Amino Acids Downregulate SIRT4 to Detoxify Ammonia through the Urea Cycle

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SUMMARY

Ammonia production via glutamate dehydrogenase is inhibited by SIRT4, a sirtuin that displays both amidase and non-amidase activities. The processes underlying the regulation of ammonia removal by amino acids remain unclear. Here, we report that SIRT4 acts as a decarbamylase that responds to amino acid sufficiency and regulates ammonia removal. Amino acids promote lysine 307 carbamylation (CP-K307) of ornithine transcarbamylase (OTC), which activates OTC and the urea cycle. Proteomic and interactome screening identified OTC as a substrate of SIRT4. SIRT4 decarbamylates CP-K307 and inactivates OTC in a NAD^+-dependent manner. SIRT4 expression was transcriptionally upregulated by the amino acid insufficiency-activated GCN2–eIF2a–ATF4 axis. *SIRT4* knockout in cultured cells caused higher CP-K307 levels, activated OTC, elevated urea cycle intermediates, and urea production via amino acid catabolism. *Sirt4* ablation decreased mouse blood ammonia levels and ameliorated CCl_4-induced hepatic encephalopathy phenotypes. We reveal that SIRT4 safeguards cellular ammonia toxicity during amino acid catabolism.

INTRODUCTION

The breakdown of amino acids, one of three major sources of cellular energy, besides glucose and fatty acids, produces toxic ammonia. Cells prevent ammonia toxicity by transferring the amine groups of branched chain amino acids to glutamine and the amine groups of other amino acids to α-ketoglutarate to form glutamate, both of which then release ammonia within the double membrane-enclosed subcellular compartments of mitochondria. Mitochondrial carbamoyl phosphate synthetase 1 (CPS1) converts ammonia to carbamoyl phosphate (CP), which reacts with ornithine to form citrulline via a process catalysed by ornithine transcarbamylase (OTC). Citrulline then channels ammonia into the urea cycle. Inefficient removal of ammonia causes hyperammonaemia as well as central neuron system toxicity, leading to conditions such as hepatic encephalopathy (HE) (*1*). Known regulatory processes of the urea cycle indicate that CPS1 is allosterically regulated (*2*) and modified (*3, 4*) by metabolites and that activities of OTC and argininosuccinate lyase (ASL) are regulated by acetylation (*5, 6*). However, the links between amino acid sufficiency and/or amino acid catabolism and ammonia removal remain largely unclear.

High ammonia levels lead to increased production of CP, which spontaneously undergoes protein lysine carbamylation (CP-K) (*7*), a posttranslational modification that can also occur via nonenzymatic binding of isocyanate, or catabolite of thiocyanate, to ε-amino groups of lysine in proteins (*8*). A widely recognized effect of CP-K is the activation of Rubisco (*9*), a protein that fixes carbon in plants and other organisms. In humans, CP-K is physiologically and pathologically significant because

high levels of carbamylated proteins are found in the plasma and tissues of patients presenting with renal diseases, inflammation (*10*), or advanced age (*11*). However, an enzyme that removes CP-K has not yet been identified. The possibility that amide-bonded CP-K is dynamically regulated cannot be ruled out because new amidase activities have been continuously detected in sirtuins (*12, 13*).

In mammals, SIRT3, SIRT4, and SIRT5 are localized in mitochondria (*14, 15*). SIRT3 mediates mitochondrial metabolism via the activities of lysine deacetylase and other amidases, such as deaminoacylase (*16, 17*). SIRT5 exhibits robust lysine desuccinylase, demalonylase (*18, 19*), and deglutarylase activities (*20*) that regulate mitochondrial functions. By contrast, SIRT4, another sirtuin in mitochondria that lacks deacetylase activity (*21, 22*), is of limited physiological significance, despite displaying ADP-ribosyltransferase and lipoamidase activities that regulate glutamate dehydrogenase (GDH) (*23*) and the pyruvate dehydrogenase complex (*24*), respectively, and reversing lysine modifications derived from reactive acyl-CoAs generated from leucine metabolism, including 3-hydroxy-3-methylglutaryl (HMG) and related modifications (*25, 26*). In mice, loss of *Sirt4* leads to dysregulated insulin secretion and accelerated age-induced insulin resistance (*23, 25*). SIRT4 regulates lipid homeostasis by coordinating the balance between lipid catabolism and synthesis (*27-30*). Furthermore, mTORC1 stimulates glutamine metabolism by repressing the transcription of *SIRT4* (*31*). Reportedly, *sirt4* knockout *Drosophila* displayed short lifespans as well as decreased fertility and activity (*32*). Loss of SIRT4 can also result in tumour formation in mice (*33, 34*). These observations indicate that SIRT4 plays a

role in regulating cellular metabolism.

RESULTS

Amino acids and SIRT4 inversely regulates protein lysine carbamylation

Carbamoyl phosphate (CP) supplementation of culture media dose-dependently increased CP-K levels in the lysates, and the fractions of mitochondria and cytosol of human hepatocellular carcinoma HepG2 cells (**Fig. 1a**), confirming that CP spontaneously forms amide-bonded CP-K in proteins (**Fig. 1b**). Overexpressing carbamoyl-phosphate synthase (CPS1) increased CP-K levels in mitochondria more than in the cytosol of HepG2 cells (**Fig. 1c**), indicating that CPS1 produces CP in mitochondria. These observations, combined with the finding that preventing CP production via *CPS1* knockout decreased CP-K levels in HepG2 cells (**Fig. 1d**), suggested that CP levels determined CP-K levels in cells.

Removing glutamine, or all proteinogenic amino acids, from culture media decreased mitochondrial CP-K levels but had a less pronounced effect on the cytosolic CP-K levels in HepG2 cells (**Fig. 1e**), consistent with that oxidative deamination of amino acids occurs in mitochondria and affects mitochondrial CP-K levels. A general inhibitor of sirtuins, nicotinamide mononucleotide (NAM), increased mitochondrial CP-K levels (**Fig. 1f**), suggesting that one or more mitochondrial sirtuins, namely SIRT3, SIRT4, or SIRT5, function as decarbamylases of mitochondrial CP-K. Overexpression of SIRT4, but not SIRT3 and

SIRT5, decreased mitochondrial CP-K levels in HepG2 cells (**Fig. 1g**), suggesting that SIRT4 exerted decarbamylase activity. This was confirmed by the observation that *SIRT4* knockout in HepG2 cells (**Fig. 1h**) and *Sirt4* knockout in C57 mouse (*Sirt4*$^{-/-}$) increased CP-K levels in the mitochondria of HepG2 cells and in the livers of mice (**Fig. 1i**), respectively. Moreover, *SIRT4* knockout in HepG2 cells abrogated glutamine or amino acid starvation induced decrease of mitochondrial CP-K levels (**Fig. 1j**), suggesting that SIRT4 acts as the downstream of amino acids to regulate CP-K levels. Furthermore, *SIRT4* deletion in mice and in HepG2 cells failed to alter acetylation levels (**Fig. 1i, j**), suggest that SIRT4 decarbamylase activity is specific to CP-K but not to other lysine acylations.

Cell-wide identification of carbamylation and SIRT4 substrates

To identify protein targets regulated by SIRT4 mediated decarbamylation, we employed both proteomic and interactome screenings. To identify CP-K modified proteins, we adapted our published approach (*35*). Pan-anti-carbamoyl lysine antibody was used to enrich CP-K modified tryptic peptides that were generated from the proteome of mice liver, followed by mass spectrometry analysis to confirm the CP-K peptide sequences and their corresponding proteins. This allowed us to identify 142 CP-K modified proteins (**Fig. 2a** and **Supplemental Table 1**). Moreover, using ectopically expressed SIRT4 as the bait, proximity-dependent biotin identification (BioID) assays (*36*) allowed us to identify 135 SIRT4-interacting proteins in mouse

hepatoma Hep1-6 cells (**Fig. 2a** and **Supplemental Table 2**). Among the 22 proteins co-identified in both approaches (**Fig. 2a** and **Supplemental Table 3**), most were mitochondrial metabolic enzymes including glutamate dehydrogenase 1 (GDH), known ADP-ribosylation substrates of SIRT4 (*23*), and pyruvate dehydrogenase E1 alpha 1 (PDHA), glutamate oxaloacetate transaminase (GOT2), and ornithine transcarbamylase (OTC), suggested that they are potential decarbamylase substrates of SIRT4. This notion was supported by that CP-K levels of these proteins (**Extended Data Fig. 1a**) and proteins of mitochondria lysate of *Sirt4*$^{-/-}$ mice (**Extended Data Fig. 1b**), were decreased by *in vitro* incubation with recombinant SIRT4, and that SIRT4 overexpression in HEK293T cells lowed CP-K levels of these proteins (**Extended Data Fig. 1c**). However, SIRT3 or SIRT5 failed to exert impact on CP-K of these samples (**Extended Data Fig. 1a-1c**). These results suggest that SIRT4 may act on multiple CP-K of mitochondrial metabolic enzymes to coordinate mitochondria metabolism.

Unbiased metabolomic analysis allowed us to compare the levels of 756 metabolites identified in wild-type and *Sirt4*$^{-/-}$ C57 mice liver tissues (**Supplemental Table 4**), in which 23 metabolites differed significantly (VIP>1, $p<0.05$) (**Fig. 2b**). Metabolic pathway enrichment analysis revealed that the “Urea Cycle” was the foremost upregulated pathway in *Sirt4*$^{-/-}$ mice (**Fig. 2c**). Targeted analysis of metabolites in mouse livers and kidneys, using LC-MS, confirmed that *Sirt4* knockout in mice increased the levels of urea cycle metabolites (**Fig. 2d** and **Extended Data Fig. 1d-1i**). These results combined with the proteomics results collectively indicated

that OTC, a mitochondrial enzyme that incorporates CP into the urea cycle and thereby initiates the urea cycle, may act as a substrate of SIRT4 (**Fig. 2e**).

SIRT4 decarbamylates OTC CP-K307

A MS/MS spectrum generated from synthetic lysine 307 carbamylation (CP-K307) OTC tryptic peptide (**Fig. 3a**) matched spectra from tryptic OTC peptide libraries of both HepG2 cell (**Fig. 3a**) and mouse liver (**Extended Data Fig. 2a**), suggesting that lysine 307 of OTC is carbamylated *in vivo*.

Incubating CP with K307 OTC peptide, but not with the same peptide with lysine switched to arginine, produced carbamylated products (**Extended Data Fig. 2b**). Moreover, CP-K307 site-specific antibody (**Extended Data Fig. 2c**) indicated that the CP-K307 levels of intact OTC peptide were elevated by CP incubation, whereas those of the OTC^{K307R} mutant were not (**Extended Data Fig. 2d**). CP-K307 levels of ectopically expressed and endogenous OTC were increased by CP supplementation of the culture media in both HEK293T cells (**Extended Data Fig. 2e**) and mouse primary hepatocytes (**Fig. 3b**). These results confirmed that K307 of OTC is carbamylated *in vitro* as well as *in vivo*.

SIRT4, but not SIRT3, SIRT5, or catalytically dead $SIRT4^{H161Y}$ (*25*), removed CP-K307 from a synthetic OTC K307 peptide in a NAD^+-dependent manner (**Fig. 3c** and **Extended Data Fig. 3a**). Decarbamylase activity of SIRT4 was confirmed via the detection of decarbamylated peptide formation from synthetic OTC CP-K307 peptide

using reverse-phase high performance liquid chromatography (RP-HPLC) (**Fig. 3d** and **Extended Data Fig. 3b**) and quantification of CP-K307 levels by dot-blot-based assays (**Fig. 3e**), respectively, after treating synthetic OTC CP-K307 peptides with recombinant SIRT4. Thus, SIRT4 appears to employ a mechanism similar to its deacetylase activity to decarbamylate CP-K307 (**Fig. 3f).** The OTC CP-K307 decarbamylase activity of SIRT4 was further supported by the interaction of OTC with ectopically expressed (**Extended Data Fig. 3c**) or endogenous SIRT4 (**Extended Data Fig. 3d**).

Decarbamylase activity of SIRT4 was quantified via monitoring the production of unmodified OTC K307 peptide from synthetic OTC CP-K307 peptide (**Extended Data Fig. 3b**). SIRT4 time-dependently removed CP-K307 and other reported amide-bonded modifications, including lipoyl and HMG modifications (*24-27*), but exerted a very low deacetylation activity, from synthetically modified OTC K307 peptides (**Fig. 3g**), with an estimated catalytic efficiency (K_{cat}/K_m) of 6.93, which was higher than that of its other amidase activities (**Fig. 3h**). This result suggested that decarbamylase activity is a major amidase activity of SIRT4.

K307 carbamylation activates OTC

K307, which is close to the active pocket of OTC where CP binds, provides a structural basis for CP-K307 to function (**Fig. 4a**). Moreover, K307 and its spanning amino acids are well conserved in OTCs from *Caenorhabditis elegans* to human (**Fig.**

185 **4b**), suggesting that K307 is important for OTC activity. Substituting OTC K307 to
186 non-carbamylable arginine (OTC^{K307R}) to conserve a positive charge at this site
187 decreased OTC specific activity (**Fig. 4c**) and abrogated CP to increase carbamylation
188 and specific activity of OTC^{K307R} as it did to OTC (**Fig. 4d, e**), suggesting that CP-
189 K307 is the major carbamylation site that activates OTC. We further conducted *in*
190 *vitro* and in cells experiments to confirm that CP-K307 itself rather than mutation at
191 K307 regulates OTC activity. We obtained differentially carbamylated OTC from
192 HEK293T cells that were cultured in media with different amino acids levels. Higher
193 carbamylated OTC from amino acids-rich media-cultured cells had higher specific
194 activity than that of lower carbamylated OTC from amino acids-starved cells;
195 however, the amino acids-effects were not seen in carbamylation-null OTC^{K307R} (**Fig.**
196 **4f**). These, together with that the activity of higher carbamylated OTC was inactivated
197 by SIRT4 incubation *in vitro*, which decreased CP-K307 levels (**Fig. 4g**), consistent
198 with that overexpressing SIRT4, but not SIRT3 and SIRT5 in cells, reduced CP-K307
199 levels and OTC activity (**Fig. 4h**), indicating that SIRT4 acts as a decarbamylase of
200 OTC CP-K307 and confirmed that CP-K307 activates OTC.

201 Varying ornithine levels produced a hyperbolic OTC kinetic curve by both OTC
202 and OTC^{K307R} (**Fig. 4i**), consistent with that ornithine is a substrate of OTC. However,
203 increasing CP levels produced a sigmoidal OTC kinetic curve, where OTC^{K307R}
204 activities increased hyperbolically with CP incremental (**Fig. 4j**). Moreover, OTC
205 decreased CP was not linear as OTC^{K307R} did (**Fig. 4k**), suggest CP regulate OTC
206 activity. These results suggest that CP functions involve more than being an OTC

substrate, but also is an OTC activator via K307.

SIRT4 shutdown the urea cycle via decarbamylating CP-K307

Both deletion of SIRT4 in mice and in HepG2 cells caused increased urea cycle intermediates (**Fig. 2d, 5a** and **Extended Data Fig. 1d-1j**), consistent with higher urea levels in culture media and lower intracellular ammonia levels in *Sirt4*$^{-/-}$ mice hepatocytes than in WT hepatocytes (**Fig. 5b**, **c**). To confirm that the increased urea cycle intermediates were indeed from ammonia, we analysed ^{15}N-traced urea cycle fluxes (**Extended Data Fig. 4a**) in mice and cells of different *Sirt4* backgrounds. Higher percentages of ^{15}N-labelled urea cycle intermediates and urea were generated from ^{15}N-ammonium in *Sirt4*$^{-/-}$ mice liver (**Fig. 5d**) and their isolated hepatocytes (**Extended Data Fig. 4b**) than in WT mice and their hepatocytes, respectively, showing that SIRT4 inactivation promoted ammonia removal via the urea cycle. Moreover, OTC knockdown using small interfering RNA (siRNA) that targets the OTC 3′-untranslated region effectively decreased urea production and increased intracellular ammonia accumulation, and abrogated *Sirt4* deletion to decrease ammonia and increase urea levels in mice hepatocytes (**Fig. 5e, f**). Overexpressing OTC at similar levels caused a more pronounced decrease in ammonia and higher urea levels in mice hepatocytes, whereas OTC^{K307R} resulted in comparable decrease in ammonia and increase in urea in mice hepatocytes (**Fig. 5g, h**). These results indicate that SIRT4 inhibits urea cycle through removal of CP-K307 and inactivation of OTC.

SIRT4 levels were regulated by intracellular amino acid levels

Regulation of ammonia removal via the urea cycle by SIRT4 prompted us to test whether SIRT4 levels are regulated by intracellular amino acids. Supplementation with amino acids, but not ammonium or CP, decreased SIRT4 levels (**Fig. 6a**). Conversely, removing either glutamine or all amino acids from culture media increased SIRT4 protein levels in mouse hepatocytes (**Fig. 6b**). These results suggested that amino acids, rather than ammonium or CP, regulated SIRT4 expression. Moreover, starvation of glutamine or amino acids, but not glucose, increased the mRNA levels of *SIRT4* (**Fig. 6c**), suggesting that SIRT4 is transcriptionally upregulated by intracellular amino acid decreasing, but not by energy deprivation.

General control nonderepressible 2 (GCN2), a general sensor of cellular amino acids (*37*), was tested for its ability to regulate SIRT4 expression. Neither protein (**Fig. 6d**) nor mRNA (**Fig. 6e**) levels of SIRT4 were upregulated by amino acid starvation in *GCN2* knockout HepG2 cells, compared to WT HepG2 cells, which increased the phosphorylation of GCN2 and presumably activated it (*38*), suggesting that amino acids regulate SIRT4 expression through GCN2. Moreover, given the GCN2–eIF2a–ATF4 pathway transmits amino acid scarcity stress (*39*) and ATF4 regulates SIRT4 transcription and expression (*31*), we tested whether amino acids regulate SIRT4 transcription via the GCN2–eIF2a–ATF4 axis (**Fig. 6f**). Knockdown of either *Eif2a* or *Atf4* inhibited glutamine starvation alone or amino acid starvation in general to upregulate SIRT4 transcription (**Extended Data Fig. 5a, b**) and SIRT4 protein (**Fig.**

6g, h) levels. Moreover, *SIRT4* KO prevented silencing of *Eif2a* and *Atf4*, as well as amino acid starvation to decreased urea levels in mouse hepatocytes (**Fig. 6i, j**). These results showed that the GCN2–eIF2a–ATF4 axis conveyed amino acid abundance signals to regulate SIRT4 expression and ureagenesis.

Deletion of Sirt4 activates mouse urea cycle

Regulation of the urea cycle by SIRT4 was further tested *in vivo* in *Sirt4*$^{-/-}$ mice. *Sirt4*$^{-/-}$ mice exhibited increased liver CP-K307 levels compared to WT mice (**Fig. 7a**). *Sirt4*$^{-/-}$ mice, though exhibiting food intake (**Extended Data Fig. 6a**) and weights (**Extended Data Fig. 6b**) comparable to those of wild-type mice, drank more water (**Extended Data Fig. 6c**) and excreted more urine (**Fig. 7b**). In addition, the urea concentrations in the urine of *Sirt4*$^{-/-}$ mice were comparable to those of wild-type C57 mice (**Fig. 7c**). These results indicated that *Sirt4* deletion promoted the urea cycle consistent with *Sirt4*$^{-/-}$ mice has elevated total urea production (**Fig. 7d**), resulting in lower levels of blood ammonia when fed with normal chow (**Fig. 7e**). Moreover, *Sirt4* deletion elevated specific activity of OTC in mouse but exerted negligible effects on activities of other urea cycle enzymes (**Fig. 7f**), consistent with that SIRT4 inactivates OTC showed in Figure 5.

Inhibiting SIRT4 prevented hepatic encephalopathy induction in mice

High blood ammonia caused by impaired ammonia removal promotes hepatic

encephalopathy (HE) (*1*). Thus, we tested whether *Sirt4* ablation in mice partially prevents CCl_4, a hepatic toxin, from inducing HE (*40*). Wild-type and *Sirt4*$^{-/-}$ mice were treated with CCl_4 and analysed for metabolic, behavioural, and histological features of HE after CCl_4 treatment (**Extended Data Fig. 7a**). CCl_4 treatment caused negligible impacts on food intake (**Extended Data Fig. 7b**) and weights (**Extended Data Fig. 7c**) of both wild-type and *Sirt4*$^{-/-}$ mice, but *Sirt4*$^{-/-}$ mice drunk more water (**Fig. 7g**) and produced more urine (**Fig. 7h**). Moreover, CCl_4 treatments resulted in similar degrees of hepatic damage in wild-type and *Sirt4*$^{-/-}$ mice, as evidenced by similar CCl_4-induced increase in bilirubin (**Extended Data Fig. 7d**) and activity of alanine aminotransferase (AST) (**Extended Data Fig. 7e**), which are established indicators of hepatic damage (*41*). However, *Sirt4*$^{-/-}$ C57 mice displayed lower mortality rates than wild-type C57 mice following HE induction (**Fig. 7i**), suggesting that mice became resistant to CCl_4 treatments upon the ablation of *Sirt4*. Magnetic resonance imaging (MRI) analysis revealed that CCl_4 treatments induced brain oedema, a histologic marker of HE that caused by astrocytes swelling (*42*), in both wild-type and *Sirt4*$^{-/-}$ mice. However, it induced more moderate brain oedema in *Sirt4*$^{-/-}$ mice than in wild-type C57 mice (**Fig. 7j** and **Extended Data Fig. 8**), suggesting that *Sirt4* knockout partially prevented HE induction by CCl_4. Thus, both open field (**Fig. 7k** and **Extended Data Fig. 9a-c**) and Y-maze (**Fig. 7l** and **Extended Data Fig. 9d-g**) tests indicated that *Sirt4*$^{-/-}$ mice displayed increased exploratory and locomotor activity and desire to explore new environments following CCl_4 treatments. These results, combined with higher urea production and lower

blood ammonia in *Sirt4*$^{-/-}$ mice than in wild-type mice after CCl_4 treatments (**Fig. 7m, n**), demonstrated that *Sirt4*$^{-/-}$ mice have stronger ability for ammonia detoxification and are more tolerant to CCl_4-induced brain oedema (i.e., HE induction).

DISCUSSION

Mitochondria are the major sites where amino acid catabolism occurs and ammonia is produced in cells. The current study revealed that amino acids-regulated SIRT4 controls the scavenging of ammonia, the toxic by-product of amino acid catabolism. As certain levels of CP need to be preserved for pyrimidine *de novo* biosynthesis under low amino acid catabolism (*43*), and ammonia toxicity need to be avoided when amino acid catabolism is high, both inhibition and activation of the urea cycle are of physiologic significance. SIRT4, which regulates both ammonia production and scavenging, is a coordinator of ammonia homeostasis. When amino acids are scarce, high SIRT4 ADP-ribosylates and inactivates GDH, the major ammonia generator in mitochondria (*23, 44*), and removes CP-K307 to inactivates OTC and the urea cycle. When amino acids are abundant and amino acids catabolism are activated, low SIRT4 activates GDH and ammonia production, whereas excess ammonia is removed by activated OTC via the urea cycle (**Extended Data Fig. 10**). This elegant mechanism ensures cellular nitrogen supply from amino acids and avoids ammonia toxicity.

In addition to activate Rubisco (*9*), CP-K displays its ability to activate OTC. Although both ammonia and CP act as signalling molecules that regulate ammonia

levels, CP carries extra advantages, such as greater chemical reactivity. This allows CP to modify proteins to covalently convey ammonia abundance signals to proteins because proteins are unable to noncovalently bind to ammonia due to the extreme hydrophilicity of ammonia. Moreover, CP is a direct substrate of OTC and therefore a better activator of the urea cycle and urea production. Furthermore, K307 of OTC, which is used to sense CP levels by CP-K, is located adjacent to the CP-binding and catalytic pocket of OTC, which allows OTC activity to be accurately regulated by its substrate levels.

The central ammonia-coordinating role of SIRT4 is further supported by the fact that SIRT4 prevents the differentiation of radial glial cells into astrocytes (*45*), the functions of which are important for preventing HE (*46-49*). These findings are consistent with our results indicating that *Sirt4* knockout prevented HE induction by CCl_4. Moreover, CP-K levels are correlated with ageing (*11*), suggesting that inefficient ammonia removal associated with aging and may cause degenerative diseases. Elucidation of ammonia toxicity-regulating abilities of SIRT4 may enable its inhibition, leading to enhancement of ammonia detoxification and containment of diseases, such as renal failure, aging, and HE.

Limitations of the Study

To better understand why SIRT4 is needed as a decarbamylase to inhibit OTC and the urea cycle, it is essential to answer questions about how this regulation is synchronized to other OTC- and the urea cycle-regulating mechanisms, such as

acetylation-regulated OTC and the urea cycle (*5*). Moreover, anti-HE effects had been observed in *Sirt4*$^{-/-}$ mice, whether inhibiting SIRT4 would be useful in intervening HE need to be further investigated, because altered the CP levels and the urea cycle will not only affect ammonia disposal but also affect nucleotides synthesis, given that CP is nitrogen sources of both de novo and salvage generation of nucleotides.

METHODS

Antibodies and reagents

The antibodies against the following proteins/epitopes were purchased from the indicated sources: SIRT4 (Sigma-Aldrich, Cat# HPA029691), CPS1 (Abcam, Cat# ab3682), OTC (Abcam, Cat# ab91418, Cat# ab203859), ASS1 (Abcam, Cat# ab170952), ASL (Abcam, Cat# ab154182), ARG1 (Abcam, Cat# ab124917), Carbamyl-lysine (Abcam, Cat# ab175132), OTC K307cp (This work), GCN2 (Cell Signaling Technology, Cat# 3302), phospho-GCN2(T899) (R&D Systems, Cat# AF7605), EIF2a (Cell Signaling Technology, Cat# 5324), phospho-EIF2a(S51) (Cell Signaling Technology, Cat# 3398), ATF4 (Proteintech, Cat# 10835-1-AP), COX-IV (Cell Signaling Technology, Cat# 5662), SIRT3 (Cell Signaling Technology, Cat# 5490), SIRT5 (Cell Signaling Technology, Cat# 8782), GAPDH (Cell Signaling Technology, Cat# 5174), Flag tag (Sigma-Aldrich, Cat# F3165), HA tag (Sigma-Aldrich, Cat# H6908), Actin (Sigma-Aldrich, Cat# A2066).

The following reagents were purchased from indicated companies: Glutamine (Sigma-Aldrich, Cat# E6627), NAD+ (Sigma-Aldrich, Cat# V900401), Nicotinamide (Sigma-Aldrich, Cat# V900517), NH4Cl (Sigma-Aldrich, Cat# A9434), NH4Cl(15N) (Sigma-Aldrich, Cat# 299251), Carbamyl phosphate disodium salt (Sigma-Aldrich, Cat# C4135), L-ornithine (Sigma-Aldrich, Cat# 06503), L-citrulline (Sigma-Aldrich, Cat# C7629), Arginosuccinic acid disodium salt hydrate (Sigma-Aldrich, Cat# A5707), L-arginine (Sigma-Aldrich, Cat# A8094), Urea (Sigma-Aldrich, Cat# U5387), RPMI 1640 amino acids solution (50x) (Sigma-Aldrich, Cat# R7131), Penicillin-

Streptomycin (Invitrogen, Cat# 15070063), α-Ketoglutaric acid sodium salt dihydrate (Sigma-Aldrich, Cat# 75892), NADPH (Sigma-Aldrich, Cat# N7505), DTT (Sigma-Aldrich, Cat# 43819).

Cell Lines

HEK293T (human embryonic kidney), HepG2 (human hepatocellular carcinoma), Hep3B (human hepatocellular carcinoma) and Hepa 1-6 cells (mouse hepatoma) were cultured in normal DMEM (HyClone) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 units/ml penicillin (Invitrogen) and 100 μg/ml streptomycin (Invitrogen). SNU-449 (human hepatocellular carcinoma) cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS. Mouse primary hepatocytes were cultured in DMEM supplemented with 10% FBS and 100 nM insulin. All cells were cultured in an incubator at 37°C and 5% CO_2.

siRNA transfections and CRISPR/Cas9-mediated deletion of SIRT4

siRNA transfections were performed with Lipo2000 (Life technology) according to manufacturer's instruction. Cells were cultured in 6-well plate (ThermoFisher Scientific) and siRNAs were used at a concentration of 20nM. Cells were harvested at 24h post-transfection. siRNAs targeting OTC, eIF2a and ATF4 used in this study are listed in **Supplementary Tab 5.**

SIRT4-knockout HepG2 were generated by a CRISPR/Cas9 plasmid targeting

SIRT4. The plasmid was constructed by cloning the annealed sgRNA into px459 vector. The sgRNAs were designed by CRISPR Design website (http: //crispr.mit.edu) and the sequences are listed in **Supplementary Tab 5.**

Cultured cells treatments

To treat cultured cells, reagents are added to the culture media to reach final concentrations as indicated. For CP treatment, CP was added in culture medium to reach 5 mM final concentration; for NH_4Cl treatment, NH_4Cl was added in culture medium to reach 5 mM final concentration 12 h before cell harvesting; for amino acids starvation, cells were transferred to 1640 medium lack of either glutamine or proteinogenic amino acids for 12 h; for NAM treatment, NAM was added into culture medium to reach 5 mM final concentrations 3-5 h before cell harvesting.

Mice

Four-week-old C57BL/6 *Sirt4*$^{-/-}$ mice (constructed using CRISPR-mediated deletion of exons 3 and 4 of the mouse *Sirt4* gene) were purchased from GemPharmatech Co., Ltd. (Shanghai, China). Genotypes were determined via PCR using genomic DNA obtained from tails and two primer pairs (sequences are provided in **Supplementary Tab 5**). All mice were housed in a specific pathogen-free facility at 20–22 °C on a 12 h light/dark cycle with *ad libitum* access to food and water. Only male mice were used, and all experiments were started when mice were 6 weeks old.

Adult male mice were euthanized, and liver and kidney tissues were collected according to standard procedure. Briefly, livers were perfused with saline to remove blood cells before isolation, and the tissues were quickly removed and snap frozen in liquid nitrogen for further analysis. All animal procedures were conducted in accordance with the animal care committee at Fudan University, Shanghai, China.

$^{15}NH_4Cl$ incorporation was carried out by tail veil injection employing an intravenous visible mouse tail injection fixator (Cat# GD-100, Dermen Instrument). $^{15}NH_4Cl$ dissolved in saline was injected in adult male mice to reach 50 mg/kg body weight with saline as control. The incorporation was allowed for 30 min before liver and kidney were perfused with saline and quickly removed for further metabolites extraction and LC-MS/MS analysis.

Metabolomics analysis

Metabolite extraction

Frozen tissues were homogenized in 80% methanol (methanol:water = 4:1) and centrifuged at 14,000 × *g* for 15 min (4 °C). The supernatant was transferred to a new tube and lyophilized into a pellet. The sample was resuspended in 1500 μL of extract solution (acetonitrile:methanol:water = 2:2:1, with isotopically labelled internal standard mixture). After 30 s of vortexing, the samples were sonicated for 10 min in an ice-water bath. Next, the samples were incubated for 1 h at –40 °C and centrifuged at 12,000 g for 15 min at 4 °C. The resulting supernatant was transferred to a fresh

glass vial for analysis.

LC-MS/MS analysis

LC-MS/MS analyses were performed using a UHPLC system (Vanquish, ThermoFisher Scientific) with an UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μM) coupled to a Q Exactive HFX mass spectrometer (Orbitrap MS, ThermoFisher Scientific). The mobile phase consisted of 25 mmol/L ammonium acetate and 25 mmol/L ammonia hydroxide in water (pH = 9.75) (A) and acetonitrile (B). The analysis was carried out with an elution gradient as follows: 0~0.5 min, 95% B; 0.5~7.0 min, 95%~65% B; 7.0~8.0 min, 65%~40% B; 8.0~9.0 min, 40% B; 9.0~9.1 min, 40%~95% B; 9.1~12.0 min, 95% B. The column temperature was 30 °C. The temperature of the autosampler was 4 °C, and the injection volume was 2 μL.

The QE HFX mass spectrometer was used due to its ability to acquire MS/MS spectra in information-dependent acquisition mode under the control of acquisition software (Xcalibur, ThermoFisher Scientific). In this mode, the acquisition software continuously evaluates the full-scan MS spectrum. The ESI source conditions were set as follows: sheath gas flow rate of 30 Arb, Aux gas flow rate of 25 Arb, capillary temperature of 350 °C, full MS resolution of 60,000, MS/MS resolution of 7500, collision energy of 10/30/60 in NCE mode, and spray voltage of 3.6 kV (positive) or –3.2 kV (negative).

Data analysis

Raw data were converted to the mzXML format using ProteoWizard and processed

using an in-house programme, which was developed using R and based on XCMS, for peak detection, extraction, alignment, and integration. An in-house MS2 database (BiotreeDB) was applied for metabolite annotation. The cut-off for annotation was set at 0.3.

^{15}N labelling of metabolites

Cells were plated in 10 cm dishes for 24 h before labelling. The next day, cells were incubated in labelling media containing 5 mM $^{15}NH_4Cl$ for 6 h before collection. At the time of collection, cells were washed with prechilled saline, lyzed with (40%) methanol:(40%) acetonitrile:(20%) water with 0.1 M formic acid, and processed as described above (Metabolomics). Metabolites were reconstituted in 100 µL of 0.1% formic acid in LC/MS-grade water, vortex-mixed, and centrifuged to remove debris. The supernatants were analyzed using LC-MS/MS.

Proteomics procedures

Protein digestion

The procedures for protein digestion were performed according to the filter-aided sample preparation (FASP) technique (*50*) with slight modifications. Briefly, FASP digestion was adapted for the following procedures in Microcon PL-10 filters. Following a three-time buffer displacement with 4 M guanidine hydrochloride and

100 mM Tris-HCl, pH 8.0, proteins were reduced using 10 mM DTT at 37 °C for 30 min, followed by alkylation with 30 mM iodoacetamide at 25 °C for 45 min in the dark. Digestion was carried out with trypsin (enzyme:protein = 1:50) at 37 °C overnight after washing with 20% acetone nitrile and digestion buffer (30 mM Tris-HCl, pH 8.0). After digestion, the solution was filtrated out, the filter was washed with 15% ACN, and all filtrates were pooled, vacuum-dried, and kept at –80 °C until use.

Carbamyl peptide enrichment

Carbamyl peptide enrichment was performed using pan-anti-carbamyl lysine antibody (Abcam, Cat# ab175132) conjugated with protein A beads (Sigma, Cat# 16-156). Peptides were mixed with antibody-conjugated beads at 4 °C for 6–8 h under rotary shaking. The mixture was briefly centrifuged at 1,000 × *g* (<10 s), and the supernatant was carefully removed. Antibody-conjugated beads were subsequently washed thrice with PBS buffer. Finally, the bound carbamyl peptides were eluted with 30% ACN/0.1% TFA and concentrated in a SpeedVac. Before MS analysis, all carbamyl peptides were desalted.

LC-MS/MS analysis

LC-MS analysis was performed using a nanoflow EASYnLC 1200 system (ThermoFisher Scientific, Odense, Denmark) coupled to an Orbitrap Fusion Lumos mass spectrometer (ThermoFisher Scientific, Bremen, Germany). A one-column system was adopted for all analyses. Samples were analyzed on a C18 analytical

column (75 μm i.d. × 25 cm, ReproSil-Pur 120 C18-AQ, 1.9 μm (Dr. Maisch GmbH, Germany)) constructed in-house. The mobile phases consisted of Solution A (0.1% formic acid) and Solution B (0.1% formic acid in 80% ACN). The derivative peptides were eluted using the following gradients: 2–5% B in 2 min, 5–35% B in 100 min, 35–44% B in 6 min, 44–100% B in 3 min, 100% B for 10 min, at a flow rate of 200 nL/min. Data-dependent analysis was employed in MS analysis: the time between master scan was 3 s, and fragmented in HCD mode, normalized collision energy was 30.

Data processing and analysis

Raw MS files were searched using Protein Discoverer (version 2.4, ThermoFisher Scientific) with Mascot (version 2.7.0, Matrix Science). Data were processed using the UniProt mouse protein database (55,462 entries). The mass tolerances were 10 ppm for precursor and fragment mass tolerance 0.05 Da. Up to two missed cleavages were allowed. The search engine set protein N-acetylation, pyroglutamate on peptide N termini, oxidation on methionine, and carbamylation on Lys as variable modifications and cysteine carbamidomethylation as a fixed modification.

Proximity-dependent biotinylation (BioID) assay

BioID was performed to identify SIRT4-interacting proteins. Full-length SIRT4 was subcloned into a pLVXyu-BirA vector (with a C-terminal tag). The construct was transfected into cells and incubated for 36–48 h in complete media supplemented with

50 μL biotin. Following three PBS washes, cells were lyzed with NP40 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP40) supplemented with protease and phosphatase inhibitor cocktail and centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was then transferred to a new tube and incubated with 25 μL streptavidin-Sepharose beads overnight at 4 °C. Beads were collected and washed twice with NP40 buffer and thrice with 50 mM ammonium bicarbonate, pH 8.0. Proteins on beads were digested in 100 mM ammonium bicarbonate overnight with 1:100 (w/w, protein/enzyme) trypsin at 37 °C. The LC-MS/MS experiment was performed as described above.

Recombinant SIRT4 expression and purification

Recombinant SIRT4 expression was performed as previously described with slight modifications (*51*). The NAD^+-dependent deacetylase domain encompassing residues 27–314 of SIRT4 was subcloned into a pSUMOH10 vector (modified based on pET28b) containing an N-terminal 10xHIS-SUMO tag. The $SIRT4^{H161Y}$ mutant was generated using a fast mutagenesis kit (Vazyme, China) and verified via sequencing. Wild-type $SIRT4_{27–314}$ and HY mutant $SIRT4_{27–314}$ were expressed in *Escherichia coli* BL21 (DE3) strain (Transgene, China). After induction with 0.2 mM isopropyl β-D-thiogalactoside at 16 °C in LB medium supplemented with 0.1 mM $ZnCl_2$ overnight, cells were harvested by centrifugation at 4,000 g for 20 min at 4 °C. The pellets were resuspended in buffer A containing 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5%

glycerol, and 20 mM imidazole and were then disrupted using an EmulsiFlex-C3 homogenizer (Avestin) at 4 °C. The lysate was further cleared by centrifugation at 14,000 × *g* for 60 min at 4 °C, and the supernatant was loaded onto a His-trap affinity column (GE Healthcare). After washing with buffer A, bound proteins were subjected to on-column cleavage by ULP1 SUMO protease. The tag-free $SIRT4_{27–314}$ protein was collected as flow-through, centrifuge-concentrated, and then purified on an anion-exchange QHP column followed by size exclusion chromatography on a Superdex 75 column (GE Healthcare). Purified peak fractions were pooled, concentrated, aliquoted, and stored at −80 °C for future use. Other sirtuin family proteins were purified using the same procedure.

Preparation of CP-K and CP-K307 antibody

Anti-carbamyl-lysine (CP-K) antibody was purchased from Abcam (Cat# ab175132) to study the pan-carbamylation levels. Anti-OTC K307-CP (CP-K307) antibody was custom-made in abclonal (Shanghai, China) to detect the OTC K307 site-specific carbamylation levels. CP-K307 antibody was developed by using KLH-conjugated carbamylated OTC K307 peptides (sequence 303-310) as antigen. The resulted antigen was subjected to immunize rabbit to obtain polyclonal antibody.

In vitro decarbamylation reaction

Mitochondria of *Sirt4*$^{-/-}$ mice liver were obtained using the mitochondrial isolation

protocol mentioned above. After washed with SIRT deacylation buffer (50 mM Tris-HCl, pH 7.8, 1 mM DTT, and 1 mM $MgCl_2$, 2 mM NAD^+) for two times, mitochondria were resuspended with deacylation buffer then sonicated (SCIENTZ sonifier, output 40%, duty cycle 10 × 30sec) to rupture the mitochondrial membrane. Lysate were collected after the disrupted mitochondria were centrifuged for 15 min at 3500 g at 4°C, before the supernatant was used for decarbamylation reaction with 5μM recombinant SIRT4, SIRT3 and SIRT5 at 37°C for 2h. All samples were then analyzed by SDS-PAGE and probed with CP-K antibody (Abcam Cat# ab175132).

MS-based decarbamylation assay

The ability of SIRT4 to hydrolyze carbamyl lysine was measured using MALDI TOF/TOF. Carbamyl peptide (200 μM) was incubated with 5 μM SIRT3, SIRT4, or SIRT5 in reaction buffer containing 50 mM Tris-HCl, pH 7.8, 1 mM DTT, and 1 mM $MgCl_2$ without or with 2 mM NAD^+ for 2 h at 37 °C. Before MS analysis, all carbamyl peptides were desalted and eluted using C18 tips. Samples were analyzed using a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems/MDSSCIEX) and operated in Reflector Positive mode using 4000 Series Explorer Software. The laser intensity was set at 3500 V.

HPLC-based deacylation assay

SIRT4 de-acylation assays were conducted by incubating 5 μM recombinant SIRT4 with different concentrations (0-1500μM) of K307-modified OTC peptides (acetyl-,

carbamy-, HMG-, lipoyl-lysine) in the reaction buffer as described above. Reactions were initiated by addition of 1 mM NAD^+ and incubated at 30°C for 120 min before they were quenched with a final concentration of 5% (v/v) TFA. The formation of deacylated peptides were assayed by HPLC by monitoring UV_{215} signals. The amounts of products were obtained by comparing the areas of products with standard curves that were generated with standard commercial standard compounds of known concentrations.

SDS-PAGE and Western Blot

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed following standard protocols. Briefly, cells were harvested with loading buffer containing 50 mM Tris-HCl pH 6.8, 10% glycerol (v/v), 2% SDS (w/v), 4% β-mercaptoethanol (v/v), and 0.0012% bromophenol blue (w/v). For western blot analysis, each sample was subjected to SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare Life Science). The membranes were blocked in 5% (w/v) skim milk in Tris-buffered saline with 0.1% (v/v) Tween-20 (TBST) for 1 hr at RT and were then probed with primary antibodies in antibody dilution buffer (QuickBlock™, Beyotime) at 4°C overnight. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies in TBST (containing 5% skim milk), membranes were developed using ECL-Plus (Thermo Fisher Scientific) and visualized using Typhoon (GE Healthcare Life Science).

RT-PCR

RNA was isolated from cells by extraction with TRIzol (Transgene, China) according to the manufacturer's instructions, and the concentration/purity was determined using a NanoDrop Spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using the HiScript III 1st Strand cDNA Synthesis Kit (Vazyme) according to the manufacturer's instructions. Quantitative real-time PCR was performed with SYBR qPCR Master Mix (Vazyme) on a Real-Time Thermocycler (BioRad). qPCR analyses in cells or tissues are relative to β-actin. The primer sequences are listed in **Supplementary information Tab S5.**

Mitochondrial isolation

Cells were washed with prechilled PBS, harvested with isolation buffer (20 mM HEPES, pH 7.4, 140 mM KCl, 10 mM EDTA, 5 mM $MgCl_2$ with a protease and phosphatase inhibitor cocktail), and homogenized using a chilled glass-Teflon homogenizer with 20 strokes. The homogenate was centrifuged twice at 700 × *g* for 10 min at 4 °C. The supernatant was transferred to a new tube and centrifuged at 9,000 × *g* for 30 min at 4 °C to obtain the mitochondrial pellet. The pellet was collected and washed thrice with washing buffer (20 mM HEPES, pH 7.4, 800 mM KCl, 10 mM EDTA, and 5 mM $MgCl_2$).

Urea cycle enzymes assays

615 Fresh tissues were weighed and transferred to solubilization buffer (1% Triton X-100,
616 150 mM NaCl, 10 mM KCl, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0) containing
617 protease and phosphatase inhibitors in 5 μL of extraction buffer per mg tissue and
618 homogenized using ceramic beads. The samples were centrifuged at 14,000 × *g* for 15
619 min (4 °C). The supernatant was transferred to a new tube for immunoprecipitation
620 and enzymatic activity measurements, and protein concentrations were determined
621 using a BCA protein assay kit (Beyotime, China). The activities of urea cycle
622 enzymes were determined as described previously with slight modifications (*52, 53*).

623 **OTC:** 10 μL of Flag peptide-eluted ectopic OTC-Flag or 10 μL of OTC antibody-
624 immunoprecipitated bead solution was added to 120 μL of reaction buffer containing
625 5 mM ornithine, 15 mM CP, and 270 mM triethanolamine (pH 7.8) and incubated at
626 37 °C for 20–30 min. Reactions were terminated by adding 48 μL acid solution
627 (phosphoric acid:sulfuric acid = 3:1, v/v). Citrulline production was evaluated by
628 incubating 7 μL of 3% 2,3-butanedionemonoxime for 15 min at 95 °C in the dark.
629 Absorbance at 490 nm was determined using a microplate reader (Spectramax M2^e,
630 Molecular Devices).

631 **CPS1:** 10 μL of CPS1 antibody-immunoprecipitated bead solution was added to
632 reaction buffer containing 50 mM NH_4HCO_3, 5 mM ATP, 10 mM magnesium acetate,
633 5 mM N-acetyl-L-glutamate, 1 mM dithiothreitol, and 50 mM triethanolamine (pH
634 8.0) and incubated at 37 °C for 10 min. The product, CP, was converted to
635 hydroxyurea by adding 10 μL of 2.0 M hydroxylamine and incubating for 10 min at
636 95 °C. Hydroxyurea was quantified by adding chromogenic reagent followed by

incubation at 95 °C for 15 min, and the absorbance was read at 458 nm.

ASS1: 10 μL of ASS1 antibody-immunoprecipitated bead solution was added to reaction buffer containing 20 mM Tris-HCl, pH 7.8, 2 mM ATP, 2 mM citrulline, 2 mM aspartate, 6 mM $MgCl_2$, 20 mM KCl, and 0.2 units of pyrophosphatase to a final volume of 0.2 ml. Samples were incubated at 37°C in 96-well microtiter plates, and the reactions were stopped after 30 min by the addition of an equal volume of molybdate buffer (10 mM ascorbic acid, 2.5 mM ammonium molybdate, 2% sulfuric acid). The accumulation of phosphate was determined spectrophotometrically at 650 nm.

ASL: 10 μL of ASL antibody-immunoprecipitated bead solution was added to reaction buffer containing 0.2 mM fumarate, 1 mM arginine, and 50 mM Tris/HCl buffer, pH 7.5. The consumption of fumarate in the forward direction was assayed by monitoring the OD240 (absorbance of fumarate) in an Amersham Biosciences Ultrospec 3100 Pro spectrophotometer.

ARG: 10 μL of ARG antibody-immunoprecipitated bead solution was added to reaction buffer containing 5 mM arginine and 50 mM Tris/HCl buffer, pH 7.5. Urea production was quantified by the addition of 3% 2,3-butanedionemonoxime with 15 min of incubation at 95°C in the dark and reading the absorbance at 498 nm.

OTC kinetic assay

OTC and $OTCK^{307R}$ was ectopically-expressed in HEK293T cell and purified with

Anti-flag M2 beads (Sigma, Cat# A2220). To study the enzyme kinetics, 0.5 μM OTC and $OTCK^{307R}$ protein was added to the OTC enzymatic reaction buffer with different concentration of CP and ornithine (0-1 mM). Reactions were initiated by addition of recombinant OTC and OTCK307R protein at 37°C for 5 min before they were quenched with phosphoric and sulfuric acid (3:1, v/v) solution. The production of citrulline were measured by enzymatic assay of OTC as described above and the kinetics data were calculated according to Briggs–Haldane equation.

Ammonia and urea measurements

Cellular ammonia was analyzed using an Ammonia Quantification Kit (BioVision, Cat#K470) according to the manufacturer's instructions. Levels of urea in mouse primary hepatocytes, culture medium, and mouse urine were determined using a Urea Quantification Kit (BioVision, Cat#K376) according to the manufacturer's instructions.

Mice behavioral experiments

The open field and Y-maze tests were performed to test the locomotor function and spatial working memory, respectively, of HE mice.

To assess locomotor activity, mice were placed in a blue plexiglass case (40 × 40 cm). The floor was divided into two parts, with an inner zone in the middle of the arena (25% of the total arena) and an outer zone comprising the remaining area (75%

of the total arena). Initially, animals were placed in the inner zone, and their behaviour was monitored for 15 min using a video camera mounted on the ceiling above the centre of the arena. The motion trajectory was analyzed using Ethovision XT software.

To assess spatial working memory, mice were placed in a Y-maze consisting of three identical arms mounted in the shape of a “Y”. Each arm was 15 inches long and 3.5 inches wide, with 3-inch-high walls made of opaque material. The procedure consisted of two sessions, each lasting approximately 30 min. During the first session, 15 min in duration, one arm (the novel arm) was blocked, but the mouse could inspect the other two open arms. During the second session, all arms were open. The position of the animal in the maze was recorded using a video camera. The motion trajectory was analyzed using Ethovision XT software.

MRI

Animals were anesthetized using 1.5–2% isoflurane in 100% oxygen for all MRI experiments. During MRI experiments, the head of each mouse was fixed in a stereotaxic system (bite bar and a pair of ear bars). Brain MRI was performed on a 7 T animal MRI and MRS scanner (ClinScan, Bruker).

Quantification and statistical analysis

Statistical tests were performed using GraphPad Prism 8 software and two-tailed unpaired Student's *t*-test to compare two groups. All data are presented as the mean ± standard error of the mean (SEM). P values were calculated assuming a normal distribution and were corrected for multiple hypotheses using the Benjamini–Hochberg procedure. P values are denoted in figures as: ^{ns} $p > 0.05$, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.01$; ****$p < 0.001$.

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AUTHOR CONTRIBUTIONS

S.M. Zhao and J.Y. Zhao conceived the concept, designed and supervised the experiments; S.H. Hu, Y.X. Yang, H.D Ma, S.X Zou, K.H. Zhang, Y.N Qiao performed the biological experiments; L. Zhang performed LC-MS/MS-based target

metabolites measurement and metabolomics experiment; L. Huang performed proteomics experiment. Y.Y Yuan, Y. Lin, W. Xu, Y. Li, H.T. Li participated in the discussion. S.M. Zhao and J.Y. Zhao wrote the manuscript. All authors read and discussed the manuscript.

COMPETING INTEREST DECLARATION

The authors have declared that no conflict of interest exists.

SUPPLEMENTAL TABLES

Supplemental Table 1, List of protein substrates of CP-K identified in mice liver.

Supplemental Table 2, List of SIRT4-interactome identified in mice hepatoma (Hepa1-6) by Bio-ID assay.

Supplemental Table 3, Co-identified proteins in both K-CP substrates and SIRT4-interactome.

Supplemental Table 4, Untargeted metabolomics analysis of liver tissues of wild-type and *Sirt4*$^{-/-}$ mice.

Supplemental Table 5, Oligonucleotide.

Data availability

All the image source data and Excel files of all data presented in graphs within the

figures and extended figures will be provided in source data before published. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code availability

Data was analysed using Graphpad Prism 8.0 (Graphpad Software). Raw MS files were searched using Protein Discoverer (version 2.4, ThermoFisher Scientific) with Mascot (version 2.7.0, Matrix Science). The motion trajectory was analyzed using Ethovision XT software (Mice behavioral experiments).

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