 °C (2 min), ramp to 114 °C at 2 °C/min, 194 °C at 10 °C (hold 3 min). Injection was performed with split mode (10:1), 1 μ L volume. DL-leucic acid-methyl ester (T9509, TargetMol Chemicals, Shanghai, China) served as the external standard. Data were analysed using MassHunter Qualitative Analysis software v. 10.0 (Agilent Technologies).

Stable isotope tracing of leucic acid production

Bacterial culture and isotope labelling

E. coli BL21 harbouring pGEX-4T-1-HicDHs were grown in LB medium (with 100 μ g/mL ampicillin) at 37 °C to OD₆₀₀ = 0.6. Protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), followed by incubation for 3 h at 37 °C. After centrifugation, the cells were resuspended to OD₆₀₀ = 0.4 in fresh LB containing 1 mM L-leucine-5,5,5-D₃ (HY-N0486S9, MedChemExpress) and cultured for 3 h.

Metabolite extraction and derivatisation

A 500 µL aliquot of the culture supernatant was dried using centrifugal freeze-drying and extracted with 200 µL methanol, sonicated (30 s, 20 kHz, 40 W) and centrifuged (18,000 *g*, 10 min). The supernatant (50 µL) was transferred to a glass vial, dried under nitrogen gas, and derivatised by sequential treatment with 50 µL pyridine containing 20 mg/mL methoxyamine hydrochloride (89803, Sigma-Aldrich; 37 °C, 1.5 h; for methoximation of carbonyl groups) and 50 µL N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA; M-132, Merck, Darmstadt, Germany; 37 °C, 1 h; for trimethylsilylation).

GC-MS analysis

Derivatised samples were analysed using a quadrupole GC-MS system (Agilent 7890B GC system and 5977B MSD System) equipped with an HP-5ms capillary column (30 m × 0.25 mm × 0.25 µm; Agilent Technologies). Helium carrier gas was maintained at a constant flow rate of 1 mL/min. The temperature program was as follows: 70 °C (2 min), ramp to 140 °C at 7 °C/min, then ramp to 310 °C at 30 °C/min (hold 9 min). Samples (1 µL) were injected in split mode (10:1 ratio). Electron impact ionisation (70 eV) was performed with mass spectra acquired in full-scan mode (*m/z* 50-650). Data were analysed using MassHunter (Agilent Technologies). Isotopomer distributions were quantified by integrating ion chromatograms (leucic acid: *m/z* 159; leucic acid-5,5,5-D3: *m/z* 162) corresponding to the trimethylsilylated leucic acid fragment, normalised to an external calibration curve generated using synthetic leucic acid-5,5,5-D3. The standard was synthesised via diazotisation of d3-leucine as previously described⁵⁹.

Tissue proteomic analysis by mass spectrometry

Gonadal WAT (gWAT) of 8-week-old male mice were used for proteomic analysis. Mice were adapted to HFD-feeding for one week, following administration of 50 mM leucic acid in drinking water or acidified water with equal pH as a control. Mice were euthanised 2 weeks post-treatment, and tissues were immediately removed for preparation of protein extracts. Tissues were homogenised in 1% cholic acid (V900388, Sigma-Aldrich) at a 1:3 (w/v) tissue-to-buffer ratio using a homogeniser. Homogenates were sonicated (15 cycles, 30 Hz) and clarified by centrifugation at 14,000 × *g* for 15 min at 4 °C. The lipid layer was carefully discarded, and the infranatant was transferred to a new tube and centrifuged again under identical conditions. The final protein supernatant was collected, and its concentration was determined using BCA assay.

For each sample, 10 µg of protein was subjected to in-solution trypsin digestion. Dried peptide samples were reconstituted in 10 µL of 0.1% formic acid. Peptide concentration was estimated by measuring OD₂₁₄. Subsequently, 400 ng of peptides from each sample were separated online using a nanoElute nanoflow LC system coupled to a timsTOF HT mass spectrometer (Bruker corporation, Billerica, MA, USA) equipped with a CaptiveSpray ion source. Peptides were loaded onto a homemade C18 column (75 µm inner diameter × 35 cm, 1.9 µm particle size, 100 Å pore size) and eluted with a 60-min linear gradient from 3% to 35% acetonitrile in 0.1% formic acid at a constant flow rate of 300 nL/min.

Mass spectra were acquired in data-independent acquisition parallel accumulation serial fragmentation (DIA-PASEF) mode. Raw data files were processed using Spectronaut 17.0 (Biognosys) in a directDIA+ mode against the UniProtKB *Mus musculus* proteome database (release 2023_01). Search parameters were set as follows: enzyme, trypsin/P; specificity, full; maximum missed cleavages, 2; fixed modification, carbamidomethylation (C); variable modifications, oxidation (M) and protein N-terminal acetylation. Cross-run normalisation was enabled based on the total peptide signal. All other parameters were set to the software default. Missing values were imputed with the lower detection limit, and log₂-transformed intensities were used for fold-change calculation and volcano plot generation. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<https://proteomecentral.proteomexchange.org>) via the iProX partner repository^{60,61} with the dataset identifier PXD068013.

Transcriptomic profiling of adipose tissues

gWAT and iWAT of 8-week-old male WT and *Aida*^{-/-} mice were subjected to transcriptomic analysis. RNA-seq was performed by Novogene (Beijing, China) on the Novaseq platform (Illumina, San Diego, CA, USA) with paired-end 150 bp reads. Raw reads were trimmed of adapter sequences and filtered to remove low-quality bases (Phred score < 20) and contaminants using FastQC (v0.11.9). High-quality reads were aligned to the GRCm39 mouse reference genome (*Mus musculus*) using HISAT2 (v2.2.1), and transcript counts were generated with featureCounts (v2.0.6) using the following flags: featureCounts -p --countReadPairs -t exon -g gene_id. Differential gene expression analysis was performed in R (v4.3.2) using the *limma-voom* pipeline. Genes with |log₂(fold change)| > 0.5 and *P* < 0.05 were classified as significantly differentially expressed. The RNA-sequencing data have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE306938.

Functional enrichment of differentially expressed genes

Differentially expressed genes (DEGs) were defined as those with |log₂(fold change)| > 0.5 and *P* < 0.05. DEGs underwent functional enrichment analysis using ShinyGO (v. 0.82)⁶² with the following parameters: enrichment fold FDR < 0.05, pathway size 2-500 genes, organism database *Mus musculus*. Selected enriched GO terms and Reactome⁶³ pathways were visualised. Gene set enrichment analysis (GSEA)⁶⁴ was performed on the full expression dataset using the gseKEGG function in clusterProfiler R package (v4.8.3). To ensure statistical robustness, we conducted the analysis with the default number of permutations while maintaining default weight settings. After the analysis, we used gseaplot2 function to visualise the selected enrichment patterns.

Immunoblot analysis of adipose tissues

Gonadal and inguinal WAT from HFD-fed male mice treated with 50 mM leucic acid in drinking water for 6 weeks were minced and homogenized in ice-cold RIPA lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate,

1 mM β -glycerophosphate) supplemented with 0.1% SDS and Phosphatase Inhibitor Cocktail I/II (HY-K0021/ HY-K0022, MedChemExpress), and Protease Inhibitor Cocktail (B14002, Selleck, Shanghai, China) using a tissue homogenizer (OSE-TH-02, TIANGEN, Beijing, China) at 7 m/s linear velocity for 5 cycles (40 s run/20 s pause per cycle). Homogenates were sonicated and centrifuged. Protein concentrations in the supernatant were determined by BCA assay (20201ES86, Yeasen, Shanghai, China), and proteins were denatured in SDS sample buffer at 70 °C for 15 min. 15 μ g protein per lane were resolved by SDS-PAGE and analysed via immunoblotting. Primary antibodies were ACSS2 (1:3,000, 16087-1-AP, RRID: AB_2878216, Proteintech, Wuhan, China), DGAT2 (1:1,000, 17100-1-AP, RRID: AB_2918049, Proteintech), FABP4 (1:10,000, 15872-1-AP, RRID: AB_2102440, Proteintech), FASN (1:5,000, A22462PM, RRID: AB_2862543, ABclonal, Wuhan, China), MOGAT2 (1:500, 19514-1-AP, RRID: AB_10638917, Proteintech) and UCP1 (1:1,000, 72298, RRID: AB_2936479, Cell Signaling Technology, Danvers, MA, USA). ACSS2, FASN and FABP4 antibodies have been validated by knockdown/knockout, DGAT2 and MOGAT2 antibodies by overexpression positive controls, and UCP1 antibody by tissue-specific expression. Secondary antibodies were goat anti-rabbit IgG HRP (1:5,000, 31460, RRID: AB_228341, Thermo Scientific, Waltham, MA, USA) and goat anti-mouse IgG HRP (1:5,000, 31430, RRID: AB_228307, Thermo Scientific). Integrated intensities of protein bands were quantified by ImageJ.

Metabolic flux analysis using [U-¹³C]oleic acid

Metabolic flux analysis was performed in beige-like adipocytes and MEFs using [U-¹³C]oleic acid tracing. For beige-like adipocytes, 3T3-L1 cells were treated with 50 μ M sodium salts of D-leucic acid, L-leucic acid, or NaCl vehicle control throughout differentiation and beigeing induction. MEFs were treated with the same compounds for 24 h. One hour prior to harvest, the medium was replaced with fresh medium containing 250 μ M [U-¹³C]oleic acid (#490431, Sigma-Aldrich, Taufkirchen, Germany) alongside the respective leucic acid enantiomers or control.

After incubation, cells were washed twice with 30 mL ice-cold saline, and culture dishes were flash-frozen in liquid nitrogen for 10 min. Cells were subsequently scraped into pre-chilled Eppendorf tubes using 1 mL ice-cold 80% methanol containing 10 μ g/mL 2-hydroxyoctanoic acid (H862015, Macklin Biochemical Technology) as internal standard. Lysates were sonicated and centrifuged at 12,000 $\times$ g for 10 min at 4 °C. An 800 μ L aliquot of the supernatant was lyophilised to complete dryness.

The dried residue was derivatised by resuspension in 50 μ L pyridine containing 20 mg/mL methoxyamine hydrochloride (89803, Sigma-Aldrich), followed by sonication and incubation at 37 °C for 1.5 h. Next, 50 μ L N-Methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA; M-108, Cerilliant, Round Rock, Texas, USA) was added, and the mixture was incubated at 55 °C for 1 h. After centrifugation, 60 μ L of the derivatised supernatant was transferred for GC-MS analysis.

GC-MS was conducted using an Agilent 7890B/5977B system. Samples (2 μ L) were injected in split mode (1:10 ratio) with an inlet temperature of 250 °C. The GC oven temperature program initiated at 70 °C (2-min hold), ramped to 140 °C at 3 °C/min, then to 310 °C at 10 °C/min. Mass spectrometry operated

in SIM mode, targeting $[M-57]^+$ fragments (loss of *tert*-butyldimethylsilyl group) and isotopologue-specific $M+n$ ions (n = number of ^{13}C atoms) of *tert*-butyldimethylsilyl (TBDMS) derivatives. Isotopic natural abundance was corrected using IsoCor software (v. 2.2.3)⁶⁵.

Integrative target identification using orthogonal computational platforms

Computational target prediction for L- and D-leucic acid enantiomers was performed using two orthogonal algorithmic platforms, Ensemble TargetNet³¹ and SwissTargetPrediction³², to ensure robust identification of potential targets. Ensemble TargetNet is a deep learning model that generates binding probabilities based on three-dimensional molecular features, including ECFP6, MACCS, and Daylight fingerprints, where targets exceeding the probability threshold of 0.7 were retained as high-confidence candidates. Complementary to this, SwissTargetPrediction employs a hybrid approach combining two-dimensional and three-dimensional ligand similarity matching with machine learning algorithms, but due to its systematic underestimation of probability values, only the top 100 ranked targets were considered for further analysis. Lipinski's Rule of Five parameters, which evaluate drug-likeness through molecular weight, hydrogen bond donors, hydrogen bond acceptors, and $\log P$ ³³ were computed directly from the TargetNet framework. The stereospecific structures of both enantiomers were defined using canonical SMILES strings with explicit stereochemistry annotations. L-leucate represented as CC(C)C[C@@H](C(=O)O)O and D-leucate as CC(C)C[C@H](C(=O)[O-])O, which were submitted as input for both prediction platforms to account for chiral specificity in target engagement.

Molecular docking

Molecular docking simulations were performed using AutoDock Vina⁶⁶ (v. 1.2.7) to elucidate the binding interactions between leucic acid enantiomers and the human HCAR2. The HCAR2 (PDB ID: 8H2G) cryo-EM structure was prepared by removing crystallographic water molecules, adding polar hydrogens, and assigning Kollman united-atom charges via AutoDockTools (v. 1.5.7)⁶⁷. Leucic acid enantiomers as well as niacin and β -hydroxybutyrate were energy-minimised using the MM2 force field in Chem 3D (v. 22.0.0, Revvity, Waltham, MA, USA), with Gasteiger charges assigned using AutoDockTools (v. 1.5.7)⁶⁸. A docking grid box ($20 \times 20 \times 20 \text{ \AA}^3$) was centred on the orthosteric binding site (coordinates: $x = 115.895$, $y = 114.032$, $z = 160.702$) to encompass key residues. Vina parameters included an exhaustiveness of 64, energy range of 4 kcal/mol, and 10 binding poses generated per run. The top-scoring pose (lowest predicted ΔG) for each target was selected. All visualisations and interaction analyses were conducted in PyMOL (v. 3.1.6.1, Schrödinger).

Ligand-receptor binding assay via ^{19}F -NMR in live cells

Ligand-receptor interactions were analysed using real-time ^{19}F NMR in live cells, adapted from established protocols^{35,36}. Cells were harvested by EDTA-mediated detachment, washed twice with ice-cold PBS, and resuspended in DMEM supplemented with 2 mM EDTA and 10% OptiPrep (D1556, Sigma-Aldrich). 4-fluoroleucic acid was synthesised via diazotisation of 4-fluoroleucine (BD271440, Bidepharm,

Shanghai, China), purified by following a previously reported protocol⁶⁰. The yield of 4-F-leucic acid from 4-F-leucine was found to be equivalent to the yield of leucic acid from leucine, as determined by GC-MS. The compound was purified via methyl *tert*-butyl ether extraction and dried under nitrogen, dissolved and neutralised with KOH to a 30 mg/mL stock (pH 7.0). For binding assays, 400 μ L cell suspension was mixed with 50 μ L fluorinated ligand, 5 μ L potassium trifluoroacetate (100 mg/mL internal standard), and 50 μ L D₂O, incubated (10 min, 37 °C), and analysed on a high-resolution NMR spectrometer (AVANCE-NEO-600, Bruker, Billerica, USA) equipped with a broadband ¹⁹F probe. Acquisition parameters: 16 scans, 12 μ s pulse width, no decoupling, ambient temperature. ¹⁹F NMR spectra were processed in MestReNova software v. 15.0.0-34764 (Mestrelab Research, Santiago de Compostela, Spain) using automated phase correction, Whittaker smoothing, and internal reference calibration.

Cellular respiration monitoring (Resipher)

Respiration of intact cells under normal culture conditions (37 °C, 5% CO₂) was performed using a non-invasive oxygen consumption rate monitoring system (Resipher 32X, Lucid Scientific Inc., Atlanta, GA, USA)⁶⁸. Briefly, 5×10^3 cells were seeded in 96-well microplates. Cells were treated with 50 μ M D- or L-leucic acid (sodium salt), or NaCl (control) for 36 h. The Resipher oxygen sensing lid was positioned upon the plate before monitoring. Oxygen concentration was measured in each well every minute at 37 °C, and OCR was calculated based on the oxygen concentration during 15 min. Data were exported using the Resipher web application and OCR normalised to cell count.

High-resolution respirometry (Oxygraph-O2k)

High-resolution respirometry with intact cells was performed using the Oroboros Oxygraph-O2k (Oroboros Instruments, Innsbruck, Austria)⁶⁹. 3T3-L1 preadipocytes were seeded into 6-well plate. Cells were grown to confluence and differentiated into mature adipocytes following the protocol as described above, with 50 μ M D- or L-leucic acid (sodium salt), or NaCl as a control in the induction and maintenance media. Following full differentiation, these adipocytes were washed twice with PBS, trypsinised and centrifuged at $300 \times g$ for 5 min, resuspended in DMEM (supplemented with non-essential amino acids, GlutaMax and sodium pyruvate). The Oroboros O2k chambers were pre-oxygenated, and the chamber temperature was set to 37 °C. The oxygen concentration was calibrated before the experiment. An aliquot containing 2×10^6 cells was added to each chamber and allowed to stabilise for 10 min before measurement began. Baseline oxygen consumption rates were recorded. Sequentially, 2.5 μ M oligomycin (s1478, Selleck), 0.5 μ M FCCP (s8276, Selleck), 0.5 μ M rotenone (s2348, Selleck) and 2.5 μ M antimycin A (ab141904, Abcam, Cambridge, UK) were sequentially added into the chambers to characterise maximum mitochondrial oxygen consumption. Data were recorded and analysed using DatLab software (v. 7.4.0.4, Oroboros Instruments). Oxygen consumption rates (OCR) were normalised to the protein concentrations of the respective samples and expressed as pmol O₂/s/mg protein.

Unilateral AAV-mediated *Hcar2* knockdown in iWAT

AAV2/9 vectors were generated for *in vivo* knockdown of *Hcar2* using a triple-plasmid system. The target sequences were *Renilla* as a control are 5'-GCTGTGTGTTCCGAGATGA-3' for murine *Hcar2* and 5'-GTAGCGCGGTGTATTATAC-3' for *Renilla* (control). The transgene plasmid, bearing AAV2 inverted terminal repeats, contained *mU6* promoter for shRNA expression. This was co-transfected into HEK293T cells alongside the pAAV2/9 rep-cap plasmid with sequences coding for REP (from type 2 AAV) and CAP (from type 9 AAV), and the pAdΔF6 adenoviral helper plasmid. Viral particles were harvested 60-72 h post-transfection. Cells underwent three cycles of freeze-thaw lysis (liquid nitrogen/37 °C), followed by precipitation of medium-derived virions with polyethylene glycol (PEG; final concentration of 8 % PEG-8000, 0.5 M NaCl; 4 °C overnight). Lysates and PEG pellets were combined, purified via discontinuous OptiPrep (D1556, Sigma-Aldrich) density gradient ultracentrifugation (17%, 25%, 40%, 60 %), and the 40% fraction was collected. Viruses were washed three times with PBS using 100 kDa ultrafiltration centrifugation (UFC910008, Millipore, Billerica, MA, USA). Genomic titers were quantified by qPCR.

Twelve 6-week-old male mice were housed under standard conditions. Bilateral iWAT depots received differential injections. The right depot was administered 2×10^{11} genomic copies of AAV2/9-*mU6-shRenilla* (control) in 50 µL PBS across four sites, and the left depot received AAV2/9-*mU6-shHCAR2* (knockdown) identically. Following a 12-day recovery period, mice were acclimated to HFD for 4 days. Animals were then randomised into two weight-matched groups. One group received 50 mM leucic acid in drinking water, and the controls received pH-matched acidified water. Tissues were harvested 13 weeks post-injection for analysis.

Statistics and reproducibility

Statistical analyses were performed using GraphPad Prism (v. 9.0, GraphPad Software, La Jolla, CA, USA). Normality was assessed using D'Agostino-Pearson omnibus or Shapiro-Wilk tests to guide test selection. For two-group comparisons, two-tailed unpaired Student's *t*-tests or Mann-Whitney tests were applied (depending on normality). Multi-group comparisons involving a single variable employed one-way ANOVA (parametric) or Kruskal-Wallis tests (non-parametric), with Tukey's, Dunn's or Dunnett's post-hoc corrections as detailed in figure legends. For experiments with two independent variables, two-way ANOVA with Dunnett's or Holm-Šídák's post-hoc testing was utilised. Statistical significance between growth curves was determined by comparing fits using an extra sum-of-squares F-test for curve divergence⁷⁰. To assess the statistical significance of differences among groups in Principal Coordinates Analysis (PCoA) plots, Analysis of Variance Using Distance Matrices (ADONIS), also known as Permutational Multivariate Analysis of Variance (PERMANOVA), was employed by using ClustVis tool⁷¹. Pearson's correlation coefficient (*r*) or Spearman's rank correlation coefficient (ρ) (depending on normality), along with their associated *P*-value, was utilised to evaluate the relationship between two variables. A significance threshold of $P < 0.05$ was applied throughout. All experiments included ≥ 3 biological replicates, with full statistical details provided in figures and source data files.

Declarations

Data availability

The 16S rRNA sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) GenBank database under BioProject accession number PRJNA1311729. Genome assemblies were deposited in NCBI GenBank under BioProject accession PRJNA1311729. The metabolomics data have been deposited to MetaboLights repository with the study identifier MTBLS12947. The RNA-sequencing data have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE306938. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<https://proteomecentral.proteomexchange.org>) via the iProX partner repository with the dataset identifier PXD068013. Statistical source data and image source data are provided with this paper. Bacteria strains LDE-1 and LDE-2, and all other data that support the findings of this study, are available from the corresponding author upon reasonable request. The uncropped immunoblot and microscopy images (PDF) and statistical source data (Excel) have been deposited in a private Figshare repository and can be accessed via this link for review: <https://figshare.com/s/3dea33aa5b47932a9a7b>

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Conceptualisation: SCL and SYL. Methodology: SJL, CL, QZ, CW⁶, FG, ZYC, KW, HYQ, XC, HG, YS, MXZ, CZ, LY, AL, LZ, YW, MZ. Resources: SJL, CL, RL, JR, YS, HX, MZ. Investigation: ZYC, KW, DTL, HYQ, YAY, SHY, LC, MJ, PCZ. Formal analysis: ZYC, KW, DTL, HYQ, YAY, SHY, QQH, CW^{3, 4}, TYL, SYL. Validation: SYL, ZYC, KW, HYQ, YAY. Visualisation: SYL, ZYC, XC, WJW. Writing - Original Draft: SYL, ZYC, HYQ, YAY. Writing - Review & Editing: SCL, SYL, SJL, HG, CL. Supervision: SYL, SCL, SJL, HX. Project administration: SYL, SCL, TYT, WH. Funding acquisition: SCL, SYL, JR, CL, HG, SHY.

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Ethics declarations

Competing interests

The authors declare the following financial interests: The bacterial strains LDE-1 and LDE-2 are the subject of a patent deposition. The findings regarding the use of leucic acid against obesity are also intended for patent protection. The authors may potentially file related patent applications in the future, which could lead to the commercialisation of technology.

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Figures

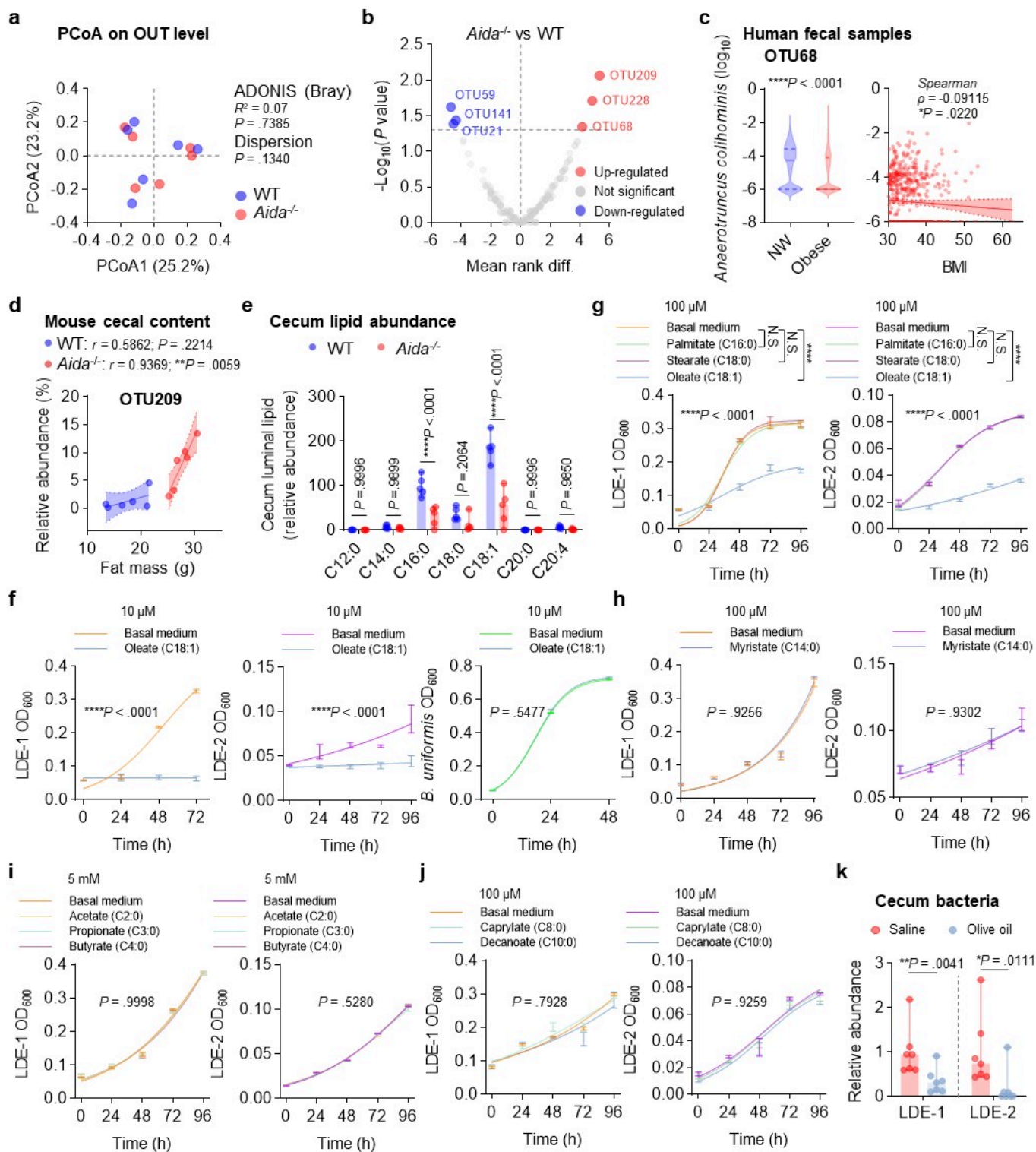


Figure 1

AIDA depletion enriches gut bacterial taxa linked to obesity.

a, Beta-diversity of cecal microbiota in WT and $Aida^{-/-}$ mice visualised by principal coordinates analysis (PCoA) of Bray-Curtis distances at the operational taxonomic unit (OTU) level. Statistical significance was tested by Analysis of Variance Using Distance Matrices (ADONIS).

b, Volcano plot of differentially abundant OTUs (*Aida*^{-/-} vs. WT). Significance of differences was assessed by multiple two-tailed unpaired Mann-Whitney test.

c, Clinical relevance of *Anaerotruncus colihominis* (OTU68). Faecal metagenomic data from 355 normal-weight (BMI 18.5-24.9) and 631 obese (BMI > 30) individuals were analysed. Violin plots (left) depict the relative abundances of *A. colihominis*. Medians (solid lines) and interquartile ranges (dashed lines) are shown. Significance was assessed using two-tailed unpaired Mann-Whitney test. Correlation analyses (right) show Spearman's rank correlation between BMI and taxon abundances in obese individuals. Correlation coefficients (ρ) and *P* values are annotated.

d, Pearson correlation analysis between fat mass (%) and relative abundance of OTU209. Shaded regions indicate 95% confidence intervals (CI). Correlation coefficients (*r*) and *P* values are annotated.

e, Abundance of fatty acid species in the luminal content of the mice cecum. Cecal contents were subjected to GC-MS analysis. The abundances of major fatty acid species in the luminal content were expressed as relative ratios to D-fructose. Data represent individual mouse (*n* = 5) with median $\pm$ 95% CI. Statistical differences were assessed by two-way ANOVA followed by Holm-Šidák's post-hoc testing.

f, Growth kinetics of LDE-1 (left), LDE-2 (middle), and *Bacteroides uniformis* (right) in basal medium with or without 10 μ M sodium oleate. Vertical error bars indicate standard error of the mean (SEM) of three biological replicates. Solid lines denote best-fit logistic growth curves derived from nonlinear regression. Curve differences relative to vehicle controls are assessed by an extra sum-of-squares *F*-test.

g-j, Growth kinetics of LDE-1 and LDE-2 in basal media supplemented with **g**, 100 μ M sodium palmitate (C16:0), stearate (C18:0), or oleate (C18:1); **h**, 100 μ M sodium myristate (C14:0); **i**, 5 mM sodium acetate (C2:0), propionate (C3:0), or butyrate (C4:0); **j**, 100 μ M sodium caprylate (C8:0) or decanoate (C10:0). Vertical error bars indicate SEM of three biological replicates. Solid lines denote best-fit logistic growth curves derived from nonlinear regression. Curve differences relative to vehicle controls are assessed by extra sum-of-squares *F*-test.

k, Olive oil gavage reduced OTU68 and OTU209 in *Aida*^{-/-} mice. Mice received daily oral gavage of 200 μ L saline or olive oil for 14 days. OTU abundances in cecal faeces were quantified by quantitative PCR (qPCR) and normalised to total bacterial 16S rRNA gene levels. Data represent individual mice (*n* = 7 per group), with median $\pm$ 95% CI. Statistical significance was determined by two-tailed unpaired Mann-Whitney test.

*****P* < 0.0001, ***P* < 0.01, **P* < 0.05, N.S. not significant.

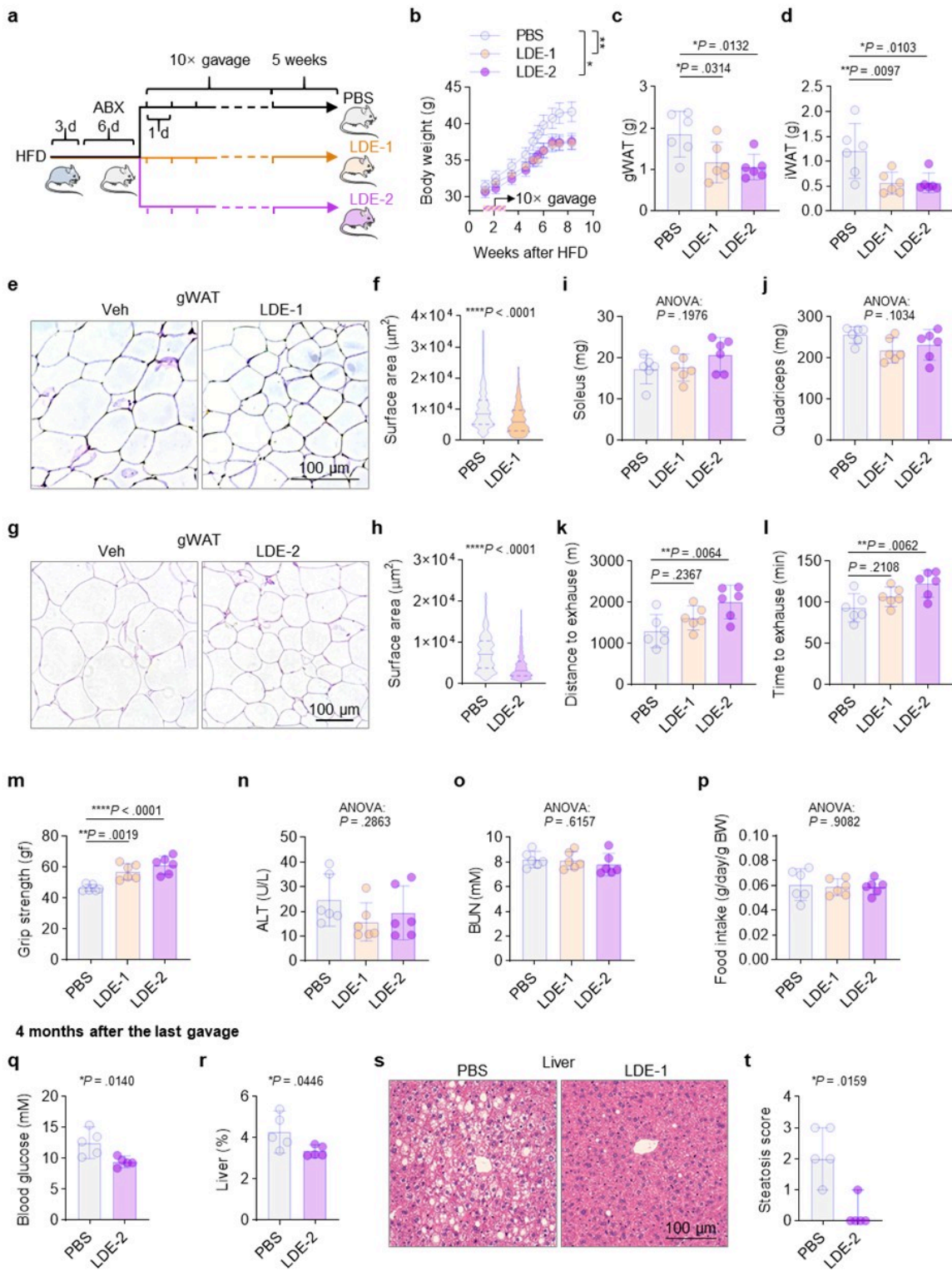


Figure 2

LDE-1 and LDE-2 ameliorate diet-induced obesity and metabolic dysfunction in mice.

a, Experimental design for oral gavage of LDE-1 and LDE-2, or phosphate-buffered saline (PBS) in high-fat diet (HFD)-fed mice.

b, Body weight trajectories of mice treated with LDE-1, LDE-2, or PBS. Slopes were analysed by simple linear regression analysis. Data are mean $\pm$ SEM, $n = 6$ mice per group.

c,d, Mass of inguinal white adipose tissue (iWAT; **c**) and gonadal white adipose tissue (gWAT; **d**). Data represent individual mice ($n = 6$ per group), with mean $\pm$ 95% CI. Differences were assessed by one-way ANOVA followed by Dunnett's test.

e-h, Adipocyte morphology in gWAT. Representative hematoxylin and eosin (H&E)-stained sections from LDE-1- (**e**) or LDE-2-treated in (**g**) mice. Adipocyte cross-sectional areas were quantified for LDE-1 (**f**; $n = 7$ or 8 mice) and LDE-2 (**h**; $n = 5$ mice). Violin plots show median (solid line) and quartiles (dashed lines). Statistical significance was determined by two-tailed unpaired Mann-Whitney test.

i,j, Soleus (**i**) and quadriceps (**j**) muscle mass. Data are individual values ($n = 6$ mice per group) with mean $\pm$ 95% CI. Differences were analysed by one-way ANOVA.

k-m, Exercise capacity assessment. Tolerance to exhaustion (distance, **k**; time, **i**) and forelimb grip strength (**m**). Data represent individual mice ($n = 6$ per group) with mean $\pm$ 95% CI. One-way ANOVA followed by Dunnett's test.

n,o, Serum alanine aminotransferase (ALT; **n**) and blood urea nitrogen (BUN; **o**) levels. Data are individual values ($n = 6$ mice per group) with mean $\pm$ 95% CI. One-way ANOVA.

p, Daily food intake per cage. Food intake was measured per cage (2 cages per group, each housing 3 mice) daily for 3 consecutive days. Individual mouse values were calculated by dividing cage-level intake by the number of mice per cage. Data are estimated individual mouse values with mean $\pm$ 95% CI. One-way ANOVA.

q-s, Long-term metabolic effects of LDE-2 treatment. Fasting blood glucose (**q**) and liver-to-body weight ratios (**r**) at 12 weeks post-treatment. Data are individual values ($n = 5$ mice per group) with mean $\pm$ 95% CI. Differences were assessed by two-tailed unpaired Student's *t*-test. (**s**) Representative H&E-stained liver sections.

t, Hepatic steatosis quantification. Steatosis scores reflect the percentage of hepatocytes containing lipid droplets. Data represent individual mice ($n = 5$ per group), with median $\pm$ 95% CI. Statistical significance was determined by two-tailed unpaired Mann-Whitney test.

**** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$.

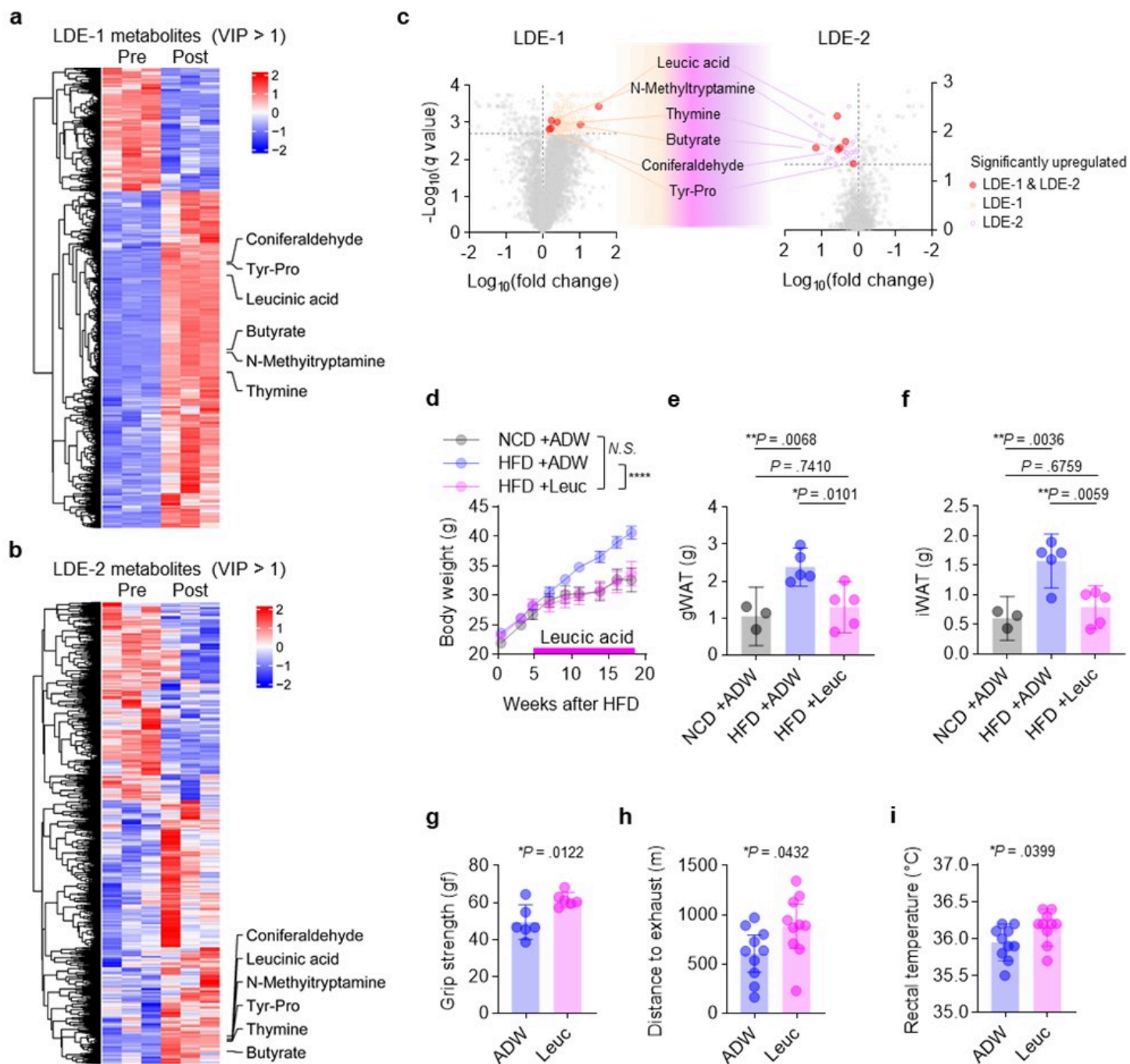


Figure 3

Leucic acid is the active metabolite that phenocopies bacterial benefits.

a,b, Heatmaps of metabolites detected in pre- and post-culture media of LDE-1 (**a**) and LDE-2 (**b**) by untargeted metabolomics. Metabolites with variable importance in the projection (VIP) score > 1 are shown. Values are z-score normalised (row-wise mean-centred and scaled by standard deviation). Metabolites significantly enriched in post-culture medium of both LDE-1 and LDE-2 are indicated.

c, Volcano plots of shared and strain-specific metabolite production by comparing pre- and post-culture media of LDE-1 and LDE-2 by untargeted metabolomics. Metabolites significantly increased (FDR < 1) in

post-culture media of LDE-1 and LDE-2 ($n = 3$ biological replicates per group). The differences in the metabolite abundance between the pre-culture and post-culture media were assessed using two-tailed unpaired Student's t -test with false discovery rate (FDR) correction (LDE-1: $< 0.2\%$; LDE-2: $< 2\%$).

d, Body weight changes in mice fed a normal chow diet (NCD), HFD, or HFD supplemented with leucic acid (initiated 5 weeks post-HFD). Data are mean $\pm$ SEM, $n = 3$ (NCD + ADW), 5 (HFD + ADW), and 5 (HFD + leucic acid) mice. Slopes compared by simple linear regression analysis.

e,f, Gonadal white adipose tissue (gWAT; **e**) and inguinal white adipose tissue (iWAT; **f**) mass. Data represent individual mice ($n = 3$ -5 per group) with mean $\pm$ 95% CI. Differences assessed by one-way ANOVA followed by Tukey's multiple comparisons test.

g,h, Functional metabolic assessments. **g**, Forelimb grip strength. **h**, Treadmill exhaustion distance. Data are individual values ($n = 6$ -10 mice per group) with mean $\pm$ 95% CI. Unpaired two-tailed Student's t -test.

i, Leucic acid increases basal body temperature in mice. Basal rectal temperature was measured in HFD-fed WT mice after 1 week of treatment with leucic acid or ADW ($n = 10$ mice per group). Data are means of two measurements per mouse $\pm$ 95% CI. Unpaired two-tailed Student's t -test.

**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

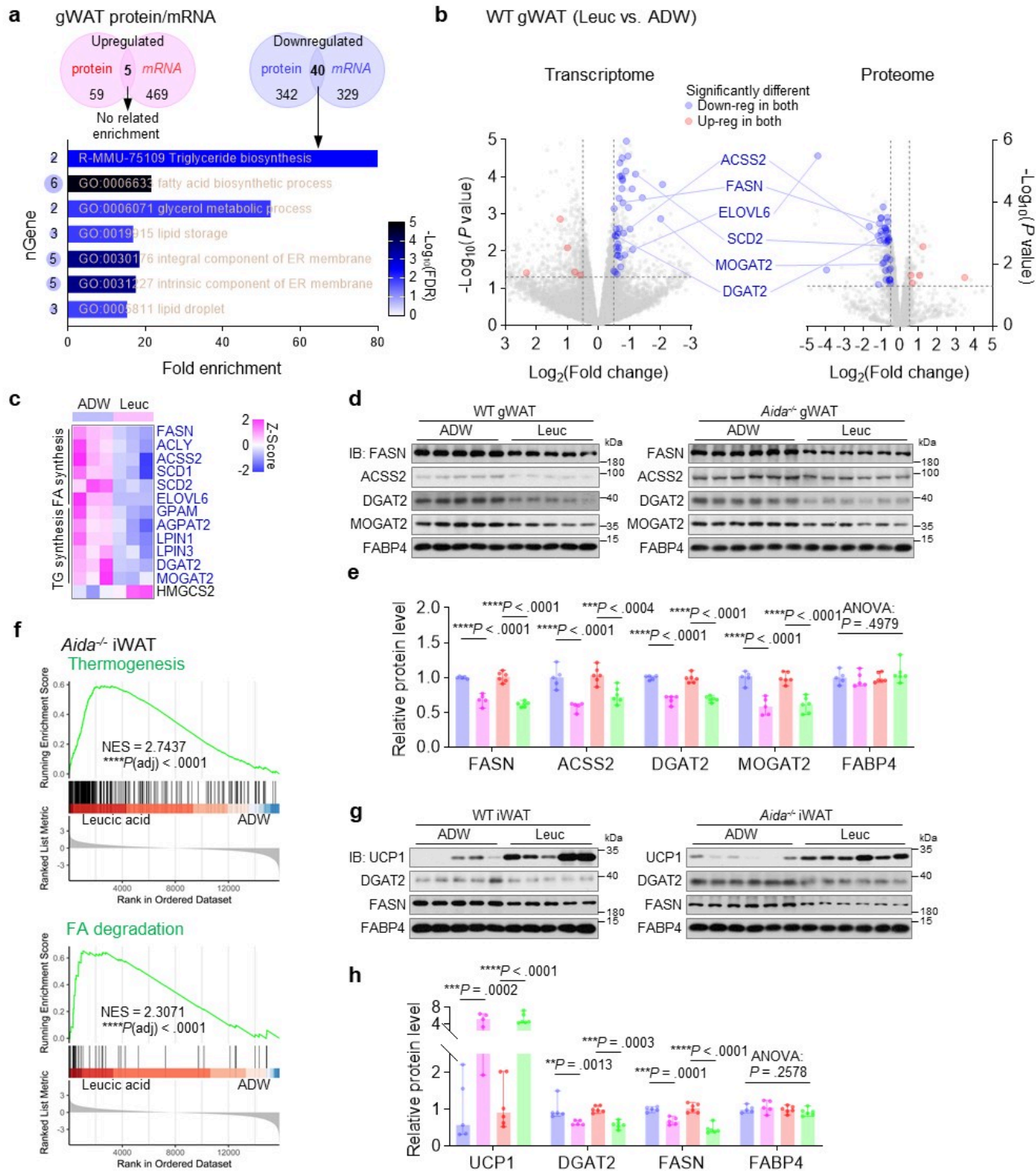


Figure 4

Leucic acid suppresses lipid storage through depot-divergent effects.

a, Multi-omics analysis of leucic acid-responsive pathways in gWAT. Upper: Venn diagram of differentially expressed genes (DEGs; $|\log_2(\text{fold change})| > 0.5$, $P < 0.05$) from proteomic and transcriptomic profiles of gWAT from HFD-fed WT mice treated with leucic acid (Leuc) or ADW (control)

for 2 weeks. Lower: enrichment analysis (ShinyGO) of downregulated candidates in lipid anabolic pathways.

b, Volcano plots of mRNA (left; $n = 4$ per group) and protein (right; $n = 3$ per group) abundance changes in gWAT (Leuc vs. ADW). Significantly altered molecules (*limma-voom*; $|\log_2(\text{fold change})| > 0.5$, $P < 0.05$) in red (up) or blue (down).

c, Heatmap of fatty acid and triglyceride synthesis enzymes in gWAT of leuc- or ADW-treated WT mice.

d,e, Leucic acid suppresses lipid synthesis machinery in gWAT. **d**, Immunoblots of key lipogenic proteins (FASN, ACSS2, DGAT2, MOGAT2) in gWAT lysates from HFD-fed WT and *Aida*^{-/-} mice treated with 50 mM leucic acid or ADW control for 6 weeks. FABP4 served as loading control (15 µg protein per lane). **e**, Quantification of immunoblot signals expressed relative to the mean of respective ADW control groups. Data represent individual mice ($n = 5$ WT, $n = 6$ *Aida*^{-/-}) with median $\pm$ 95% CI. Statistical significance for protein levels was determined by one-way ANOVA followed by Holm-Šídák's test when ANOVA indicated significant differences ($P < 0.05$).

f, Gene Set Enrichment Analysis (GSEA) of thermogenesis pathway in leuc-treated *Aida*^{-/-} iWAT. Normalised Enrichment Score (NES) and statistical significance shown.

g,h, Leucic acid modulates lipid synthesis and thermogenic programs in iWAT. **g**, Immunoblots of lipid synthesis regulators (FASN, DGAT2) and thermogenic marker UCP1 in iWAT lysates from mice described in **d**. FABP4 served as loading control (15 µg protein per lane). **h**, Quantification of immunoblot signals using methodology identical to **e**, with statistical analysis of DGAT2 levels performed via Kruskal-Wallis test followed by Dunn's test due to violation of normality assumptions.

**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$.

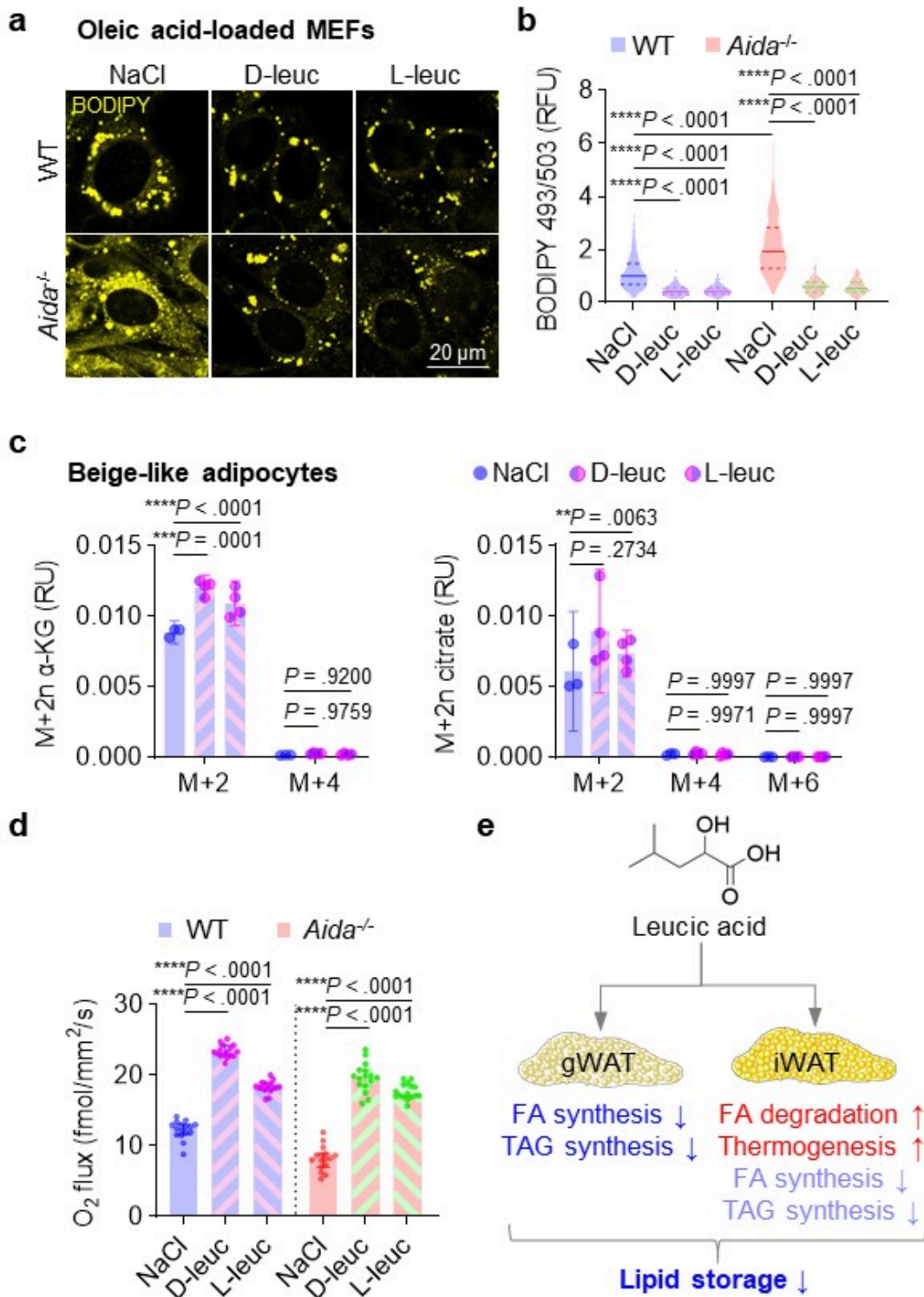


Figure 5

Leucic acid directly enforces lipid catabolism in an enantioselective manner.

a, Lipid droplet accumulation in WT and *Aida*^{-/-} mouse embryonic fibroblasts (MEFs) pre-treated with 50 μM D- or L-leucic acid (sodium salt) or NaCl (control) for 36 h, then 250 μM oleic acid for 12 h. Stained with BODIPY 493/503. Scale bar: 20 μm.

b, Quantification of lipid droplet fluorescence intensity ($n = 326-419$ cells per group from 2 fields). Data normalised to WT control. RFU: relative fluorescence intensity. Violin plots show median (solid line) and quartiles (dashed lines). Kruskal-Wallis followed by Dunn's test.

c, Isotopic enrichment (M+2 species) in 3T3-L1 derived beige-like adipocytes. Cells pre-treated with 50 μM D- or L-leucic acid or NaCl for 24 h, then 250 μM [U- ^{13}C]oleic acid for 1 h. Data are presented as individual values with mean $\pm$ 95% CI, two-way ANOVA followed by Dunnett's test.

d, Oxygen consumption rate (OCR) in MEFs treated with 50 μM D- or L-leucic acid. MEFs were monitored using the Resipher continuous oxygen consumption reader every 15 min for 1 h, and shown as individual values (4 replicate wells for each group, each with 4 time points recorded) with mean $\pm$ 95% CI, two-way ANOVA followed by Dunnett's test.

e, Schematic diagram of the effect of leucic acid on lipid metabolism across adipose depots.

**** $P < 0.0001$, *** $P < 0.001$, * $P < 0.05$.

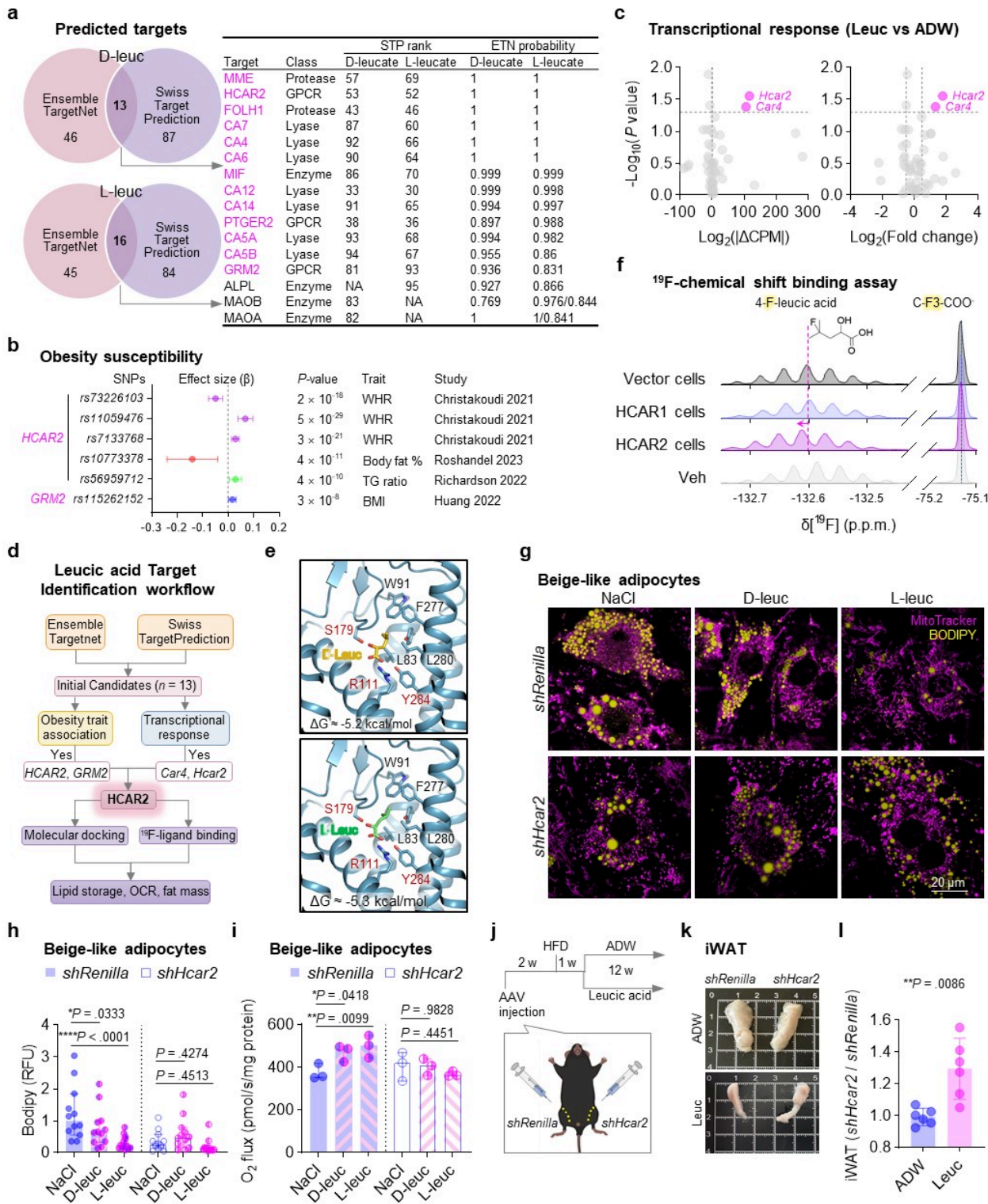


Figure 6

HCAR2 mediates leucic acid-induced suppression of lipid accumulation.

a, Integrative target prediction for leucic acid enantiomers. Venn diagram shows 13 consensus targets shared by Ensemble TargetNet (ETN; probability > 0.7) and SwissTargetPrediction (STP; top 100 ranked targets). Magenta labels: high-confidence candidates.

b, Genetic associations of *HCAR2* (12q24.31) and *GRM2* (3p21.2) loci with obesity traits. Forest plot showing effect sizes (β) and 95% CI of intergenic variants identified through GWAS Catalogue screening. Traits: Waist-to-hip ratio (WHR), Body fat percentage (Body fat %), ratio of triglycerides to phosphoglycerides (TG ratio), and body mass index (BMI).

c, Transcriptional response to leucic acid in adipose tissue. Dual volcano plots assess differential expression by fold-change magnitude ($|\log_2(\text{fold change})| > 0.5$, $P < 0.05$) and absolute abundance change ($|\Delta\text{CPM}| > 10$, $P < 0.05$). *Hcar2* and *Car4* (magenta) are the only significantly altered genes by both metrics (*limma-voom*; $n = 3$ -4 per group).

d, Target identification workflow.

e, Predicted binding modes of D-leucate (yellow) and L-leucate (green) within the orthosteric pocket of HCAR2. Molecular docking simulations were performed using the cryo-EM structure of niacin-bound human HCAR2 in complex with the Gi heterotrimer (PDB ID: 8H2G) as the structural template. Key interacting residues are labelled, with polar residues highlighted in red. Binding free energies were calculated using AutoDock Vina.

f, ^{19}F -NMR ligand-detected binding assay in intact cells. Spectra of 4-fluoroleucic acid with HCAR2-expressing cells show a significant chemical shift. Reference: trifluoroacetic acid (TFA).

g, *Hcar2* knockdown abolishes lipid reduction in beige-like adipocytes. Representative BODIPY (lipids, yellow) and MitoTracker (mitochondria, red) images. Scale bar: 20 μm .

h, Quantification of lipid contents in *Hcar2*-knockdown cells. Data are presented as individual cells ($n = 12$ -13 per group) with median $\pm$ 95% CI. Two-way ANOVA followed by Holm-Šídák's test.

i, *Hcar2*-dependent oxygen consumption. Maximum mitochondrial oxygen consumption in beige-like adipocytes. Data are presented as individual experiments ($n = 3$) with median $\pm$ 95% CI, two-way ANOVA followed by Dunnett's test.

j, Experimental design for unilateral AAV-mediated *Hcar2* knockdown in iWAT. WT mice received in situ injections of AAV-*shHcar2* or AAV-*shRenilla* as a control at four sites per iWAT pad (contralateral control). Two weeks post-injection, mice were switched from NCD to HFD. After 1 week of HFD adaptation, leucic acid or vehicle ADW treatment was administered for 12 weeks prior to tissue collection.

k,l, *Hcar2*-dependent attenuation of leucic acid-induced iWAT mass reduction and browning. **k**, Representative images for iWAT depots. **l**, Mass ratios of *Hcar2*-knockdown iWAT to contralateral *shRenilla*-treated iWAT in the same animals. Data shown as individual points with mean $\pm$ 95% CI; $n = 6$ mice; statistical significance determined by two-tailed Student's *t*-test with Welch's correction.

**** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$.

Supplementary Files

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- [SupplementaryTable118.xlsx](#)
- [Additionalinformation.docx](#)