Phenylalanine impairs insulin signaling and inhibits glucose uptake through modification of IRβ

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Whether amino acids act on cellular insulin signaling remains unclear, given that increased circulating amino acid levels are associated with the onset of type 2 diabetes (T2D). Here, we report that phenylalanine modifies insulin receptor beta (IRβ) and inactivates insulin signaling and glucose uptake. Mice fed phenylalanine-rich chow or phenylalanine-producing aspartame or over-expressing human phenylalanyl-tRNA synthetase (hFARS) develop insulin resistance and T2D symptoms. Mechanistically, FARS phenylalanylate lysine 1057/1079 of IRβ (F-K1057/1079), inactivating IRβ and preventing insulin from promoting glucose uptake by cells. SIRT1 reverse F-K1057/1079 and counteract the insulin-inactivating effects of hFARS and phenylalanine. F-K1057/1079 and SIRT1 levels in white blood cells from T2D patients are positively and negatively correlated with T2D onset, respectively. Blocking F-K1057/1079 with phenylalaninol sensitizes insulin signaling and relieves T2D symptoms in hFARS-transgenic and db/db mice. These findings shed light on the activation of insulin signaling and T2D progression through inhibition of phenylalanylation.

To facilitate glucose uptake, insulin is secreted by beta cells in islets upon glucose exposure, and insulin signaling is activated in other cells, such as muscle cells, to promote glucose uptake after glucose consumption. Insulin signaling is initiated by the binding of insulin to the α subunit of the insulin receptor (IRα), which activates the tyrosine kinase activity of β subunit of the insulin receptor (IRβ) by phosphorylating tyrosine residues in the cytoplasmic domain of IRβ. Insulin signaling proceeds through the recruitment of the IR substrate (IRS) family proteins via phosphorylated IRβ and subsequent activation of insulin signaling components, namely, PI3K, Akt, and AS160, to promote GLUT4-mediated glucose uptake. Impairments in any step of the insulin cascade cause defects in glucose uptake and result in diabetes. For example, defects in insulin secretion cause maturity-onset diabetes in the young (MODY). Impairments in the components...
of the insulin signaling cascade, such as competition between isofoms of IR that disrupt insulin signaling generation or deletion of AKT2 resulting in disruption of insulin signaling transmission, cause reduced insulin sensitivity and type 2 diabetes (T2D).

Glycerol from triglyceride and amino acids are two other major energy sources that can be converted to glucose through gluconeogenesis when the glucose supply is low. Physiologically, when cells sense high levels of intracellular fatty acids or amino acids, they may suppress glucose uptake to avoid an oversupply of energy nutrients and a waste of resources, because glucose uptake consumes energy and other cellular resources. Fatty acids have been shown to suppress insulin signaling and glucose uptake through various mechanisms. Free fatty acids inhibit insulin signaling by inhibiting AKT kinase activity; unsaturated C18 fatty acid elaidate suppresses the insulin-induced fusion of GLUT4 storage vesicles in the plasma membrane, and palmitic acid induces sustained JNK activation and insulin resistance in primary mouse hepatocytes and pancreatic β-cells.

In contrast to fatty acids, the roles of amino acids in regulating insulin signaling and glucose uptake are unclear, although increased blood levels of aromatic amino acids, branched-chain amino acids, and other amino acids have been repeatedly found in T2D patients, suggesting that deregulated amino acid levels either cause or are a consequence of T2D. Moreover, habitually high-protein intake, which is often employed to decrease body weight, increases amino acid levels and is associated with insulin resistance and an increased risk of developing T2D, supporting the hypothesis that upregulated amino acids may be an underlying cause of the disease. Mechanistically, amino acid infusion or excessive protein ingestion reduces insulin sensitivity independent of mTOR signaling activation, suggesting that amino acids may not impair insulin-mediated glucose disposal mainly by activating anabolism, such as protein synthesis, although essential amino acids stimulate skeletal muscle mitochondrial protein synthesis, which accounts for a minor fraction of total cellular protein synthesis, and decreases insulin sensitivity in healthy humans. Moreover, amino acids may induce insulin resistance in human skeletal muscle by inhibiting glucose transport. These results link amino acid oversupply to insulin resistance, inhibition of glucose uptake, and progression to T2D through mostly unknown mechanisms.

Amino acids may modify proteins as substrates to alter cell signaling. Amino acids are newly identified modifiers of proteins. Aminoacyl tRNA synthetases (ARSs) are aminoacyl transferases that catalyze the lysine aminoacylation of its cognate amino acid to proteins to regulate a number of physiological and pathological processes, including mTORC1 signaling, apoptosis, and neuron loss. In the current study, we provide evidence that phenylalanine, one of the amino acids exhibiting significantly increased levels in the sera of T2D patients, induces insulin resistance and T2D symptoms by modifying IRβ.

Results
Phenylalanine-rich and aspartame-containing chow induced T2D symptoms in mice
To elucidate whether phenylalanine elevation is a cause or consequence of T2D, we challenged C57BL/6J mice (C57 mice) with a reported posttranslational modification that is catalyzed by phenylalanyl-tRNA synthetase (FARS) and employing phenylalanine as a substrate, we checked whether cytosolic FARS, mitochondrial phenylalanyl-tRNA synthetase 2 (FARS2), or both are involved in phenylalanine-rich and aspartame-containing chow feeding. To mimic a more physiologically relevant phenylalanine exposure, we used 0.1% phenylalanine chow, which increased blood phenylalanine by 22.9% (Supplementary Fig. 1d), and this manipulation resulted in insulin resistance and T2D phenotypes after 6 months of feeding (Supplementary Fig. 1e–k), slower than the 1% phenylalanine chow treatments. These results suggest that phenylalanine promotes T2D onset in mice in a dose-dependent manner. Further confirming this notion, a chow containing 1% aspartame, which elevated mouse serum phenylalanine levels by 36% to reach approximately 120 μM (Supplementary Fig. 1i) and increased blood weight (Supplementary Fig. 1m) and increased glucose tolerance (Supplementary Fig. 1j) and blood insulin (Supplementary Fig. 1p) levels and reduced glucose tolerance (Supplementary Fig. 1q, r), insulin tolerance (Supplementary Fig. 1s), and elevated HOMA-IR value (Supplementary Fig. 1l) that phenocopied those of phenylalanine-rich chow after 12 weeks of feeding (Figs. 1c–f).

Intracellular phenylalanine blocked insulin signaling in cultured cells
To elucidate the underlying T2D-promoting mechanism of phenylalanine, we tested the effects of phenylalanine supplementation on cultured cells. Methyl-phenylalanine (Me-Phe) supplementation of 3T3-L1 murine adipocytes and L6 rat skeletal myoblasts in 2 mM culture media, which can be converted to phenylalanine by intracellular esterase, successfully increased intracellular phenylalanine levels approximately six-fold (Supplementary Fig. 2a, 2b). Moreover, Me-Phe supplementation severely prevented insulin from stimulating glucose uptake in these cells (Fig. 1g, Supplementary Fig. 2c), as measured by 2-deoxy-D-glucose (2-DG) uptake assays, suggesting that phenylalanine may blunt insulin signaling. This notion was confirmed by Me-Phe supplementation, which prevented insulin from increasing the phosphorylation levels of IR tyrosine 1150/1151, insulin receptor substrate 1 (IRS1) tyrosine, AKT threonine 308 and serine 473, and AS160 threonine 642, indicators of insulin signaling activation, in 3T3-L1 adipocytes (Figs. 1h, i) and L6 cells (Supplementary Fig. 2d, 2e) in a time- and dose-dependent manner before the self-decay of insulin signaling (Fig. 1h, Supplementary Fig. 2d). Furthermore, membrane enrichment of GLUT4, a consequence of insulin signaling activation, was prevented by Me-Phe treatment in 3T3-L1 adipocytes (Fig. 1j).

Insulin signaling is initiated in the extracellular compartment and is transmitted intracellularly. Me-Phe treatment blocked insulin signaling, indicating that phenylalanine acts intracellularly to block insulin signaling. This conclusion was further supported by the fact that, in 3T3-L1 cells, small interfering RNA (siRNA)-mediated silencing of phenylalanine dehydrogenase (PAH), the first phenylalanine catalyzing enzyme, to increase intracellular phenylalanine levels (Supplementary Fig. 2f), effectively desensitized insulin to activate insulin signaling in 3T3-L1 cells (Fig. 1k), but did not noticeably change the protein levels of the insulin signaling pathway (Supplementary Fig. 2g). These results suggest that intracellular phenylalanine acts on one or more components of and inactivates insulin signaling.

FARS overexpression phenocopied phenylalanine treatment efficacy
To examine whether phenylalanine inhibits insulin signaling by augmenting lysine phenylalanylation (phenylalanylation hereafter), a reported posttranslational modification that is catalyzed by phenylalanyl-tRNA synthetase (FARS) and employing phenylalanine as a substrate, we checked whether cytosolic FARS, mitochondrial phenylalanyl-tRNA synthetase 2 (FARS2), or both are involved in
insulin signaling regulation. Ectopically co-expressing both the $\alpha$ and $\beta$ subunits of FARS (FARSA and FARSB), but not FARS2 (Supplementary Fig. 3a), blunted insulin signaling (Fig. 2a). Insulin signaling inactivation by FARS was substantiated by the insulin-stimulated 2-DG uptake ability of 3T3-L1 cells, which was partially abrogated by FARS overexpression (Fig. 2b) and further by silencing catalytic FARSA but not FARS2 (Supplementary Fig. 3b) with siRNA, sensitizing insulin to activate insulin signaling (Fig. 2c), and promoting 2-DG uptake (Fig. 2d) in 3T3-L1 cells. Moreover, knockout of FARSA in HepG2 cells or knockdown of FARSA in 3T3-L1 cells sensitized insulin signaling (Fig. 2e) and insulin-promoted 2-DG uptake (Fig. 2f), abrogating Me-Phe to inhibit insulin signaling and 2-DG uptake (Figs. 2e, 2f). These results suggest
that phenylalanine inactivates insulin signaling through FARS, and that phenylalanylation underlies increased phenylalanine levels to inactivate insulin signaling.

We confirmed the role of phenylalanylation in the inactivation of insulin signaling in mice. As human FARS a subunit (hFARSa) overexpression resulted in more than 80% inactivation of insulin signaling (Supplementary Fig. 3c). FARS was overexpressed in C57 mice (hFARSa mice) to examine the insulin signaling-inhibiting effects of FARS (Supplementary Fig. 3d). Although the food intake (Supplementary Fig. 3e) of hFARSa mice was comparable to that of the mother mouse strain, moderate body weight gain (Supplementary Fig. 3f), higher serum (Supplementary Fig. 3g) and hepatic (Supplementary Fig. 3h) triglyceride levels, and energy expenditure under comparable respiratory quotient (RQ) (Supplementary Fig. 3i) were observed in hFARSa mice fed standard chow. Blood glucose levels of hFARSa mice were significantly higher than those of the mother mouse strain starting four weeks after birth (Fig. 2g), and eight-week-old adult male hFARSa mice exhibited increased fasting blood glucose levels (Fig. 2h), reduced glucose tolerance (Supplementary Fig. 3j), reduced insulin tolerance (Fig. 2i), increased blood insulin levels (Fig. 2j), and increased HOMA-IR values (Fig. 2k), all of which phenocopied the consequences of phenylalanine chow feeding. Moreover, the adipose, liver, and muscle tissues of hFARSa mice showed decreased insulin signaling (Fig. 2l). Furthermore, insulin had a lower potency to stimulate the membrane localization of GLUT4 in hFARSa mouse muscle cells, in which GLUT4 was distributed mainly in the cytosol (Fig. 2m). These results are consistent with FARS-catalyzed phenylalanylation inhibiting insulin signaling.

FARS phenylalanylated Lys1057 and Lys1079 of IR

To identify the proteins involved in insulin signaling that are regulated by phenylalanylation, we screened for how changes in phenylalanine targets, including SHC, ERK1/2, S6K, and GSK3β are affected by phenylalanine level variation. These results suggest that the levels of F-K1057 and F-K1079 are substantially altered by phenylalanine level variation.

Abrogating IR F-K1057/1079 prevented phenylalanine and FARS from inhibiting insulin signaling

To elucidate the importance of F-K1057 and F-K1079 in insulin signaling, we mutated either K1057 or K1079 or both residues to arginine (IRβK1057R, IRβK1079R, IRβK1057/1079R) to mimic non-phenylalanylated IRβ, and we mutated either K1057 or K1079 or both residues to phenylalanine (IRβK1057F, IRβK1079F, IRβK1057/1079F) to mimic phenylalanylated IRβ. When these mutants were each knocked in HepG2 cells and were expressed at comparable levels as those of wild-type IRβ, insulin had similar potency to increase IRβ1150/1151 phosphorylation and activate insulin signaling in wild-type and non-phenylalanylation mimetic IRβ mutant knock-in HepG2 cells (Fig. 4a), but had a much weaker potency to activate insulin signaling in phenylalanylation mimetic IRβ mutant knock-in HepG2 cells (Fig. 4b). Moreover, IRβK1057F-expressing cells responded more strongly, and IRβK1079F-expressing HepG2 cells responded more weakly to insulin stimulation than wild-type IRβ-expressing cells (Fig. 4a). It was confirmed that IRβK1057F, IRβK1079F, and IRβK1057/1079F showed stronger, weaker, and weaker insulin responses than wild-type IRβ-silenced 3T3-L1 cells (Fig. 4c), consistent with insulin having much weaker potency to activate 2-DG uptake in IRβK1079F-expressing IRβ knockdown 3T3-L1 cells (Fig. 4d). These results suggest that phenylalanylation negatively regulates IRβ and insulin signaling.

Notably, Me-Phe supplementation failed to decrease the phosphorylation levels of indicators of insulin activation in both IRβK1057F and IRβK1079F knock in cells as it did in wild-type HepG2 cells (Supplementary Fig. 7b) and in simulated IRβK1057F and IRβK1079F-expressing in IRβ-silenced
3T3L1 cells (Fig. 4e), consistent with the notion that Me-Phe supplementation is unable to decrease insulin-stimulated 2-DG uptake in both mutant IRβ-expressing IRβ knockdown 3T3-L1 cells (Fig. 4f). This suggests that K1057 and K1079 phenylalanine-mediated phenylalanine-induced insulin signaling inactivation. This notion is substantiated by the finding that FARS overexpression increased IRβ phenylalanation, inactivating insulin signaling in wild-type but not in IRβ<sup>p1150/1151</sup> or IRβ<sup>p308</sup> knock in HepG2 cells (Supplementary Fig. 7c), observations that were recaptured in IRβ knockdown 3T3-L1 cells, of which siRNA-resistant wild-type IRβ, but not IRβ<sup>p1150/1151</sup> or IRβ<sup>p308</sup>, inactivated insulin signaling (Fig. 4g) and decreased 2-DG uptake (Fig. 4h). Lastly, consistent with the fact that F-K1057 and F-K1079 underlie
insulin resistance, a high-fat diet, an established risk factor of insulin resistance, increased circulating phenylalanine levels (Supplementary Fig. 7d), decreased SIRT1 levels (Supplementary Fig. 7e) as reported but had negligible effects on FARSA levels, consistently induced K1057/F-K1079 levels, and inhibited insulin signaling in the liver, muscle, and adipose tissues of mice (Supplementary Fig. 7e). However, treatment with the P3K inhibitor wortmannin inhibited downstream insulin signaling in FARSA-knockout HepG2 cells, which had diminished F-K1057 and F-K1079 levels (Supplementary Fig. 7f), suggesting that phenylalanylation on IRβ is sufficient but not necessary for inactivating insulin signaling.

**SIRT1 removed F-K1057/1079 and sensitized insulin signaling**

We tested whether F-K1057 and F-K1079 were dynamically regulated. Given that sirtuins have deaminoacylation activity and that the sirtuin inhibitor nicotinamide rather than the histone deacetylase inhibitor trichostatin A (TSA) elevated F-K1057 and F-K1079 levels and inhibited insulin signaling in hepatocytes of wild-type C57 mice but not in IRβ KO mice. These results were consistent with previous reports that SIRT1 KO causes insulin resistance, which was confirmed by our findings that elevated fasting blood glucose (Supplementary Fig. 5c), reduced glucose tolerance (Supplementary Fig. 5d), insulin tolerance (Supplementary Fig. 5e), higher blood insulin levels (Supplementary Fig. 5f), and increased HOMA-IR values (Supplementary Fig. 5g) were observed in SIRT1 KO mice. F-K1057 and F-K1079 levels were high in type 2 diabetes patients to investigate whether IRβ F-K1057 and F-K1079 levels are associated with T2D onset, we collected and analyzed blood samples from 60 healthy individuals and 62 patients with T2D matched in age and gender (Supplementary Table 1). We first confirmed that the blood phenylalanine levels of T2D samples were higher than those of non-T2D subjects (Fig. 6a) and positively correlated with HbA1c, a key diabetes marker (Fig. 6b), confirming previous findings. F-K1057 and F-K1079 levels and insulin signaling of human white blood cells (WBCs) were dose-dependently increased and inhibited, respectively, by phenylalanine (Fig. 6c), and the phenylalanine effects were recapitulated in the WBCs of phenylalanine-fed mice (Fig. 6d), suggesting that the WBCs may be used to detect phenylalanylation in clinical samples.

The levels of F-K1057 and F-K1079 in WBCs of patients with T2D were not only higher than those of the non-T2D subjects but also positively correlated with HbA1c levels (Fig. 6e–h), consistent with F-K1057 and F-K1079 levels underlying insulin signaling inhibition and insulin resistance. Moreover, SIRT1 protein levels, estimated by western blot analysis, were moderately lower in WBCs of T2D patients than in normal subjects (Fig. 6i), and negatively correlated with HbA1c levels (Fig. 6j), consistent with SIRT1 inactivation causing insulin resistance. However, FARSA protein levels were unexpectedly indistinguishable between patients with T2D and normal subjects (Fig. 6k), and there was no correlation between FARSA protein levels and HbA1c levels (Fig. 6l). These results suggest that WBC F-K1057 and F-K1079 levels are good clinical markers for T2D.

Decreasing F-K1057 and F-K1079 levels restored insulin signaling

The effects of decreasing F-K1057 and F-K1079 levels on insulin signaling activation and glucose uptake were determined in cells and mice to confirm that F-K1057 and F-K1079 may cause T2D. Phenylalaninol is a structural analog of phenylalanine (Fig. 7a) that may inhibit F-K1057 and F-K1079 synthesis by occupying the phenylalanine binding pocket of FARSA (Fig. 7b, Supplementary Fig. 9a). This hypothesis was confirmed by the decrease in F-K1057 and F-K1079 levels in a dose-dependent manner and the activation of insulin signaling in HepG2 cells, but not in IRβKO knock-in HepG2 cells (Fig. 7c), suggesting that phenylalaninol activates insulin signaling by inhibiting F-K1057 and
F-K1079 synthesis, which is consistent with phenylalaninol promoting 2-DG uptake by 3T3-L1 and L6 cells independent of insulin (Fig. 7d, Supplementary Fig. 9b).

Feeding mice with phenylalaninol-supplemented chow (PN chow) decreased the levels of F-K1057 and F-K1079 and increased phosphorylation levels of insulin signaling components in hFASA mouse livers (Fig. 7e, Supplementary Fig. 9c), and in the livers, adipose tissues, and muscles of aspartame Chow-fed mice (Fig. 7f, Supplementary Fig. 10a). PN chow had no observable effects on food intake (Supplementary Fig. 3e), and moderately decreased serum and hepatic TG levels (Supplementary Fig. 3g, h), and energy expenditure (Supplementary Fig. 3i), consistent with phenylalaninol being an F-K1057 and
F-K1079 synthesis inhibitor. Although PN chow did not affect caloric intake in mice (Supplementary Fig. 10b), it decreased body weight by 10% (Fig. 7g) and body fat (Fig. 7h) after 12 weeks of feeding. Moreover, the body weight- and body fat-reducing effects of phenylalaninol were more pronounced in hFARSA mice (Figs. 7g, 7h), which had higher body weights and body fat (Figs. 7g, 7h) than C57 mice. Furthermore, PN chow effectively reduced blood glucose levels in C57 and hFARSA mice (Fig. 7f). These results are consistent with the finding that F-K1057 and F-K1079 contribute to obesity, high blood glucose, and T2D phenotypes. The effects of phenylalaninol were also tested in C57BL/6j-db/db mice (db/db mice). Although F-K1057 and F-K1079 levels in db/db mice were only slightly higher than those in C57 mice, PN chow feeding significantly reduced liver and muscle F-K1057 and F-K1079 levels in both strains (Fig. 7i) and resulted in enhanced glucose tolerance (Fig. 7j) and insulin tolerance (Fig. 7k), lowered blood insulin and glucose levels (Figs. 7m, n), decreased HOMA-IR values (Fig. 7o), and decreased body weight by 30% (Fig. 7p) after 6 weeks of feeding. These results suggest that F-K1057 and F-K1079 are major contributors to insulin resistance and that reversing them is beneficial for relieving T2D symptoms.

Discussion

Using phenylalanine and aspartame to mimic extremes for serum Phe elevation in human samples, we demonstrate that FARS senses phenylalanine levels and converts them into a phenylalanine signal by modifying proteins. For IRβ, the phenylalanine signal F-K1057/1079 inactivates insulin signaling and inhibits glucose uptake, providing a protective mechanism of excess glucose uptake when intracellular amino acids are abundant. Therefore, phenylalanyl transferase, together with already reported mechanisms including phosphorylation, sumoylation, myristylation, OGlcylation, ubiquitination of IRβ, provided another integrated response to nutrient excess. Moreover, SIRT1, a metabolic regulator that employs the cellular energy level indicator NAD+ as a cofactor, integrates cellular energy levels into insulin signaling and glucose uptake regulation. This elegant design prevents insulin signaling and glucose uptake activation when either high amino acid or cellular energy levels are detected by cells. A question that needs to be answered is whether every amino acid can signal its abundance. Interestingly, the most significantly elevated amino acids in patients with T2D are essential amino acids, including aromatic amino acids and branched-chain amino acids. This suggests that essential amino acids are good indicators of amino acid abundance. It makes physiological sense because essential amino acids rely on uptake from the environment to fulfill cellular needs, and intracellular levels of essential amino acids are more accurate indicators of amino acid abundance than nonessential amino acids, which can be generated intracellularly from other sources. This is consistent with the observation that fatty acids, palmitate, and myristate have more signaling roles than other fatty acids.

Although elevated phenylalanine and F-K1057/1079 or reduced SIRT1 levels are negatively correlated with insulin signaling activation and glucose uptake and positively correlated with 2D onset, consistent with SIRT1 ameliorating insulin resistance, no correlations were found between 2D onset and FARS levels, a positive regulator of F-K1057/1079, whose overexpression promoted insulin resistance in mice. This implies two possibilities. First, intracellular phenylalanine levels are the major determining factors of F-K1057/1079 levels, and second, FARS overexpression is toxic and is under negative selection during evolution. Notably, inhibiting F-K1057/1079 levels significantly alleviated T2D symptoms in db/db mice, in which insulin resistance is not a direct consequence of phenylalanine overloading, highlighting the importance of F-K1057/1079 in T2D development and suggesting an alternative strategy to contain 2D. Of note, phenylalaninol seemed to moderately decrease mice body weights, which may complicate the explanation of 2D symptoms relieving by phenylalaninol in db/db mice, and ask further study to validate this manipulation. The limitations of the current study include that 1% in dietary exposure of Phe or aspartame to induce type 2 diabetes phenotypes is beyond real life exposures, and this may reduce the reliability of the conclusion. Second, we noticed that F-K1057/1079 slightly affect energy balance, as evidenced by altered weight gain and energy expenditure was found in overexpressing hFARSA tg and PN-fed mice (Supplementary Fig. 3e-i), which leaves a possibility that altered energy balance may also contribute to F-K1057/1079-mediated insulin sensitivity. High-protein diets are often suggested for patients with T2D, despite studies indicating that prolonged high-protein intake induces insulin resistance. High-protein diets, such as high-fat ketogenic diets, may have transient blood glucose-controlling effects but may impair insulin signaling after prolonged exposure and thus have mixed outcomes. Moreover, aspartame is a widely used artificial sweetener that decreases sugar intake and is thought to be beneficial for T2D prevention. Our study suggests that, although aspartame is safe under the suggested doses, over-dosing or prolonged exposure may have adverse effects on insulin signaling, as previously reported.
Antibodies against PAH (ab178430, 1:3000), IRβ (ab69508, 1:1000), GLUT4 (ab654, 1:3000) was purchased from Abcam (Cambridge, UK). The antibodies against FARSA (18121-1-AP, 1:1000), SIRT1 mouse (60303-1-lg, 1:1000), FARS2 (16436-1-AP, 1:1000) was purchased from Proteintech (Rosemont, USA). The antibody against Actin(#A00702, 1:5000), anti-rabbit secondary antibody and anti-mouse secondary antibody were purchased from Genscript (Piscataway, USA). The antibodies against Flag(#M20008, 1:3000), Myc(#M20002, 1:3000), HA(M20003, 1:3000) were purchased from Abmart (Berkeley Heights, USA). Alexa Fluor Plus 488 donkey anti-mouse IgG secondary antibody (#A32766TR, 1:5000), Alexa Fluor Plus 555 donkey anti-rabbit IgG secondary antibody (#A32794TR, 1:5000), Alexa Fluor Plus 488 goat anti-

rabbit IgG secondary antibody (#A11008, 1:5000) were purchased from Invitrogen (California, USA).

The Anti-F-K1057 (1:500) and Anti-F-K1079 (1:500) antibodies were generated by Abmart Shanghai Co., Ltd during this study. Briefly, synthetic peptides (KGEAETRVAVK1057PheTVNESASLRE) and IEFL-

**Plasmids constructs and transfection**

Whole length human FARS2, FARS2, IR, SIRT1, SIRT2, SIRT6 were amplified from HEK293T cDNA and cloned into the Xho I and EcoR I restriction sites of the pcDNA3.1-Flag/HA vector using Plasmids constructs and transfection.

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**Cell lines and treatments**

**Cell lines.** HEK293T cells and HepG2 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibico) supplemented with 10% fetal bovine serum (FBS) (Gibico, Carlsbad, USA), 100 units/ml penicillin (Invitrogen, Carlsbad, USA) and 100 mg/ml streptomycin (Invitrogen, Carlsbad, USA).

3T3-L1 fibroblasts were cultured and differentiated into adipocytes by established protocols. In brief, 3T3-L1 fibroblasts were cultured in DMEM supplemented with 10% newborn calf serum (NCS). Two days after confluence, cells were switched into differentiation medium containing 10% FBS, 1 mM insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 1 mM dexamethasone for 2 days. The differentiation medium was then replaced with media containing 10% FBS and 167 nM insulin for another 2 days. Cells were then maintained in DMEM with 10% FBS. Adipocytes were used for experiments at day 10 post differentiation.

L6 rat skeletal myoblasts were cultured in DMEM with 10% FBS and antibiotics. After cultures reached 80%–90% confluency, medium was switched to 2% FBS to allow differentiation and myotube formation. After 8–10 days, cultures were used for experiments.

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Primary hepatocytes isolation was performed according to published methods. Briefly, D-Hanks’ buffer (Calcium and magnesium-free Hanks’ Balanced Salt Solution (HBSS) (Gibico, Carlsbad, USA) that contained 0.2% BSA and Hanks’ buffer (HBSS, calcium and magnesium containing 0.2% BSA and collagenase 0.5 mg/ml (Gibico, Carlsbad, USA) was prewarmed at 40 °C in a water bath. Mice were anesthetized by intramuscular injection of pentobarbital sodium. Hepatocytes were prepared by collagenase digestion via catheterization of the inferior vena cava (IVC) using a 24 G needle catheter. Prior to collagenase infusion, the liver was perfused (3–4 min) with D-Hanks’ buffer via IVC after severing the portal vein. The color of the liver was observed, which changed to a beige or light brown color after perfusion. The liver was then perfused with collagenase in Hanks’ buffer until the appearance of cracking on the liver surface. Collagenase perfused livers were excised out into DMEM and cells from the digested livers were teased out, suspended in DMEM, filtered through 70 μm nylon filter and centrifuged at 600g for 6 min. The cell pellet was then mixed with Percoll (adjusted to physiological ion strength with 10×PBS) to a final concentration of 30% and centrifuged at 2500 rpm for 10 min. Hepatocytes were collected and then cultured on collagen-coated plates (100,000 cells per well in 24-well plate) in Williams Medium E (Life Technologies, Carlsbad, USA) containing penicillin and streptomycin, 10 nM dexamethasone (all from Life Technology, Carlsbad, USA) and 10% FBS, before they can be cultured in serum-free medium.
**Cell treatments.** Methyl-phenylalanine or aspartame treatment to cells was achieved by incubating cells in phenylalanine-free RPMI 1640 (Gibico, Carlsbad, USA) without serum for 60 min, followed by treatment with Methyl-phenylalanine or aspartame for 15–120 min.

Phenylalaninol treatment to cells was achieved by incubating cells in serum-free DMEM for 2 h, followed by treatment with indicated phenylalaninol for 2 h. To measure the inhibiting effects of phenylalaninol or FARSA to insulin signaling, 100 nM insulin was used to stimulate insulin signaling for 5 min before harvest of cells.

Trichostatin A treatment was carried out by adding 10 μM TSA to the culture medium 16-20 h before harvesting cells. Nicotinamide treatment was carried out by adding 5 mM nicotinamide to the culture medium 16-20 h before harvesting cells.
Fig. 5 | SIRT1 dephenylalanylates K1057/1079 phenylalanylation and sensitizes insulin signaling. a Sirt1 inhibition elevates F-K1057 and F-K1079 levels and inactivates insulin signaling. F-K1057/1079 levels and phosphorylation of components of insulin signaling were detected in HepG2 cells that were not untreated and treated with 5 mM TSA. b SIRT1 decreases F-K1057/1079 levels and activates insulin signaling. F-K1057/1079 levels and phosphorylation of components of insulin signaling were detected in HepG2 cells and HepG2 cells overexpressing SIRT1, SIRT2, SIRT6, and SIRT7. c SIRT1 overexpression decreases F-K1057/1079 levels and activates insulin signaling. F-K1057/1079 levels and phosphorylation levels of components of insulin signaling pathway were detected in mouse primary hepatocytes and mouse primary hepatocytes overexpressing SIRT1. SIRT1 inhibitor fails to alter F-K1057/1079 levels and insulin signaling in Sirt1−/− mouse primary hepatocytes. Effects of EX527 (10 μM) on F-K1057/1079 levels and phosphorylation levels of components of the insulin signaling pathway were detected in wild-type and Sirt1−/− C57 mouse primary hepatocytes. g SIRT1−/− and SIRT1 mouse hepatocytes (F-K1057/1079) respond differently to phenylalanine. F-K1057/1079 levels and insulin-stimulated insulin signaling responses to phenylalanine were detected in SIRT1−/− and SIRT1 mouse primary hepatocytes. h Absence of K1057/1079 abrogates SIRT1-mediated activation of insulin signaling and 2-DG uptake. The ability of SIRT1 overexpression to activate insulin signaling (h) in wild-type, IR−/−, and IR−/- knock-in HepG2 cells, and 2-DG uptake (h) in IR knockdown 3T3-L1 adipocytes expressing IRβ, IRβ−/−, and IRβ−/- was assessed. Significance was indicated as one-way ANOVA. Values are expressed as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. j Sirt1 knockdown increases F-K1057/1079 levels and impairs insulin signaling. F-K1057/1079 levels and phosphorylation of components of insulin signaling were detected in the muscles of wild-type and Sirt1−/− mice. K Sirt1 knockout decreases membrane enrichment of GLUT4. Cellular GLUT4 distribution was detected by immunofluorescence in the muscles of wild-type and Sirt1−/− mice.

The guide sequences targeting the IR K1057 were: Forward, 5′-CACCCTGACCGTTCTTCAAGCCGACCG-3′ Reverse, 5′-AAACCGTGCGGGTGAAGACGTCACC-3′ The guide sequences targeting the IR K1079 were: Forward, 5′-CACCCTGAGCCCTCTGAAGCCGACCC-3′ Reverse, 5′-AAACCGGTCAAGCCCTCTGACCCG-3′.

CRISPR/Cas9 generation of knockout cells. To generate FARSα and knockout HepG2 cells, the following guide sequence targeting the human FARSA forward: 5′-CAC CGC CGC CAA CTC GGC GCT GTC C-3′, reverse: 5′-AAA CGG ACA CGG CTC TGG CGG C-3′, and the human IR forward: 5′-CAG CGG CTC TGG CGG CGC TGC TAC T3′, reverse: 5′-AAA CAG TAG CAG CGG CCC CAC CGG C-3′ were used, following standard CRISPR/Cas9 gene editing protocols.

Mice

All animal procedures were in accordance with the animal care committee at Fudan University.

Mice maintenance and treatments. Male C57BL/6J mice about six-week-old were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Male db/db mice were purchased from Gem Pharmatech Co., Ltd. (Nanjing, Jiangsu). Mice were housed in cages under a 14 h light/14 h dark cycle in a temperature- and humidity-controlled room, and freely fed standard laboratory chow with water ad libitum.

Mice were randomly divided into control and experimental groups when testing the phenylalanine or phenylalaninol efficacies. The control was fed with standard chow (Research Diet, AIN93G) and the experimental group was fed with either 1% or 0.1% phenylalanine chow or 1% phenylalaninol chow (modified from AIN-93G, Research Diet) that were manufactured from Shanghai SLAC Laboratory Animal Co., Ltd for 12 weeks.

Mice were randomly divided into control and experimental groups when testing the aspartame efficacies. The control was fed with standard chow (Research Diet, AIN93G) and the experimental group was fed with either 1% aspartame chow or 1% aspartame plus phenylalaninol chow (modified from AIN-93G, Research Diet) that were manufactured from Shanghai SLAC Laboratory Animal Co., Ltd for 12 weeks. Food intake measurements followed published protocol19.

In high-fat diet animal models, male C57 mice freely fed with the standard laboratory chow (Research Diet, D12450) and the high-fat diet chow (Research Diet, D12492).

Transgenic mice. The hFARSA-transgenic mice were generated in a C37BL/6 genetic background by inserting CAG-LSL-hFARSA-WPRE-PA.
in Gt (ROSA) 26S or (ENSMUSG00000086429) gene using a CRISPR-Cas9-mediated genome editing system by Shanghai Model Organisms Center, Inc. Cas9 mRNA was in vitro transcribed with mMESSAGE mMACHINET7 Ultra Kit (Ambion, TX, USA) according to the manufacturer’s instructions, and subsequently purified using the MEGAclearTM Kit (Thermo Fisher, USA). 5′-GGGGACACACTAAGGGAGCT-3′ was chosen as Cas9 targeted guide RNA (sgRNA) and in vitro transcribed using the MEGAshortscript Kit (Thermo Fisher, USA) and subsequently purified using MEGAclearTM Kit. The donor vector with sgRNA and Cas9 mRNA was microinjected into C57BL/6J fertilized eggs. F0 generation mice positive for homologous recombination were identified by long PCR. The PCR products were further confirmed.
Fig. 6 | Elevation of F-K1057/1079 levels in patients with T2D. a Phenylalanine levels are elevated in plasma samples from patients with T2D. Phenylalanine levels were measured in plasma samples from patients with T2D ($n = 62$, red spots, hereafter in this figure) and matched control samples ($n = 60$, black spots, hereafter in this figure). b Phenylalanine levels are positively associated with HbA1c levels in plasma samples from patients with T2D. Phenylalanine levels in patients with T2D and healthy subjects were plotted against their corresponding HbA1c levels. c F-K1057/1079 levels and insulin signaling are inversely regulated in human WBCs. F-K1057/1079 levels and insulin signaling were detected in human WBCs treated with the indicated levels of phenylalanine in the culture media. d Phe-fed mice have higher F-K1057/1079 levels and lower insulin signaling. F-K1057/1079 levels and insulin signals were detected in Phe-fed and normal chow-fed mice ($n = 6$). e hF-K1057/1079 levels are correlated with T2D. F-K1057 (a) and F-K1079 levels (b) were compared between patients with T2D and matched control samples, and F-K1057 (g) and F-K1079 (h) were plotted against their corresponding HbA1c levels. $I_{.*}$ SIRT1 protein levels are low in patients with T2D. SIRT1 levels in patients with T2D and healthy subjects were measured ($I_{.*}$) and their values were plotted against their corresponding HbA1c levels ($J_{.*}$). $k_{.}$ FARS protein levels are negligibly altered in patients with T2D. The FARSA levels of patients with T2D and healthy subjects were measured ($k_{.}$), and their values were plotted against their corresponding HbA1c levels ($k_{.*}$). Student’s $t$ tests (unpaired, two-tailed) are applied for all statistical analyses in this figure. Values are expressed as the mean ± SEM. Significance was indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

by sequencing. F0 mice were crossed with C57BL/6j mice to obtain hFARS heterozygous mice. Sirt1fl/fl mice (C57BL/6-Sirt1fl/fllox/lox containing two loxP sites flanking exon 3) was obtained from Shanghai Model Organisms Center, Inc. (Shanghai, China). The Sirt1 knockout mice were created by cross-breeding Sirt1fl/fl and UBC-cre/ERT2 mice. The resulting UBC-cre/ERT2 Sirt1fl/lox-flox mice (Jackson stock number #007001) were mated with Sirt1fl/+ mice to obtain Sirt1 conditional knockout mice (Sirt1fl/fl; Sirt1fl/+). and the corresponding Sirt1fl/fl mice (Sirt1fl/fl; Sirt1fl/fl) as the knockouts. CRE activity was induced by intraperitoneal injection of tamoxifen (100 mg/kg) daily for a total of 7 days. Genotyping was performed by PCR analysis of tail DNA. The primer sequences used to detect UBC-cre/ERT2 transgene were as follows, UBC-transgene: forward 5′-GAC GTC ACC GTG TCT GTT G-3′, reverse 5′-AGG CAA ATT TTG GTG TAC GG-3′, UBC-WT: forward 5′-GTA GGG CAC AGA ATT GAA AGA TCT T-3′, reverse 5′-GTA GGT GGA AAT TCT AGC ATC ATC C-3′. Primer sequences used for genotyping Sirt1fl/fl mice were as follows: forward 5′-GGA CCT GGT GTG GAA AGG CAG GAA AA-3′, reverse 5′-TGG CCT ACA TCT GAC TAA CTC AAA TCC CT-3′.

Clinical samples
Participants. Human IR K1057/K1079 phenylalanine studies were conducted using fresh clinical Blood samples of T2D patients and matched healthy subjects were obtained from volunteers of Huashan hospital, Shanghai with known age, height, fasting blood glucose, insulin, HbA1c, triglyceride, cholesterol, LDL cholesterol, HDL cholesterol and type 2 diabetes status. Exclusion criteria were active cardiac, liver, or renal disease or long-term complications from diabetes. None of the nondiabetic subjects were taking medications that may alter glucose tolerance. Diabetic subjects taking anti-diabetes medications (sulfonlurea or metformin) were asked to discontinue their medications for at least two weeks prior to participation.

The study was approved by the Human Investigation Ethics Committee of Huashan Hospital. With a full understanding of the study, each participant signed the informed consent form voluntarily.

Isolation of white blood cells. ebioscience™ 10X RBC Lysis Buffer (00-4300, Invitrogen, Carlsbad, USA) was used to isolate PBMCs according to the manufacture instruction. Briefly, 20 mL 1x RBC lysis buffer was mixed with 2 mL blood (with EDTA anticoagulant) and incubated 10 min at room temperature. After centrifuging at 500g at RT for 5 min, the pellets were resuspended with 1x RBC lysis buffer and centrifuged again. The pellets were then washed with PBS and finally used for ELISA.

Isolated WBCs were cultured in 6-well plates, using supplemented RPMI-1640 medium with 10% FBS, and incubated at 37 °C in a humidified, 5% CO2 atmosphere.

Assays
ITT and GTT. Assays were performed on male C57BL/6 mice after 12 weeks of treatments. For GTTs, mice were intraperitoneally injected with glucose (1 g/kg) after 16 h of fasting, and blood was sampled at 0, 15, 30, 60, 90, and 120 min after glucose injection. For ITTs, mice were intraperitoneally injected with 0.4–0.5 units/kg of insulin after 6 h of fasting, and blood was sampled at 0, 15, 30, 60, 90, and 120 min after insulin injection.

Blood insulin and HOMA-IR. Plasma insulin levels were measured by ELISA (Abcam, Cambridge, UK). The HOMA-IR was calculated as follows: Fasting blood glucose level (FPG, mmol/L) × Fasting insulin level (FINs, μU/mL) / 22.5.

Glucose uptake. The non-radioactive 2-deoxyglucose uptake was detected using a Glucose Uptake-Glo™ Assay Kit (J1341, Promega, Madison, USA) according to manufactured instruction. Briefly, cells were seed on 96-well plates and serum starved for 3 h before the assay. Cells were washed with PBS three times then replaced the medium with DMEM without serum or glucose and treatment with 2 mM methyl-phenylalanine 2 h, then stimulated with 100 nM insulin for 30 min. Cells were then incubated with 1 mM 2-DOG in PBS for additional 10 min at 25 °C. Cells were then lysed in stop buffer and neutralized with neutralization buffer. Lysates were then centrifuged at 15000 g for 15 min at 4 °C, and the supernatant was incubated with 2DG6P detection reagent for 1 h at 25 °C, then recorded luminescence with 0.3 μL Luminometer (Glomax96, Promega, Madison, USA).

Myc-GLUT4-mCherry assay. Cells were seeded on poly-D-lysine coated overslips were transfected with Myc-GLUT4-mCherry plasmid by electroporation. Cells were harvested and fixed with 4% paraformaldehyde for 10 min, then washed with cold PBS twice and blocked with PBS containing 5% donkey serum for 45 min at room temperature. Cells were then incubated with primary rabbit anti-Myc antibody overnight at 4 °C, followed by Alexa Fluor 488-conjugated Donkey anti-rabbit secondary antibody at least 1 h at room temperature. After washing, the overslips were mounted with DAKO mounting medium. Mounted samples were subjected to confocal imaging using a Zeiss LSM710 confocal laser microscope system with a x63 NA/1.40 CFI Plan APO VC oil-immersion objective.

Immunofluorescence. Cells were harvested and washed with PBS twice to remove the remaining medium. Paraformaldehyde (4%) was used to fix the cells at room temperature, 0.5% Triton X-100 in PBS was added and incubated for 20 min at room temperature. 3% BSA was added for 30 min at room temperature. The primary antibodies used were: anti-mouse IRβ (1:100, Abcam, Cambridge, UK) and anti-rabbit FARSA (1:100, Proteinotech, Rosemont, USA). The secondary antibodies (1: 1000, Thermo Fisher Scientific) used were: Alexa Fluor 355 donkey anti-rabbit and Alexa Fluor 488 donkey anti-mouse. DAPI was subsequently added, for nuclear staining. Cells were observed under a fluorescence microscope.

Immunostaining of GLUT4. Mice muscle tissues were fixed in 4% paraformaldehyde in PBS for 4 h and in 30% sucrose overnight, embedded in opti-mum cutting temperature (O.C.T.) compound
(Sakura) and cut into 5μm thick sections. Sections were fixed in 4% PFA at RT for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After washing with PBS, sections were blocked in PBS containing 5% goat serum for 1 h at RT, and then incubated with the GLUT4 antibody (1:100 dilution in 5% goat serum) overnight at 4 °C. After washing with PBS, sections were incubated with Alexa Fluor 488 goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, USA) for 1 h at RT in dark. Sections were washed and then incubated with DAPI (P36962, Thermo Scientific, Carlsbad, USA) for 1 minute to display the nuclei. Sections were mounted in a fluorescent mounting medium and images were captured using a confocal laser scanning microscope (Olympus, FV3000).
**ELISA for F-K1057, F-K1079, SIRT1 and FARSA quantitation.** ELISA-based quantitation methods were developed for quantifying the F-K1057 and F-K1079 of PBMC in clinical samples. Microtiter-plate wells were coated with IRβ antibody (#3020, CST, Danvers, Massachusetts, USA). The PBMC lysate, F-K1057 or F-K1079 antibody and GHRP was loaded into the wells. After 2 h of incubation at 37 °C, the wells were washed and 3,30,5,50-tetramethylbenzidine (TMB) substrate solution was added into the wells. The F-K1057 and F-K1079 concentrations were obtained by comparing the absorbance of samples at 450 nm with that of standards.

For SIRT1 or FARSA quantification, we coated microtiter-plate wells with SIRT1 antibody (#60363-1-Ig, Proteintech, Rosemont, USA) or FARSA antibody (#sc-100987, Santa Cruz, Dallas, USA) and followed the same procedures as described for F-K1057 and F-K1079. SIRT1 (#13161-1-AP, Proteintech, Rosemont, USA) or FARSA (#18121-1-AP, Proteintech, Rosemont, USA) was used as the standards for each assay, respectively.

**Immunoprecipitation.** Cells or tissues were homogenized in lysis buffer cells were lysed with 0.5% NP-40 buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 mM PMFS). Protein was incubated with anti-IRS1 antibody. The binding complexes were washed with 0.5% NP-40 buffer and mixed with loading buffer for SDS-PAGE. Presence of IRS1 in the precipitates was detected by a rabbit anti-IRS1 and anti-pan-phospho-tyrosine antibody.

**Western blot.** Cultured cells or cells extracted from mice tissue and patient-matched PBMC were homogenized with 0.5% NP-40 buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, and a mixture of protease inhibitors (Sigma-Aldrich). After centrifugation at 12,000 rpm and 4 °C for 15 min, the supernatant of the lysates was collected for western blotting according to standard procedures. Detection was performed by measuring chemiluminescence on a Typhoon FLA 9500 (GE Healthcare, Little Chalfont, UK).

**In Vitro Phenylalanylation Reaction.** In vitro phenylalanylation reactions were carried out in a 30 μl reaction mix that contained 50 mM HEPES (pH 7.5), 6 mM MgCl₂, 1 mM DTT, 1 mM NAD+ , 0.05 mg/mL synthetic phenylalanylated peptide or recombinant IR protein, 1 mg/ml SIRT1 and 1 mM PMFS. The reaction was allowed continue for 4 h at 37 °C. The peptide was desalted by passing through a C18 ZipTip (Millipore) before subjecting to analysis by a MALDI-TOF/TOF mass spectrometer (SCIEX-S800) and the resulted IR was subjected to western blot analysis.

**In vitro de-phenylalanylation reaction.** In vitro de-phenylalanylation reactions were carried out in a 30 μl reaction mix that contained 50 mM HEPES (pH 7.5), 6 mM MgCl₂, 1 mM DTT, 1 mM NAD+ , 0.05 mg/mL synthetic phenylalanylated peptide or recombinant IR protein, 1 mg/ml SIRT1 and 1 mM PMFS. The reaction was allowed continue for 4 h at 37 °C. The peptide was desalted by passing through a C18 ZipTip (Millipore) before subjecting to analysis by a MALDI-TOF/TOF mass spectrometer (SCIEX-S800) and the resulted IR was subjected to western blot analysis.

**Nuclear magnetic resonance analysis of sera metabolites.** Nuclear magnetic resonance (NMR) analysis was adapted from published methods (4). Briefly, Metabolites in 200 μl sera samples were extracted by adding 800 μL prechilled methanol. Supernatants were collected after centrifugation (12,000g, 4 °C) for 10 min. The extract was repeated twice and the supernatants were combined and lyophilized after vacuum dry to remove methanol. The extracted metabolites were redissolved in 600 μL phosphate buffer (0.15 M, pH 7.4) by vortex. The resulted solution was centrifuged at 16,099 g (4 °C) for 10 min before 550 μL supernatant was transferred into NMR tubes for analysis. All the one-dimensional 1H NMR spectra were acquired at 298 K on a Bruker Advance III 600 MHz NMR spectrometer (600.13 MHz for proton frequency) equipped with an inverse cryogenic probe (Bruker Biospin, Germany) using the software package TOPSPIN (V3.6.0 software package TOPSPIN (V3.6.0)) Bruker Biospin, Bruker Biospin, Karlsruhe, Karlsruhe, Germany). For 1H NMR spectra, an exponential window function was employed with a line broadening factor of 1 Hz and zero-filling was performed 100 times, each spectrum was multiplied by cosine squared window function and zero-filling was done up to 128 K prior to Fourier transformation. Each spectrum was then filled to 128 K prior to Fourier transformation. The characteristic and least-overlapping NMR signals (tyrosine: δ 6.89; phenylalanine: δ 7.43) were used to calculate absolute concentration of metabolites with the known concentration of TSP. For NMR signals tyrosine and phenylalanine were used to study the concentration of metabolites with the known concentration of TSP, software package TOPSPIN soft.

**MS/MS Identification of F-K1057 and F-K1079**

**Sample preparation.** Purified FAS were digested with trypsin, followed by LC-MS/MS analysis. MaxQuant (version 1.4.1.2) was employed to search MS/MS spectra against the SwissProt-human database (Release 2014-04-10) that contained forward and reverse sequences and oxidation of methionine, using the Andromeda search engine. Mass and fragment mass had an initial mass tolerance of 5 ppm and 0.05 Da. Minimal peptide length was set to seven amino acids and a maximum of four mis-cleavages was allowed. The false discovery rate (FDR) was set to 0.01 for peptide and protein identifications.
LC-MS/MS analysis. LC peaks eluted at the same time as those of standard synthetic peptides were subject to MS analysis with Orbitrap Fusion mass spectrometer. Peptide counting and/or areas of samples were compared to those of standard synthetic peptides of known concentrations. The MS machine was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 350–1600) were acquired in Orbitrap with a mass resolution of 60,000 at m/z 200. The AGC target was set to 300,000, and the maximum injection time was 50 ms. MS/MS acquisition was performed in Orbitrap with 3 s cycle time, the resolution was 15,000 at m/z 200. The intensity threshold was 50,000, and the maximum injection time was 200 ms. The AGC target was set to 200,000, and the isolation window was 2 m/z. Ions with charge states 2+, 3+, and 4+ were sequentially fragmented by higher energy collisional dissociation with a normalized collision energy of 30%, fixed first mass was set to 120. In all cases, one micro scan was recorded using dynamic exclusion of 30 s.

Raw mass spectrometry files were searched using Protein Discoverer (version 1.4.0.288, Thermo Fisher Scientific) with Mascot (version 2.7.0, Matrix Science). The data were processed with UniProt Human protein database (70,727 entries, download in 20161202). The data were processed with the MASCOT search engine (version 2.4.0.128, Thermo Fisher Scientific) and Scaffold (version 4.2.5.1, Proteome Software, Inc., San Diego, CA, USA) and Excel (Microsoft Corp., Redmond, CA, USA). Two-tailed Student’s t test was performed for the two-group analysis (Figs. 1a-g, 2b, 2d, 2g, 2h, 2i-k, 3j-k, 6a, 6e, 6g, 6i, 6k, 7d, 7k-p and Supplementary Figs. 1b-e, 1g-m, 1o-t, 2a-c, 2f, 3j-k, 7d, 8c-g, 9b and Table S1). One-way Welch’s ANOVA to compare more than two groups (Figs. 2f, 3b-c, 4d, 4f, 4h, 5i, 7e-i and Supplementary Fig. 3f–i). Pooled results were expressed as the means ± SEM. Differences were considered statistically significant if the p value was less than 0.05. Significance was indicated as *p < 0.05; **p < 0.01; ***p < 0.001.

Western blot assays were performed at least two independent times. Five or more regions of interest were randomly assessed for each microscopy image, and representative images were shown. To enhance reproducibility, no less than five animals were assigned per group.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD035076. All experimental data that support the findings of this study are included as follows: 7 main figures, 10 Supplementary figures, and Source data. Source data are provided with this paper.

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Author contributions

S.M.Z. and W.X. conceived the project and designed the experiments. S.M.Z. and W.X., and Q.Z. supervised experiments, analyzed the data and wrote the manuscript. Q.Z. executed molecular biological experiment and analysis and interpretation of data. Q.Z., J.C.C., P.C.L., W.X.S.
supervised animal experiments. W.W.S., Q.Z., J.C.C., H.L.Z., J.L., Y.L.
supervised clinical sample experiments. Q.Z., Y.P.A., L.H., and X.W.Z.
performed the NMR, LC-MS/MS, and MALDI-TOF/TOF mass analysis.
B.X.W. conducted the computer modeling. Y.M.L., Y.Y.Y., and J.Y.Z.
discussed and contributed to the interpretation of the data. All the
authors discussed the results and commented on the manuscript.

Competing interests
The authors declare no competing interests.

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