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USP48 Is Upregulated by Mettl14 to Attenuate Hepatocellular Carcinoma via Regulating SIRT6 Stabilization

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ABSTRACT

Exploiting cancer metabolism for the clinical benefit of patients with hepatocellular carcinoma (HCC) is a topic under active investigation. Ubiquitin-specific peptidase 48 (USP48), a member of the ubiquitin-specific protease family, is involved in tumor growth, inflammation, and genome stability. However, the role of USP48 in HCC tumorigenesis remains unknown. In this study, we report that expression of USP48 is downregulated in diethylnitrosamine-induced liver tumorigenesis in mice as well as in human HCC. USP48 physically bound and stabilized SIRT6 by K48-linked deubiquitination at the K33 and K128 sites of SIRT6, which impeded metabolic reprogramming to hamper HCC tumorigenesis. Moreover, methyltransferase-like 14 (Mettl14)–induced m^{6}A modification participated in the regulation of USP48 in HCC by maintaining USP48 mRNA stability. Our work uncovers the tumor-suppressive function of the Mettl14–USP48–SIRT6 axis via modulation of glycolysis, providing new insights into the critical roles of metabolic activities in HCC and identifying an attractive target for future treatment studies.

Significance: These findings demonstrate that USP48 is regulated by Mettl14-induced m^{6}A modification and stabilizes SIRT6 to attenuate HCC glycolysis and malignancy.

Introduction

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related death worldwide and accounts for over 80% of primary liver cancers (1, 2). Despite advances in systemic therapy for HCC in the decade since the approval of sorafenib, the treatment options for intermediate and advanced stages of HCC are still limited. In this case, it is paramount to identify new druggable targets for the development of novel therapies.

The sirtuins (SIRT) are a nicotinamide adenine dinucleotide (NAD^+)–dependent family of class III histone deacetylases, which play diverse roles in aging, metabolism, and cancer. The seven mammalian SIRTs are implicated in cancer through the regulation of cancer-associated metabolism, affecting maintenance of genome integrity and modifying the tumor microenvironment (3). Among them, SIRT6 is located in the nucleus and is bound to chromatin, acting as a tumor suppressor via its potent inhibition of aerobic glycolysis in cancer cells (4). In addition, SIRT6 also regulates lipid metabolism by repressing triglyceride synthesis and fatty acid uptake, as well as promoting fatty acid β oxidation (5, 6). Liver-specific deficiency of SIRT6 leads to fatty liver formation in mice, whereas loss of SIRT6 correlates with chronic liver disease and poor clinical outcome in human HCC (7–9). Although SIRT6 was reported to attenuate HCC malignancy through inhibiting ERK, cyclin D1, or ZEB2 (10–12), conflicting evidence also showed that SIRT6 could serve as a tumor promoter by preventing DNA damage and cellular senescence (13–15), potentiating apoptosis evasion (16–18), or promoting epithelial-to-mesenchymal transition (EMT) in HCC (19). Accordingly, further investigation is needed to decipher the mechanisms that control SIRT6 with regard to the fate of cancer.

Ubiquitination, a common form of posttranslational modification, is a tightly controlled and reversible process that regulates stability, activity, and localization of target proteins, and is involved in various biologic processes, like cell-cycle control, DNA damage repair, immune response, and signal transduction (20, 21). Deubiquitinases or deubiquitinating enzymes (DUB) can reverse this effect by hydrolytically removing ubiquitin from protein adducts and determining the target proteins' fate with a downstream impact on cellular processes. In humans, ubiquitin-specific proteases (USP) are the most numerous classes of DUBs, with approximately 60 proteases. Dysfunction or dysregulation of ubiquitination and deubiquitination is closely related to the initiation and development of multiple cancers (20). Multiple USP family members have been implicated with HCC, such as USP4, USP5, USP7, USP10, USP11, and USP22 (22–27). As a member of the USP family, USP48 has been reported in glioblastoma and acute promyelocytic leukemia (28, 29). However, so far, the specific roles of USP48 in hepatocytes and its functional mechanism in tumorigenesis remains elusive.

In this study, we demonstrate that USP48 expression is decreased in the livers of patients with HCC. By using xenograft mice and liver-specific USP48-knockout mice, we revealed the protective effects of

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USP48 in HCC tumorigenesis. We also found that USP48 stabilized SIRT6 protein levels by deubiquitylation, thereby negatively regulating glycolysis and HCC tumorigenesis. Moreover, aberrant expression of USP48 in HCC is related to methyltransferase-like 14 (Mettl14)–induced N^6-methyladenosine (m^6A) modification, which maintains the stability of USP48 mRNA. Overall, we clarify that USP48 attenuates human HCC malignancy through SIRT6 stabilization, and Mettl14-induced m^6A modification is involved in the regulation of USP48 mRNA stability.

Materials and Methods

Additional materials and methods are provided in the Supplementary Materials and Methods.

Cell lines

HCC cell Huh-7 and BEL-7404 were purchased from the ATCC. Hepatoblastoma cell Sk-hep-1 and endothelial-derived cell Sk-Hep1 were purchased from the ATCC. All the above cell lines of HCC were cultured in DMEM (Biological Industries) + 10% FBS (Biological Industries) + 1% penicillin/streptomycin at 37°C with 5% CO_2 and saturated humidity. All cell lines used were cultured within 35 generations and regularly tested for *Mycoplasma* contamination by the Plasmo Test kit (InvivoGen, rep-pt1). The short tandem repeat analysis method was used to verify the identity of cell lines twice a year at the core institution.

Animals and animal model

Hepatocyte-specific knockout USP48 was obtained by crossing Alb-Cre mice with *USP48*$^{flox/flox}$ mice. All animal experimental procedures followed the guidelines of the NIH and the guidelines of Guangxi Medical University (Nanning, Guangxi, P.R. China). The *USP48*$^{flox/flox}$ mice were generated by Cyagen Biosciences Inc.

Lactate assay, pyruvate assay, glucose uptake assay, and ATP assay

The lactate assay (Abcam, ab65331), pyruvate assay (ab65342), glucose uptake assay (Abcam, ab136955), and ATP assay (Abcam, ab83355) were performed according to the manufacturer's recommended protocol.

RNA sequencing analysis

Total RNA was acquired from hepatocytes isolated from USP48^{WT} and *Alb-Cre*; *USP48*$^{flox/flox}$ mice using TRIzol, and high-throughput sequencing was performed with Illumina HiSeq. TopHat2 software was used to match the genome, gene-level expression was quantified by applying HTSeq, and related differential genes were analyzed using DESeq2 software.

In vitro ubiquitination assay

USP48 and SIRT6 proteins were expressed with a TNT Quick Coupled Transcription/Translation System (Promega). Ubiquitination was analyzed using a ubiquitination kit (Boston Biochem) according to the manufacturer's recommended protocol.

Patient sample

Tissue sections of patients with HCC were obtained from The Second Affiliated Hospital of Shandong University (Jinan, P.R. China) and the Pathology Department of The First Affiliated Hospital of Guangxi Medical University. The use of patient samples followed the rules of the Ethics Committee of the Second Affiliated Hospital of Shandong University on the use of human specimens, and the patient's written informed consent was obtained according to the Declaration of Helsinki.

Statistical analysis

All data were statistically analyzed using the Student *t* test. Data are presented as mean ± SD, and $P < 0.05$ was considered statistically significant.

Data and materials availability

All data needed to evaluate the conclusions in the article are present in the article and/or the Supplementary Materials and Methods. Additional data related to this article may be requested from the authors.

Results

USP48 expression is downregulated in HCC

DUBs hydrolyze and remove ubiquitin from protein adducts, and determine the effect of the target protein's fate on downstream cell processes. To explore the potential function of DUBs in liver cancer, diethylnitrosamine (DEN) was used to induce HCC in mice (30) to detect the expression levels of USP family members, which contain approximately 60 members of the DUB species of protease. As shown in **Fig. 1A**, mRNA expression of USP48 was significantly decreased in DEN-induced mice compared with control mice. IHC staining and immunoblotting also showed a notable reduction of USP48 protein levels in liver tissues from DEN-induced mice (**Fig. 1B** and **C**; Supplementary Fig. S1A and S1B). Moreover, tumor-suppressive USP1 and USP10 diminished in HCC (31, 32), whereas oncogenic USP4 and USP7 increased in the same context (22, 24, 25, 33). Next, we analyzed putative copy-number alterations and mRNA expression of the USP48 gene across HCC using the GISTIC tool. As a result, the frequent shallow deletion of this locus was identified (**Fig. 1D**). USP48 protein levels and mRNA expression are consistently found to be significantly lower in hepatocarcinoma tissues than in paracarcinoma tissues, as measured by quantifying immunostaining (**Fig. 1E**), immunoblotting (**Fig. 1F**), and qRT-PCR (**Fig. 1G**). In addition, the analysis of 213 patients with liver cancer also found that the expression level of USP48 is closely related to tumor size, microvessel invasion, tumor size, and tumor differentiation (Supplementary Table S1). Overall, the results derived from DEN-induced mice, human datasets, and tissues indicate that the expression level of USP48 is correlated with the occurrence of HCC.

USP48 deficiency promotes HCC tumorigenesis

To further investigate the role of USP48 in hepatocytes, we generated mice with USP48 deficiency specifically in the liver (*Alb-Cre; USP48*$^{flox/flox}$ mice) by mating *USP48*$^{flox/flox}$ with albumin-Cre mice. USP48 expression was effectively silenced in the liver-specific knockout mice (**Fig. 2A–D**). Next, male Alb-Cre; USP48$^{flox/flox}$ mice were exposed to a single dose of DEN. At 9 months of age, *Alb-Cre; USP48*$^{flox/flox}$ mice developed visible tumors, which were barely observed in USP48^{WT} mice (**Fig. 2E**). Ki67 immunostaining and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) apoptosis staining were performed on tumor tissues. The results showed that Ki67 was significantly expressed in tumors in Alb-Cre; USP48$^{flox/flox}$ mice, while TUNEL apoptosis staining was not (Supplementary Fig. S1C and S1D). In addition, *Alb-Cre; USP48*$^{flox/flox}$ mice displayed promoted liver tumorigenesis with increased tumor numbers, areas, and maximal sizes (**Fig. 2F** and **G**). Furthermore, strikingly reduced survival rates were noted in *Alb-Cre; USP48*$^{flox/flox}$

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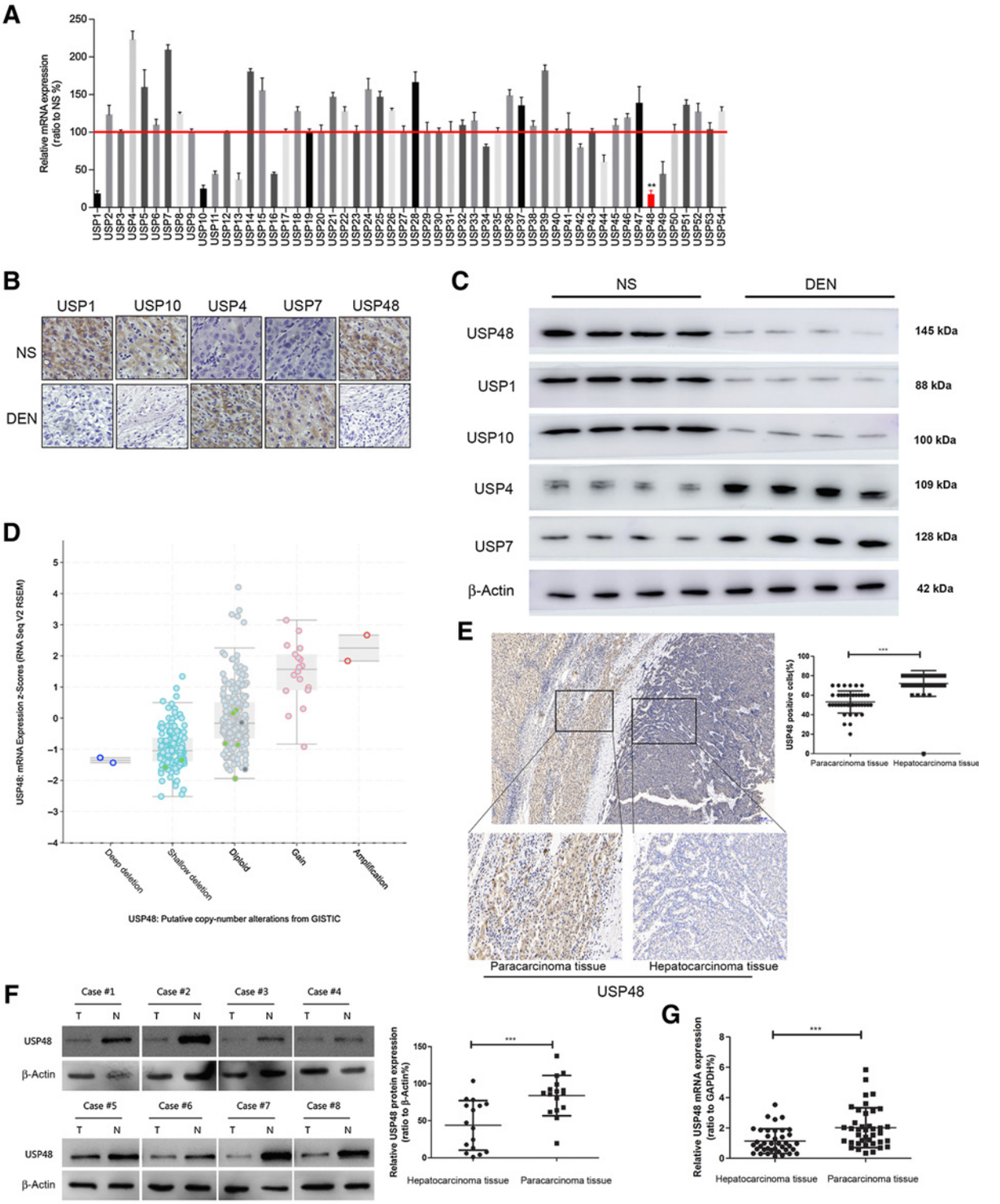

Figure 1.

USP48 expression is downregulated in HCC. **A,** qRT-PCR to measure the expression of USPs in the liver tissues of NS or DEN-induced mice. **B** and **C,** IHC staining (**B**) and immunoblotting (**C**) to measure the protein levels of USP1, USP10, USP4, USP7, and USP48. **D,** Gene expression of USP48 in human HCC compared with normal tissues from GISTIC database. **E** and **F,** Representative images (left) and quantification (right) of IHC staining (**E**) and immunoblotting (**F**) for USP48 in sections from human hepatocarcinoma or paracarcinoma tissue. **G,** mRNA expression of USP48 in sections from human hepatocarcinoma or paracarcinoma tissue. Data are shown as mean ± SD of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$.

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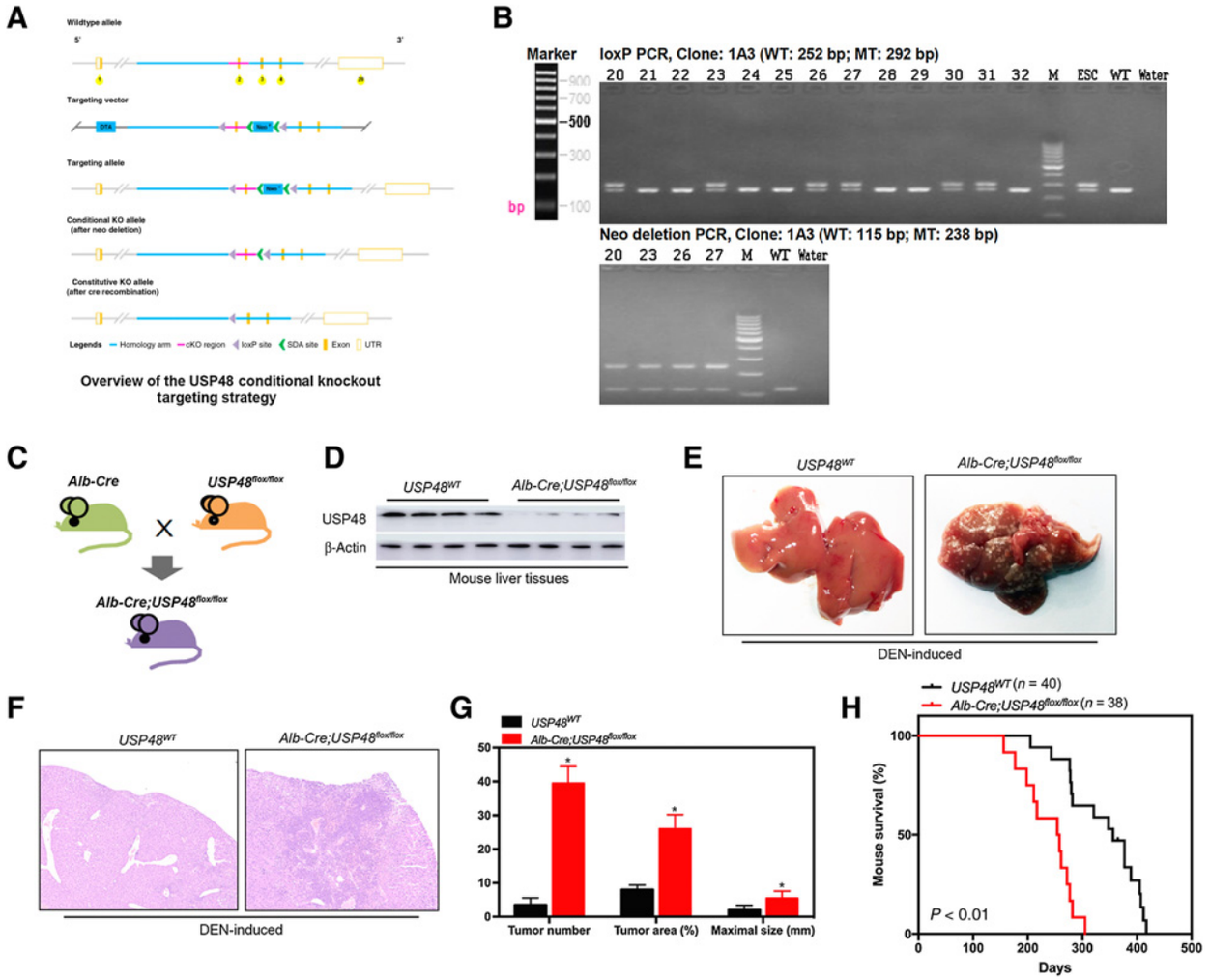

Figure 2.

USP48 deficiency promotes HCC tumorigenesis in liver-specific knockout mice. **A,** Overview of the USP48 conditional knockout targeting strategy. **B,** PCR analysis of loxP and Neo deletion genes in genomic DNA analysis of liver extracts from mice. **C,** The scheme of generation and characterization of the liver-specific USP48 knockout mice used in this study. **D,** Immunoblotting to measure USP48 protein levels in liver tissues from USP48^{WT} and *Alb-Cre; USP48$^{flox/flox}$* mice. **E,** Typical liver of 8-month-old DEN-induced USP48^{WT} mice (left) and *Alb-Cre; USP48$^{flox/flox}$* mice (right). **F,** Hematoxylin and eosin staining of liver sections from the DEN-induced mice described in **E**. **G,** Quantification of tumor number, area (%), and maximal size (mm) of the DEN-induced mice described above. **H,** Kaplan–Meier analysis indicating overall survival of the DEN-induced mice described above. Data are shown as mean ± SD of three independent experiments. *, $P < 0.05$.

mice compared with the control USP48^{WT} mice (**Fig. 2H**). Collectively, the above observations from liver-specific USP48 knockout mice indicate that USP48 depletion promotes HCC tumorigenesis *in vivo*.

To further explore the role of USP48 in HCC cell malignant behavior, we overexpressed USP48 ectopically in Huh-7 and Sk-hep-1 cell lines (Supplementary Fig. S2A). Overexpression of USP48 significantly reduced the cell viability, cell proliferation ability, and invasion/migration ability of HCC cells (Supplementary Fig. S2B–S2D). In addition, we also knocked out USP48 in BEL-7404 and HepG2 cells (Supplementary Fig. S2E). Knockout of USP48 significantly increases the cell viability, cell proliferation ability, and invasion/migration ability of HCC cells (Supplementary Fig. S2F–S2H). In addition, USP48 was overexpressed in BEL-7404 cells knocked out of USP48 to test cell viability, cell proliferation ability, invasion, and migration. The results showed that overexpression of USP48 reversed cell viability, clone formation, and invasion and migration (Supplementary Fig. S2I–S2K). Consistent with the above *in vitro* observations, xenograft mice injected with Huh-7 cells bearing USP48 inserts exhibited reduced tumor formation and proliferation compared with control models, as shown by bioluminescence imaging and Ki67 staining (Supplementary Fig. S3A and S3B). On the other hand, there were excessive tumor formation and elevated Ki67 levels in xenografts established with the injection of USP48-deficient BEL-7404 cells compared with the control mice (Supplementary Fig. S3C and S3D). Taken together, these data imply that USP48 might suppress HCC cell malignant behavior by inhibiting cell viability, colony formation, and proliferation.

USP48 mediates glucose homeostasis in HCC

To gain an insight into the mechanism by which USP48 regulates liver tumorigenesis, we performed RNA sequencing with hepatocytes isolated from USP48^{WT} and *Alb-Cre; USP48$^{flox/flox}$* mice (**Fig. 3A**). As a

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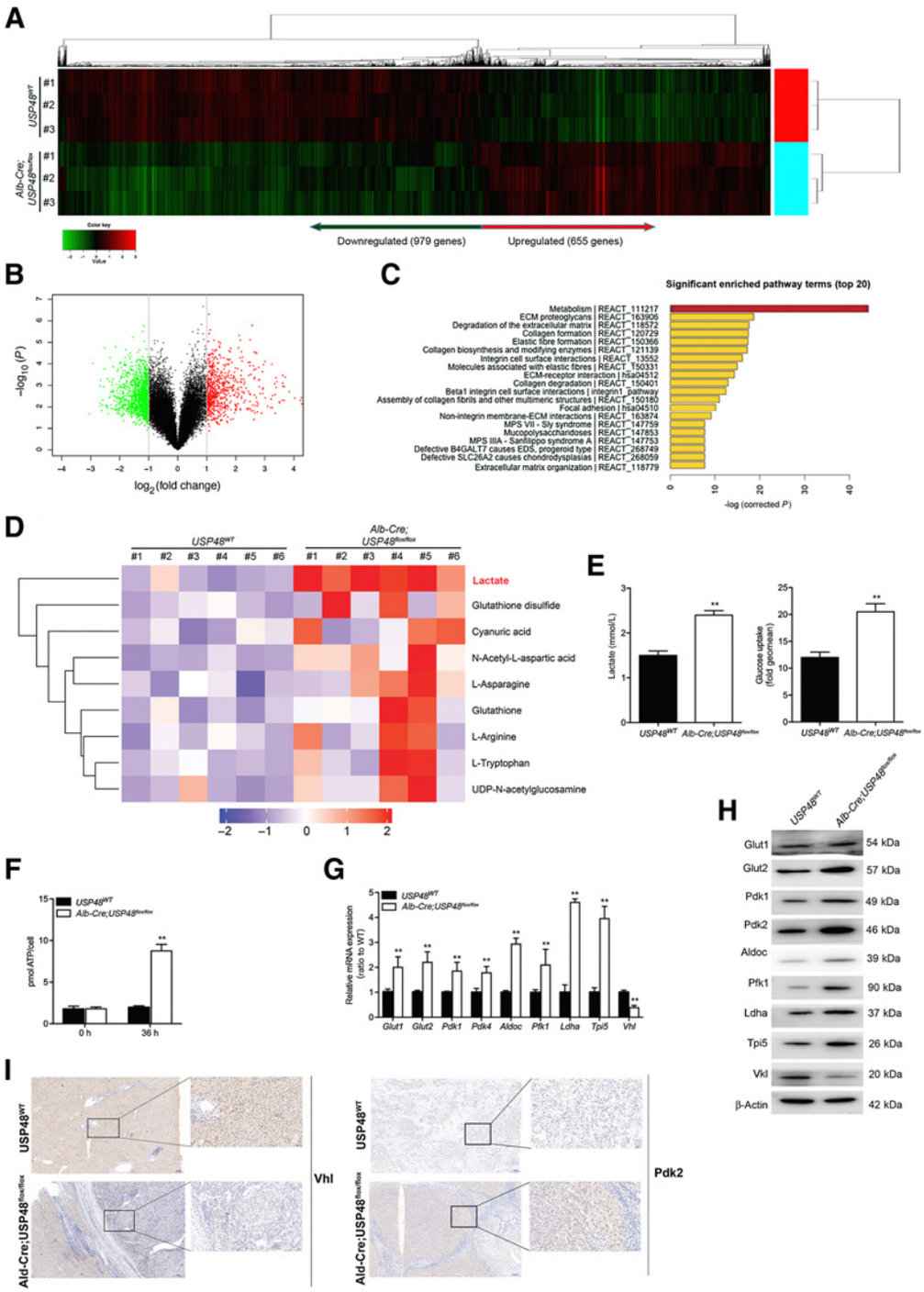

Figure 3.

USP48 mediates glucose homeostasis in HCC. **A,** The heatmap summarizes the differentially expressed genes in USP48^{WT} and Alb-Cre; USP48$^{flox/flox}$ mice induced by DEN for 8 months. **B,** Volcano plot displaying differentially expressed genes. Upregulated genes (655) are highlighted in red. Downregulated genes (979) are highlighted in green. Black dots represent genes not differentially expressed. **C,** Functional enrichment analysis of differentially expressed genes (showing the signal pathway of the top 20) in USP48^{WT} and Alb-Cre; USP48$^{flox/flox}$ mice. **D,** Heatmap showing the changes in liver metabolite levels between USP48^{WT} and *Alb-Cre; USP48$^{flox/flox}$* mice. Upregulated metabolites are highlighted in red. Downregulated metabolites are highlighted in purple. **E,** Measurement of lactate production (left) and glutamine uptake (right) in liver cells from USP48^{WT} and *Alb-Cre; USP48$^{flox/flox}$* mice. **F,** Measurement of ATP in liver cells from USP48^{WT} and *Alb-Cre; USP48$^{flox/flox}$* mice. **G,** qRT-PCR to measure the expression of genes related to glucose metabolism in liver cells from USP48^{WT} and *Alb-Cre; USP48$^{flox/flox}$* mice. **H,** Immunoblotting to measure the proteins related to glucose metabolism in liver cells from USP48^{WT} and *Alb-Cre; USP48$^{flox/flox}$* mice. **I,** Representative images of IHC staining for Vhl and Pdk2 in liver sections from USP48^{WT} and *Alb-Cre; USP48$^{flox/flox}$* mice. Data are shown as mean ± SD of three independent experiments. **, $P < 0.01$.

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result, a total of 655 genes was found to be upregulated, while 979 genes were negatively correlated with liver-specific USP48 depletion (**Fig. 3B**). The top 20 enriched pathways are listed in **Fig. 3C**, among which, metabolism stands out as the most significantly enriched pathway, suggesting the vital role USP48 might play in metabolism. Furthermore, we conducted LC/MS-MS analysis of metabolites in hepatocytes from USP48^{WT} and *Alb-Cre; USP48$^{flox/flox}$* mice ($n = 6$; **Fig. 3D**). *Alb-Cre; USP48$^{flox/flox}$* mouse hepatocytes exhibited significantly increased lactate production and glucose uptake (**Fig. 3E**), whereas excessive expression of USP48 resulted in a dramatic decrease in lactate levels, pyruvate levels glucose consumption in the HCC cells (Supplementary Fig. S4A). Consistently, under low glucose conditions, the same trend of higher ATP content was detected in the *Alb-Cre; USP48$^{flox/flox}$* hepatocytes compared with the control (**Fig. 3F**). In comparison, USP48 overexpression led to a reduction of the ATP levels in HCC cell lines (Supplementary Fig. S4B). To further characterize the glycolytic phenotype of these cells, we measured the expression levels of key glycolytic genes, such as glucose transporter *Glut1* and *Glut2*, pyruvate dehydrogenase kinase (*Pdk1*) and *Pdk4*, aldolase C (*Aldoc*), phospho-fructo kinase (*Pfk1*), lactate dehydrogenase A (*Ldha*), triose phosphate isomerase (*Tpi5*), and von Hippel-Lindau tumour suppressor (*Vhl*; **Fig. 3G**; Supplementary Fig. S4C). As expected, glycolysis promoting genes were upregulated in USP48-deficient hepatocytes, and downregulated upon USP48 overexpression in Huh-7 cells. Meanwhile, the gene Vhl, which inhibits glycolysis (29, 34), is regulated in the opposite way. Similar results were obtained at the protein levels, further supporting this observation, as shown in **Fig. 3H** and **I** and Supplementary Fig. S4D. Overall, these data signified that USP48 deletion enhanced metabolic flux into aerobic glycolysis, implying the suppressive role of USP48 in the metabolic reprogramming of HCC cells.

USP48 is physically associated with SIRT6 and regulates the SIRT6 signaling pathway by affecting its protein levels

To explore the signaling pathway affected by USP48, gene set enrichment analysis (GSEA) was performed, which revealed the enriched signatures related to endogenous USP48-dependent transcription in HCC, namely, the SIRT6 signaling pathway (**Fig. 4A**), known for its important function in regulating aerobic glycolysis and lipid metabolism. To figure out the proteins physically associated with USP48, we used anti-Flag affinity purification mass spectrometry to identify potential USP48-interacting proteins in 293T cells expressing Flag-tagged USP48. Consistent with the enrichment result, SIRT6 was identified as a USP48-associated protein (**Fig. 4B**; Supplementary Table S2). Reciprocal coimmunoprecipitation (co-IP) experiments were performed to corroborate the interactions between USP48 and SIRT6. IP with antibodies against USP48-Flag followed by immunoblotting with antibodies against SIRT6-HA demonstrated that USP48 was physically associated with SIRT6 and vice versa (**Fig. 4C** and **D**). In addition, SIRT6 or USP48 antibodies were used for co-IP in BEL-7404 cells, and the immunoblotting results showed that SIRT6 also interacts with USP48 at the cellular level (**Fig. 4E**). USP48 consists of a catalytic hydrolase domain (aa 89–422) in the N-terminal, a core domain (aa 423–1021) including a domain in USPs (aa 460–824) and a ubiquitin-like domain in the C-terminal of the protein (28, 35). To determine the fragments involved in the physical association of USP48 with SIRT6, we mapped potential binding regions of USP48 by generating multiple truncated mutants and found that the 1–200 fragment of USP48 is required for the interaction between these two proteins (**Fig. 4F** and **G**). In addition, we also found that the 1–200aa truncation mutant of USP48 significantly increased cell viability and clone-forming ability in HCC cells (Supplementary Fig. S5A–S5D). We also found that 1–200aa truncation mutant of USP48 significantly inhibited the glycolysis rate, while the 201–1035aa truncation mutant of USP48 had no effect on the glycolysis rate (Supplementary Fig. S6A–S6C). To address the functional significance of the physical interaction between USP48 and SIRT6, we subsequently examined the effect of USP48 on the expression of SIRT6. Immunoblotting assays established that USP48 ablation resulted in a dramatic decrease in the protein level of SIRT6 in hepatocytes from liver-specific knockout mice (**Fig. 4H**) and HCC cell lines (**Fig. 4I**), whereas the level of SIRT6 protein was remarkably elevated upon USP48 overexpression in Huh-7 and Skhep-1 cells (**Fig. 4J**). Altogether, these findings strongly support the notion that USP48 directly interacts with SIRT6 and controls its related signaling pathway by affecting the protein abundance of SIRT6.

USP48 protects SIRT6 protein from degradation by deubiquitination

Given the known impact of USP48 on the stability of its substrates, such as Gli1 (28), TRAF2 (36), or Mdm2 (37), as a deubiquitinase, we next examined whether USP48 contributed to SIRT6 stabilization. Indeed, the reduction in SIRT6 protein level caused by USP48 deficiency was probably through a proteasome-mediated protein degradation mechanism, as the effect could be effectively abolished by a proteasome-specific inhibitor, MG132 (**Fig. 5A**). These observations indicate that USP48 has an evident effect on SIRT6 stability and that SIRT6 is a putative substrate of USP48. To further support this deduction, the potential of USP48 to modulate the turnover rate of SIRT6 protein was evaluated by cycloheximide chase assays, which revealed that USP48 depletion was clearly associated with a decreased half-life of SIRT6, because the gradual decline in SIRT6 protein levels throughout the time course of cycloheximide treatment was accelerated by USP48 knockdown (**Fig. 5B**). Considering the above observations, which illustrate that USP48 controls the stability of SIRT6, we next explored whether USP48-triggered SIRT6 stabilization is a consequence of USP48-catalyzed deubiquitination. To this end, BEL-7404 and HepG2 cells with doxorubicin-inducible expression of wild-type USP48 (USP48/WT) and a catalytically inactive mutant of USP48 (USP48/C98A; ref. 28) were established, respectively. Only the USP48/WT gradually increased the SIRT6 level in a doxorubicin dose–dependent manner (**Fig. 5C**), whereas no discernible alteration of SIRT6 protein level was detected in cells expressing USP48/C98A (**Fig. 5D**). In addition, knockdown of USP48 by RNAi in Hela and HepG2 cells led to the accumulation of ubiquitinated SIRT6 (**Fig. 5E**). Ectopic expression of USP48/WT significantly decreased the amount of SIRT6 ubiquitination, whereas the presence of USP48/C98A had no effect (**Fig. 5F**). Taken together, these results provide convincing evidence that SIRT6 is a substrate of the deubiquitinase USP48, which protects SIRT6 from degradation by deubiquitination. In addition, we also found that USP48 inhibited K48-linked ubiquitination of SIRT6, but had no effect on K63-linked ubiquitination of SIRT6 (**Fig. 5G**). An *in vitro* ubiquitination assay also showed that USP48 inhibited K48-linked ubiquitination modification of SIRT6 (Supplementary Fig. S7A). To further clarify the effect of USP48 on SIRT6, K48 and K63 ubiquitin mutants containing arginine substitutions (K48R, K63R) were applied. Both *in vivo* and *in vitro* ubiquitination experiments showed the inhibitory effect of USP48 on K48-linked ubiquitination of SIRT6 (Supplementary Fig. S7B and S7C). We found seven possible ubiquitination sites of SIRT6, by predicting potential sites (K33, K81, K128, K230, K245, K296, and K300; **Fig. 5H**; Supplementary Fig. S7D). To examine the K48-linked ubiquitination sites, HEK293T cells were cotransfected with the seven Myc-tagged mutants

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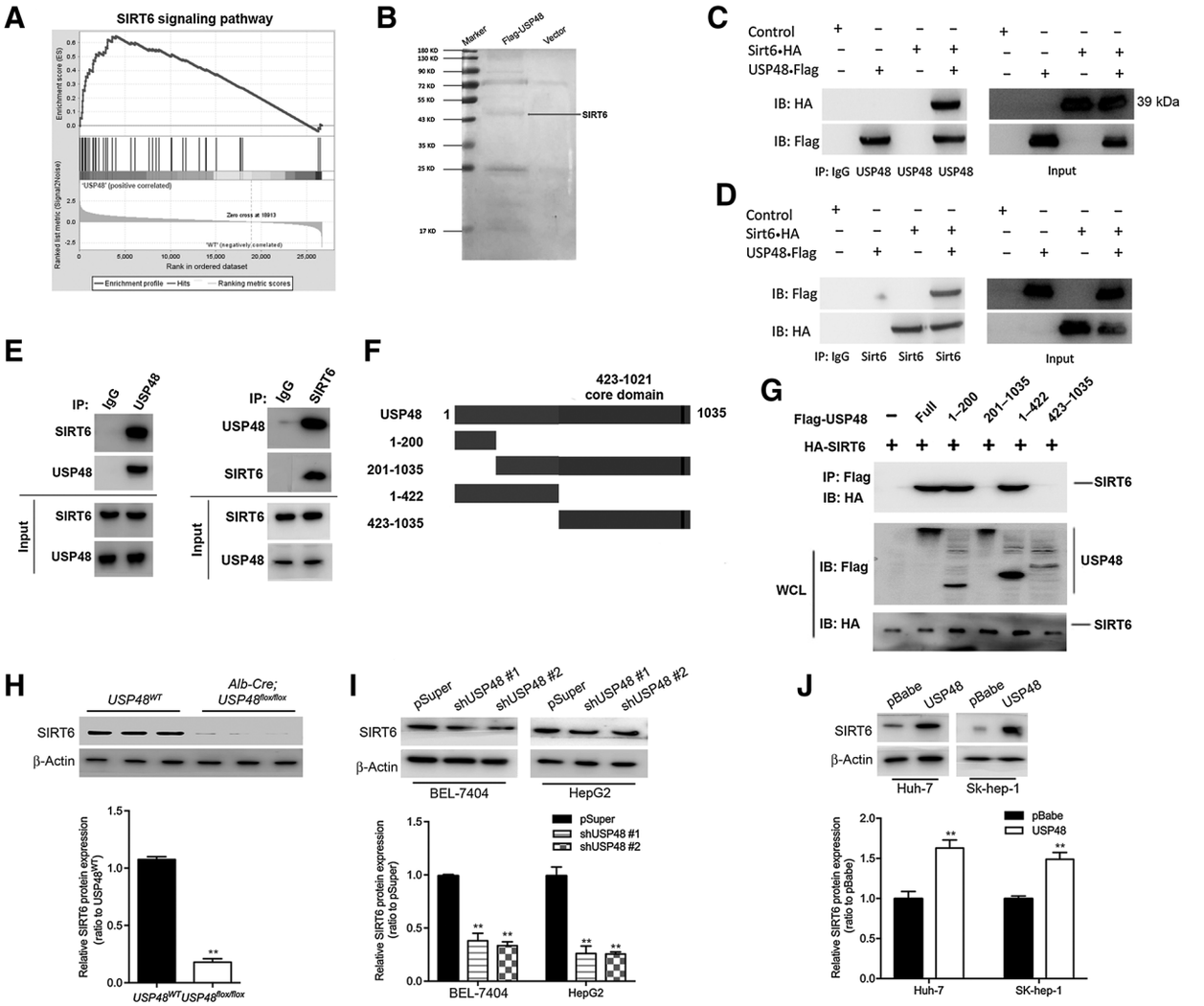

Figure 4.

USP48 is physically associated with SIRT6 and regulates the SIRT6 signaling pathway by affecting its protein levels. **A,** Enrichment of SIRT6 signaling in GSEA of genes altered as described above. **B,** Flag-USP48 pull-down products from 293T cells were separated by SDS-PAGE and visualized by silver staining. USP48-interacting proteins were identified by mass spectrometry. **C,** Lysates from 293T cells expressing control or Flag-USP48 and HA-SIRT6 were immunoprecipitated with IgG or anti-USP48, and then immunoblotted with anti-HA and anti-Flag. **D,** Lysates from 293T cells expressing control or Flag-USP48 and HA-SIRT6 were pulled down with IgG or anti-SIRT6, and then immunoblotted with anti-HA and anti-Flag. **E,** Lysates from BEL-7404 cells expressing control or USP48 and SIRT6 were pulled down with IgG or IRT6, and then immunoblotted with SIRT6 and USP48. **F,** Domain structures of USP48 and its truncated mutants. A conserved catalytic domain in the N-terminus of USP48 protein is indicated. **G,** Lysates from 293T cells expressing indicated full-length or truncated constructs were pulled down with anti-Flag and then immunoblotted with anti-HA (top). Input was immunoblotted with anti-Flag and anti-HA (bottom). WCL, whole-cell lysates. **H–J,** Immunoblotting to measure SIRT6 protein levels in liver tissues from USP48^{WT} and *Alb-Cre; USP48$^{flox/flox}$* mice (**H**), and BEL-7404 and HepG2 cells transfected with control or shUSP48 vectors (**I**), and Huh-7 and Sk-hep-1 cells expressing control vehicle or USP48 vector (**J**). Data are shown as mean ± SD of three independent experiments. **, $P < 0.01$. IB, immunoblot; IP, immunoprecipitation.

of SIRT6 and HA-ubiquitin K48. An *in vitro* ubiquitination assay showed that ubiquitin K48 ubiquitinated SIRT6 at K33, K128, and K230 sites (**Fig. 5I**). To identify the target residue within SIRT6 that is modified by USP48, corresponding point mutants were constructed at K33, K128, and K230 sites (**Fig. 5J**), and HEK293T cells were cotransfected with the described mutants of SIRT6 tagged with Myc, HA-ubiquitin K48, and Flag-USP48. As shown in **Fig. 5K**, USP48 inhibited K48-linked ubiquitination of SIRT6 at K33 and K128 sites. In addition, only the 1–200aa region of USP48 has a suppressing role on the ubiquitination of SIRT6 at K33 and K128 sites (**Fig. 5L**), implying that the physical association of USP48 and SIRT6 is required at the region of 1–200aa for the function of USP48. Taken together, these results show that USP48 prevented SIRT6 degradation by inhibiting K48-linked ubiquitination at the K33 and K128 sites of SIRT6, through physical binding at the 1–200aa fragment.

USP48-inhibited metabolic reprogramming is SIRT6 dependent

The inhibitory effects of USP48 on aerobic glycolysis in hepatocytes prompted us to investigate the underlying mechanisms. Because

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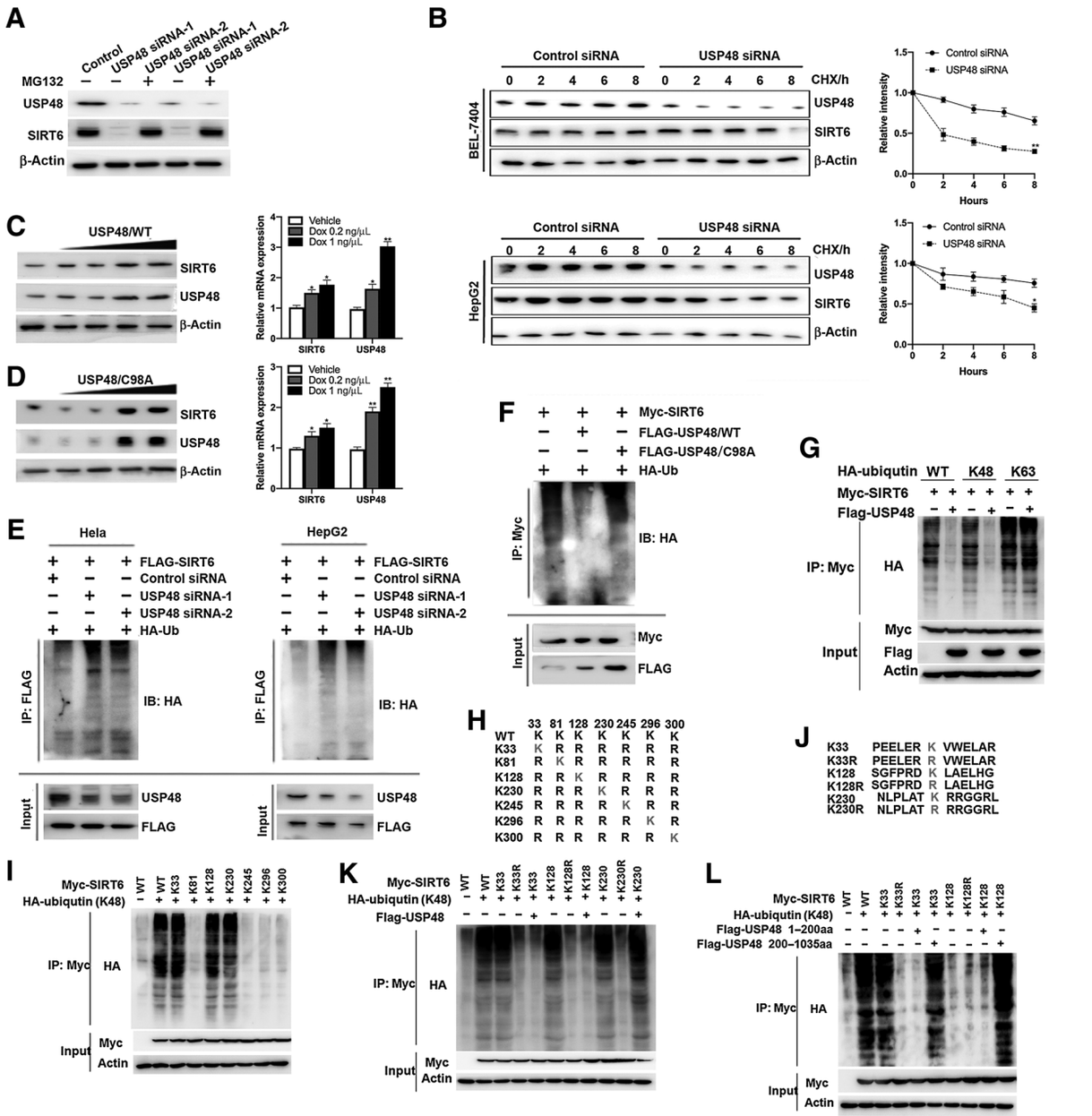

Figure 5.

USP48 protects SIRT6 protein from degradation by deubiquitination. **A,** Immunoblotting to measure USP48 and SIRT6 protein levels in cells transfected with control or USP48 siRNAs in the absence or presence of MG132 (50 μg/mL), a proteasomal inhibitor. **B,** Immunoblotting to measure USP48 and SIRT6 protein levels in BEL-7404 (top) and HepG2 (bottom) cells transfected with control or USP48 siRNAs in the absence or presence of the protein synthesis inhibitor cycloheximide (CHX; 50 μg/mL) for the indicated time. **C** and **D,** Immunoblotting (left) and qRT-PCR (right) to measure USP48 and SIRT6 protein levels and mRNA expression in cells with doxorubicin-inducible expression of USP48/WT (**C**) and a catalytically inactive mutant of USP48 (USP48/C98A; **D**). **E,** Lysates from Hela (left) and HepG2 (right) cells expressing indicated constructs were pulled down with anti-Flag and then immunoblotted with anti-HA (top). Input was immunoblotted with anti-USP48 and anti-Flag (bottom). β-Actin was used as loading control. **F,** Lysates from cells expressing indicated constructs were pulled down with anti-Myc and then immunoblotted with anti-HA (top). Input was immunoblotted with anti-Myc and anti-Flag (bottom). β-Actin was used as loading control. **G,** Lysates from cells expressing indicated SIRT6, USP48, and ubiquitin mutants were pulled down with anti-Myc and then immunoblotted with anti-HA (top). Input was immunoblotted with anti-Myc and anti-Flag (bottom). β-Actin was used as loading control. **H,** A series of SIRT6 ubiquitination site mutants. **I,** Lysates from cells expressing indicated SIRT6 ubiquitination site mutants were pulled down with anti-Myc and then immunoblotted with anti-HA (top). Input was immunoblotted with anti-Myc and anti-Flag (bottom). β-Actin was used as loading control. **J,** The SIRT6-related lysine site is mutated to arginine. **K,** Lysates from cells expressing indicated SIRT6 mutants were pulled down with anti-Myc and then immunoblotted with anti-HA (top). Input was immunoblotted with anti-Myc and anti-Flag (bottom). β-Actin was used as loading control. **L,** Lysates from cells expressing indicated SIRT6 mutants and truncated USP48 were pulled down with anti-Myc and then immunoblotted with anti-HA (top). Input was immunoblotted with anti-Myc and anti-Flag (bottom). β-Actin was used as loading control. Data are shown as mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$. IB, immunoblot; IP, immunoprecipitation.

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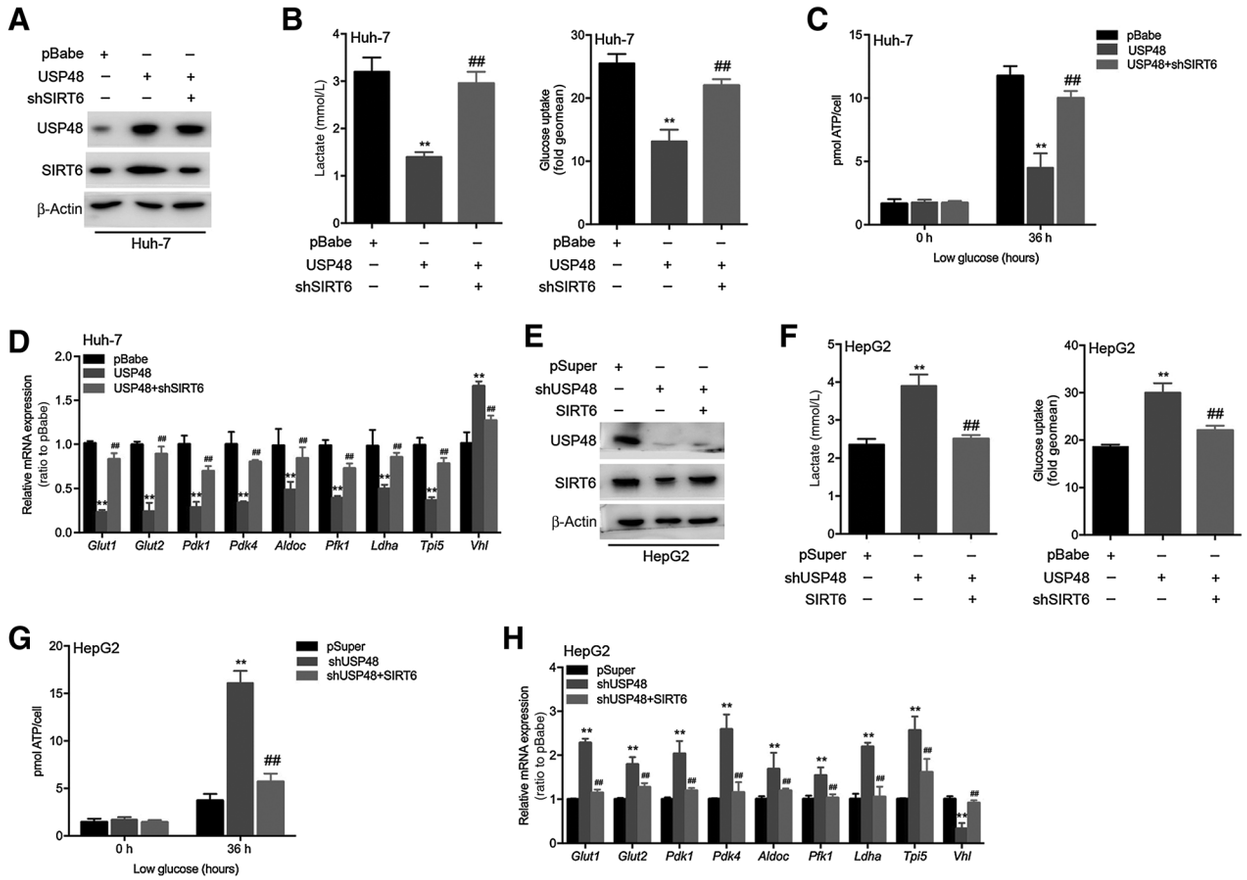

Figure 6.

USP48-inhibited metabolic reprogramming is SIRT6 dependent. **A,** Immunoblotting of USP48 and SIRT6 in Huh-7 cells transfected with different combinations of control vector, USP48 vector, and/or sh-SIRT6. **B,** Measurement of lactate production (left) and glutamine uptake (right) in transformed cells described in **A**. **C,** Measurement of ATP in transformed cells described in **A** at indicated time points. **D,** qRT-PCR to measure the expression of genes related to glucose metabolism in transformed cells described in **A**. **E,** Immunoblotting of USP48 and SIRT6 in HepG2 cells transfected with different combinations of control vector, sh-USP48, and/or SIRT6 vector. **F,** Measurement of lactate production (left) and glutamine uptake (right) in transformed cells described in **E**. **G,** Measurement of ATP in transformed cells described in **E** at indicated time points. **H,** qRT-PCR to measure the expression of genes related to glucose metabolism in transformed cells described in **E**. Data are shown as mean ± SD of three independent experiments. **, $P < 0.01$; $^{\#\#}$, $P < 0.01$.

USP48 could interact with and stabilize SIRT6, which is a critical modulator of cancer metabolism, we therefore set out to determine whether USP48 exerts its functions depending on SIRT6 in HCC. For this purpose, we established stable Huh-7 cell lines expressing the USP48 insert, as well short hairpin RNAs that target SIRT6 (**Fig. 6A**). As anticipated, downregulation of SIRT6 substantially rescued lactate production, glucose uptake, and ATP levels in Huh-7 cells overexpressing USP48 (**Fig. 6B** and **C**). In addition, our results also showed that SIRT6 K33R/K128R rescued lactate production, glucose uptake, and ATP levels in Huh-7 cells overexpressing USP48 (Supplementary Fig. S8A and S8B), suggesting the necessity of K33/K128 residue in the function of USP48. Simultaneously, our results also showed that SIRT6 K33/K128 rescued the cell viability and colony formation in Huh-7 cells overexpressing USP48 (Supplementary Fig. S8C and S8D). Likewise, SIRT6 depletion also successfully reversed the alterations of key glycolytic genes elicited by USP48 overexpression in Huh-7 cells (**Fig. 6D**). Conversely, the forced expression of SIRT6 almost completely abrogated the glycolytic phenotypes induced by USP48 deficiency in HepG2 cells (**Fig. 6E–H**). Taken as a whole, these observations indicate that SIRT6 is indispensable for the regulation of glucose metabolism by USP48 in HCC.

Upregulated pyruvate dehydrogenase kinase 1 (PDK1) was found in USP48-deficient cells, as shown in **Fig. 3**, dominating the pivotal switch in the Warburg effect by phosphorylating pyruvate dehydrogenase (PDH) in cancer cells (38). To test whether the glycolytic phenotypes observed in the USP48-deficient cells contribute to HCC cell malignant behavior, we treated USP48-deficient BEL-7404 and HepG2 cells with the PDK1 inhibitor BX-795 to suppress glycolysis (Supplementary Fig. S9A). As expected, treatment with the drug strikingly attenuated PDH phosphorylation, as well as the proliferative and migratory capacity of the USP48-deficient cells (Supplementary Fig. S9B–S9D). Furthermore, inhibition of PDK1 severely diminished tumor formation and cell proliferation *in vivo* (Supplementary Fig. S9E and S9F). Together, these results demonstrate that the inhibition of glycolysis in USP48-deficient cells is sufficient to revert the oncogenic phenotype. Accordingly, USP48 may act as a tumor suppressor by blocking a switch toward aerobic glycolysis, which relies on SIRT6 stabilization.

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SIRT6 is required for the suppressive role of USP48 in HCC tumorigenesis

Metabolic reprogramming from mitochondrial respiration toward aerobic glycolysis, the Warburg effect, is one of the most prominent distinctions of cancer cells. In light of our observation that USP48-mediated deubiquitination and stabilization of SIRT6 regulates the glycolytic phenotypes in hepatocytes, it is thus reasonable to postulate that the USP48/SIRT6 signaling pathway plays a role in cell proliferation and carcinogenesis. To test this hypothesis, we decided to explore the possible role of SIRT6 in USP48-mediated HCC tumorigenesis *in vitro* and *in vivo*. In agreement with previous findings, the downregulation of SIRT6 expression fully recovered cell viability, colony formation, migration, and invasion in Huh-7 cells overexpressing USP48 (Supplementary Fig. S10A–S10C). Coincident observations that SIRT6 deficiency in Huh-7 cells completely abrogated the alterations of USP48 overexpression on EMT markers, like E-cadherin, β-catenin, N-cadherin, and vimentin, were achieved by immunoblotting (Supplementary Fig. S10D). Whereas the overexpression of SIRT6 almost completely abolished the cell viability, colony formation, migration, and invasion induced by USP48 deficiency in HepG2 cells (Supplementary Fig. S11A–S11C). Overexpression of SIRT6 in HepG2 cells reversed the alteration of EMT markers (such as E-cadherin, β-catenin, N-cadherin, and vimentin) by knocking out USP48 (Supplementary Fig. S11D). *In vivo* evidence from mouse xenografts injected with double transfected cells revealed the consistent result that ablation of SIRT6 expression overrides the growth-inhibitory effect of USP48 overexpression, whereas the effects of USP48 decline on tumor growth and metastasis was impaired by SIRT6 overexpression (Supplementary Fig. S12A–S12D). These data altogether indicate that the stabilization of SIRT6 by USP48 is vital for blocking the oncogenic phenotype and tumor metastasis in HCC.

Mettl14-induced m^{6}A modification is involved in the upregulation of USP48

m^{6}A regulates RNA stability through methylation modification, thereby affecting the expression and function of RNAs (39). Deregulation of m^{6}A modification has also been implicated in HCC. One of the components of a multisubunit m^{6}A writer complex, Mettl14 was found to have reduced expression in HCC, which was related to the HCC tumorigenesis (40). IHC staining of Mettl14 in tissue samples from patients with HCC also showed the low expression of Mettl14 in HCC (Supplementary Fig. S13A). However, whether USP48 mRNA could be modified by m^{6}A is still unclear. In an effort to better understand the upstream regulation mechanism of USP48 in HCC, we evaluated the expression levels of Mettl14 and USP48 in The Cancer Genome Atlas (TCGA) database of patients with HCC. Notably, Mettl14 expression was positively correlated with the USP48 expression level ($R = 0.55$; **Fig. 7A**). Consistently, overexpression of Mettl14 significantly increased USP48 mRNA and protein levels in Huh-7 and HepG2 cells (**Fig. 7B** and **C**). In contrast, Mettl14 depletion apparently reduced USP48 mRNA and protein levels (**Fig. 7D** and **E**). RNA-binding protein immunoprecipitation (RIP) assays also showed that overexpression of Mettl14 significantly increased the m^{6}A level of USP48 mRNA (**Fig. 7F**), while knocking out Mettl14 significantly decreased the m^{6}A level of USP48 mRNA (**Fig. 7G**). To investigate whether Mettl14 contributes to the mRNA decay of USP48, an mRNA stability assay was conducted to measure the stability of USP48 mRNA in HCC cells. In response to the transcription inhibitor actinomycin D, Mett114 deficiency reduced the stability of USP48 mRNA, while overexpression of Mett114 increased the stability of USP48 mRNA (**Fig. 7H**). Similarly, overexpression of Mettl14 also significantly increased SIRT6 expression levels, while the knockout of Mett14 suppressed SIRT6 expression levels (**Fig. 7I**). In addition, overexpression or knockout of Mettl14 reversed the regulation of glycolysis by the knockout or overexpression of USP48, respectively (**Fig. 7J**). In addition, we also analyzed the expression levels of Mettl14, USP48, and SIRT6 in HCC tissues: Mettl14 was positively correlated with USP48 and SIRT6, respectively (Supplementary Fig. 13B and S13C). These results indicate that Mettl14 stabilizes USP48 mRNA through m^{6}A modification and increases its expression level, thereby inhibiting the ubiquitination of SIRT6 and subsequent glycolysis regulated by SIRT6 (**Fig. 7K**).

Discussion

Although a variety of therapeutic strategies and diagnostic modalities have been developed for HCC treatment, HCC still remains a most difficult malignancy to cure due to an incomplete understanding of the molecular mechanisms of this disease. In this study, we found that the expression level of USP48 in HCC was significantly reduced. By regulating the stability of SIRT6 protein to prevent metabolic reprogramming, USP48 has a protective effect on HCC tumorigenesis. Moreover, this is also the first publication reporting that Mettl14 regulates USP48 mRNA m^{6}A modification and increases USP48 protein expression (**Fig. 7K**).

Compared with the other USP family members, USP48 is a poorly characterized protein with few literature studies regarding its function and regulation. Several reports have implicated the deubiquitinated activity of USP48 in the regulation of NFκB signaling, which is critical for diverse cellular processes including inflammation, immunity, differentiation, and cell survival (35, 41). With respect to cancer, besides its connection to NFκB signaling, USP48 has been linked to tumorigenesis of glioblastoma by stabilizing Gli1 (28), the stability of oncoprotein Mdm2 in a deubiquitylation-independent manner (37) and the reduction of E cadherin–mediated adherents junctions (36). Apart from the above oncogenic functions, USP48 also promotes genome stability by antagonizing BRCA1 E3 ligase function (42), and aids ATRA-induced granulocytic differentiation of acute promyelocytic leukemia cells (29), serving tumor-suppressive roles. However, its abundance and biological activity in HCC are completely unknown. In this study, we found that the expression of USP48 was significantly reduced in DEN-induced mouse HCC and clinical patient tissues. Recent studies have focused on molecular regulation of the stability of SIRTs and activity by the ubiquitin system. The USP22–SIRT1 axis (43) and USP10–SIRT6 axis (44) have been identified in HCC and nonalcoholic fatty liver disease, respectively. In line with these findings, our study found that SIRT6 is a bonafide substrate of USP48, and USP48 regulates SIRT6 relevant signaling through the stabilization of this protein.

We discovered that metabolism was the most significantly enriched pathway altered by USP48 ablation, which is consistent with the subsequent observations that the depletion of USP48 enhanced metabolic flux into aerobic glycolysis in the liver. Because SIRT6 represses glycolysis to restrain tumorigenesis and is a substrate of USP48, it is not surprising to find that SIRT6 is essential in USP48-inhibited metabolic reprogramming and HCC tumorigenesis. The exploitation of the high glucose flux in cancer cells for targeted therapy may render innovative treatment options. However, compensatory and adaptive responses will probably lead to the resistance of cancer cells to glycolytic inhibitors, like PDK1 or GLUT1 inhibitors (45). It is thus necessary to define the pathways limiting tumor progression and investigate the context specificity of metabolic preferences and liabilities in malignant

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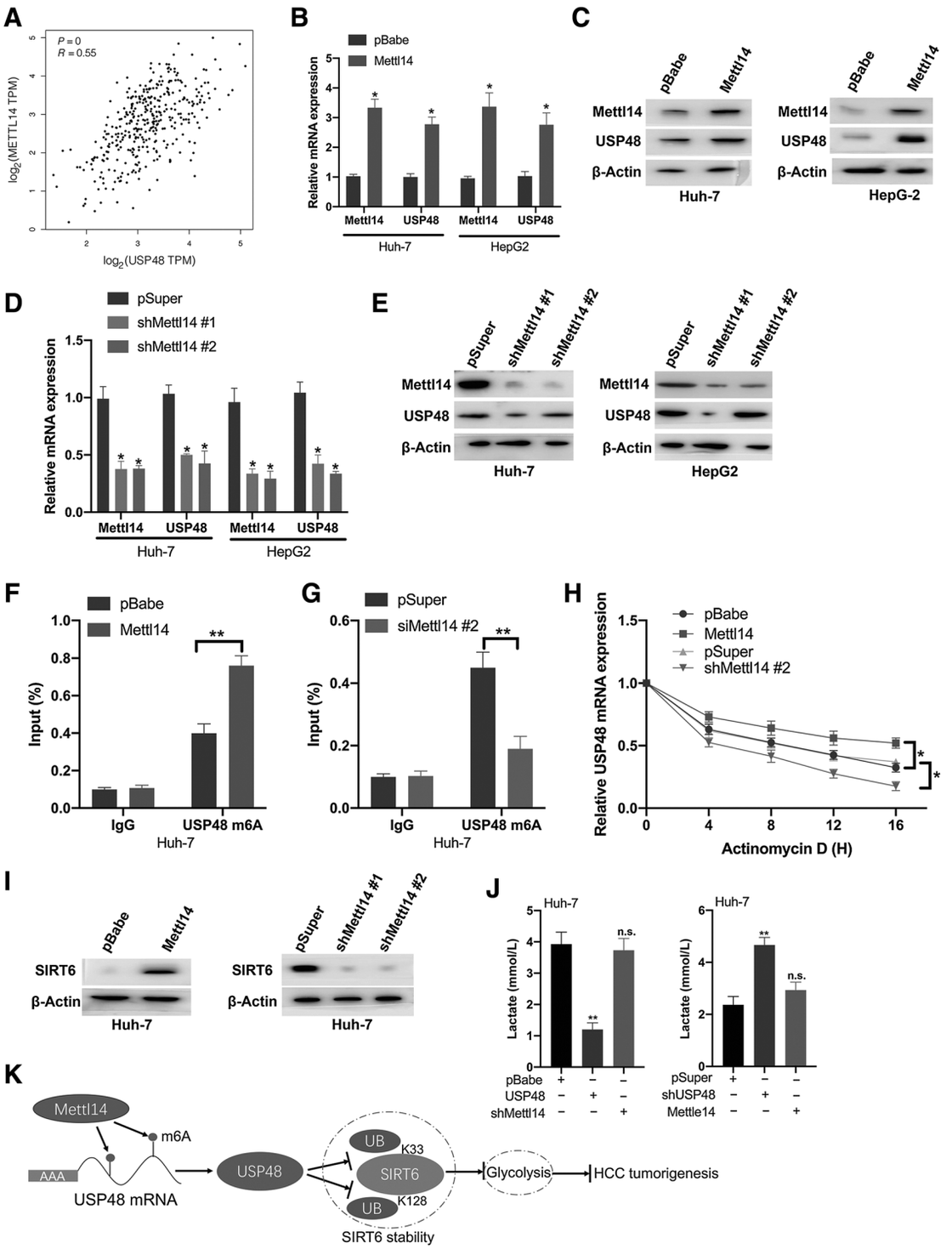

Figure 7.

Mettl14-induced m^{6}A modification is involved in the upregulation of USP48. **A,** USP48 expression was positively correlated with Mettl14 expression in an analysis of TCGA data. **B–E,** qRT-PCR and Western blot analysis of Mettl14 and USP48 after Mettl14 knockout and overexpression. **F** and **G,** RIP-qPCR showing the enrichment of USP48 m^{6}A after Mettl14 knockout and overexpression. **H,** The decay rate of USP48 mRNA after treatment with 2.5 μmol/L actinomycin D for indicated times, with Mettl14 knockout and overexpression. **I,** Western blot analysis of SIRT6 after Mettl14 knockout and overexpression. **J,** The lactate secretion level in Huh-7 cells that overexpress USP48 and knockout Mettl14 or overexpress Mettl14 and knockout USP48. **K,** The schematic figure shows that Mettl14 upregulates the m^{6}A level of USP48 mRNA; overexpression of USP48 inhibits the stability of SIRT6, thereby inhibiting glycolysis and HCC tumorigenesis. Data are shown as mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; n.s., no significance.

cells. In addition, the competition between tumor-infiltrating T cells and tumor cells might dampen the ability of the former to respond to the latter (46), suggesting a promising strategy of a combination of glycolytic inhibitors with immunotherapy, such as PD-1– or PD-L1–targeted antibodies. Intriguingly, recent reports have shown that PD-L1 activates the ITGB4/SNAI1/SIRT3 signaling pathway to promote the growth and metastasis of cervical cancer (47), and anti–PD-1 treatment caused elevated SIRT1 levels in xenograft mice (48). Given the impact of USP48 and SIRT6 on NFκB signaling and inflammation, as well as reprogrammed glucose metabolism, the clinical benefit of targeting this axis deserves further exploration.

The m^6A modification is an important epigenetic regulatory mechanism, which has been reported to affect polyadenylation, pre-mRNA splicing and RNA stability (49). Disorders of RNA modification are related to obesity, diabetes, infertility, and cancer (50, 51). Mettl14 is one of the key members of the m^6A methyltransferase complex and acts as the "writer" of m^6A (40, 52). Mettl14 expression has also been found to be significantly lower in HCC, and promotes miR-126 expression in an m^6A-dependent manner to inhibit HCC metastasis (40). However, it is not clear whether there is another mechanism implicated in the protective role of Mettl14 in HCC. Our results indicate that Mettl14 modifies USP48 to increase mRNA stability, thereby mediating SIRT6 stabilization and glycolysis.

In conclusion, the current study provides evidence that USP48 inhibits the glycolysis that supports HCC tumorigenesis, and the hepatoprotective role of USP48 is dependent on SIRT6 via deubiquitylation and stabilization of this protein. Furthermore, Mettl14 upregulated the expression level of USP48 through m^6A modification. Therefore, specifically targeting hepatocyte USP48 or the USP48-SIRT6 axis may be a potential therapeutic strategy for HCC treatment in the future.

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

L. Du: Conceptualization, resources, data curation. **Y. Li:** Conceptualization, resources, data curation. **M. Kang:** Conceptualization, resources, data curation. **M. Feng:** Resources, data curation. **Y. Ren:** Resources, data curation. **H. Dai:** Resources, data curation. **Yumin Wang:** Resources, data curation. **Yunshan Wang:** Resources, data curation, methodology. **B. Tang:** Resources, data curation.

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USP48 Is Upregulated by Mettl14 to Attenuate Hepatocellular Carcinoma via Regulating SIRT6 Stabilization

Lutao Du, Yang Li, Min Kang, et al.

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