



SIRT5 Potentiates Hepatocarcinogenesis by Modulating Protein Acylation in Mice*

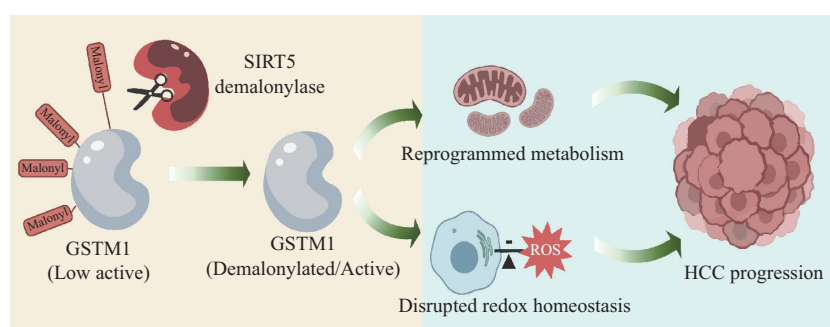
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Graphical abstract



Abstract Objective Hepatocellular carcinoma (HCC) represents 90% of all primary liver cancers. The main risk factors associated with HCC include viral hepatitis (B and/or C), alcohol abuse, and metabolic dysfunction-associated steatotic liver disease (MASLD), which progressively advance to liver fibrosis, cirrhosis, and ultimately evolve into HCC. Surgical resection represents the most effective treatment for HCC, while recent advances in immunotherapy, including immune checkpoint inhibitors and adoptive cell therapies, have provided improved treatment prospects for patients with unresectable HCC. However, the complex metabolic heterogeneity of HCC limits the therapeutic efficacy. Metabolic intermediates acyl-CoA not only provide energy and substrates for numerous biochemical reactions but also serve as donors for protein lysine acylation, a major class of post-translational modification (PTM). Therefore, a deeper understanding of the molecular mechanisms underlying protein lysine acylation and hepatocarcinogenesis is urgently needed. **Methods** The levels of protein lysine acylation and silence information regulator 5 (SIRT5) expression levels in clinical HCC samples were analyzed by Western blot. Quantitative malonylome and succinylome of HCC samples were analyzed by antibody-based affinity enrichment coupled with tandem mass spectrometry. The proliferation of HCC cells was analyzed with Cell Counting Kit-8 (CCK-8) assays, the apoptosis was quantified by Annexin V-FITC/propidium iodide (PI) staining coupled with flow cytometry, and the ability of cells to migrate was assayed by Transwell assays. The enzymatic activity of glutathione S-transferase Mu 1 (GSTM1) was quantified. Transgenic mice with hepatic overexpression of SIRT5 were

* This work was supported by grants from the National Key R&D Program of China (2022YFA1603702) and The National Natural Science Foundation of China (92254304).

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Received: February 28, 2026 Accepted: May 8, 2026

constructed using CRISPR-Cas9, and primary hepatocarcinogenesis was induced by administration of diethylnitrosamine. **Results** Western blot analysis indicated that the expression level of SIRT5 was elevated in clinical samples from HCC patients, and the levels of lysine malonylation, glutarylation, and succinylation were significantly reduced in HCC tissues. Knockout of SIRT5 in MHCC-97H and MHCC-97L hepatoma cells suppressed cell proliferation, and increased the percentage of apoptotic cells significantly. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the differentially malonylome and succinylome of HCC samples revealed significant enrichment in two major classes of biological processes: core energy metabolism (*e.g.*, glycolysis/gluconeogenesis, tricarboxylic acid metabolic process, fatty acid beta oxidation) and detoxification and oxidative stress response (*e.g.*, response to toxic substance, chemical carcinogenesis, reactive oxygen species (ROS)). SIRT5 removes malonylation from lysine residues in GSTM1 and restores its detoxification activity, which is crucial for the survival of hepatocytes under stressed conditions. More importantly, *in vivo* experiment indicated that hepatic-specific overexpression of SIRT5 in mice accelerated diethylnitrosamine-induced liver fibrosis and hepatocarcinogenesis, indicating the critical role of SIRT5 in HCC progression. **Conclusion** This study highlights the previously unrecognized SIRT5-GSTM1 axis as a key regulator in hepatocarcinogenesis, and suggests a potential target for the treatment of patients with HCC.

Key words hepatocellular carcinoma, silence information regulator 5, glutathione S-transferase Mu 1, post-translational modification, malonylation, succinylation

DOI: 10.3724/j.pibb.2026.0098

CSTR: 32369.14.pibb.20260098

As the critical metabolic hub of the body, the liver orchestrates the processing, partitioning, and metabolism of macronutrients, during which a wide array of metabolic intermediates^[1-2], including acyl-coenzyme A (acyl-CoA)^[3], are generated. Acyl-CoA not only provides energy and substrates for numerous biochemical reactions but also serves as donors for protein lysine acylation, a major class of post-translational modifications (PTMs)^[4]. Lysine acylation, including acetylation, malonylation, succinylation, and lactylation, dynamically regulates protein function and stability, and influences the metabolism and signaling pathways within cells^[5]. In previous work, we established that lysine malonylation and succinylation levels are elevated in type 2 diabetes mellitus (T2DM)^[6]; correspondingly, the elimination of malonylation and succinylation attenuates abnormal glucose and lipid metabolism in mice^[7]. Recently, we uncovered that succinylation of TANK-binding kinase 1 (TBK1), a key mediator linking metabolism and inflammation, is a driving force for metabolic dysfunction-associated steatotic liver disease (MASLD)^[8].

MASLD can progressively advance to liver fibrosis, cirrhosis, and ultimately evolve into hepatocellular carcinoma (HCC). Globally, HCC represents 90% of all primary liver cancers and was the sixth most commonly diagnosed cancer and the third leading cause of cancer-related mortality in 2022^[9-10]. Despite advances in surgical resection and immunotherapies that have improved survival of patients diagnosed with HCC, the complex genetic, metabolic and inflammatory interactions remain a barrier towards effective treatment^[11]. Therefore, a deeper understanding of the molecular mechanisms underlying hepatocarcinogenesis is urgently needed.

In this study, we report decreased protein lysine malonylation, glutarylation and succinylation levels in HCC clinical samples, while deacylase silence information regulator 5 (SIRT5) is upregulated. Bioinformatic analysis revealed the enrichment of malonylation and succinylation in proteins related to cell metabolism and redox homeostasis. SIRT5 removes malonylation from multiple lysine residues of glutathione S-transferase Mu 1 (GSTM1) directly, restoring its activity, which is crucial for hepatocyte function. More importantly, hepatic-specific overexpression of SIRT5 in mice accelerated diethylnitrosamine (DEN)-induced liver fibrosis and hepatocarcinogenesis, indicating the critical role of SIRT5 in HCC progression. These results highlight the previously unrecognized SIRT5-GSTM1 axis as a key regulatory pathway in hepatocarcinogenesis and suggest a potential novel therapeutic target for patients diagnosed with HCC.

1 Materials and methods

1.1 Antibodies and reagents

Antibodies against SIRT5 (15122-1-AP), β -tubulin (10068-1-AP) and β -actin (66009-1-Ig) were purchased from ProteinTech (Cambridge, UK). Anti-malonyllysine (PTM-901), anti-succinyllysine (PTM-419), anti-glutaryllysine (PTM-1125), anti-malonyllysine antibody conjugated agarose beads (PTM-902) and anti-succinyllysine antibody conjugated agarose beads (PTM-402) were purchased from PTM Biolabs (Hangzhou, China). Protease inhibitor cocktail (K4001), phosphatase inhibitor cocktail (K1014), and deacetylase inhibitor cocktail (K1017) were purchased from APExBIO (TX, USA). radio immunoprecipitation assay (RIPA) lysis buffer (R0020, R0030), protein loading buffer (5 $\times$) (P1040)

were purchased from Solarbio (China). A list of other chemicals used for liquid chromatography (LC) - tandem mass spectrometry (MS/MS) can be found in our previous publication^[6].

1.2 Cell culture

The human HCC cell lines MHCC-97H and MHCC-97L were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified atmosphere containing 5% CO₂.

The *SIRT5* targeting sgRNA (F: 5'-GCTTCAG-GCCACAATATAGC-3', R: 5'-GGTGCAGCTCATC-GATGTTC-3') was cloned into the lentiCRISPR v2 plasmid, and an empty vector was used as a control. The human *SIRT5* coding sequence was amplified by PCR from a cDNA template and cloned into the lentiviral vector pLVS-Puro, and an empty vector was used as a control. Stable *SIRT5*-overexpressing (OE) and knockout (KO) cell lines were established *via* lentiviral transduction as previously described^[12].

1.3 Western blot

Tumor samples from liver cancer patients were collected from the Department of Hepatobiliary Surgery, Cancer Hospital, Chinese Academy of Medical Sciences. The study was approved by the Ethics Committee of Cancer Hospital, Chinese Academy of Medical Sciences (approval No. 21/197-2868) and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient prior to surgery. The procedures related to human subjects were approved by the Ethics Committee of the Institute of Biophysics, Chinese Academy of Sciences. For analysis of protein acylation and *SIRT5* expression, 10 pairs of HCC and adjacent non-cancerous tissues were homogenized in RIPA lysis buffer (complete protease inhibitor cocktail and deacetylase inhibitors were added before use)^[13]. For cell lines, the cells were cultured and directly lysed in RIPA lysis buffer. Western blot analysis of protein expression levels was carried out as described previously^[14].

1.4 Cell proliferation and apoptosis assays

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8; 40203ES, Yeasen Biotechnology)^[14]. In brief, cells in the logarithmic growth phase were seeded into 96-well plates. After cell attachment, the time point was designated as 0 h, and the absorbance at 450 nm of the CCK-8 working solution was measured immediately. Cells were then cultured for an additional 24, 72, and 120 h. At each indicated time point, the absorbance was measured following the same procedure. Cell proliferation was normalized to the 0 h baseline absorbance.

Cell apoptosis was detected using the Annexin V-FITC/PI apoptosis detection kit (40302ES, Yeasen Biotechnology) following the manufacturer's instructions. Briefly, harvested cells were washed twice with pre-cooled PBS and resuspended in 1× binding buffer. Annexin V-FITC and PI staining solutions were added, gently mixed, and incubated at room temperature for 15 min in the dark. Subsequently, 1×binding buffer was added, and the samples were kept on ice. Apoptotic cells were analyzed by BD FACSCalibur within 1 h.

1.5 Migration and invasion assays

Cell migration and invasion were assessed using Transwell chambers with 8 μm pore size membranes^[15]. For invasion assays, the upper chambers were pre-coated with Matrigel, while for migration assays, the chambers were left uncoated. Cells were seeded in the upper chambers in serum-free medium, and medium containing 10% FBS was added to the lower chambers as a chemoattractant. After incubation for the indicated time, cells remaining on the upper surface of the membrane were removed with cotton swabs, and cells that had migrated or invaded to the lower surface were fixed, stained with crystal violet, and photographed under a microscope. The number of migrated or invaded cells was counted in five randomly selected fields per chamber.

1.6 Mass spectrometry

Mice liver tissues were homogenized in RIPA buffer containing Protease Inhibitor Cocktail, then centrifuged at 12 000g at 4°C for 15 min. Proteins in the supernatant were precipitated with acetone and resolved in 8 mol/L urea containing 50 mmol/L Tris-HCl, pH 8.0. Tryptic digestion and affinity enrichment of acylated peptides were performed as previously described^[6]. The malonyllysine and succinyllysine peptides were analyzed by reverse phase LC-MS/MS against the UniProt mouse proteome database (release 03/2025) using the SEQUEST HT search engine implemented in Thermo Proteome Discoverer (version 2.2.0.388). The normalization to the protein median of each sample was used to correct for experimental bias, with total peptide amount selected as the normalization mode. Protein ratios were calculated as the median of all possible pairwise ratios. Gene Ontology (GO) functional enrichment analysis ($P < 0.05$) was performed using the clusterProfiler package in R (version 3.6.1).

1.7 Purification and *in vitro* malonylation of GST proteins

C57BL/6J mice were purchased from Vital River Co., Ltd. (Beijing, China). Liver tissues from mice

were homogenized in IP buffer and centrifuged at 12 000g for 10 min at 4°C. The supernatants were incubated with glutathione (GSH)-conjugated agarose beads for 2 h at room temperature. After washing twice with IP buffer, bound GST proteins were eluted with elution buffer for 30 min at room temperature. The eluates were dialyzed against dialysis buffer to remove glutathione, concentrated, and protein concentrations were determined using the BCA protein assay kit.

To enhance malonylation *in vitro*, purified GST proteins were incubated with 1 mmol/L malonyl-CoA in reaction buffer (pH 8.0) at 37°C for 8 h. The reaction mixture was subjected to mass spectrometry and immunoblotting to detect malonylation levels. Equal amounts of GST proteins from each group were used for GST activity assay using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. The formation of the CDNB-GSH conjugate was monitored spectrophotometrically by measuring the absorbance at 340 nm. Enzyme activity was expressed as units (U). All assays were performed in triplicate and repeated at least three times independently.

1.8 Animal and DEN-induced HCC model

Hepatic SIRT5-overexpressing mice on a C57BL/6 background were established by using the CRISPR/Cas9 gene editing tool we described previously^[16]. Mouse studies were approved by the ethics committee of the Institute of Biophysics, Chinese Academy of Sciences (approval number: SYXK2020064), following the National Health and Medical Research Council of China Guidelines on Animal Experimentation. To study DEN-induced HCC, mice on postnatal day 14 were intraperitoneally injected with a single dose of DEN (25 mg/kg). The mice were sacrificed at 9 months of age to enable analysis of their tumor burdens.

1.9 Masson's trichrome staining

Masson's trichrome staining was performed on mouse liver tumor tissues using a commercial kit according to the manufacturer's instructions (G1340, Solarbio). Briefly, Formalin-fixed, paraffin-embedded sections (4 µm) were deparaffinized, rehydrated, and mordanted with Bouin's solution. Sections were sequentially stained with Weigert's iron hematoxylin and Biebrich scarlet-acid fuchsin, differentiated in phosphomolybdic-phosphotungstic acid, and stained with aniline blue. After differentiation in 1% acetic acid, sections were dehydrated, cleared in xylene, and mounted for microscopic examination.

1.10 Statistical analysis

All experiments were performed independently

at least three times. Data are presented as the mean ± standard deviation (SD). Unless otherwise specified, statistical comparisons between two groups were performed using an unpaired two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant. Significance levels are indicated as: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

2 Results

2.1 Reduced protein acylation and elevated SIRT5 levels in clinical samples from patients diagnosed with HCC

We first examined the protein malonylation, glutarylation and succinylation levels in paired clinical samples from patients diagnosed with HCC. Results of Western blot analysis revealed that in comparison with adjacent non-cancerous tissues, the levels of lysine acetylation were significantly reduced in HCC tissues (Figure 1a). Since SIRT5 is the primary deacylase responsible for the removal of malonyl, glutaryl and succinyl groups from lysine residues, we further examined the expression of SIRT5 in HCC samples and found a significant elevation of SIRT5 in 9 out of 10 samples (Figure 1b, c).

The reduced protein malonylation, glutarylation and succinylation, and the elevated SIRT5 in HCC implied the possibility that SIRT5 is crucial for protein acylation. To verify this hypothesis, we overexpressed SIRT5 in MHCC-97H hepatoma cells and detected the levels of protein acylation. As shown in Figure 1d, overexpression of SIRT5 led to a marked reduction in protein malonylation, glutarylation and succinylation. Collectively, these data indicate that elevated SIRT5 is likely a principal driver of the reduction in protein acylation observed in tumor tissues.

2.2 SIRT5 deletion suppresses the proliferation and migration of hepatoma cells

Next, we investigated the impact of SIRT5 expression in hepatoma cells *in vitro* by generating SIRT5-knockout (KO) cell lines using CRISPR/Cas9 (Figure 2a, b). Knockout of SIRT5 in both MHCC-97H and MHCC-97L cells significantly suppressed the cell proliferation (Figure 2c, d); moreover, a moderate but significant increase in the percentage of apoptotic cells was also observed in SIRT5-KO cells (Figure 2e, f). Considering that metastasis contributes to the poor prognosis of HCC, we evaluated the influence of SIRT5 on the migration of hepatoma cells *in vitro*. Transwell assays indicated reduced cell migration capacity in SIRT5-KO cells (Figure 2g, h).

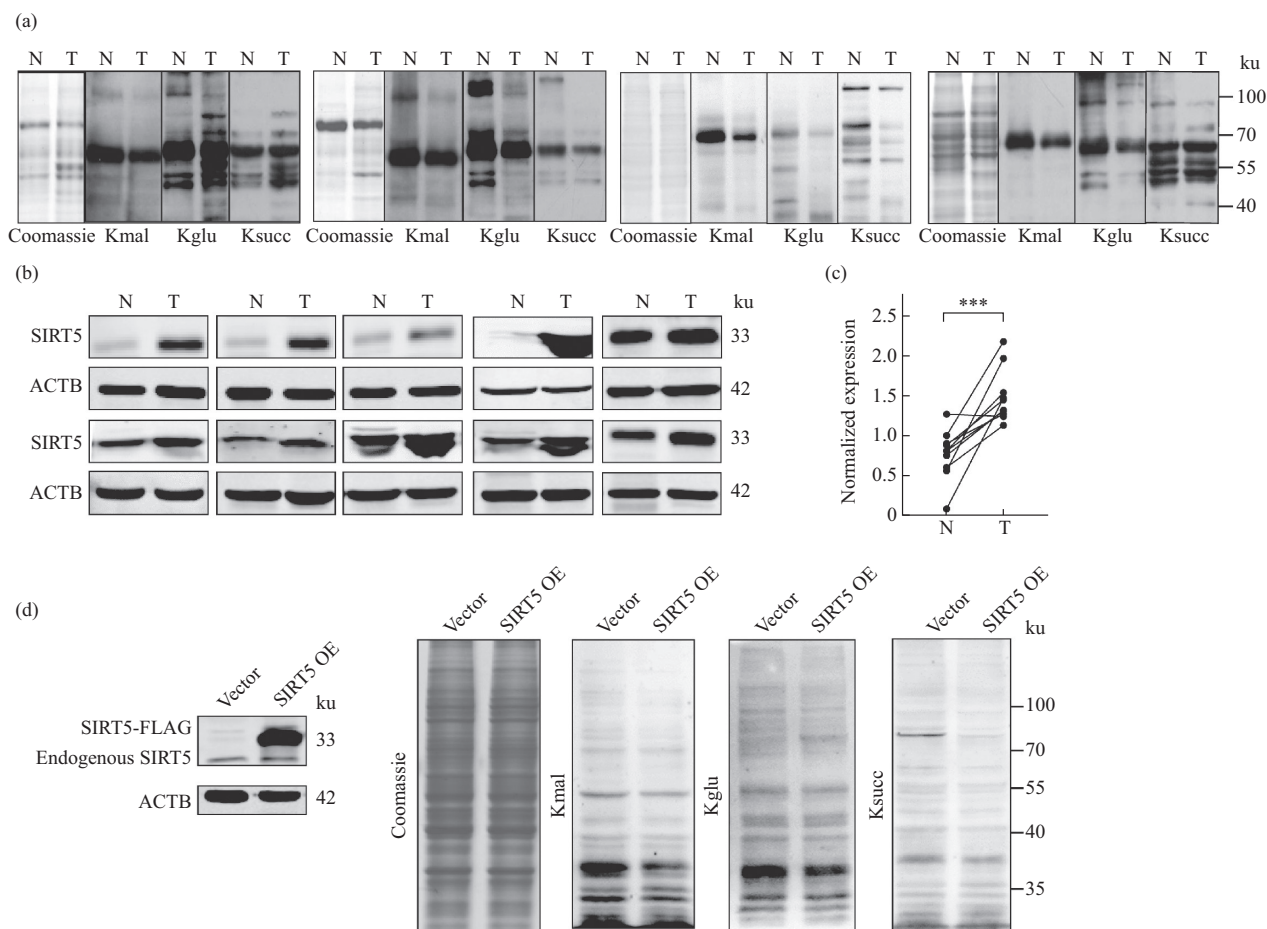


Fig. 1 SIRT5 is up-regulated in HCC and negatively associated with the protein lysine malonylation, glutarylation and succinylation

(a) Malonylation (Kmal), glutarylation (Kglu), and succinylation (Ksucc) levels were detected in four paired clinical samples of hepatocellular carcinoma (T) and adjacent non-cancerous tissues (N). Coomassie blue staining was used as a loading control. (b) SIRT5 expression levels were detected in ten paired clinical specimens of hepatocellular carcinoma (T) and adjacent non-cancerous tissues (N). β -Actin (ACTB) was used as a loading control. (c) Quantitative analysis of SIRT5 expression levels. *** $P < 0.001$ compared with adjacent non-cancerous tissues ($n = 10$) by paired two-tailed Student's t -test. (d) Establishment of SIRT5-overexpressing cells with Flag-tag, showing endogenous and exogenous SIRT5 expression (left). Malonylation (Kmal), glutarylation (Kglu), and succinylation (Ksucc) levels in Vector and SIRT5-OE cells. Coomassie blue staining was used as a loading control (right).

2.3 SIRT5 reprograms the malonylome and succinylome of metabolic pathways

To identify the specific protein targets and pathways regulated by SIRT5 in HCC patients, we performed quantitative malonylome and succinylome analyses using HCC and adjacent non-cancerous liver tissues with antibody-based affinity enrichment coupled with tandem mass spectrometry^[17] (Figure 3a). In total, we identified and quantified 395 unique succinylated peptides (corresponding to 156 proteins) and 59 unique malonylated peptides (corresponding to 34 proteins). Further analysis revealed that 29 malonylated peptides and 184 succinylated peptides were exclusively detected in adjacent non-tumor

tissues but absent in HCC tissues (Figure 3b). GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the differentially modified proteins revealed significant enrichment in two major classes of biological processes: core energy metabolism (*e. g.*, glycolysis/gluconeogenesis, tricarboxylic acid metabolic process, fatty acid beta oxidation) and detoxification and oxidative stress response (*e. g.*, response to toxic substance, chemical carcinogenesis, reactive oxygen species (ROS)) (Figure 3c, d). These findings suggest that SIRT5 may actively promote HCC progression by selectively removing malonyl and succinyl groups from key metabolic and antioxidative enzymes, thereby

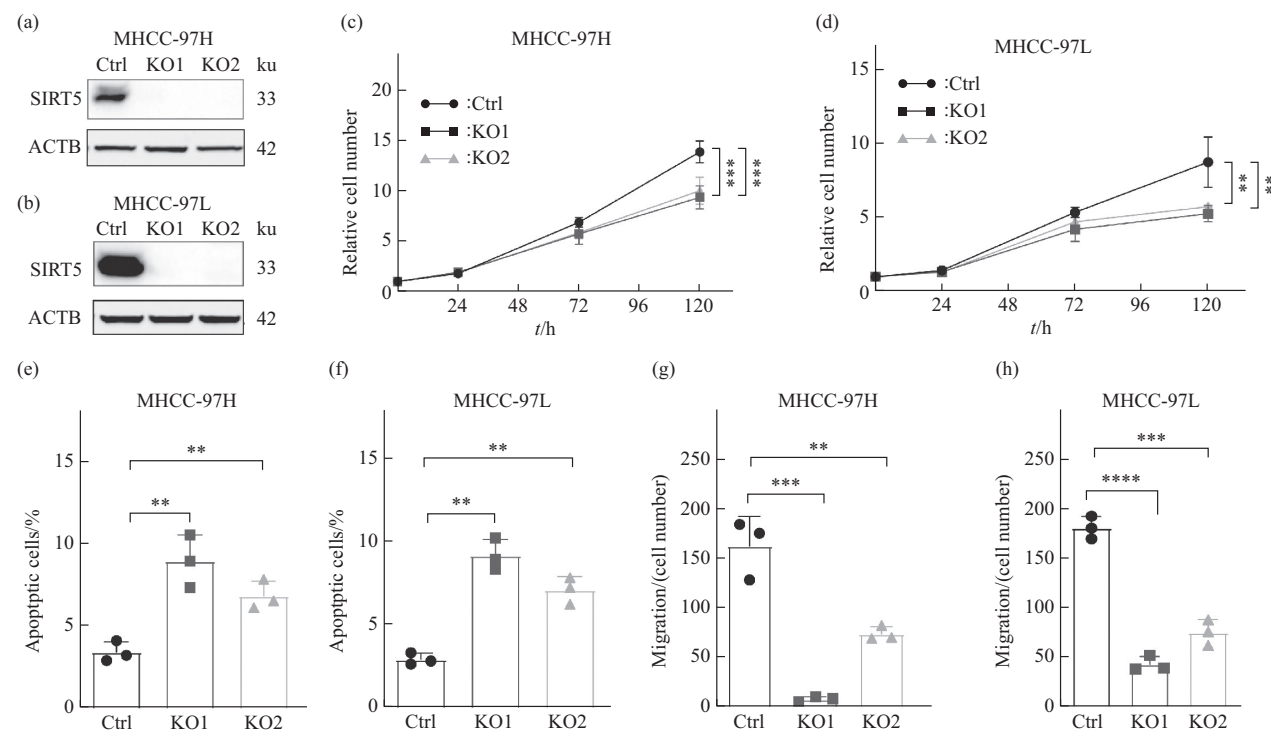


Fig. 2 Knockout of SIRT5 suppressed proliferation and migration, and induced apoptosis in hepatoma cells

(a) SIRT5 knockout was confirmed in MHCC-97H cells. (b) SIRT5 knockout was confirmed in MHCC-97L cells. (c,d) The relative cell number of Ctrl and SIRT5-knockout MHCC-97H cells (c) and the relative cell number of Ctrl and SIRT5-knockout MHCC-97L cells (d) were assessed at the indicated time points. ** $P < 0.01$, *** $P < 0.001$ compared with Ctrl ($n = 6$) by two-way ANOVA. (e,f) Quantification of apoptosis in Ctrl and SIRT5-knockout MHCC-97H cells (e) and quantification of apoptosis in Ctrl and SIRT5-knockout MHCC-97L cells (f). Data are presented as mean $\pm$ SD from three independent experiments. ** $P < 0.01$ compared with Ctrl by unpaired t -test. (g, h) Quantification of cell migration in Ctrl and SIRT5-knockout MHCC-97H cells (g) and quantification of cell migration in Ctrl and SIRT5-knockout MHCC-97L cells (h). Data are presented as mean $\pm$ SD from three independent experiments. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared with Ctrl for KO1 and KO2 by an unpaired t -test, respectively.

reshaping metabolic pathways, cellular detoxification, and ROS defense networks in malignant cells.

Among SIRT5-regulated proteins, GSTM1 is one of the most prominently expressed glutathione S-transferase isoforms in the liver and plays a central role in cellular detoxification and antioxidant defense by catalyzing the conjugation of glutathione to electrophilic substrates^[18-19]. To elucidate the functional impact of malonylation on GSTM1 activity, we conducted a biochemical reconstitution assay. Purified GSTM1 protein was incubated with malonyl-CoA, which induced nonenzymatic malonylation of lysine residues (Figure 3e). Malonylation markedly inhibited GSTM1 activity (Figure 3f). However, the malonylation of GSTM1 could be effectively removed by recombinant SIRT5, and the enzymatic activity of GSTM1 was recovered upon demalonylation. These results demonstrate that the activity of GSTM1 can be reversibly regulated by malonylation and demalonylation. Integrated mass

spectrometry and structural analyses suggested that this regulation likely targeted a critical and putative lysine residue (K129) adjacent to the substrate-binding pocket of GSTM1 (Figure 3g). This finding implied that SIRT5-dependent demalonylation of GSTM1 might act as a molecular switch, linking upstream metabolic signals to downstream detoxification and redox homeostasis, and ultimately influence the progression of HCC.

2.4 Hepatic overexpression of SIRT5 exacerbates hepatocarcinogenesis and liver fibrosis in mice

Next, we treated mice with DEN to induce HCC in mice (Figure 4a) and analyzed SIRT5 expression in murine HCC tissues and adjacent non-tumorous liver tissues. Consistent with clinical human HCC samples, SIRT5 was markedly upregulated in murine HCC samples (Figure 4b). To investigate the possible tumor-promoting function of SIRT5 *in vivo*, we generated hepatocyte-specific SIRT5-overexpressing mice (referred to as Liver *Sirt5* OE mice) by crossing

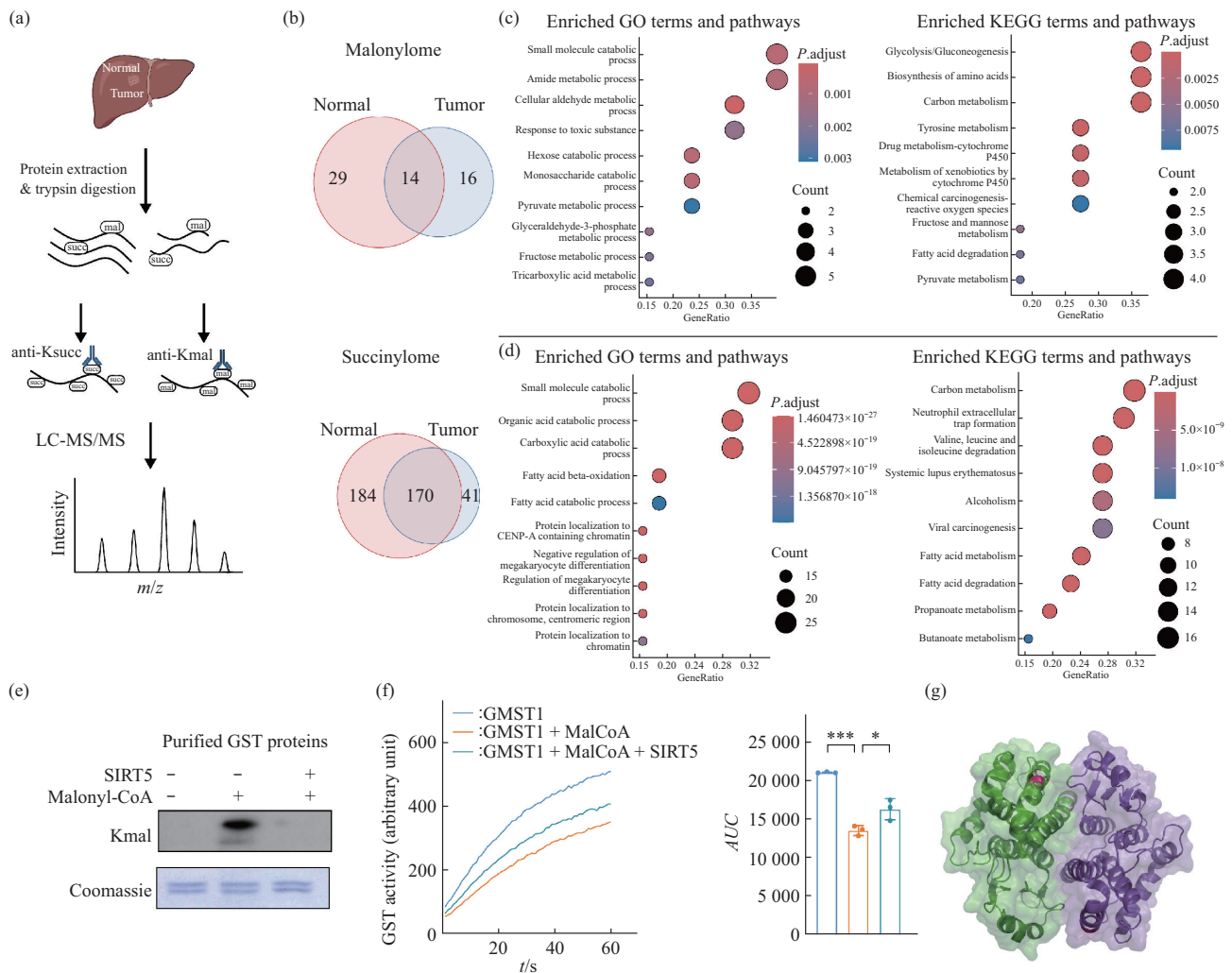


Fig. 3 SIRT5 reprograms the malonylome and succinylome of metabolic pathways and regulates the activity of GSTM1

(a) Workflow of quantitative malonylome and succinylome analysis. (b) Venn diagrams showing the number of malonylated (up) and succinylated (down) proteins identified in HCC (tumor, blue) and adjacent non-cancerous tissues (normal, pink). (c) GO and KEGG pathway enrichment analyses of differentially malonylated proteins. (d) GO and KEGG pathway enrichment analyses of differentially succinylated proteins. (e) *In vitro* malonylation and demalonylation of GSTM1. Purified GSTM1 protein was incubated with malonyl-CoA alone or together with recombinant SIRT5. Coomassie blue staining was used as a loading control ($n=3$). (f) GST activity of malonylated or demalonylated GSTM1. GSTM1 was treated with malonyl-CoA alone or together with SIRT5, and then GST activity was measured (left). Quantification of area under the curve (AUC) from the time course data. Data are presented as mean $\pm$ SD ($n=3$). Unpaired two-tailed Student's *t*-test, * $P<0.05$, *** $P<0.001$ (right). (g) Structure of the mouse GSTM1 dimer. Lysine 129 (K129) is highlighted in red.

Sirt5 $KI^{fllox/fllox}$ with *Alb-cre* mice (*Alb-cre*) (Figure 4c). Both Liver *Sirt5* OE mice and wild-type mice (WT) were challenged with DEN to induce HCC. Nine months after DEN injection, Liver *Sirt5* OE mice exhibited markedly accelerated tumor development, evidenced by increases in both tumor mass and nodule multiplicity compared to WT mice (Figure 4d–f). Furthermore, Masson's trichrome staining revealed extensive fibrosis and collagen deposition in the tumors from Liver *Sirt5* OE mice (Figure 4g, h). These findings provide *in vivo* evidence that

overexpression of SIRT5 facilitates HCC tumorigenesis.

3 Discussion

Lysine acylation is involved in the regulation of protein structures and functions, but its possible roles in tumor progression remain unclear. Here, we demonstrate decreased protein lysine acylation and elevated SIRT5 in HCC clinical samples, while hepatic overexpression of SIRT5 causes marked progression of DEN-induced hepatocarcinogenesis in

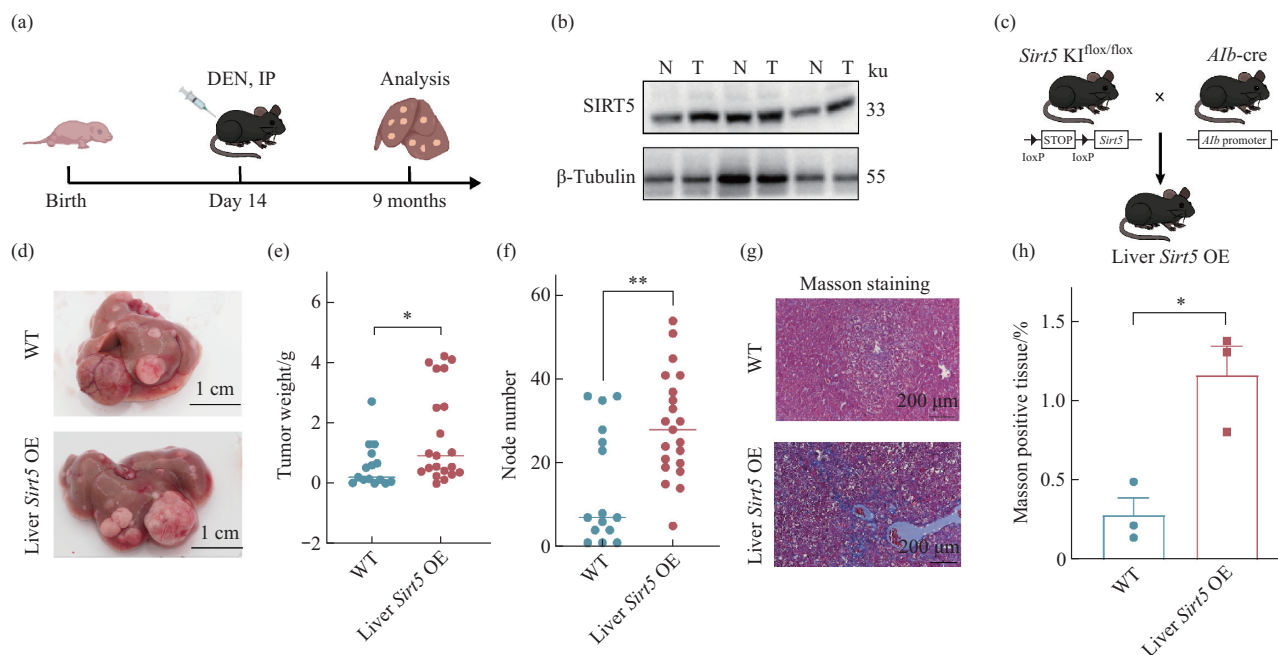


Fig. 4 Hepatic overexpression of SIRT5 exacerbates hepatocarcinogenesis and liver fibrosis in mice

(a) Schematic diagram of the DEN-induced HCC mouse model. Mice were injected with DEN at 14 d after birth, and liver tissues were collected at 9 months of age. (b) Western blot analysis of SIRT5 expression in liver tissues from control and DEN-treated mice. β -Tubulin was used as a loading control ($n=3$). (c) Strategy for generating liver-specific SIRT5 overexpressing mice. (d) Representative photographs of livers from wild-type (WT) and liver-specific SIRT5 overexpression (Liver *Sirt5* OE) mice subjected to DEN-induced HCC. (e) Tumor weight was measured from dissected livers of WT and Liver *Sirt5* OE mice. Horizontal lines represent the mean. $*P<0.05$ compared with WT ($n=15$ in WT and $n=21$ in Liver *Sirt5* OE) by unpaired t -test. (f) Tumor node number per liver in WT and Liver *Sirt5* OE mice. Horizontal lines represent the mean. $**P<0.01$ compared with WT ($n=15$ in WT and $n=21$ in Liver *Sirt5* OE) by unpaired t -test. (g) Representative images of Masson's trichrome staining of liver tissues from WT and Liver *Sirt5* OE mice. Collagen fibers are stained blue. (h) Quantitative data were derived from individual mice ($n=3$ each group), while the Masson staining images shown are representative of one mouse per group. Data are presented as mean $\pm$ SD. $*P<0.05$ compared with WT by unpaired t -test.

mice. Mass spectrometry and biochemical analysis indicate that SIRT5 removes the malonyl modifications from GSTM1 and restores its activity, which is crucial for the survival and proliferation of HCC cells. Collectively, these data reveal the importance of the SIRT5-GSTM1 axis as a key regulator in the progression of HCC.

HCC represents 90% of all primary liver cancers^[10]. The main risk factors associated with HCC include viral hepatitis (hepatitis B virus (HBV) and hepatitis C virus (HCV))^[20-21], alcohol abuse^[22-23], and MASLD^[20, 24]. With the widespread use of hepatitis vaccines and antiviral therapies, the proportion of HCC cases driven by HBV/HCV infection has steadily declined^[25]. On the other hand, MASLD is estimated to affect about one-third of adults worldwide^[26]. The increasing prevalence of MASLD, and its progression to metabolic dysfunction-associated steatohepatitis (MASH) raises the incidence of progressive liver fibrosis, cirrhosis, and HCC^[27]. The incidence of HCC related to MASLD is

projected to increase by 47% to 130% by the year 2030^[28].

Hepatocarcinogenesis is a complex, multifactorial process involving various risk factors including metabolic alterations, chronic inflammation, oxidative stress, mitochondrial dysfunction, and altered immune response^[27]. Recently, we uncovered the important role of SIRT5 in the progression of MASLD, by coupling metabolism and inflammation^[8]. SIRT5 is an enzyme with highly efficient demalonylation and desuccinylation activities. It dynamically regulates the acylation status of various metabolic enzymes and is deeply involved in cellular glycolysis^[29], fatty acid oxidation^[30], and the urea cycle^[31].

The biological role of SIRT5 in HCC remains academically controversial. Multiple studies support SIRT5 as an oncogenic factor. For example, SIRT5 can promote HCC progression by promoting cell proliferation (targeting E2F1)^[32], inhibiting mitochondrial apoptosis^[33], and driving aerobic

glycolysis^[34]. In contrast, other studies have reported tumor-suppressive effects. Peroxisomal SIRT5 inhibits ACOX1 activity through desuccinylation, thereby playing a role in liver protection and the inhibition of HCC development^[34]. In addition, downregulated SIRT5 in human HCC also contributes to the immunosuppressive microenvironment, thereby cooperating with oncogenes to drive hepatocarcinogenesis^[33]. By using both cellular and animal models, our study supports that SIRT5 is essential for maintaining the malignant phenotypes of HCC cells—including proliferation, migration, and apoptosis resistance—and for driving hepatocarcinogenesis *in vivo*. These apparently conflicting conclusions may arise from several aspects. First, differences in research models, including HCC induction methods and sample sources, are likely to be important. The etiology of HCC formation is highly heterogeneous, mainly driven by different factors such as HBV, HCV infection^[21], or MASLD^[22, 24], resulting in significant differences in gene expression profiles. In our study, due to biosafety considerations, we analyzed primary surgical HCC specimens from patients without HBV or HCV infection, thereby enriching for cases with a metabolic disease background. Additionally, our study used DEN induction, whose pathological process is different from other models (such as hydrodynamic injection) that focus on oncogene-driven mechanisms. Finally, and most importantly, the biological function of SIRT5 may exhibit significant “substrate selectivity” and “environmental dependency”. Under different etiologies, different cellular microenvironments, or subcellular localizations, SIRT5 may regulate different downstream substrates, thereby mediating completely different or even opposite biological outputs.

To further elucidate the mechanism by which SIRT5 promotes hepatocarcinogenesis, we performed a proteomics analysis to uncover the key substrates of SIRT5. Among multiple metabolic and detoxification-related proteins regulated by SIRT5, we focused on GSTM1. GST is a superfamily of cytosolic soluble detoxification enzymes, which conjugate reduced glutathione to various xenobiotics and endobiotics and thus play an important role in cellular protection against oxidative stress and exogenous toxins. It has been reported that GSTM1/glutathione S-transferase theta 1 (GSTT1) null genotypes are associated with an increased risk of HCC in the Chinese population^[35]. GSTM1 also plays a dual role in the development and metastasis of HCC, by modulating redox homeostasis^[36]. With the aid of mass spectrometry and

enzymatic assays, we provide direct evidence that SIRT5 directly regulates GSTM1 activity *via* demalonylation, potentially at K129. Alterations in the modification state at this site may directly influence substrate binding affinity, thereby dynamically tuning enzymatic activity.

4 Conclusion

In this study, we identified that SIRT5 is elevated in clinical samples from HCC patients, and is responsible for the hypoacylation (malonylation, glutarylation and succinylation) of proteins related to metabolism, biosynthesis, detoxification and redox homeostasis. More importantly, SIRT5 removes malonylation from the lysine residues of GSTM1 and restores its activity, which is crucial for the survival of hepatocytes under stressed conditions. Hepatic-specific overexpression of SIRT5 in mice accelerated diethylnitrosamine-induced liver fibrosis and hepatocarcinogenesis, indicating the critical role of SIRT5 in HCC progression. This study highlights the previously unrecognized SIRT5-GSTM1 axis as a key regulator in hepatocarcinogenesis, and suggests a potential target for the treatment of patients with HCC.

Acknowledgements The authors are indebted to Profs. LIU Ping-Sheng and YANG Fu-Quan for valuable discussions, and Drs. DU Yi-Peng, DU Kang, CAI Tan-Xi and WANG Ji-Feng (Institute of Biophysics, Chinese Academy of Sciences) for technical assistance.

Data Availability The data associated with this paper (DOI: 10.57760/sciencedb.34962/CSTR: 31253.11.sciencedb.34962) are available from Science DataBank (ScienceDB) (<https://www.sciadb.cn/anonymous/MzZaQmYy>).

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沉默信息调节因子5调控蛋白质酰基化修饰 并促进小鼠肝细胞癌发生发展*

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摘要 目的 肝细胞癌占原发性肝癌90%以上, 外科手术是治疗肝细胞癌的主要治疗方法。近年来, 免疫治疗(包括免疫检查点抑制剂和过继性细胞治疗)的突破为不可手术的肝细胞癌患者提供了替代治疗方案, 但肝细胞癌患者的复杂遗传异质性和代谢异质性制约了治疗效果。本研究旨在探究沉默信息调节因子5(silence information regulator 5, SIRT5)介导的赖氨酸去酰基化修饰在肝细胞癌代谢重编程中的调控作用, 并明确其通过去酰基化激活谷胱甘肽硫转移酶M1(glutathione S-transferase Mu 1, GSTM1)解毒功能, 从而促进肝细胞癌发生的关键分子机制。**方法** 通过蛋白质印迹法分析了肝细胞癌临床标本中的蛋白质赖氨酸酰基化水平及SIRT5表达水平, 通过基于CRISPR-Cas9的基因编辑技术构建了在肝组织中特异性过表达SIRT5的转基因小鼠, 并以此建立原发肝癌模型, 以分析肝组织SIRT5在原发性肝癌发生发展中的作用。**结果** SIRT5在肝细胞癌临床标本中表达水平升高, 其表达水平与蛋白质赖氨酸酰基化水平降低相关。蛋白组学分析及生物信息学分析表明, 肝细胞癌组织中与代谢、生物合成、解毒及氧化还原稳态相关蛋白质的酰基化修饰(包括丙二酰化修饰、琥珀酰化修饰)发生改变, 进一步通过蛋白质修饰分析及酶活性分析证明GSTM1的活性受丙二酰化修饰影响。在肝组织中特异性过表达SIRT5加速二乙基亚硝胺引发的肝组织纤维化, 促进肝细胞癌的发生和发展。**结论** SIRT5通过擦除GSTM1等关键酶的酰基化修饰而影响肝细胞代谢及氧化还原稳态, 从而促进肝细胞癌的发生发展。干预蛋白质酰基化修饰可能为肝细胞癌的免疫治疗带来新的机遇。

关键词 肝细胞癌, 沉默信息调节因子5, 谷胱甘肽硫转移酶M1, 蛋白质翻译后修饰, 丙二酰化修饰, 琥珀酰化修饰

中图分类号 Q51, R73

DOI: 10.3724/j.pibb.2026.0098

CSTR: 32369.14.pibb.20260098

* 国家重点研发计划(2022YFA1603702)和国家自然科学基金(92254304)资助项目。

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收稿日期: 2026-02-28, 接受日期: 2026-05-08