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ORIGINAL ARTICLE

Caveolin-1 promotes Rfng expression via Erk-Jnk-p38 signaling pathway in mouse hepatocarcinoma cells

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Abstract

Caveolin-1 (Cav-1) is a critical structural protein of caveolae and plays an oncogene-like role by participating in abnormal protein glycosylation in hepatocellular carcinoma (HCC). However, the mechanism by which Cav-1 regulates glycosylation and glycosyltransferase expression has not been completely defined. Here, we show that Cav-1 promotes the expression of Rfng, which is a β-1,3-N-acetylglucosaminyltransferase included in the Fringe family. In this study, we showed that the mouse HCC cell line, Hepa1–6, with low Rfng transcription and protein levels, lacked Cav-1 expression, whereas strong Rfng expression was found in the mouse HCC cell line Hca-F, with high transcription and protein levels for Cav-1. Subsequently Cav-1 overexpression in Hepa1–6 was found to activate mitogen-activated protein kinase (MAPK) signaling and induce phosphorylation of the transcription factors Hnf4a and Sp1, which bind to the *Rfng* promoter region to promote its transcription. On the contrary, when knocking down Cav-1 expression in Hca-F, the activity of the MAPK pathway was significantly inhibited, and phosphorylation of Hnf4a, Sp1 and the expression of Rfng were attenuated. These data reveal that Cav-1 promotes phosphorylation of transcription factors Hnf4a and Sp1, which bind to the *Rfng* promoter region, via the MAPK signaling pathway, to induce the transcription of Rfng. Our current findings provide molecular genetic evidence that Cav-1 plays an important role in regulating glycosyltransferase expression and may participate in the abnormal glycosylation that mediates the invasion and metastasis of HCC.

Keywords Caveolin-1 · Rfng · MAPK · Hnf4a · Sp1 · HCC

Introduction

Caveolae are flask-shaped plasmalemmal invaginations formed by lipids and proteins that function as trafficking vesicles and organized signal-transduction compartments [25]. Caveolin-1 (Cav-1) is an essential caveolar component that has a variety of biological activities, such as cholesterol transport, maintenance of lipid balance, the processes of malignant transformation and metastasis of tumors [15, 19, 25]. Cav-1 contains a scaffolding domain for binding to multiple signaling proteins, and is widely expressed in various tissues. The middle transmembrane region of Cav-1 forms a hairpin-like structure to allow Cav-1 to be embedded in the membrane, making Cav-1 a key regulator of signal transduction [15, 19, 20, 25, 28]. In addition, there is increasing evidence that Cav-1 is associated with glycosylation and glycosyltransferase expression. Cav-1 has been reported to induce the glycosylation of CD147 and to up-regulate the expression of the sialyltransferase, ST6Gal-1, to promote adhesion and invasion of hepatocellular carcinoma (HCC) cell lines [8, 13, 29], indicating that association of Cav-1 with abnormal tumor glycosylation may be achieved by regulating the expression of glycosyltransferases.

Protein glycosylation is an important post-translational modification of proteins, and it has been reported that more than 50% of proteins undergo glycosylation in mammals. Glycosylation abnormalities often occur in tumors, due mainly to disordered metabolism of sugar donors, or abnormal expression of

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glycosyltransferases [21]. Fringe proteins are β-1,3-N-acetylglucosaminyltransferases that transfer N-acetylglucosamine to O-linked fucose present at a particular consensus site of EGF-like repeats in Notch [10]. Three Fringe proteins in mammals have been identified, including Lunatic Fringe (Lfng), Manic Fringe (Mfng), and Radical Fringe (Rfng) [12]. Lfng and Mfng inhibit the Notch pathway by impeding Jagged1 ligand binding, and can also trigger the Notch pathway by enhancing Delta-like (DLL1) ligand binding, whereas Rfng enhances binding of both Jagged1 and DLL1 to activate the Notch pathway [11]. Rfng is important for the glycosylation and activation of Notch receptors and Notch ligands, thus directly affecting the activity of the Notch signaling pathway [5, 11]. It was reported that abnormal expression of Rfng was associated with aberrant activation of the Notch pathway in tumors [1]. Therefore, Rfng could participate in tumorigenesis and progression through its catalytic function in Notch glycosylation.

This study aimed to investigate the mechanism by which Cav-1 regulates the expression of the glycosyltransferase, Rfng, and to explain the details of this regulation in mouse HCC cells at all levels, from the initiation of gene transcription through to protein translation. The results of the present study provide a new theoretical basis for how Cav-1 participates in the regulation of abnormal glycosyltransferase expression and glycosylation, suggesting that Cav-1 may be further used as a potential therapeutic target in HCC.

Materials and methods

Cell culture

The mouse HCC cell line, Hca-F, with high Cav-1 expression levels, (established and stored by Department of Pathology, Dalian Medical University, Dalian, China) was grown in mouse abdominal cavities for 7 days [24]. The cells were then cultured in 90% RPMI 1640 (Gibco, Thermo Fisher Scientific, Waltham, MA) medium supplemented with 10% fetal bovine serum for 24 h. The mouse HCC cell line, Hepa1–6, with no Cav-1 expression (obtained from Cell Center of Peking Union Medical College, Beijing, China) was maintained in DMEM supplemented with 10% fetal bovine serum. All cultures were maintained in medium supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin (1% antibiotic) and grown at 37 °C in a humidified incubator with 5% CO_2.

Stable clone selection and transfection

Hca-F cells were harvested by centrifugation (1000 rpm, 5 min), the supernatant discarded and the cells resuspended in RPMI 1640 cell culture medium without serum. The resuspended cells were adjusted to a concentration of 10^6 cell/ml and placed in the 4-mm gap electroporation cuvettes along with the appropriate plasmid DNA and electroporated twice at 500 V. Cells transfected with a plasmid containing an unrelated sequence were used as the negative control (NC), while those transfected with the pGPU6/shCav-1 plasmid constituted the experimental group. Both plasmids were purchased from GenePharma (Shanghai, China). Hepa1–6 cells were plated uniformly on a 10 cm cell culture plate, and transfected with the pcDNA3.1/Cav-1 plasmid after reaching 80% confluence. Media supplemented with G418 at a concentration of 800 μg/ml were used to select cells stably transfected with the Cav-1 expression plasmid.

Immunohistochemical staining

Paraffin-embedded liver tissue sections from wild-type or Cav-$1^{-/-}$ C57BL/6 J mice were dewaxed and treated with boiling citrate buffer, pH 6.0, for 3 min. The sections were incubated in hydrogen peroxide (3%) for 20 min at room temperature to block endogenous peroxidase activity and then incubated with anti-goat serum for 15 min at room temperature. The sections were incubated with primary antibodies specific for Cav-1 (Thermo Fisher) and Rfng (Thermo Fisher) overnight at 4 °C. Immunohistochemical analyses were performed using a Histostain Kit (Beyotime, Nantong, China) and the stained sections were examined using a LEICA DMI4000 B microscope.

Quantitative real-time PCR

Mouse livers were harvested from either wild-type C57BL/6 J mice or Cav-$1^{-/-}$mice C57BL/6 J (three mice each), and total cellular RNA was isolated from the samples using TRIzol (Invitrogen, Carlsbad, CA). qPCR procedures were carried out as previously described [7]. The sequences of the upstream and downstream primers for *Rfng*, *Mfng*, *Lfng*, *Cav-1* and *Gapdh* are shown in Table 1. All target gene transcripts were normalized to *Gapdh* transcript levels, and the relative fold-change in expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot

Total protein extraction and western blotting procedures were carried out as previously described [7]. Cav-1 and Rfng antibodies were purchased from Invitrogen (Carlsbad, CA), phospho-Erk was purchased from Bioworld (Shanghai, China), Sp1, Erk, Jnk, p38 and Gapdh were purchased from Proteintech (Wuhan, China), Hnf4a, phospho-Hnf4a, phospho-Sp1, phospho-Jnk, phospho-p38 were purchased from Bioss Antibodies (Beijing, China).

Table 1 The sequences of primers used

Gene	Forward	Reverse
Cav-1	5'-GGTCAATCTCCTTGGTGTGC-3′	5'-ATGTCTGGGGGCAAATACG-3′
Lfng	5'-TCTGCTGTTCGAGACCTGGAT-3′	5'-GTGAGCACCACATTGCCTGT-3′
Mfng	5'-GGTCTCCAGGATCAGGCAAC-3′	5'-TTGCAGGACAGAGCAGGATG-3′
Rfng	5′- GGCTGTGTTGCTGCTACTGC-3′	5′- CCGAGTGGTCTTGACTGCAA-3'
GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3'	5'-GGGGTCGTTGATGGCAACA-3'

Immunofluorescence staining

Cells were seeded onto 10-mm coverslips. After washing twice with phosphate-buffered saline (PBS), cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 before being incubated with the specific primary antibodies for Cav-1 and Rfng (identical to the antibodies described in the Western blotting experiments above) overnight at 4 °C. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and the stained cells were viewed with a microscope system.

Vector construction and luciferase reporter assays

Extraction of genomic DNA from Hepa1–6 cells was performed according to the manufacture's instructions using GenElute™ Mammalian Genomic DNA Miniprep Kits (Sigma, St. Louis, MO). PCR primers were designed to amplify the *Rfng* promoter region sequence: 5′-AGTG TGGCTCCTCTTGTCATACATGA-3′ and 5′-TCCT GGCTACCTGATGCTTAGGTTAC-3′ and contained *Nhe*I and *Bgl*II restriction sites. The PCR products were ligated into the pGL3-control luciferase expression vector using T4 ligase. Hepa1–6 cells, Hepa1–6/Cav-1 cells, Hca-F and Hca-F cells transfected with pGPU6/shCav-1 were then co-transfected with the reporter plasmid or the control plasmid pRL-TK. After 48 h, luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were carried out as previously described [7]. Antibodies specific for Hnf4a were purchased from Abcam (Cabridge, UK) and those for Sp1 were purchased from Cell Signaling Technology (Tokyo, Japan). Two pairs of ChIP primers listed in Table 2 were designed based on the predicted binding sites. PCR was performed using ChIP products or input samples as the template, and the PCR products then were analyzed by 2.0% agarose gel electrophoresis, followed by ethidium bromide staining.

Primer analysis of RNA-seq data and analysis of differentially expressed genes

After filtering RNA-seq data using the HISAT and Bowtie tools, the RNA-Seq by Expectation Maximization (RSEM) tool was applied for quantitative gene expression analysis; quantitative expression is calculated as fragments per kilobase million (FPKM). We used the Poisson distribution method to analyze the differentially expressed genes (DEGs). For pathway analysis, all DEGs were mapped to terms in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and then scrutinized for significantly enriched pathway terms compared to the background genome. KEGG pathways fulfilling the criterion of a Bonferroni corrected $P \leq 0.05$ were defined as significantly enriched in DEGs.

Statistics

All data represent at least three experiments, and are expressed as the mean ± S.E.M. Differences between groups were compared using the Student's *t* test (when comparing two groups), one-way ANOVA with Tukey's multiple comparison test (when comparing more than two groups and considering one independent variable) or two-way ANOVA with Bonferroni *post hoc* test (when comparing differences between groups considering two independent variables). All statistical tests were conducted using GraphPad Prism (La Jolla, CA). Differences were considered statistically significant at $*P<0.05$, $**P<0.01$ and $***P<0.001$.

Table 2 Chromatin immunoprecipitation (ChIP) primer sequences

Gene	Sequence (5′–3′)
ChIP-HNF4A-F	TGGGCACGTGGGTTATATGAAA
ChIP-HNF4A-R	ATGTGCCCGTGTACCCAAG
ChIP-Sp1-F	TAGAAGGGTGAGGGCTGAGG
ChIP-Sp1-R	TGAGCCATCTCTACAGCAGC

Results

Cav-1 upregulates the transcription and protein levels of Rfng

To study the function of Cav-1 in the development of HCC, especially tumor behavior caused by the abnormal glycosylation and glycosyltransferase expression induced by Cav-1, we first performed RNA-seq experiments in the mouse HCC cell line, Hepa1–6 (that lacks Cav-1 expression), and Cav-1 expressing stably transfected Hepa1–6/Cav-1 cells. The results showed that the expression levels for each the three members of the Fringe family were different in these cells. *Mfng* was not substantially expressed in Hepa1–6 and Hepa1–6/Cav-1 cells, while *Lfng* expression was not significantly different between Hepa1–6 and Hepa1–6/Cav-1. Nonetheless, the expression of *Rfng* was significantly increased after Cav-1 overexpression (Fig. 1a). This result was subsequently verified by qPCR assay (Fig. 1b). Moreover, the immunohistochemistry experiments showed that the expression of Rfng was significantly lower in the liver tissues of Cav-1 knockout mice (Cav-1$^{-/-}$ C57BL/6 J) compared with wild type mice liver cells (Fig. 1c). These data demonstrate that Rfng transcription was positively correlated with Cav-1 expression.

To further analyze the relationship between Cav-1 and Rfng in mouse HCC, we analyzed the expression of Rfng at both the transcription and translation levels by using qPCR and immunoblotting assays of extracts from Hca-F and Hepa1–6 cells. The results in Fig. 1d show that Rfng expression was significantly higher in Hca-F cells, which have high levels of Cav-1, than in Hepa1–6 cells, which lack Cav-1 expression. Furthermore, Cav-1 overexpression accompanied the elevation in Rfng mRNA and protein levels in Hepa1–6 (Fig. 1e). Cav-1 shRNA-transfected Hca-F cells exhibited attenuated Rfng expression (Fig. 1f). Immunofluorescence assay data were also consistent with the results described above (Fig. 1g). Together, these results implicate Cav-1 in mediating upregulated Rfng expression in mouse HCC cells.

Cav-1 activates transcription factors Hnf4a and Sp1 by phosphorylation

To explore the mechanism by which Cav-1 modulates Rfng mRNA levels in mouse HCC cell lines, a dual-luciferase reporter gene assay was performed. The results revealed a significant increase in the luciferase signal from the Rfng promoter in Hepa1–6/Cav-1 cells compared with that in Hepa1–6 control cells. In contrast, an attenuation luciferase signal from the Rfng promoter was observed in Hca-F cells transfected with Cav-1 shRNA (**P<0.01, Fig. 2a). We then used online software JASPAR [14] to analyze the sequence 2500 bp upstream of the *Rfng* promoter region, and which predicted binding sites for transcription factors Hnf4a and Sp1 (Fig. 2b). Region I in the *Rfng* promoter region (from −790 to −900 bp) include one predicted Hnf4a binding site and Region II (from −251 to −351 bp) includes two predicted Sp1 binding sites (Fig. 2b). Based on these JASPAR predictions, we designed primers to amplify Regions I and II in a ChIP-PCR assay (Table 2). ChIP experiments confirmed that Hnf4a and Sp1 could indeed bind to the *Rfng* promoter region in Hepa1–6 cells (Fig. 2c). We then examined the effect of Cav-1 in the activation and phosphorylation of transcription factors Hnf4a and Sp1. The results showed that the phosphorylation levels of Hnf4a and Sp1 were enhanced after overexpression of Cav-1 in Hepa1–6 cells. Knocking down the expression of Cav-1 decreased the phosphorylation levels of Hnf4a and Sp1 in Hca-F, indicated that Cav-1 was able to activate Hnf4a and Sp1 via phosphorylation. Taken together, these results suggested that Cav-1 upregulates *Rfng* transcription by increasing the phosphorylation levels of Sp1 and Hnf4a, which were then able to bind to the *Rfng* promoter region (Fig. 2d and e).

Cav-1 triggers the Erk-Jnk-p38 signaling pathway

To further investigate the signaling pathway by which Cav-1 regulates *Rfng* expression, we performed RNA-seq experiments on Hepa1–6 and Hepa1–6/Cav-1 cells in order to integrate differential signaling pathways in these two cell lines. RSEM [17] tools were used for quantification of gene expression, and quantitative expression is expressed in FPKM (Supplementary Table 1). To find the specific enrichment functional terms for the Cav-1 modulated signaling pathway, KEGG analysis were performed.

Fig. 1a–g Overexpression of caveolin-1 (Cav-1) enhances expression of Rfng. **a** RNAseq results expressed as Fragments per kilobase million (FPKM). **b** Expression levels of *Mfng*, *Lfng* and *Rfng* as defined by qRT-PCR normalized to *Gapdh* levels that served as a loading control. **P<0.01. **c** Translation levels of Rfng protein were examined by immunohistochemical staining. 4′,6-Diamidino-2-phenylindole (DAPI) *blue staining* represents the nucleus, and *brown staining* represents Cav-1 and Rfng [the secondary antibody was labeled with horseradish peroxidase, which produces a brown color with the substrate 3,3'-diaminobenzidine (DAB)]. Representative sections of liver tissues from wild-type and Cav-1$^{-/-}$ C57BL/6 mice. Original magnification, 100 ×. **d** Endogenous Cav-1 and Rfng mRNA and protein levels in Hepa1–6 and Hca-F cell lines were determined by qRT-PCR and Western blotting. Gapdh served as a loading control. **P<0.01. **e** qRT-PCR and Western blotting assays were used to detect the mRNA and protein expression levels of Rfng after overexpression of Cav-1 in Hepa1–6 cells. Gapdh served as a loading control. **P<0.01. **f** qRT-PCR and Western blotting assays were used to detect the mRNA and protein expression levels of Rfng after knockdown of Cav-1 expression in Hca-F cells. Gapdh served as a loading control. **P<0.01. **g** Immunofluorescence staining was performed to further detect protein expression of Rfng after overexpression of Cav-1 in Hepa1–6 cells and after knockdown of Cav-1 expression in Hca-F cells. Nuclei were counterstained with DAPI (40 ×). Data (mean ± SEM) from three separate experiments are presented as the median with error bars (*P<0.05, **P<0.01 and ***P<0.001)

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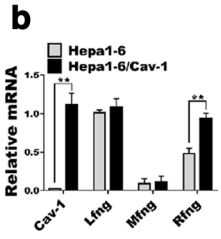
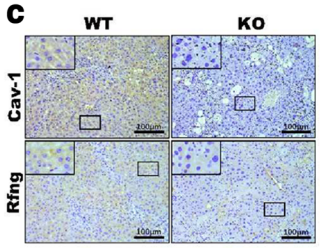
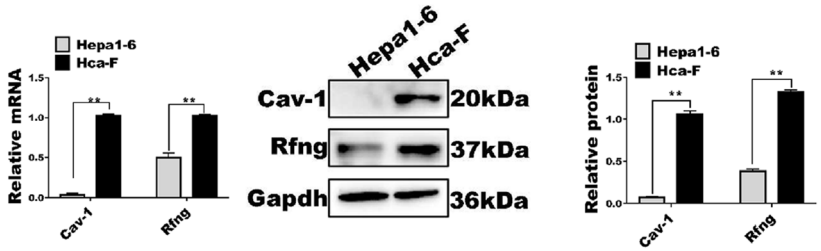
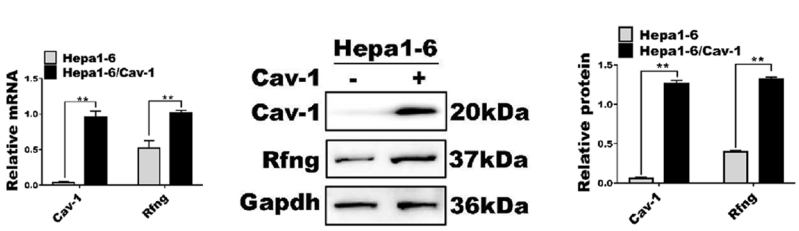
FPKM values of genes (RNA-seq)

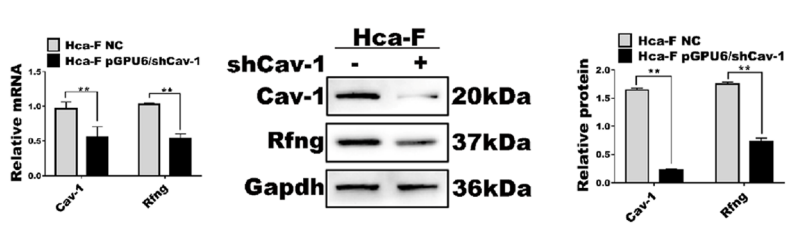
	Hepa1-6	Hepa1-6/Cav-1		
		1#	2#	3#
Cav-1	0.2195	1.57	1.128271	1.568751
Lfng	1.879473	1.87	1.900781	1.529532
Mfng	0.192063	0.16	0.487901	0.254922
Rfng	9.987272	16.7	17.39164	14.33446

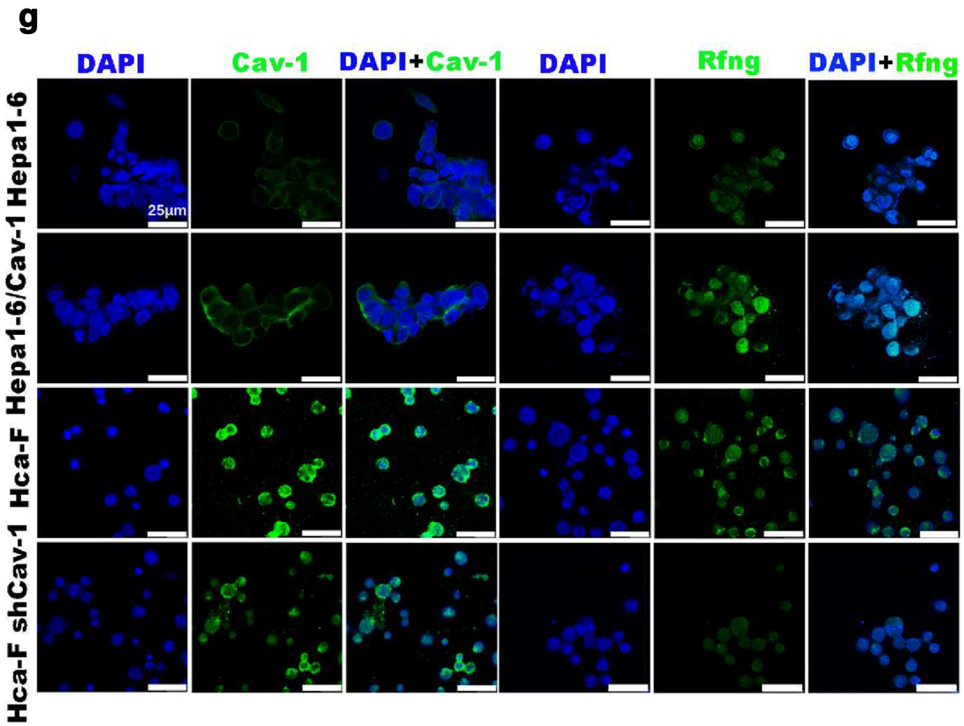












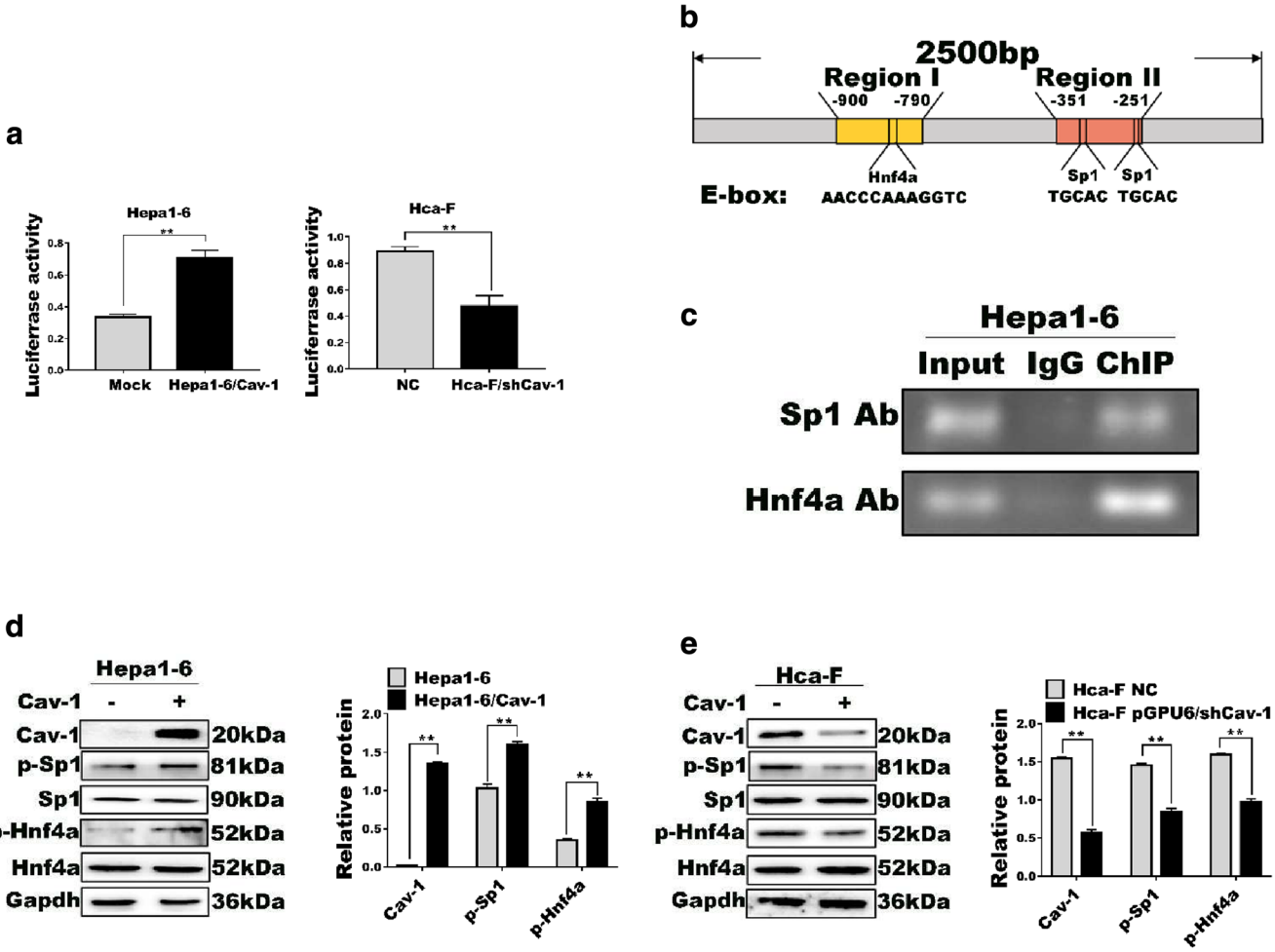


Fig. 2 Cav-1 activates transcription factors Hnf4a and Sp1 by phosphorylation. **a** Luciferase activity in Hepa1–6, Hepa1–6/Cav-1, Hca-F and Cav-1-knockout Hca-F cells was assayed at 48 h after transfection. Experiments were performed in triplicate, and the statistical significance of the difference was determined using the t-test (**P<0.01 vs. control). **b** Schematic representation of predicted binding sites for Hnf4a and Sp1. **c** Chromatin immunoprecipitation (ChIP) assays showing the binding of Hnf4a and Sp1 to the following regions: region 1 (from −790 to −900 bp) and region 2 (from −251 to −351 bp). **d** A Western blot assay was used to detect the phosphorylation levels of Hnf4a and Sp1 after overexpression of Cav-1 in Hepa1–6 cells. Gapdh served as a loading control. **P < 0.01. **e** A Western blot assay was used to detect the phosphorylation levels of Hnf4a and Sp1 after knockdown of Cav-1 expression in Hca-F cells. Gapdh served as a loading control. **P < 0.01. Data (mean ± SEM) from three separate experiments are presented as the median with error bars (*P<0.05, **P<0.01 and ***P < 0.001)

In the KEGG pathway analysis, the dysregulated mRNAs were found to be enriched in 306 pathways. Among the top six enriched signal pathways, the most enriched pathway was mitogen-activated protein kinase (MAPK) signaling. The expression of 36 genes in MAPK signaling pathway were altered, with 30 of them being up-regulated, suggesting that Cav-1 overexpression activates the MAPK pathway (Fig. 3a). To verify this result, we selected the Erk, Jnk, and p38 classical transduction pathways in the MAPK family for analysis by an immunoblotting assay. The results showed that the phosphorylation levels of Erk, Jnk and p38 were significantly increased in Hepa1–6/Cav-1 cells compared with Hepa1–6 cells, confirming that the MAPK pathway is significantly activated by Cav-1 expression. Conversely, the phosphorylation levels of Erk, Jnk and p38 were found to be significantly decreased in Hca-F cells that were transfected with Cav-1 shRNA, indicating that the MAPK pathway was inhibited by Cav-1 downregulation (Fig. 3b and c). These results demonstrated that Cav-1 activates MAPK signaling pathway in HCC cells.

Cav-1-mediated MAPK activation contributes to *Rfng* transcription

In the results described above, Cav-1 was demonstrated to upregulate the phosphorylation and activation of Hnf4a and Sp1, which bind to the *Rfng* promoter region in mouse HCC cells. To further verify the participation of MAPK signaling and transcription factors Hnf4a and Sp1 in Cav-1-mediated increases in *Rfng* expression, Hepa1–6 and Hepa1–6/Cav-1 cells were treated with

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Signaling pathway	Up-regulated gene number	Down-regulated gene number	Total changed gene number
MAPK	30	6	36
TNF	22	7	29
IL-17	18	2	20
FoxO	12	3	15
p53	12	3	15
HIF	7	4	11

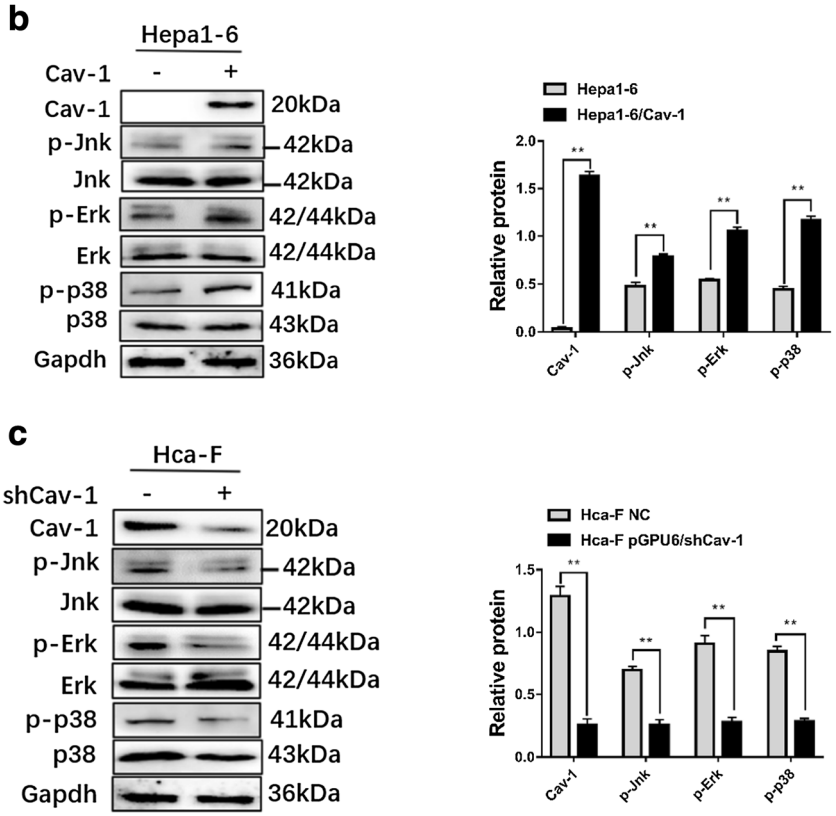
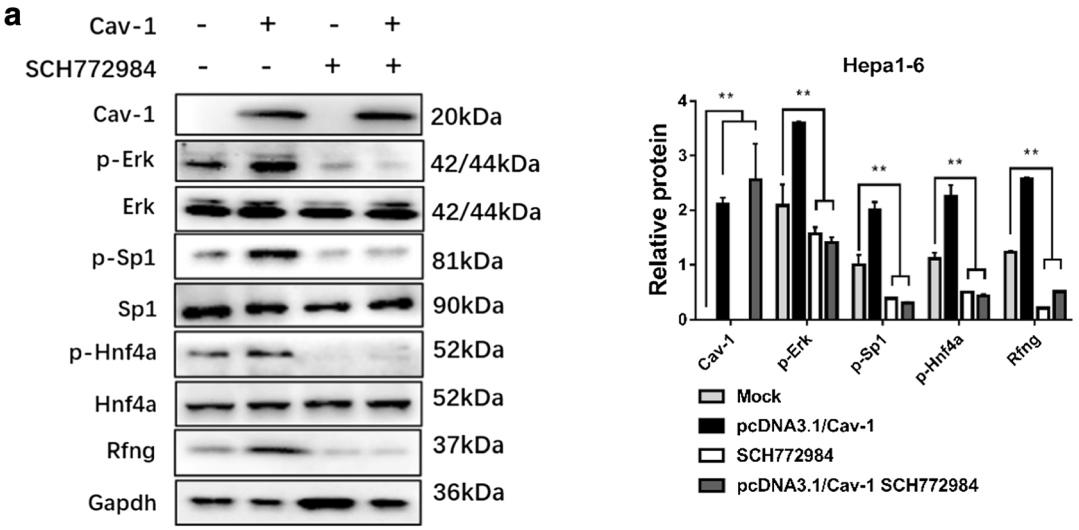


Fig. 3a–c Cav-1 activates the Erk-Jnk-p38 signaling pathway. **a** A total of 36 genes in the mitogen-activated protein kinase (MAPK) pathway had expression changes, 30 of which were up-regulated in Hepa1–6/Cav-1 cells. **b** Western blot assay was used to detect phosphorylation levels of Erk, Jnk and p38 after overexpression of Cav-1 in Hepa1–6 cells. Gapdh served as a loading control. $^{**}P<0.01$. **c** A Western blot assay was used to detect phosphorylation levels of Erk, Jnk and p38 after knockdown of Cav-1 expression in Hca-F cells. Gapdh served as a loading control. $^{**}P<0.01$. Data (mean ± SEM) from three separate experiments are presented as the median with error bars ($^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$)

specific MAPK signaling inhibitors (Erk inhibitor, SCH772984, 20 nM for 6 h; Jnk inhibitor, SP600125, 30 nM for 6 h; p38 inhibitor, SB203580, 20 nM for 6 h). Immunoblotting was used to detect the phosphorylation levels of Hnf4a and Sp1. The results showed that the phosphorylation levels of Hnf4a and Sp1 decreased significantly after the addition of Erk inhibitors, while Cav-1 was overexpressed, indicating that Cav-1 regulates the phosphorylation of Hnf4a and Sp1 through MAPK/Erk signaling pathways (Fig. 4a). After the addition of Jnk inhibitor, Hnf4a and Sp1 phosphorylation were significantly decreased in both Hepa1–6 and Hepa1–6/Cav-1 cells, and Rfng expression was significantly decreased (Fig. 4b). Similar to the results of these MAPK inhibitors, we also found that the p38 inhibitor decreased the activation of transcription factors and attenuated Rfng expression (Fig. 4c). These results suggested that Cav-1 promoted the expression of Rfng by regulating the phosphorylation of Hnf4a and Sp1 via Erk-Jnk-p38 MAPK signaling pathways.

Discussion

Cav-1, the structural protein of caveolae in the plasma membrane, modulates several molecular pathways leading to the regulation of hepatic lipid accumulation, lipid and glucose metabolism, mitochondrial biology, and hepatocyte proliferation [3]. Cav-1 participates in a variety of life processes, such as ocular neovascularization, and promotes atherosclerosis and tumor invasion and metastasis through regulation of the Jnk signaling pathway [9, 25, 26]. It was reported that Cav-1 reverse-regulates gastric tumor cell growth by negative feedback regulation of Raf-Erk [22]. In embryonic stem (ES) cells, a high glucose environment causes high expression of Cav-1, which activates the p38 signaling pathway and promotes the proliferation of ES cells [16]. In this research, we demonstrated that Cav-1 up-regulates the expression of the glycosyltransferase, Rfng, by activating the Erk-Jnk-p38 MAPK pathways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p-Sp1 81kDa
Sp1 90kDa
p-Hnf4a 52kDa
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Rfng 37kDa
Gapdh 36kDa
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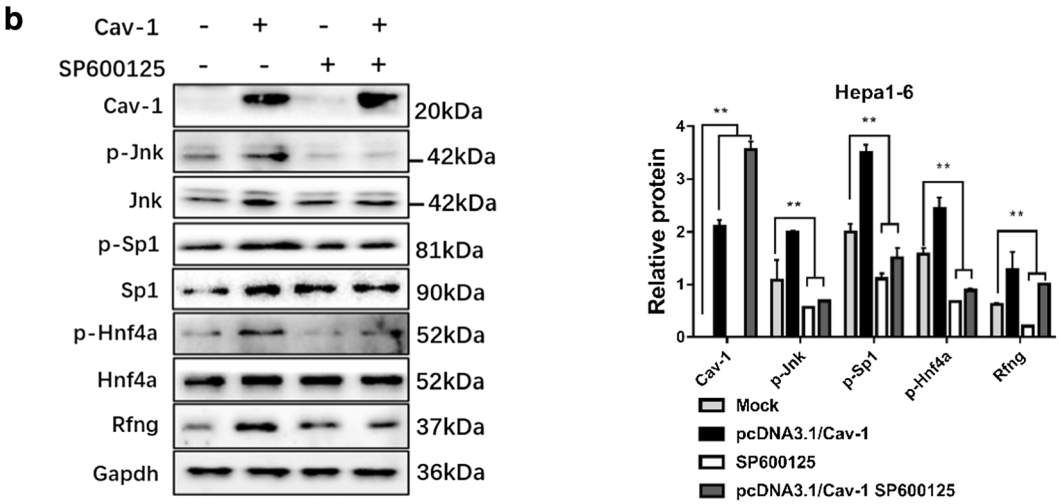
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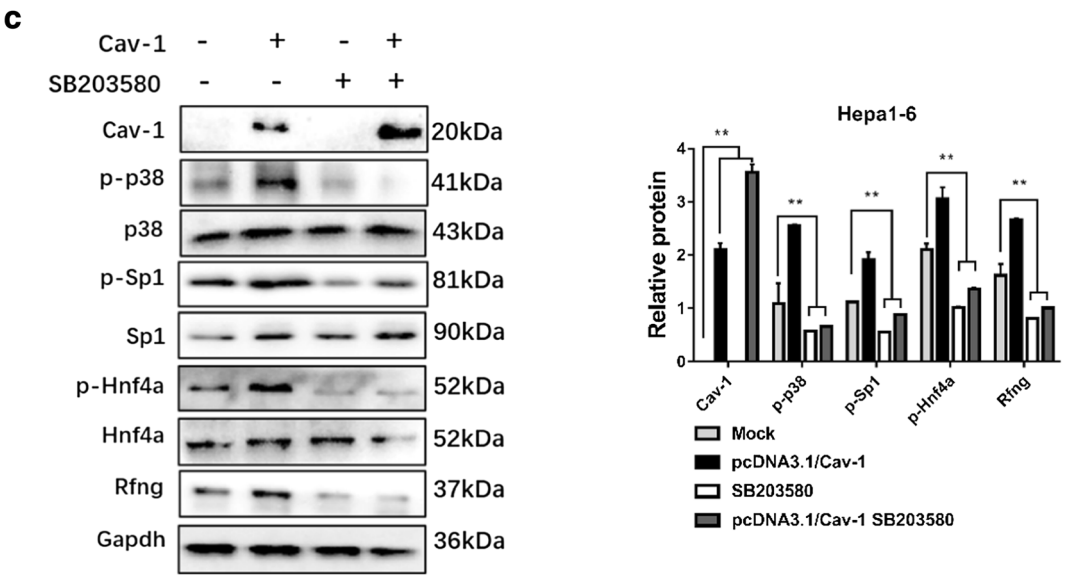
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p38 43kDa
p-Sp1 81kDa
Sp1 90kDa
p-Hnf4a 52kDa
Hnf4a 52kDa
Rfng 37kDa
Gapdh 36kDa
Hepa1-6
Relative protein
Cav-1
p-p38
p-Sp1
p-Hnf4a
Rfng
Mock
pcDNA3.1/Cav-1
SB203580
pcDNA3.1/Cav-1 SB203580

Fig. 4 Cav-1 regulates transcription factors Hnf4a and Sp1 via Erk-Jnk-p38 to moderate Rfng expression. **a** Western blot analysis of Hepa1–6/Cav-1 or control Hepa1–6 cells treated for 6 h with 20 nM Erk inhibitor (SCH772984). Gapdh served as a loading control. $^{**}P<0.01$. **b** Western blot analysis of Hepa1–6/Cav-1 or control Hepa1–6 cells treated for 6 h with 30 nM Jnk inhibitor (SP600125). Gapdh served as a loading control. $^{**}P<0.01$. **c** Western blot analysis of Hepa1–6/Cav-1 or control Hepa1–6 cells treated for 6 h with 20 nM p38 inhibitor (SB203580). Gapdh served as a loading control. $^{**}P<0.01$. Data (mean ± SEM) from three separate experiments are presented as the median with error bars ($^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$)

to induce phosphorylation of transcription factors Hnf4a and Sp1 (Fig. 5). This suggests that the Erk-Jnk-p38 pathway is involved in modulating glycosyltransferase expression and may even contribute to glycosylation abnormalities. In our RNA-seq transcriptome assay results, many genes in other signaling pathways have also undergone expression changes after overexpression of Cav-1 in Hepa1–6 cells (Fig. 3a), suggesting that other pathways might also affect the expression of *Rfng* and further exploration is needed.

It has been reported that the transcription factors Hnf4a and Sp1 are downstream of the MAPK signaling pathway and are also regulated by this pathway [6, 23]. In this investigation, we also confirmed that Hnf4a and Sp1 are transcription factors that bind to the transcriptional promoter region of many genes (Fig. 4), and a large number of other transcription factor binding sites are also present in the *Rfng* promoter region to regulate Rfng transcription. However, Hnf4a and Sp1 may play important roles in the regulation of Rfng transcription. We proved that MAPK inhibition mediated Hnf4a and Sp1 inactivation and drastically decreased Rfng expression in cells overexpressing Cav-1. Nevertheless, whether Hnf4a and Sp1 play a decisive role in the transcription of *Rfng* needs further investigation.

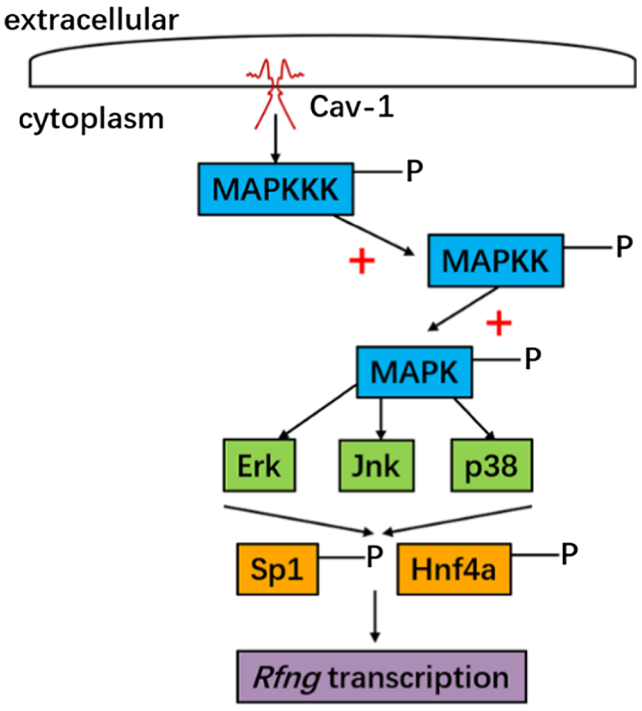

Fig. 5 Schematic showing how Cav-1 promotes Rfng expression via the Erk-Jnk-p38 signaling pathway in mouse hepatocellular carcinoma (HCC) cells. Cav-1 up-regulates Rfng by increasing the phosphorylation of Hnf4a and Sp1 via the MAPK pathway in mouse HCC cells

Abnormal protein glycosylation is closely related to the occurrence and development of many diseases, including cancer [18]. Fucosylation is a cancer-related glycosylation, and abnormal levels of fucose modifications are found in various tumors [2]. In recent years, an increasing number of reports have indicated that Cav-1 is associated with abnormal glycosylation in tumors. Cell surface α2, 6-sialylation was required for integrin α5, β1-dependent cell adhesion to fibronectin, Cav-1 promoted the expression of the key enzyme, ST6Gal1, that catalyzes α2, 6-sialylation and fibronectin-mediated adhesion of mouse HCC cell [21]. CD147 is a regulator of matrix metalloproteinase (MMP) production on the surface of many malignant tumor cells and exists in both highly glycosylated form and a low-glycosylated form. Cav-1 expression leads to an increased proportion of the highly glycosylated form of CD147 (HG-CD147) relative to the sparsely glycosylated form of CD147 (LG-CD147), increased production of MMP-11 and promoted a higher invasive capability in mouse HCC cells [29]. Although the studies described above have shown that Cav-1 is indeed associated with abnormal glycosylation in tumors, the specific mechanism by which Cav-1 regulates the level of glycosylation modification is not clear. This study demonstrates that Cav-1 affects expression of the glycosyltransferase, Rfng, by regulating the Erk-Jnk-p38 signaling pathways, providing a new theoretical basis and support for explaining the involvement of Cav-1 in the abnormal glycosylation observed in tumor cells.

In this study, we showed a positive correlation between Cav-1 and Rfng expression. ChIP experiments confirmed that the transcription factors Hnf4a and Sp1 can bind to the promoter region of Rfng and initiate transcription of Rfng. Cav-1 up-regulates the phosphorylation levels of Hnf4a and Sp1, thereby increasing Rfng expression at the transcriptional level. The results of RNA-seq and immunoblotting assays showed that Cav-1 could activate the Erk-Jnk-p38 signaling pathways, and, after adding specific inhibitors of these three signaling pathways, Hnf4a and Sp1 phosphorylation levels, along with Rfng expression, were markedly decreased. These data indicate that activation of Hnf4a and Sp1 by Cav-1 is achieved through the Erk-Jnk-p38 pathway, mediating upregulation of Rfng expression at both the mRNA and protein levels.

Rfng is a fucose-specific β1, 3-N-acetylcosaminyltransferase, and has been reported to catalyze the extension of O-fucosylation on Notch [27]. Analysis of the amino acid sequences of Lfng, Mfng and Rfng, predicts that Lfng is a pre-pro-protein precursor, while Mfng and Rfng are pre-protein precursors [4]. The

expression of Lfng, Mfng and Rfng is tissue specific and their functions are different. This is likely to be because of the differences in the amino acid sequences of Mfng, Lfng and Rfng and their localization, since the results were so different when we tested their expression in Hepa1–6 and Hepa1–6/Cav-1 cells. Mfng expression was extremely low in Hepa1–6 and Hepa1–6/Cav-1 cells, while Lfng was expressed in both Hepa1–6 and Hepa1–6/Cav-1 cells, but there was no significant difference in expression (Fig. 1a). Rfng expression in Hepa1–6/Cav-1 cells was significantly higher than in Hepa1–6 cells (Fig. 1a). These data indicated that Rfng is the only member of the Fringe family that is regulated by Cav-1 in mouse HCC cells. The substrates of Rfng are glycol-peptides that have O-fucose modified sugar chains. The most widely studied O-fucose modified proteins are the Notch receptors and ligands. However, the study of Rfng is currently limited. Almost all reports on Rfng function are based on its glycosylation modification of Notch. In this study, the question of whether Cav-1 mediated Rfng expression also affects the activity of the Notch pathway remains to be solved.

In summary, our results reveal the specific mechanism by which Cav-1 participates in the regulation of glycosyltransferase and glycosylation abnormalities in HCC cells, suggesting that Cav-1 may further function as a potential therapeutic target in HCC.

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