



SIRT5-mediated SDHA desuccinylation promotes clear cell renal cell carcinoma tumorigenesis

Yuanzhen Ma^{a,1}, Yijun Qi^{a,1}, Lei Wang^b, Zhaoxu Zheng^a, Yue Zhang^a, Junfang Zheng^{a,c,*}

^a Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Capital Medical University, Beijing, 100069, China

^b Department of Urology, Beijing Friendship Hospital, Capital Medical University, Beijing, China

^c Beijing Key Laboratory for Cancer Invasion and Metastasis Research, Beijing International Cooperation Base for Science and Technology on China-UK Cancer Research, Beijing, 100069, China



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ABSTRACT

Metabolic reprogramming is a prominent feature of clear cell renal cell carcinoma (ccRCC). Protein succinylation influences cell metabolism, but its effects on ccRCC tumorigenesis remain largely uncharacterized. In this study, we investigated the lysine succinylome of ccRCC tissues by using tandem mass tag labeling, affinity enrichment, liquid chromatography–tandem mass spectrometry and integrated bioinformatics analyses. Proteins involved in metabolic process, the tricarboxylic acid (TCA) cycle, oxidation-reduction and transport processes were subject to succinylation. A total of 135 sites in 102 proteins were differentially succinylated between ccRCC and adjacent normal tissues. Succinate dehydrogenase complex subunit A (SDHA), which is involved in both the TCA cycle and oxidative phosphorylation, was desuccinylated at lysine 547 in ccRCC. SDHA desuccinylation by mimetic mutation (K547R) suppressed its activity through the inhibition of succinate dehydrogenase 5 (SDH5) binding, further promoted ccRCC cell proliferation. The desuccinylase sirtuin5 (SIRT5) was found to interact with SDHA, and SIRT5 silencing led to the hypersuccinylation and reactivation of SDHA. SIRT5 was also found to be up-regulated in ccRCC tissues, and its silencing inhibited ccRCC cell proliferation. This indicates that SIRT5 promotes ccRCC tumorigenesis through inhibiting SDHA succinylation. This is the first quantitative study of lysine succinylome in ccRCC, through which we identified succinylation in core enzymes as a novel mechanism regulating various ccRCC metabolic pathways. These results expand our understanding about the mechanisms of ccRCC tumorigenesis and highlight succinylation as a novel therapeutic target for ccRCC.

1. Introduction

The molecular mechanisms underlying renal cell carcinoma (RCC) tumorigenesis have not been comprehensively elucidated, which has hindered the development of anti-RCC therapies. Metabolic reprogramming is a prominent feature of clear cell RCC (ccRCC) [1,2]. An array of metabolic processes including fatty acid β -oxidation, ketone

body production, cellular respiration and the activities of metabolic enzymes including 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), pyruvate dehydrogenase complex are regulated by protein succinylation [3,4]. Succinylation is a newly discovered post-translational modification (PTM), in which a succinyl group (–CO-CH₂-CH₂-CO₂H) is added to the lysine residue of a protein [5]. Succinylation alters the charge of the lysine and introduces a structural moiety that is

Abbreviations: ccRCC, clear cell renal cell carcinoma; TMT, tandem mass tag; LC-MS/MS, liquid chromatography–tandem mass spectrometry; TCA, tricarboxylic acid; SDHA, succinate dehydrogenase complex subunit A; SDH5, succinate dehydrogenase 5; SIRT5, sirtuin5; RCC, renal cell carcinoma; HMGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; PTM, post translational modification; HPLC, high-performance liquid chromatography; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DAVID, the Database for Annotation, Visualization and Integrated Discovery; GSEA, gene set enrichment analysis; ES, enrichment score; WT, wild type; Co-IP, coimmunoprecipitation; AL4A1, 1-pyrroline-5-carboxylate dehydrogenase; CO1A1, collagen alpha-1(I) chain; ECHA, tri-functional enzyme subunit alpha, mitochondrial; SPTN1, spectrin alpha chain, non-erythrocytic 1; FIBG, fibrinogen gamma chain; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; PPI, protein-protein interaction; NAD, nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase; DLD, dihydrolipoamide dehydrogenase; TCGA_KIRC, The Cancer Genome Atlas kidney cancer database; FAD, flavin adenine dinucleotide; HIF, hypoxia-inducible factor

* Corresponding author. Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Capital Medical University, No.10 Xitoutiao, You An Men, Fengtai District, Beijing, 100069, China.

E-mail address: zhengjf@cmmu.edu.cn (J. Zheng).

¹ These authors contributed equally to this work.

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larger than both acetylation and methylation [5]. Succinylation is implicated in numerous diseases, including hepatic, cardiac and pulmonary disorders [6]. Protein succinylation is regulated by sirtuin5 (SIRT5), an important desuccinylase. However, the roles of this enzyme in ccRCC tumorigenesis remain poorly understood.

The dysregulation of protein succinylation is associated with genome instability and cancer [7–9]. To date, succinylation has been shown to contribute to both colon and gastric cancer [8,9], but its role in ccRCC remains undefined. In this study, we analyzed changes in the succinylome that occurred during ccRCC tumorigenesis. Succinylated proteins regulating various biological processes were identified, with particular enrichment in metabolic pathways. The desuccinylation of SDHA inhibited its enzymatic activity and promoted cell proliferation through weakening its interaction with SDH5. SIRT5 was found to interact with SDHA and promote ccRCC cell proliferation through desuccinylating SDHA. These findings provide the first insight into the influence of succinylation during ccRCC tumorigenesis.

2. Materials and methods

2.1. Tissue collection

Primary ccRCC and matched adjacent normal kidney tissues from the same patient (n = 20, male, AJCC stage I) were obtained from nephrectomy specimens at the Affiliated Beijing Friendship Hospital, Capital Medical University from October 2013 to February 2014. Specimens were collected immediately after nephrectomy and frozen in liquid nitrogen prior to use. All specimens were histologically confirmed by pathologists. The study was approved by the Research Ethics Board of Affiliated Beijing Friendship Hospital and was performed according to the World Medical Association Declaration of Helsinki. All subjects included in the protocol signed a declaration of informed consent. Prior to surgery, the patients had not received chemotherapy or radiotherapy.

2.2. Protein extraction, trypsin digestion, TMT labeling, high-performance liquid chromatography (HPLC) fractionation, Ksu peptides affinity enrichment

Pooled samples were prepared by combining equal amounts of protein from individual samples and were adjusted to a final quantity of 300 µg for quantitative study and 1.875 mg for succinylation modification study, respectively. The proteins were digested with trypsin and TMT analysis were performed as previously described [9]. Peptides were labeled with the 6-plex TMT kit and were incubated with pre-washed anti-Pan-succinylation beads (PTM Biolabs Inc., Chicago, IL) at 4 °C overnight with gentle shaking. The bound peptides were eluted from the beads with 0.1% TFA. The eluted fractions were combined and vacuum-dried. The resulting peptides were cleaned with C18 ZipTips (Millipore) according to the manufacturer's instructions.

2.3. Liquid chromatography–tandem mass spectrometry analysis and database search

LC-MS/MS analysis of peptides was performed as previously described [2]. Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) was used. For MS scans, the *m/z* scan range was 350–1600. The MS/MS data were processed using MaxQuant with integrated Andromeda search engine (v.1.4.1.2) and searched against *SwissProt_Human* database concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to 4 missing cleavages, 5 modifications per peptide and 5 charges. Mass error was set to 20 ppm for first search, 5 ppm for main search and 0.02 Da for fragment ions. Succinylation on Lys was specified as the variable modification. False discovery rate (FDR) thresholds for protein, peptide and modification site were specified at 1%. Minimum peptide

length was set at 7. All the other parameters in MaxQuant were set to default values. The site localization probability was set as > 0.75. The mass spectrometry proteomics data and annotated MS/MS spectra of all modified peptides have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the iProX partner repository with the dataset identifier PXD011788.

2.4. Bioinformatics analysis

Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and InterPro domain analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) against the background of *Homo sapiens*. Protein-protein interaction (PPI) networks were constructed by STRING and visualized using Cytoscape. Gene set enrichment analysis (GSEA) was performed as previously described [2] to assess whether genes from pre-defined gene-sets were enriched amongst the highest/lowest ranked genes, through the calculation of pathway Enrichment Score (ES).

2.5. Plasmid construction

Wild type (WT) succinate dehydrogenase complex subunit A (SDHA) overexpressing plasmid and shSIRT5 plasmid were constructed by amplifying the corresponding sequences and ligation into pcDNA3.0-flag and pLKO.1 vector, respectively. Desuccinylated SDHA mutant (K547R) was obtained by point mutagenesis. Sequences were verified by PCR amplification.

2.6. Cell culture, transfection, WB, cell proliferation and colony formation

The human RCC cell line ACHN, 769-P and human embryonic kidney 293A (HEK293A) cells were grown in RPMI-1640 medium and Dulbecco's modified Eagle medium, respectively. All cell culture reagents were purchased from HyClone (Logan, UT). Transfections were performed by Lipofectamine 2000 (Invitrogen, CA) following the protocol as reported before [10]. Cell based assays were performed as previously described [11]. Anti-SIRT5, anti-Flag, anti-SDHA, anti-ALDOB, anti-FGA, pan anti-succinyllysine, anti-succinate dehydrogenase 5 (SDH5) and anti-β-actin antibodies were purchased from proteintech, Affinity Biosciences, PTM Biolabs and Sigma-Aldrich, respectively.

2.7. Determination of enzyme activity and metabolic assays

SDHA activity was determined using SDHA activity assay kit (Solarbio Science & Technology Co., Ltd, Beijing, China). Nicotinamide adenine dinucleotide phosphate NADPH/NADP⁺ ratio, glutathione/glutathione disulfide (GSH/GSSG) ratio and cellular reactive oxygen species (ROS) level were determined using NADP/NADPH Quantification Kit (S0179, Beyotime), GSH/GSSG Assay Kit (S0053, Beyotime), Fluorometric Intracellular ROS Kit (S0033, Beyotime), respectively. All these assays were performed according to the manufacturer's protocol.

2.8. Coimmunoprecipitation (Co-IP)

Co-IPs were performed as previously reported [12]. Briefly, lysates of transfected cells were solubilized, clarified, and then incubated with 10 µl of antibodies with protein A/G-agarose for 5 h with end-over-end rotation at 4 °C. After five times washes with 1 ml ice-cold PBS buffer, the immunoprecipitated proteins were eluted from the beads with SDS-PAGE sample buffer.

2.9. Statistical analysis

All data were statistically compared using Graphpad Prism 5.0

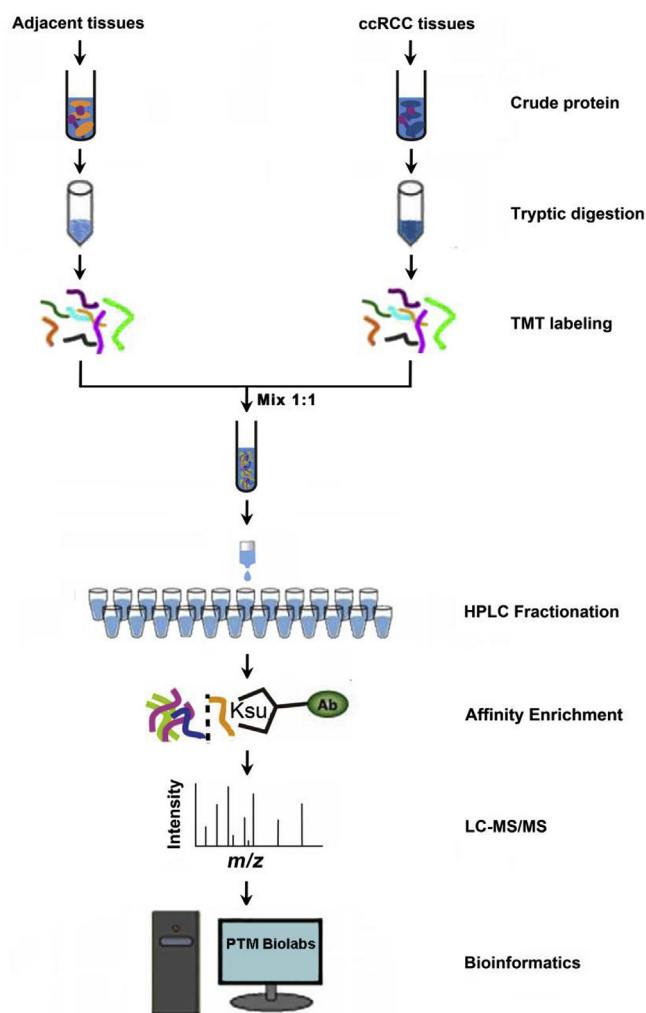


Fig. 1. Integrated strategy for the identification and quantitative profiling of succinylome between ccRCC and adjacent normal tissues. Proteins in ccRCC and adjacent normal tissues were extracted, trypsin digested, TMT labeling and mixed. Then anti-pan-succinylation antibody was used to enrich succinylated proteins followed by high-resolution LC-MS/MS and bioinformatics analyses.

(Graphpad Software, Inc., San Diego, CA) or SPSS program (version 19.0; SPSS, Chicago, IL). A p -value < 0.05 was deemed statistically significant.

3. Results

3.1. Identification and quantitation of the succinylome in ccRCC

We combined TMT labeling, HPLC fractionation, highly specific pan-antibody enrichment, high resolution Orbitrap mass spectrometry and bioinformatic analysis for the systematic quantification of the succinylome in ccRCC and adjacent normal tissues (Fig. 1). A total of 305 lysine succinylation sites on 215 proteins were identified (Table S1). The peptides ranged in length 7–22 amino acids (Table S2). Of which, 162 of the identified proteins were succinylated at a single lysine, whilst 53 proteins had multiple succinylated sites. For example, 1-pyrroline-5-carboxylate dehydrogenase (AL4A1), collagen alpha-1(I) chain (CO1A1) and mitochondrial trifunctional enzyme subunit alpha (ECHA) had five succinylation sites. Spectrin alpha chain, non-erythrocytic 1 (SPTN1) and fibrinogen gamma chain (FIBG) had four succinylation sites (Table S3).

We next quantified the succinylome in ccRCC vs healthy tissue. For TMT quantification, the ratios of the TMT reporter ion intensities in

MS/MS spectra (m/z 130 and 131) from raw data sets were used to calculate fold changes between ccRCC and healthy tissues. For a given protein, only unique peptides were considered for relative quantitation. For each sample, the quantification was normalized using the average ratio of all the unique peptides. Relative protein quantitation between groups was calculated from the median ratio of protein corresponding unique peptides when there were at least two unique peptides in a protein. Subsequently, succinylation level was normalized with protein level. To evaluate differential succinylation of protein, one-sample two-sided t -tests were performed with unique peptide ratio of corresponding protein. Quantitative data with p value < 0.05 and ratio > 1.5 or < 0.667 were considered as differential succinylation. A total of 135 sites in 102 proteins were differentially succinylated between ccRCC and adjacent healthy renal tissues (Table S4 & Fig. S1). Notably, isocitrate dehydrogenase (IDH), a critical enzyme in the tricarboxylic acid (TCA) cycle was differentially succinylated at up to four independent lysine residues. Other three enzymes in the TCA cycle were also differentially succinylated, namely: mitochondrial dihydrolipoyl dehydrogenase (DLD, a subunit of α -ketoglutarate dehydrogenase), succinate dehydrogenase (SDH) and malate dehydrogenase (MDH). These observations suggest that succinylation is an important PTM in the regulation of the TCA cycle in renal tissue.

3.2. Protein-protein interaction networks of the succinylated proteins

PPIs occur in all types of cells and are critical for the regulation of an array of biological processes. To further our understanding about the role of succinylation during ccRCC tumorigenesis, we assembled the PPI networks of the succinylated proteins based on the STRING database. This aimed to identify specific physical/functional interactions and biological processes related to succinylation. Network analysis revealed that the succinylated proteins displayed a degree of crosstalk, in which 215 proteins were identified as nodes that were connected by 221 direct physical interactions. As shown in Fig. 2A, succinylated proteins were enriched in four highly connected subnetworks including metabolic process, the TCA cycle, oxidation-reduction and transport processes. In addition, succinylated proteins were enriched in ribosomal pathway, metabolic pathway and the PI3K/AKT signaling axis (Fig. 2B–D).

3.3. Functional annotation of differentially succinylated proteins

The above data indicated that succinylation is important in the regulation of cellular metabolism. Enrichment analyses using the GO, KEGG and InterPro domains were performed to investigate the potential roles of differentially succinylated proteins. GO enrichment analysis based on the biological process category showed that succinylated proteins were significantly enriched in a wide range of cellular and metabolic processes, which may be attributed to the interaction of succinylation and metabolism. Moreover, succinylated proteins were involved in oxidation-reduction and the TCA cycle, which may relate to their function in the mitochondria. For cellular component analysis, we observed that a large number of succinylated proteins localized to the mitochondria, cytoplasm, and membrane-enclosed lumen. This confirmed the role of protein succinylation in the mitochondrial processes of ccRCC cells. Consistent with these findings, molecular function analysis revealed that succinylated proteins were enriched in oxidoreductase activity, catalytic activity, NAD binding and coenzyme binding ability (Fig. 3A & Table S5). KEGG pathway analysis also revealed that a large proportion of the metabolic enzymes including proteins involved in carbon metabolism, the TCA cycle, pyruvate metabolism, glycolysis/gluconeogenesis, amino acid synthesis and degradation, and fatty acid metabolism were differentially succinylated (Fig. 3B & Table S6).

We next employed InterPro to predict the presence of domains in the succinylome. InterProScan allows protein sequences to be scanned against InterPro signature sequences from multiple databases. Enriched

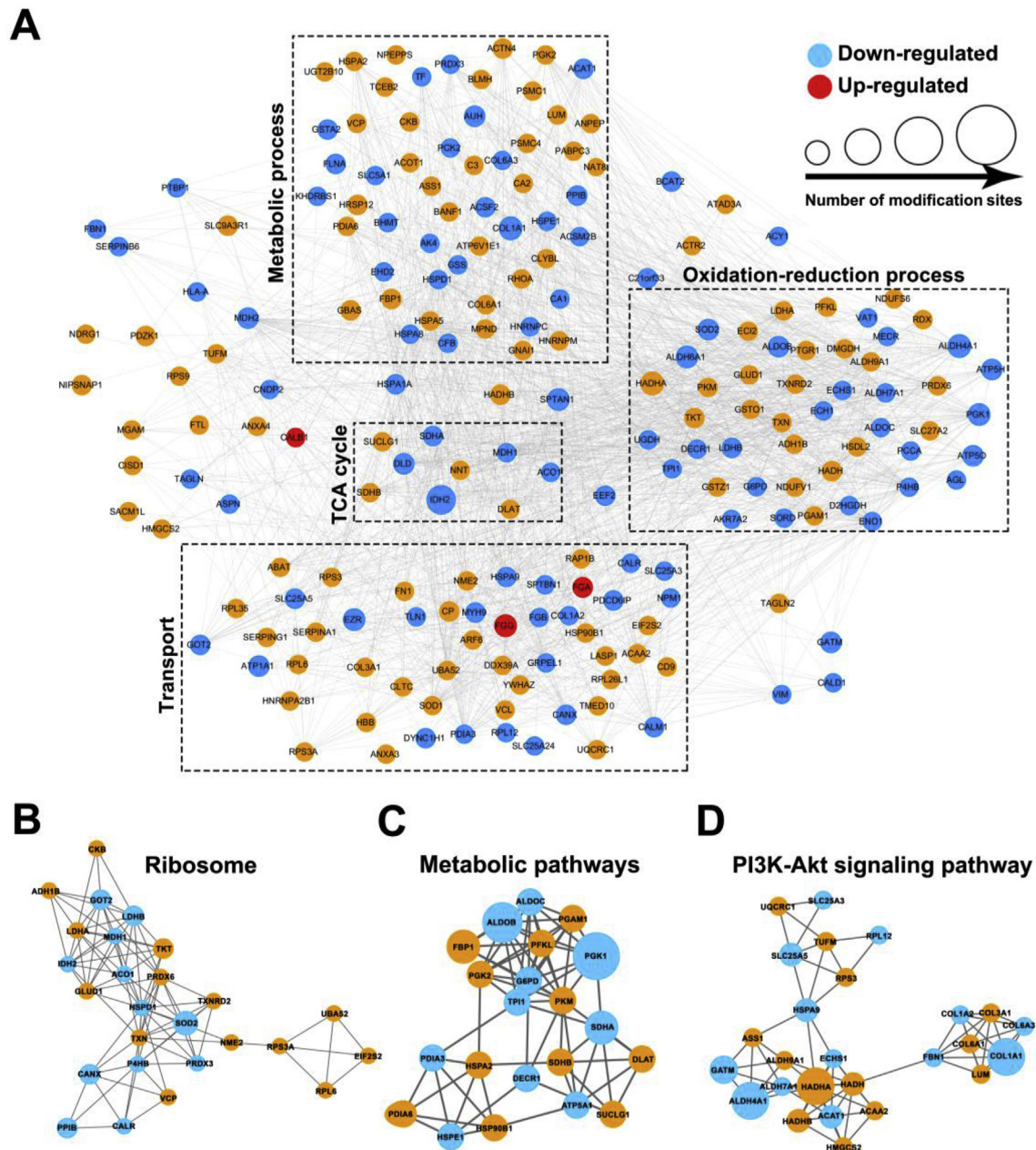


Fig. 2. PPI network and sub-networks of succinylated proteins. (A) PPI network on the basis of the STRING database (Version 10.5) was visualized in Cytoscape. Four representative biological processes enriched by Lys succinylome were circled. (B–D) PPI sub-networks of succinylated proteins clustered in ribosome, metabolic pathway and PI3K/AKT signaling pathway, respectively. Red/blue/orange nodes represent proteins in which succinylation level were upregulated/downregulated/unchanged, respectively. The size of the node corresponds to the relative number of Ksu sites identified on each protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

lysine-succinylated substrates with functional domains included NAD (P)-binding domain, aldehyde dehydrogenase/lactate dehydrogenase (LDH)/ATPase C-terminal domain, and fibrinogen coiled coil domain (Fig. 3C & Table S6). Since specific domain structure is one of the major functional features in proteins, these results confirmed the association of succinylation with cell metabolism.

3.4. Analysis of succinylated-lysine sequence motifs

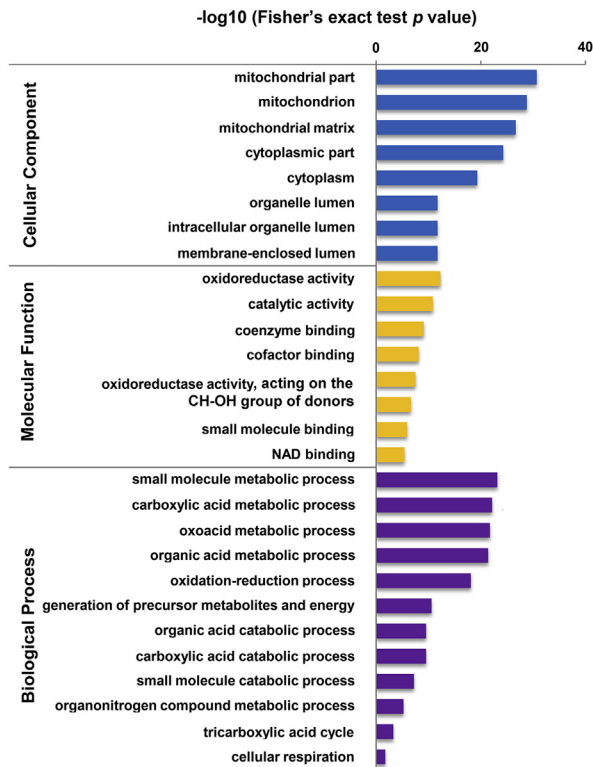
Previous studies in both eukaryotic and prokaryotic cells have observed preferences for amino acid residues surrounding the succinylated lysine [3]. To identify signature sequences, sequence motifs of the identified succinylated sites were analyzed using the Motif-X program.

Of the 305 succinylated sites identified, 115 were located on two predicted motifs, KsuD+1 and KsuP+1 (10 amino acids upstream and downstream of the site) (Fig. 3D).

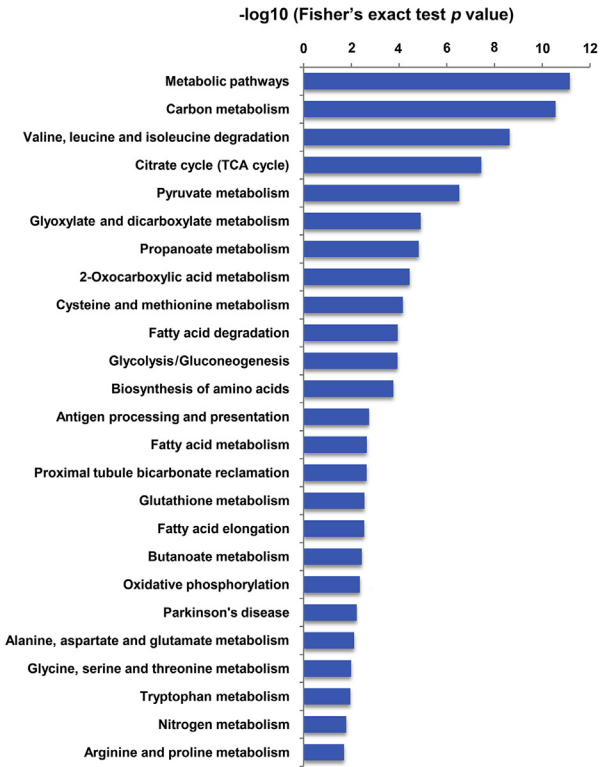
3.5. Verification of the differential succinylation of proteins found by MS/MS assay

To confirm the reliability of the differential succinylation of proteins found by MS/MS assay, we pooled every five samples from 20 patients together and got four groups of samples. We chose the representative proteins which were differentially succinylated. Then we used the Co-IP assay to validate the differential succinylation of these proteins between ccRCC and matched adjacent normal kidney tissues. All results

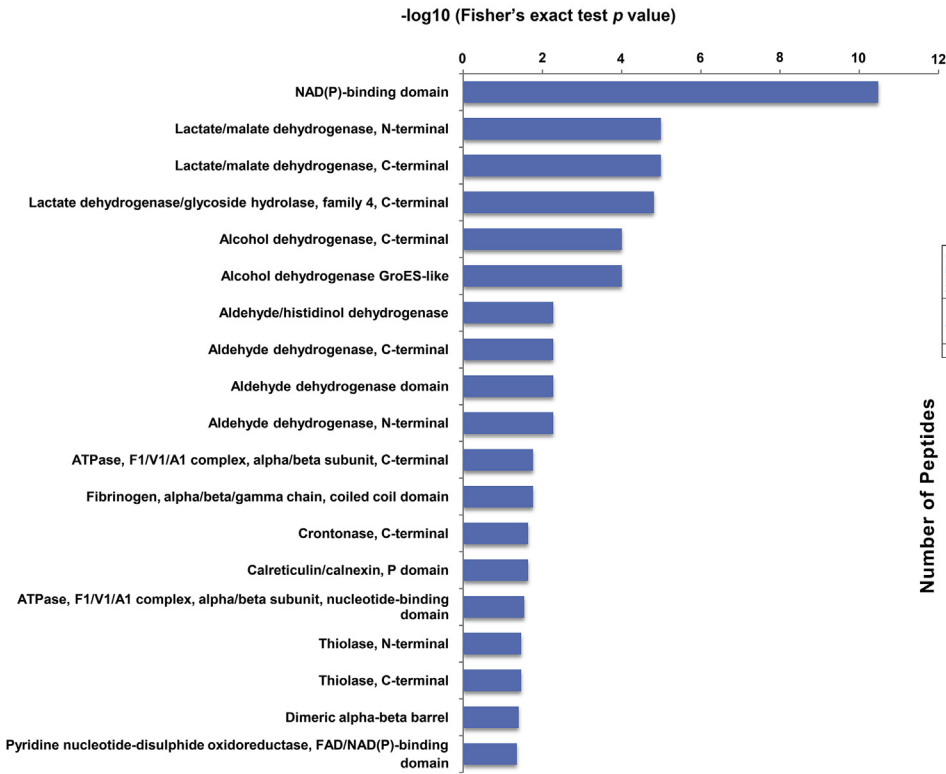
A



B



C



D

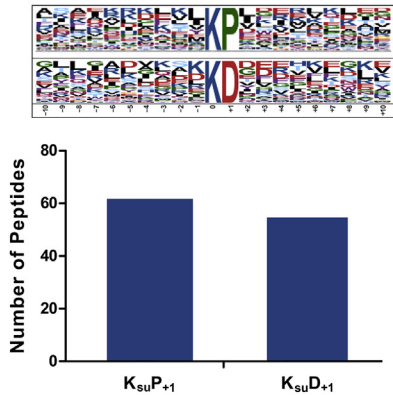


Fig. 3. Enrichment analyses of succinylated proteins. (A) Histogram according to GO (cellular component, molecular functions and biological processes) analyses of differentially succinylated proteins. (B) KEGG pathway enrichment analysis and (C) InterPro domain enrichment analysis of succinylated proteins. (D) Sequence motif around succinylated-lysine (up), the number of peptides with specific sequence motif (down).

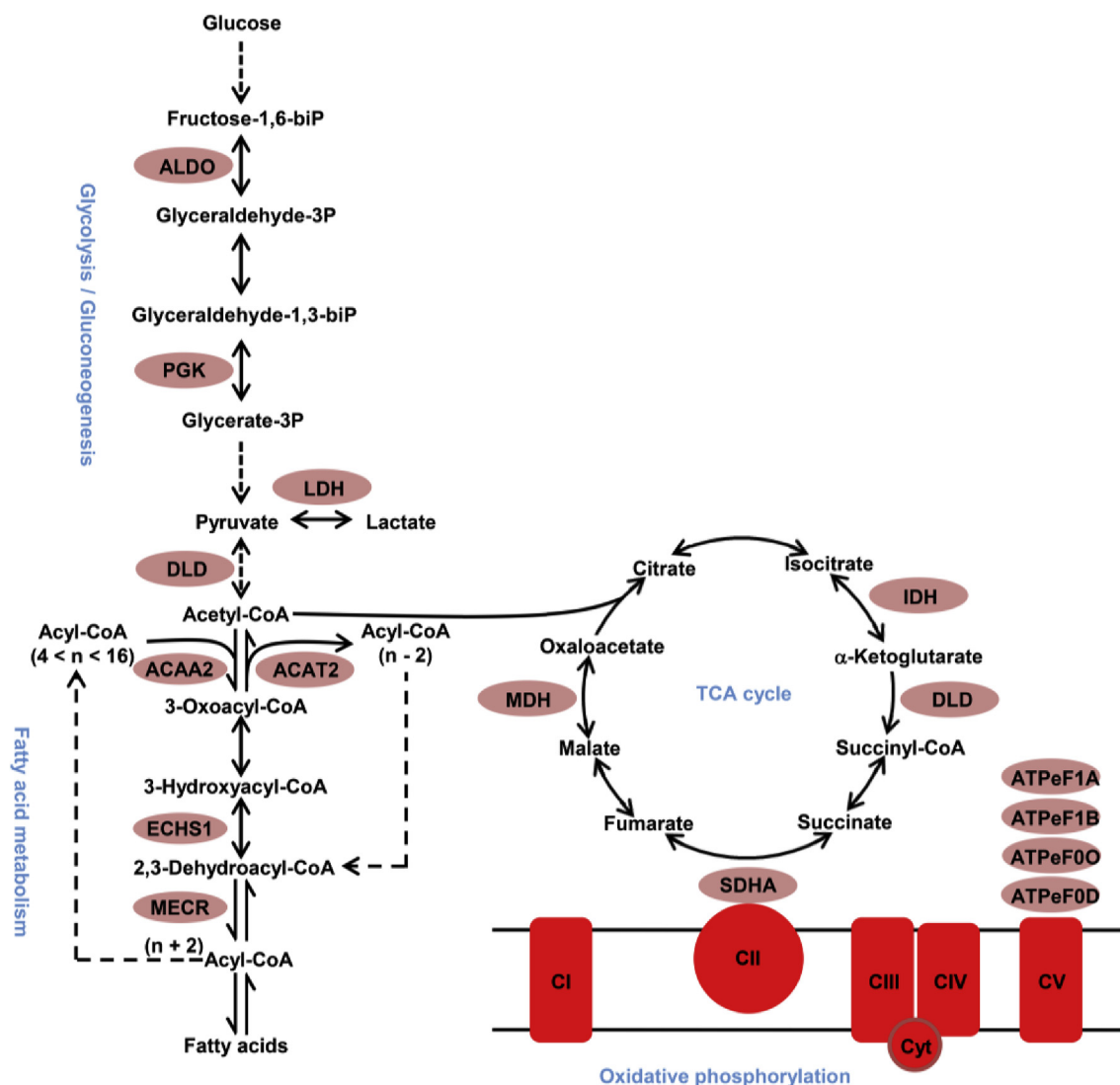


Fig. 4. Key proteins in metabolic pathways that are differentially succinylated. Succinylated key enzymes involved in core metabolic pathways (glycolysis/gluconeogenesis, fatty acid metabolism, the TCA cycle, oxidative phosphorylation) were highlighted in pink. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

validated the differential succinylation found by MS/MS assay (Fig. S2), demonstrating that the differential succinylation of proteins found by MS/MS assay were reliable.

3.6. Lysine desuccinylation promotes ccRCC cell proliferation by inhibiting SDHA activity and weakening SDH5 binding

Succinylated proteins that participate in metabolic pathways are shown in Fig. 4. Three proteins within the glycolysis/gluconeogenesis pathway, fructose-bisphosphate aldolase (ALDO), phosphoglycerate kinase (PGK) and LDH were differentially succinylated at multiple sites, indicating a potential role of succinylation in glycolysis regulation. Amongst the nine identified enzymes that participate in the TCA cycle, four were differentially succinylated, including dihydrolipoamide dehydrogenase (DLD), one of subunits for both pyruvate dehydrogenase complex that connects glycolysis to TCA cycle by catalyzing the transition of pyruvate to acetyl-CoA, and enzyme functioning during the TCA cycle by catalyzing the conversion of α-ketoglutarate into succinyl-CoA, IDH which catalyzes the conversion of isocitrate to α-ketoglutarate, SDH and MDH.

SDHA, a subunit of SDH that is important in both the TCA cycle and oxidative phosphorylation, was also differentially succinylated. Lysine

succinylation has been reported to alter enzyme activities [3,4]. To investigate the impact of succinylation on SDHA activity, we constructed an SDHA-K547R mutant to mimic desuccinylated SDHA observed in ccRCC. We found that K547R was indeed not succinylated (Fig. 5A) and K547R-overexpression led to up to 74% of decrease in cellular SDHA activity compared to WT-SDHA overexpressing cells (Fig. 5B). Overexpression of the K547R mutant also resulted in increased NADPH/NADP⁺ ratio (Fig. 5C) and the GSH/GSSG ratio (Fig. 5D). However, ROS level was significantly decreased (Fig. 5E). SDHA is a flavoprotein that catalyzes the oxidation of succinate to fumarate. For this, the covalent insertion of FAD (flavination) onto the SDHA subunit is essential. SDH5 is required for flavination which is necessary for both SDH assembly and function [13]. We therefore investigated the role of succinylation on the SDHA-SDH5 interaction through Co-IP analysis. We found that full length SDHA-WT co-immunoprecipitated with SDH5, whilst the interaction of desuccinylated SDHA-K547R with SDH5 was drastically reduced (Fig. 5F). These data reveal that SDHA desuccinylation inhibits its activity through weakening its binding to SDH5.

High SDHA expression positively correlated with oxidative phosphorylation and negatively correlated with glucose metabolism and cell proliferation (Fig. S3). This suggested that the change of SDHA activity

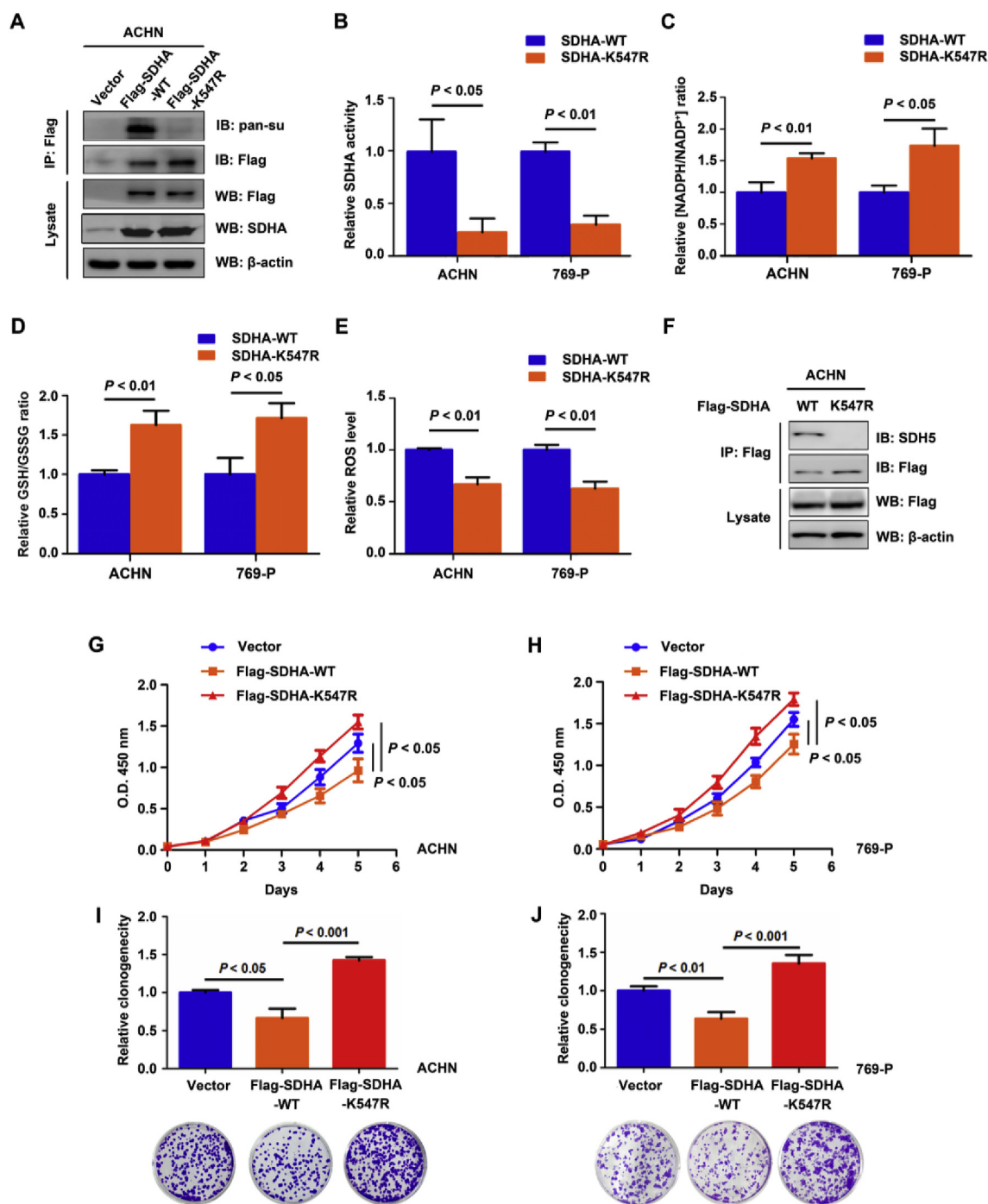


Fig. 5. Desuccinylation of SDHA promotes ccRCC cell proliferation via inhibiting its catalytic activity and weakening SDH5 binding. (A) Empty vector, Flag-tagged WT SDHA or the K547R mutant was ectopically expressed in ACHN cells, succinylation level of SDHA was detected by immunoprecipitating SDHA and blotting with anti-pan-succinylation antibody. In vector transfected control cells without flag expression, endogenous SDHA was not immunoprecipitated by flag antibody and no succinylation signal was detected. SDHA WT was succinylated and K547R mutant was hardly succinylated. (B–E) K547R mutant overexpressing cells had decreased SDHA activity, increased cellular NADPH/NADP⁺ and GSH/GSSG ratios, and decreased cellular ROS level in ACHN and 769-P cells. (F) Co-IP of Flag-tagged WT/K547R SDHA and SDH5 in ACHN cells revealed that compared with WT, K547R mutant bound with less SDH5. (G, H) K547R overexpression led to the enhanced cell proliferation and colony formation. Proliferation of ACHN and 769-P cells transfected with empty vector, SDHA WT or the K547R mutant was detected by CCK-8 assay at the indicated time points (repeated-measures analysis of variance). Colony formation was monitored in ACHN and 769-P cells for 14 days (t-test). Data were shown in mean $\pm$ S.D., $n = 3$.

regulated by succinylation influences ccRCC cell proliferation. Indeed, desuccinylated K547R-overexpressing ACHN and 769-P cells displayed increased proliferation and colony formation ability compared with vector transfected control cells or SDHA WT overexpression cells (Fig. 5G–J). These observations indicate that SDHA succinylation regulates the activity of SDHA and subsequent ccRCC cell proliferation.

3.7. SIRT5 is upregulated in ccRCC tissues and deactivates SDHA through desuccinylation

Considering the role of SDHA desuccinylation in ccRCC tumorigenesis, we next aimed to identify the enzyme that regulates SDHA succinylation. SIRT5 is the only currently recognized desuccinylase

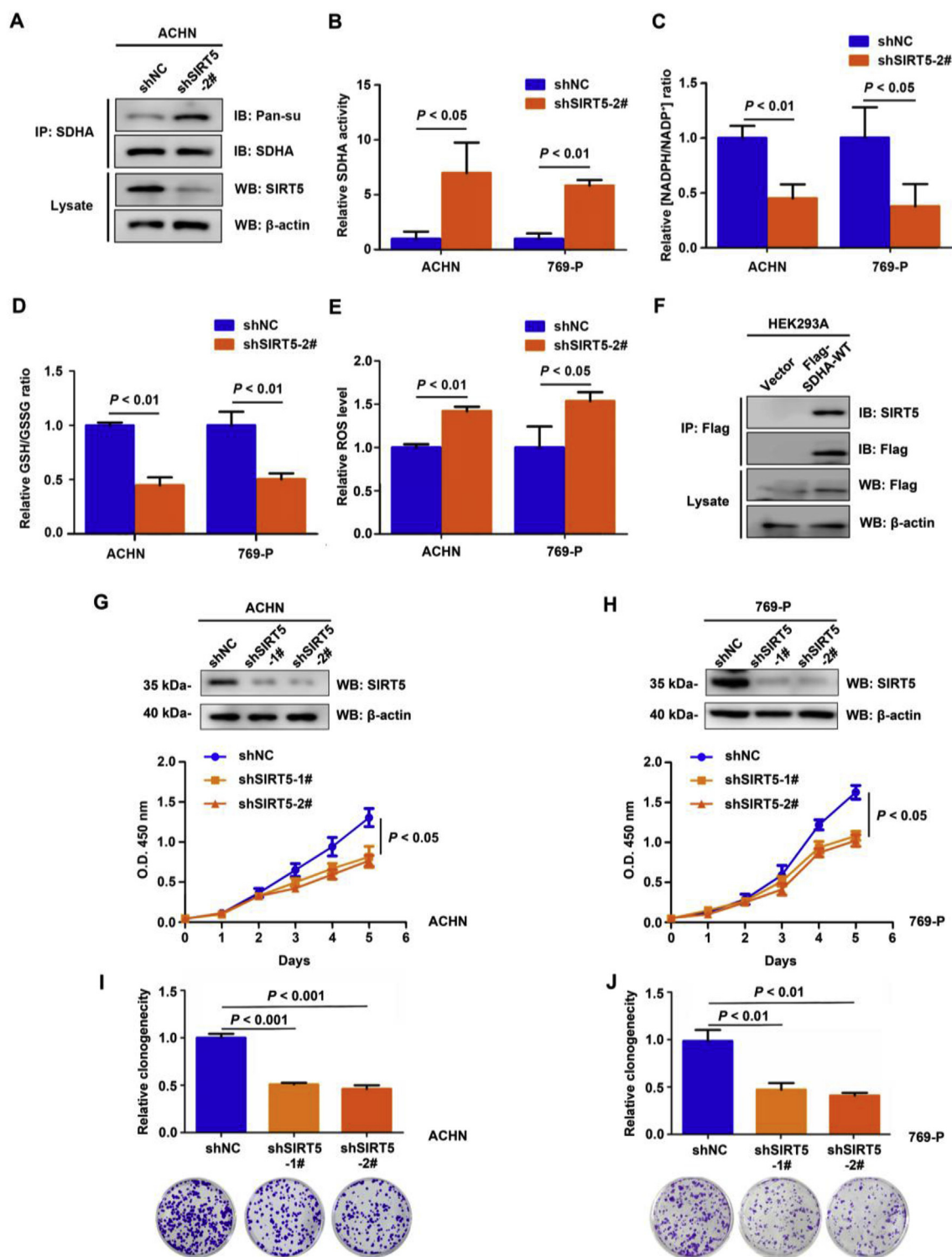


Fig. 6. SIRT5 promotes ccRCC cell proliferation by interacting with and desuccinylating SDHA. (A–B) ACHN cells were transfected with scramble shRNA or SIRT5 shRNA plasmid, SDHA was precipitated and its succinylation level was probed by anti-pan-succinylation antibody. SIRT5 silencing led to hypersuccinylation of SDHA in ccRCC cells and increased activity of SDHA. (C–E) SIRT5 silencing decreased cellular NADPH/NADP⁺ and GSH/GSSG ratio and increased cellular ROS level. (F) Co-IP of SIRT5 and flag-tagged WT SDHA in HEK293A cells revealed that SIRT5 coimmunoprecipitated with SDHA. (G–J) SIRT5 silencing repressed ACHN and 769-P cell proliferation and colony formation. Methods followed Fig. 5G–J.

[14]. We therefore assessed SIRT5 expression in clinical ccRCC samples by western blot analysis. We found that SIRT5 was upregulated in ccRCC tissues compared to adjacent normal tissues (Fig. S4A), suggesting it may contribute to SDHA desuccinylation in ccRCC. To verify this, we silenced SIRT5 expression and detected the levels of SDHA

succinylation. SIRT5 silencing led to enhanced SDHA succinylation (Fig. 6A) and a > 7-fold increase in SDHA activity (Fig. 6B). SIRT5 silencing also resulted in decreased NADPH/NADP⁺ ratio (Fig. 6C) and the GSH/GSSG ratio (Fig. 6D). However, ROS level was significantly increased (Fig. 6E). To confirm the involvement of SIRT5, we

performed co-IP assay and found that SIRT5 interacted with SDHA (Fig. 6F). Taken together, these data confirm that SIRT5 directly regulates SDHA succinylation.

3.8. SIRT5 regulates ccRCC cell metabolism and promotes ccRCC cell proliferation

Acylation associated enzymes are involved in tumor development and progression [15]. To investigate the role of SIRT5 during ccRCC development, we performed bioinformatics analyses of ccRCC clinical data from TCGA_KIRC database. We found that high SIRT5 level positively correlated to dysregulated cell metabolism and malignant proliferation (Fig. S4B–D). To confirm the involvement of SIRT5 in ccRCC tumorigenesis, we silenced SIRT5 expression in ACHN and 769-P cells and observed decreased cell proliferation and colony formation (Fig. 6G–J). These results indicate that SIRT5 promotes ccRCC cell proliferation through its ability to desuccinylate SDHA.

4. Discussion

In this study, the PPI analysis of succinylated proteins in ccRCC tissues revealed their clustering into metabolic process, the TCA cycle, oxidation-reduction and transport processes. Proteins involved in metabolic regulation, particularly carbon metabolism, were subject to differential succinylation. SDHA was desuccinylated in ccRCC tissues. *In vitro* experiments demonstrated that the desuccinylation of SDHA profoundly inhibited its activity through weakening its binding to the SDH5 subunit, further promoting cell proliferation. SDHA succinylation was regulated by the desuccinylase SIRT5 and SIRT5 silencing inhibited ccRCC cell proliferation, indicating that the SIRT5-induced desuccinylation of SDHA promotes ccRCC tumorigenesis. These findings provide the first evidence that SIRT5 plays an important role in ccRCC progression and that lysine succinylation regulates the activity of key proteins involved in various metabolic pathways and proliferation in ccRCC. This provided an insight into the potential mechanisms controlling ccRCC occurrence, and paves the way for an improved understanding of the function of succinylation in ccRCC and other tumor tissues.

In this study, we integrated chemical labeling, bioinformatics, cell based assays, and the assessment of clinical samples to study the differential succinylation of ccRCC related proteins. SIRT5 is reported to function as an oncogene or tumor suppressor dependent on its targets [16–20]. We found that SIRT5 promoted a cancer phenotype in ccRCC through its effects on SDHA. Acylation regulators are attractive anti-cancer drugs [21], including sirtuins, a new family of NAD⁺-dependent protein deacylases that have emerged as candidates to treat metabolic disorders and cancer [22]. The role of SIRT5 as a ccRCC therapeutic target warrants further studies based on our findings.

We found that metabolism related proteins were differentially succinylated in ccRCC tissues. Previous studies of the succinylome in gastric cancer and colon cancer tissue showed similar findings [8,9], highlighting the importance of succinylation in metabolism regulation and tumorigenesis. The activity of the metabolic enzymes was greatly affected by the mutagenesis of key lysine residues that undergo acylation [5,23]. SDH, also known as Complex II of the electron transport chain, catalyzes the conversion of succinate to fumarate in the TCA cycle, coupled to a reduction of NAD⁺ to NADH and the generation of ubiquinol from ubiquinone. It comprises 4 subunits, SDHA, SDHB, SDHC and SDHD. Previous studies exploring the effects of SIRT5 on Complex II SDH activity showed that SIRT5 desuccinylates and inhibits SDHA activity, restricting TCA cycling through decreased fumarate production, suggesting that succinylation activates SDH [3]. Li and coworkers also performed SIRT5 overexpression studies and observed increased SDH activity in cells [16]. The results of Zhang and colleagues suggested that SIRT5 is a positive regulator of Complex II [24]. Our work found that the desuccinylation of SDHA led to the inhibition of its

activity in ccRCC cells. The mechanism of SDH-deficient tumorigenesis appeared to involve the accumulation of succinate in the cytosol and its subsequent oncogenic effects caused by both hypoxia inducible factor (HIF)- α prolyl hydroxylase inhibition [25] and the induction of genome-wide hypermethylation due to TET enzyme inhibition [26,27]. The mechanism of SDHA demalonylation multidrug resistance also involved the accumulation of succinate. Thereby, succinate binds to and activates TrxR2 to maintain chemotherapy resistance, and inhibits α KG-dependent dioxygenases to regulate cetuximab resistance [28]. This study further found that SDHA desuccinylation ccRCC genesis was mediated by its weakened binding to SDH5 and its lower activity. In the future studies, molecular targeted therapy targeting these cellular factors may be available for these patients.

During tumor development, core metabolism switches from oxidative phosphorylation to aerobic glycolysis [29]. We observed altered succinylation levels of ALDO, PGK and LDH, coupled to enhanced glycolysis, consistent with this metabolism switch in tumor tissue. In previous studies, we found that metabolism related proteins, particularly those involved in lipid metabolism, played a key role in ccRCC occurrence and progression [2]. In this study, we further verified the importance of lipid metabolism related proteins during ccRCC genesis. As the proteins involved in acetyl-CoA transfer were succinylated, we anticipated that lysine succinylation regulates the synthesis of acetyl-CoA during lipid metabolism and the TCA cycle.

In addition, we found that succinylation extensively occurred on extra-mitochondrial proteins. The PPI network of succinylated proteins revealed their involvement in PI3K/AKT signaling. Since the PI3K/AKT/mTOR axis drives aerobic glycolysis through the transcriptional activation of HIF [1] and abnormalities in PI3K/AKT signaling play a vital role in ccRCC tumorigenesis [30], these results further verified the importance of succinylation modification in ccRCC tumorigenesis. We also identified several succinylated proteins linked to ribosomal pathways (Calnexin, 60S ribosomal protein L12, Ubiquitin-60S ribosomal protein L40, 60S ribosomal protein L26-like 1), suggesting a role for lysine succinylation in this system. These proteins may be important to ccRCC development and warrant further biological investigation.

Oxidation-reduction related proteins were identified as a further class of differentially succinylated proteins. IDH2, which regulates NADPH production and promotes the regeneration of reduced glutathione (GSH) by supplying NADPH to glutathione reductase or thioredoxin reductase, has been reported as an essential enzyme in the mitochondrial antioxidant system. Desuccinylation of IDH2 enhances cellular antioxidant defenses and maintains NADPH homeostasis [31]. Targeting IDH2 is an important cancer chemoprevention strategy [32]. We found that IDH2 is differentially succinylated in ccRCC, indicating its potential as a chemoprevention strategy for ccRCC. In addition, Mn superoxide dismutase and PRDX3 (both involved in the antioxidant system) were differentially succinylated, suggesting oxidation-reduction dysfunction is a defining feature of ccRCC tumorigenesis.

In summary, we report the first large-scale, high resolution MS-based assessment of differential lysine succinylation between adjacent normal renal tissues and ccRCC tissues. The identified succinylated proteins were involved in various biological processes and were particularly enriched in metabolic process. Gene silencing of SIRT5 showed that lysine succinylation of key metabolic enzymes could influence the ccRCC cell phenotype. Knowledge of the succinylome of renal tissue provides a rich resource to examine its biological roles in renal tissue and reveals previously unappreciated roles for lysine succinylation in the pathogenesis of ccRCC. This highlights the potential of succinylation regulators as new ccRCC therapies.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.freeradbiomed.2019.01.030>.

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