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α5 integrin regulates hepatic tight junctions through SRC-TET1-mediated DNA hydroxymethylation

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Highlights

- Decreased expression of α5 integrin in hepatocytes is associated with matrix deposition
- TJs are impaired after selective silencing of α5 integrin in hepatocytes
- α5 integrin regulates the expression of claudin 1 in an SRC signaling-dependent manner
- α5 integrin-SRC signaling regulates DNA hydroxymethylation in hepatocytes
α5 integrin regulates hepatic tight junctions through SRC-TET1-mediated DNA hydroxymethylation

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SUMMARY
The functional tight junctions' integrity plays an important role in liver physiology. A variety of liver diseases have been associated with the perturbation of tight junctions. Herein, we showed that the lower expression of α5 integrin in hepatocytes in patients with liver cirrhosis is associated with matrix deposition in the space of Disse. Selective silencing of α5 integrin in hepatocytes compromised the ultrastructure of tight junctions by downregulating claudin 1 in an SRC (proto-oncogene, non-receptor tyrosine kinase) signaling-dependent manner. α5 integrin signaling induced SRC-TET-mediated changes in 5-hydroxymethylcytosine and 5-methylcytosine levels in hepatocytes in vitro and in vivo. hMeDIP sequencing showed intergenic hypohydroxymethylation of the claudin 1 gene in hepatocytes after α5 integrin silencing in mice. Therefore, understanding the mechanisms regulating hepatic tight junction integrity in which α5 integrin-SRC signaling and epigenetic modifications cooperate might help advance the development of useful diagnostics and therapeutic approaches for liver disease.

INTRODUCTION
The establishment and maintenance of tight junctions (TJs) are essential for liver architecture and homeostasis, including sealing the apical pole of adjacent cells to form bile canaliculi and separating the basolateral domain drained by sinusoidal blood flow.1,2 TJ integrity and function require carefully orchestrated cooperation of TJ proteins, including different claudins, occludin, and junctional adhesion molecules, that interact with a cytoplasmic network of scaffolds (e.g., zonula occludens (ZO)) on the intercellular domain of the plasma membrane and with the actin cytoskeleton.3 Mutations in claudin 1, the most abundantly expressed TJ protein in the liver,4 in human hepatocytes are associated with neonatal sclerosing cholangitis.5,6 TJP1 (also called ZO-1) and ZO-2 ablation causes failure of protein localization and disruption of the TJ structure, and impairs liver zonation, and both bile ducts and sinusoids, leading to severe cholestatic liver disease.7,8

For hepatic TJs to function properly, the interaction between hepatocytes and the extracellular matrix (ECM) is of utmost importance.9,10 Our previous study showed that the endothelial cell-derived matrix, mainly including fibronectin, collagen I, and collagen IV, closely resembles the naive ECM dominant in the space of Disse in the liver and promotes the maturation of metabolic function in hepatocyte. Reducing the fibronectin content in the endothelial cell-derived matrix inhibits the effect of the matrix on hepatocyte maturation.11 The significant ECM alterations in liver fibrosis and cirrhosis result in the distortion of the liver architecture and abnormal hepatocyte function.12

Communication between cells and the ECM is achieved through integrins and the associated integrin-proximal adhesion molecules.13 Integrins are heterodimeric transmembrane proteins existing in unique combinations of noncovalently interacting α-subunits (18 types) and β-subunits (8 types).14 Via their action as cell adhesion receptors, integrins have long been recognized to serve as bidirectional hubs mediating cell and tissue function in a wide range of scenarios in health and disease.14,15 Our previous study showed that silencing the expression of α5 integrin, a specific fibronectin receptor subunit,16 in hepatocytes inhibited the effect of the endothelial cell-derived matrix on hepatocyte maturation, resulting in lower expression of dipeptidyl peptidase 4 (DPP4) located on the apical membrane of hepatocytes.11 Intracellular DPP4 trafficking to the canalicular domain is considered the basis for hepatic TJ function.17 Studies have shown that specifically deleting integrin-linked kinase from hepatocytes results in loss of differentiation.

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of hepatocytes and histologic abnormalities in the liver. However, whether and how integrin signaling regulates the structure and function of hepatic TJs remain to be explored.

Recent evidence has shown that integrin mediates DNA hydroxymethylation by regulating the expression of ten-eleven translocation (TET) methylcytosine dioxygenases. DNA hydroxymethylation is a type of DNA modification originating from the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in a process catalyzed by TET methylcytosine dioxygenases. There is increasing evidence that alterations in the patterns of 5hmC and 5mC influence gene regulation. 5hmC-mediated deregulation of genes involved in hepatic metabolism is an emerging area of research in liver disease.

In the current study, we found that the lower expression of α5 integrin in hepatocytes in patients with liver cirrhosis is associated with matrix deposition in the space of Disse. Using a model of hepatocyte-specific α5 integrin deficiency, we found that α5 integrin-SRC signaling regulates the expression of claudin 1 and the structural integrity of TJs through TET1-mediated DNA hydroxymethylation both in vitro and in vivo.

RESULTS

Decreased expression of α5 integrin in hepatocytes is associated with matrix deposition

To assess the relationship between the expression of α5 integrin in hepatocytes and matrix deposition, liver tissues from patients with cholestatic cirrhosis were analyzed. Cholestatic cirrhosis is a chronic cirrhosis disease of the liver characterized by the destruction of hepatic TJs and ECM deposition around hepatocytes in the space of Disse, which leads to fibrosis and potential cirrhosis due to complications. Masson trichrome staining showed rounded regenerative nodules separated by thick fibrous septa in liver tissue in patients with cholestatic cirrhosis (Figure 1, upper panel). ECM deposition in the space of Disse was found to be significantly increased in liver tissue in patients with cholestatic cirrhosis compared with liver tissue in control individual (Figure 1, middle panel). Immunohistochemical staining showed that α5 integrin protein level in hepatocytes in patients with cholestatic cirrhosis was lower than that in liver tissue in control individual (Figure 1, lower panel and Figure S1). This result indicates that the lower expression of α5 integrin in hepatocytes is associated with excessive matrix deposition in the space of Disse.
Figure 2. Selective silencing of α5 integrin destroys hepatic TJ
(A) Protein levels of ZO-1 and F-actin in ITGA5 siRNA- and control siRNA-treated hASC-HLCs were determined by immunofluorescence staining. Scale bars: 25 μm.
(B) Relative mRNA levels of ZO-1 in ITGA5 siRNA- and control siRNA-treated hASC-HLCs were determined by quantitative RT-PCR.
(C) The properties of hepatic TJs were determined in Itga5-sgRNA and control mice livers. Protein levels of Itga5 in Itga5-sgRNA and control mice mouse livers were determined by immunohistochemical staining. The black arrows indicate the expression of Itga5. Scale bars: 50 μm. The morphology of liver tissues in Itga5-sgRNA and control mice was evaluated by HE staining. The black arrows indicate the cell-cell junctions. Scale bars: 50 μm. The ultrastructure of the liver tissues in Itga5-sgRNA and control mice was evaluated using TEM. Mi, mitochondria; TJ, tight junctions. The yellow arrows indicate tight junctions. Scale bars: 1 μm.
(D) The distance of sinusoidal space was analyzed using ImageJ software.
Figure 2. Continued
(E) Protein levels of Zo-1 in Itga5-sgRNA and control mice liver tissue were determined by immunofluorescence staining. Scale bars: 25 μm.
(F) Relative mRNA levels of Zo-1 in Itga5-sgRNA and control mice liver tissue were determined by quantitative RT-PCR. Statistical significance from three independent experiments compared with the control groups was determined by unpaired two-tailed Student’s t test. The data are presented as the mean ± SD. ITGA5 or Itga5, α5 integrin; HE, haematoxylin-eosin; TEM, transmission electron microscopy; hASC-HLCs, human adipose stem cell-derived hepatocyte-like cells. Itga5-sgRNA, hepatocyte-specific deletion of α5 integrin, Cas9, control. See also Figures S2–S5.

Tight junctions are impaired after selective silencing of α5 integrin in hepatocytes
To investigate the effect of α5 integrin on hepatic TJs, the expression of α5 integrin was knocked down in human hepatocytes using α5 integrin siRNA (Figure S2), and the properties of hepatic TJs were determined. Immunofluorescence staining showed that the localization of ZO-1 along individual cell-cell borders was significantly decreased and that F-actin, which was colocalized with ZO-1, was organized in parallel stress fibers in α5 integrin siRNA-treated cells, unlike in control siRNA-treated cells (Figures 2A, S3A, and S3C). Quantitative RT-PCR showed that the mRNA level of ZO-1 in α5 integrin siRNA-treated human hepatocytes compared to control siRNA-treated hepatocytes (Figures 2B, S3B, and S3D).

To further explore the effect of α5 integrin on hepatic TJs in vivo, liver tissues from the hepatocyte-specific deletion of α5 integrin mice and control mice generated using adeno-associated virus 8 (AAV8) to deliver the CRISPR-Cas9 system containing the liver-specific promotor-1 (LP1) promotor were detected. The results showed that the mRNA and protein levels of Itga5 were successfully reduced by 50%–60% in liver tissue in hepatocyte-specific deletion of α5 integrin (Itga5-sgRNA) mice compared to control (cas9) mice (Figure S4). Immunohistochemical staining confirmed that the expression of α5 integrin in hepatocytes was significantly decreased in Itga5-sgRNA mice compared to control mice (Figure 2C, left panel).

To explore the effect of α5 integrin on hepatic matrix deposition, collagen levels in liver tissue in Itga5-sgRNA and control mice were determined by Sirius red staining. The results showed that there were no differences in the levels of collagen in liver tissue between Itga5-sgRNA mice and control mice (Figure S5). In addition, there were no differences in body weight or liver weights between Itga5-sgRNA mice and control mice. However, a disorganized liver architecture with increased sinusoidal space was found in liver tissue in Itga5-sgRNA mice (Figure 2C, middle panel and Figure 2D). TEM analysis revealed that the ultrastructure of TJs appeared dilated, was lost, and contained less electron-dense material in Itga5-sgRNA mice (Figure 2C, right panel). Immunofluorescence staining showed that the localization of ZO-1 along individual cell-cell borders was significantly decreased in Itga5-sgRNA mice, in contrast to control mice (Figure 2E). Quantitative RT-PCR showed that the mRNA level of ZO-1 was significantly decreased in Itga5-sgRNA mice compared with control mice:

Claudin 1, Nedd4l, and Tuba1a were confirmed by quantitative RT-PCR (Figure 3F).

The tight junction signaling pathway is downregulated after selective silencing of α5 integrin
To gain an initial perspective on global gene expression changes, RNA sequencing was performed to compare gene expression in the liver tissue of Itga5-sgRNA and control mice. The results revealed 88 significantly differentially expressed genes between the two groups. Sixty-two genes were significantly downregulated, and 26 genes were significantly upregulated in Itga5-sgRNA mice (Figure 3A). Functional annotation analysis of the downregulated genes in Itga5-sgRNA mice led to the identification of functional groups, such as “aldosterone-regulated sodium reabsorption (MMU04960),” “FoxO signaling pathway (MMU04371),” “mitophagy animal (MMU04137),” and “TJ signaling pathway (MMU04530)” (Figure 3B). In addition, gene set enrichment analysis (GSEA) revealed that among the pathways, the TJ signaling pathway was significantly downregulated in Itga5-sgRNA mice (Figure 3C). Genes associated with “apical junctions (GO0043296),” “adherent junctions (GO0005912),” “gap junctions (MMU04540),” and “ECM receptor interaction signaling (MMU04512)” were downregulated in Itga5-sgRNA mice (Figure S6).

Genes involved in the TJ signaling pathway are listed in Figure 3D. Among these genes, the following were significantly downregulated in Itga5-sgRNA mice compared with control mice: Claudin 1; neural precursor cell expressed, developmentally downregulated gene 4-like (Nedd4l); and tubulin, alpha 1A (Tuba1a) (Figure 3E). To validate the accuracy of the gene indices calculated from RNA sequencing, the mRNA levels of claudin 1, Nedd4l, and Tuba1a were confirmed by quantitative RT-PCR (Figure 3F).
**Figure 3. Selective silencing of α5 integrin decreases tight junction signaling**

(A) A heatmap showing the common genes in liver tissue from Itga5-sgRNA and control mice based on different gene subsets was generated using MeV v4.8 (http://www.tm4.org/mev/). In the heatmap, high expression is indicated in red, and low expression is indicated in green. The columns show the log10-transformed values of the normalized intensity of triplicate samples in each group.

(B) KEGG pathway analysis revealed the significantly downregulated pathways in liver tissue in Itga5-sgRNA mice.

(C) GSEA revealed that genes enriched in the tight junction pathway were downregulated in liver tissue in Itga5-sgRNA mice.

(D) GSEA revealed the core enrichment genes in the tight junction pathway in Itga5-sgRNA and control mice liver tissue.

(E) The relative FPKM values of claudin 1, Nedd4l, and Tuba1a in Itga5-sgRNA and control mice liver tissues were determined by RNA sequencing.

(F) Relative mRNA levels of claudin 1, Nedd4l, and Tuba1a in Itga5-sgRNA and control mice liver tissues were determined by quantitative RT-PCR. Statistical significance from three independent experiments compared with the control mice group was determined by unpaired two-tailed Student’s t test. The data are presented as the mean ± SD. DE: Differentially Expressed; ITGA5 or Itga5, α5 integrin; Cldn1, claudin 1; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, gene set enrichment analysis; NES, normalized enrichment score; FDR, Benjamini-Hochberg False Discovery Rate; FPKM, fragments per kilobase million. Itga5-sgRNA, hepatocyte-specific deletion of α5 integrin, Cas9, control. See also Figure S6.
In human hepatocytes, the mRNA level of claudin 1 was significantly decreased in α5 integrin siRNA-treated cells compared with control siRNA-treated cells (Figures S7A, S7C, and S7E).

**The claudin 1 protein level is decreased after selective silencing of α5 integrin in hepatocytes**

Considering that claudin 1 plays a key role in the maintenance of hepatic TJs, the protein level of claudin 1 in liver tissue was confirmed in Itga5-sgRNA and control mice. The results indicated that the claudin 1 protein level in liver tissue in Itga5-sgRNA mice was significantly decreased compared with that in control mice (Figure 4A). Immunofluorescence staining confirmed that the expression of claudin 1 localized at the borders of hepatocytes was significantly decreased in Itga5-sgRNA mice (Figure 4B).

Western blotting analysis confirmed that the claudin 1 protein level was also significantly decreased in α5 integrin siRNA-treated human hepatocytes compared with control siRNA-treated cells (Figures 4C and 4D). The localization of claudin 1 along individual cell-cell borders in α5 integrin siRNA-treated human hepatocytes was significantly decreased compared with that in control siRNA-treated cells (Figures S7B, S7D, and S7F). More importantly, the protein level of claudin 1 in hepatocytes in patients with cholestatic cirrhosis was significantly decreased in the area of matrix deposition (Figure 4E). Together, these results indicate that α5 integrin plays a key role in the maintenance of hepatic TJs by regulating the expression of claudin 1 in hepatocytes.

**α5 integrin regulates the expression of claudin 1 in an SRC signaling-dependent manner**

To determine the functional downstream signaling molecules, RNA sequencing data were further investigated. The results showed that the expression of Src in the “tight junction signaling pathway” in the liver tissue was decreased in Itga5-sgRNA mice compared with control mice (Figure 3D). Furthermore, quantitative RT-PCR and western blotting analysis confirmed that the Src mRNA and protein levels in the liver tissue were significantly decreased in Itga5-sgRNA mice compared with control mice (Figure 5B). Additionally, the protein levels of SRC and phospho-Src (Tyr416) were significantly decreased in α5 integrin siRNA-treated human hepatocytes (Figures 5A, 5B, and S9).

SRC has been reported to physically bind to claudin 1 and form a multiprotein complex with ZO-1 that serves as a component of TJs to regulate paracellular permeability. To explore the potential effects of SRC on hepatic TJs and claudin 1 expression, SRC expression was knocked down by delivering siRNA to human hepatocytes (Figure S10). Immunofluorescence staining verified that in SRC siRNA-treated cells, the localization of ZO-1 along individual cell-cell borders was significantly decreased, and F-actin, which was colocalized with ZO-1, was organized in parallel stress fibers (Figures 5C, S11A, and S11C). Quantitative RT-PCR showed that the mRNA level of ZO-1 was also significantly decreased in SRC siRNA-treated cells compared with control siRNA-treated cells (Figures 5D, S11B, and S11D). Notably, the mRNA and protein levels of claudin 1 were significantly decreased in SRC siRNA-treated cells compared with control siRNA-treated cells (Figures 5E, 5F, 5G, and S12). These data reveal that SRC may act as a downstream signaling molecule of α5 integrin, regulating the expression of claudin 1 and hepatic TJs.

**α5 integrin-SRC signaling regulates DNA hydroxymethylation in hepatocytes**

Considering the regulatory effect of integrin on DNA hydroxymethylation, the 5hmC and 5mC levels in hepatocytes were investigated. Immunofluorescence staining showed that compared with those in control siRNA-treated cells, the intensity of 5hmC was significantly decreased in α5 integrin siRNA-treated human hepatocytes (Figures 6A (middle panel), S13A, and S13C), while the intensity of 5mC was significantly increased in α5 integrin siRNA-treated human hepatocytes (Figures 6B (middle panel), S13B, and S13D). Furthermore, the intensity of 5hmC was significantly lower (Figures 6A (right panel), S13A, and S13C) and the intensity of 5mC was much higher in SRC siRNA-treated human hepatocytes than in control siRNA-treated cells (Figures 6B (right panel), S13A, and S13C). Consistent with the in vitro data, dot blot analyses showed that the density of 5hmC was significantly decreased in liver tissue in Itga5-sgRNA mice (Figure 6C), while the density of 5mC was increased in Itga5-sgRNA livers compared to the corresponding controls (Figure 4D). Moreover, immunofluorescence staining demonstrated that the intensity of 5hmC was significantly decreased in hepatocytes in Itga5-sgRNA mice (Figure 6E), while the intensity of 5mC in Itga5-sgRNA hepatocytes was increased compared to the corresponding controls (Figure 6F). The level of 5hmC in hepatocytes in patients with cholestatic cirrhosis was also significantly decreased in the area of matrix deposition (Figure 6G). Collectively, these results suggest that the α5 integrin-SRC signaling axis induces the oxidation of 5mC to 5hmC on DNA in hepatocytes.
integrin-SRC signaling induces an increase in TET1-mediated 5hmC levels in hepatocytes.

Given the critical roles of TET enzymes in facilitating demethylation by oxidizing the methyl group of 5mC to yield 5hmC, we assessed whether the regulation of 5hmC DNA levels by α5 integrin-SRC signaling depends on the catalytic action of TET dioxygenases. The results showed that the mRNA and protein levels of TET1 were significantly decreased in α5 integrin siRNA-treated human hepatocytes compared with control siRNA-treated hepatocytes.

**Figure 4. Selective silencing of α5 integrin decreases claudin 1 expression**

(A) Protein levels of claudin 1 in Itga5-sgRNA and control mice liver tissue were determined by western blotting. The relative density of each protein band was analyzed using ImageJ software.

(B) Protein levels of claudin 1 in Itga5-sgRNA and control mice liver tissue were determined by immunofluorescence staining. Scale bars: 10 μm. The white arrow indicates the location of claudin 1. The relative fluorescence intensity of claudin 1 was determined using ImageJ software.

(C) Protein levels of claudin 1 in ITGA5 siRNA- and control siRNA-treated hASC-HLCs were determined by western blotting. The relative density of each protein band was analyzed using ImageJ software.

(D) Protein levels of claudin 1 in ITGA5 siRNA- and control siRNA-treated hFHPC-HLCs were determined by western blotting. The relative density of each protein band was analyzed using ImageJ software.

(E) The ECM deposition in liver tissues in three different patients with cholestatic cirrhosis or control was determined by Masson trichrome staining. The black arrows indicate the ECM in the space of Disse (upper panel). The protein levels of claudin 1 in hepatocytes in controls and patients with cholestatic cirrhosis were determined by immunohistochemistry. The black arrows indicate the expression of claudin 1 (lower panel). Scale bars: 50 μm. The relative density of claudin 1 was analyzed using ImageJ software. Statistical significance from three independent experiments compared with the control group was determined by unpaired two-tailed Student’s t test. The data are presented as the mean ± SD. ITGA5 or Itga5, α5 integrin; CLDN1 or Cldn1, claudin 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HASC-HLCs, human adipose stem cell-derived hepatocyte-like cells; hFHPC-HLCs, human fetal hepatic progenitor cell-derived hepatocyte-like cells. Itga5-sgRNA, hepatocyte-specific deletion of α5 integrin, Cas9, control. See also Figure S7.

**α5 integrin-SRC signaling induces an increase in TET1-mediated 5hmC levels in hepatocytes**

Given the critical roles of TET enzymes in facilitating demethylation by oxidizing the methyl group of 5mC to yield 5hmC, we assessed whether the regulation of 5hmC DNA levels by α5 integrin-SRC signaling depends on the catalytic action of TET dioxygenases. The results showed that the mRNA and protein levels of TET1 were significantly decreased in α5 integrin siRNA-treated human hepatocytes compared with control siRNA-treated hepatocytes.
cells (Figures 7A, 7B, and S14). In addition, the mRNA and protein levels of TET1 were significantly decreased in SRC siRNA-treated human hepatocytes compared with control siRNA-treated cells (Figures 7C, 7D, and S14).

Furthermore, the mRNA levels of Tet1, Tet2, and Tet3 in liver tissues were significantly decreased in Itga5-sgRNA mice compared to control mice (Figure 7E). The protein level of Tet1 was also significantly decreased in Itga5-sgRNA mice (Figure 7F). These data indicate that α5 integrin-SRC signaling induces an increase in TET1-mediated dioxygenase activity to oxidize 5mC to 5hmC on DNA in hepatocytes.

Figure 5. Selective silencing of SRC downregulates the expression of claudin 1 and hepatic TJ proteins
(A and B) Protein levels of SRC (A) and phospho-Src 174 (Tyr416) (B) in ITGA5 siRNA and control siRNA-treated hASC-HLCs were determined by western blotting. The relative density of each protein band was analyzed using ImageJ software.
(C) Protein levels of ZO-1 and F-actin in SRC siRNA- and control siRNA-treated hASC-HLCs were determined by immunofluorescence staining. Scale bars: 25 μm.
(D) Relative mRNA levels of ZO-1 in SRC siRNA- and control siRNA-treated hASC-HLCs were determined by quantitative RT-PCR.
(E) Relative mRNA levels of claudin 1 in SRC siRNA- and control siRNA-treated hASC-HLCs were determined by quantitative RT-PCR.
(F) Protein levels of claudin 1 in SRC siRNA- and control siRNA-treated hASC-HLCs were determined by western blotting. The relative density of each protein band was analyzed using ImageJ software.
(G) Protein levels of CLDN1 in SRC siRNA- and control siRNA-treated hASC-HLCs were determined by immunofluorescence staining. Scale bars: 25 μm. Statistical significance from three independent experiments compared with the control siRNA-treated hASC-HLCs were determined by unpaired two-tailed Student’s t test. The data are presented as the mean ± SD. CLDN 1, claudin 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hASC-HLCs, human adipose stem cell-derived hepatocyte-like cells. See also Figures S8–S12.
Figure 6. The α5 integrin-SRC signaling axis regulates the 5hmC DNA level in hepatocytes

(A and B) 5hmC (A) and 5mC (B) DNA levels in ITGA5 siRNA-, SRC siRNA- and control siRNA-treated hASC-HLCs were determined by immunofluorescence staining. Scale bars: 25 μm. The relative fluorescence intensity of 5hmC and 5mC normalized to DAPI in each cell was determined using ImageJ software. n = 100 cells.

(C and D) The 5hmC (C) and 5mC (D) DNA levels in Itga5-sgRNA and control mice livers were determined by dot blotting. The relative density of 5hmC and 5mC was analyzed using ImageJ software.

(E and F) The 5hmC (E) and 5mC (F) levels in Itga5-sgRNA and control mice livers were determined by immunofluorescence staining. Scale bars: 25 μm. The relative fluorescence intensity of 5hmC and 5mC normalized to DAPI in liver sections was analyzed using ImageJ software. n = 20 images.

(G) ECM deposition in liver tissues in three different patients with cholestatic cirrhosis and control individual was evaluated by Masson trichrome staining. The black arrows indicate ECM in the space of Disse (upper panel). The levels of 5hmC in hepatocytes in three different patients with cholestatic cirrhosis and control individual was determined by immunohistochemistry. The black arrows indicate the expression of 5hmC (lower panel). Scale bars: 50 μm. Statistical significance from three independent experiments compared with the control group was determined by unpaired
**α5 integrin regulates the hydroxymethylation levels of genes in hepatocytes**

In an attempt to explore whether gene hydroxymethylation in liver tissue is regulated by α5 integrin, hMeDIP sequencing was performed on mouse liver tissues obtained from Itga5-sgRNA and control mice. The results showed that the differentially hydroxymethylated regions were associated with promoters, gene bodies, intergenic regions, enhancers and super-enhancers. Among these regions, 45 differentially hydroxymethylated promoter regions in mRNAs were found, including 18 mRNAs associated with hyperhydroxymethylation and 27 mRNAs associated with hypohydroxymethylation in liver tissue in Itga5-sgRNA mice (Table S4).

The GO analysis showed that hypohydroxymethylated genes were enriched in the “Biological Process” terms “Protein complex oligomerization (GO:0051259),” “Negative regulation of protein complex disassembly (GO:0043242),” “Regulation of protein complex disassembly (GO:0043242),” “Cellular component organization (GO:0022607)” in Itga5-sgRNA mice (Figure 8A). Genes involved in the “cellular component assembly” process included membrane protein, palmitoylated 7 (Mpp7); K+ voltage-gated channel subfamily S 2 (Kcns2); spectrin beta non-erythrocytic 1 (Sptbn1); phosphatidylinositol 3-kinase catalytic subunit type 3 (Pik3c3); lymphocyte antigen 6 complex locus G6C (Ly6g6c); AMP-activated beta 1 noncatalytic subunit (Prkab1); and lipocalin 2 (Lcn2) (Table S4). In addition, Sptbn1 and Katnb1 were also involved in “negative regulation of cytoskeleton organization (GO:0051494).”

hMeDIP-PCR analysis confirmed hypohydroxymethylation in the promoter of the Lcn2 gene in Itga5-sgRNA mice (Figure S15A), consistent with the decreased level of Lcn2 mRNA in liver tissue in Itga5-sgRNA mice (Figure S15B). Sequences in the hydroxymethylated regions of the Lcn2 gene were processed and analyzed by visual mapping, along with the corresponding gene promoter and CpG islands. The normalized number of reads covering each nucleotide position is shown in different tracks for liver tissue in Itga5-sgRNA mice and control mice (Figure S15C). The results showed that CpG islands in Lcn2 exhibited decreased hydroxymethylation in Itga5-sgRNA mice compared with the control mice.

Although there were no significant changes in the promoter in the Claudin 1 gene (Figures 8B and 8C and Table S5), the hydroxymethylation of the cldn1 intergenic regions located at chr16:26279061-26279820 and chr16:26325841-26326040 was decreased in liver tissue in Itga5-sgRNA mice (Figure 8D, Table S6). These results indicate that α5 integrin regulates the DNA hydroxymethylation level of genes in hepatocytes.

Overall, our findings indicate that ECM deposition around hepatocytes in patients with liver cirrhosis may downregulate the expression of α5 integrin, which reduces the level of TJ protein claudin 1 via DNA hydroxymethylation in an SRC-dependent manner.

**DISCUSSION**

Although an increasing amount of in vitro evidence indicates that the ECM plays an important role in determining the structural and functional phenotype of hepatocytes,10,26 there is little evidence that these findings apply in vivo. Here, we describe a critical role of α5 integrin in regulating hepatic tight junctions through SRC-TET1-mediated DNA hydroxymethylation.

Via their action as transmembrane matrix heterodimeric receptors, integrins have long been recognized to provide the main molecular link attaching cells to the ECM and to serve as bidirectional hubs transmitting signals between cells and their environment.13 Recent evidence has shown that the cytoplasmic tail of the β1 integrin subunit plays a key role in regulating the composition and function of the tight and adherens junctions that define the paracellular transport properties of terminally differentiated renal proximal tubule epithelial cells.27 In this study, our findings provide evidence that silencing α5 integrin expression in hepatocytes results in their conversion from a tight to a “loose” structure, characterized by an increase in the sinusoidal space and low expression of the TJ-associated proteins claudin 1 and ZO-1. Moreover, knocking down α5 integrin downregulated hepatic polarity-associated signaling pathways, including TJ signaling, apical junctions, adherens junctions, and gap junctions. Tight junction proteins expressed on hepatocytes play an important functional role as paracellular gatekeepers, enabling the production and secretion of bile.
as well as metabolic exchange and detoxification. Claudin 1 is the most abundantly expressed TJ protein in the liver, and plays crucial roles in TJ function, cell growth and differentiation. Our data indicate a critical role for \( \alpha_5 \) integrin in maintaining liver structural homeostasis. Previously, we established that inhibiting the phosphorylation and expression of SRC dramatically decreases the effect of \( \alpha_5 \) integrin in hepatocyte maturation in vitro. Herein, the results showed that the α5 Integrin-SRC signaling induced increases in TET1-mediated 5hmC modification in hepatocytes.

**Figure 7.** \( \alpha_5 \) Integrin-SRC signaling induced increases in TET1-mediated 5hmC modification in hepatocytes
(A) Relative mRNA levels of TET1, TET2, and TET3 in ITGA5 siRNA- and control siRNA-treated hASC-HLCs were determined by quantitative RT-PCR. The relative density of each protein band was analyzed using ImageJ software.
(B) Relative mRNA levels of TET1 in ITGA5 siRNA- and control siRNA-treated hASC-HLCs were determined by western blotting. The relative density of each protein band was analyzed using ImageJ software.
(C) Relative mRNA levels of TET1, TET2, and TET3 in the SRC siRNA- and control siRNA-treated hASC-HLCs were determined by quantitative RT-PCR. The relative density of each protein band was analyzed using ImageJ software.
(D) Protein levels of TET1 in SRC siRNA- and control siRNA-treated hASC-HLCs were determined by western blotting. The relative density of each protein band was analyzed using ImageJ software.
(E) Relative mRNA levels of Tet1, Tet2, and Tet3 in Itga5-sgRNA and control mice livers were determined by quantitative RT-PCR.
(F) Protein levels of Tet1 in Itga5-sgRNA and control mice livers were determined by immunohistochemical staining. Scale bars: 50 μm. The relative density of Tet1 in each image was analyzed using ImageJ software. Statistical significance from three independent experiments compared with the control group was determined by unpaired two-tailed Student’s t test. The data are presented as the mean ± SD. N.S., not significant. ITGA5 or Itga5, \( \alpha_5 \) integrin; hASC-HLCs, human adipose stem cell-derived hepatocyte-like cells; TET, ten-eleven translocation. Itga5-sgRNA, hepatocyte-specific deletion of \( \alpha_5 \) integrin, Cas9, control. See also Figure S14.
mRNA and protein levels of Src in the liver tissue were decreased in Itga5-sgRNA mice compared with control mice. Furthermore, we confirmed that knocking down SRC impaired the localization of ZO-1 and F-actin in human hepatocytes, and decreased the expression of claudin 1. Studies have shown that SRC is a key molecule involved in the regulation of cell polarity. Therefore, we suggest that α5 integrin maintains hepatic TJs by regulating claudin 1 expression through the SRC signaling pathway.

α6 integrin signaling has been reported to induce STAT3-TET3-mediated hydroxymethylation of genes critical for maintaining glioma stem cells. However, it remains unknown whether α5 integrin signaling regulates the DNA hydroxymethylation level to maintain hepatic TJs. Notably, ShmC, a DNA demethylation intermediate and a marker of DNA hydroxymethylation, is frequently associated with transcriptional activation, and its formation is catalyzed by TET1-3 dioxygenases, which are α-ketoglutarate- and Fe2+-dependent dioxygenases (α-KGDDEs). Given the essential functions of TET proteins and ShmC epigenetic

Figure 8. α5 Integrin regulates the hydroxymethylation of genes critical for hepatic TJs
(A) The top 10 GO biological processes enriched with hypohydroxymethylation in Itga5-sgRNA mice livers.
(B) Visualization of Claudin 1 hydroxymethylation. The normalized number of reads covering each nucleotide position is shown in different tracks for Itga5-sgRNA and control mice liver tissue analyzed by hMeDIP, with three biological replicates each. The RefSeq gene models are shown in blue below the tracks, with arrowed lines indicating introns and the direction of the gene on the chromosome, narrow boxes representing UTRs, and wide boxes indicating exons. The tracks below the gene model track show the annotations of the promoter region and the CpG islands as identified using the UCSC Genome Browser database.
(C) The hydroxymethylation level of the Claudin 1 promoter in Itga5-sgRNA and control mice liver tissue was determined by hMeDIP-PCR.
(D) The two locations of Claudin 1 mRNA-associated hydroxymethylated intergenic regions were determined by hMeDIP-seq. chr16: start-end position of Claudin 1 on chr16; loc: start-end, the start-to-end location of Claudin 1 on chr16; type: Down, downregulated in Itga5-sgRNA vs control mice, log2FC: fold change in the normalized tag count between the two groups (log2 transformed); p value: p value of the DhMR, (a lower value indicates higher significance). Statistical significance from three independent experiments compared with the control mice group was determined by unpaired two-tailed Student’s t test. The data are presented as the mean ± SD. N.S., not significant. Itga5, α5 integrin; hMeDIP, hydroxymethylated DNA immunoprecipitation; Cldn 1, claudin 1; GO, gene ontology; FPKM, fragments per kilobase million. Itga5-sgRNA, hepatocyte-specific deletion of α5 integrin, Cas9, control. See also Figure S15, Tables S4, S5 and S6.
marks, numerous studies have shown their importance in gene regulation in the liver. DNA methylation and hydroxymethylation are known modifications affecting the expression and function of polarity-related genes. Our results showed that knocking down α5 integrin or SRC expression downregulated TET1 protein expression, decreased the 5hmC level, and increased the 5mC level in human hepatocytes. This finding confirmed that α5 integrin-SRC signaling regulates the expression of TET1 dioxygenase to oxidize 5hmC to 5mC on DNA in hepatocytes.

To identify the gene whose hydroxymethylation in liver tissue is regulated by α5 integrin, hMeDIP sequencing was performed on mouse liver tissues obtained from Itgα5-sgRNA and control mice. Promoter hypohydroxymethylation was found in genes such as Lcn2, Kcns2, Ly6g6c, Mpp7, Pik3c3, Prkab1, Sptbn1, and Katnb1, which are critical genes for maintaining cell polarity. Interestingly, knocking down α5 integrin significantly decreased both the mRNA and hydroxymethylation levels of Lcn2. Lcn2 has been described as an acute-phase protein in the liver and is currently considered a potential effective biomarker of pathogenic changes occurring during liver disease, including hepatic damage, steatosis, inflammation, and fibrosis. Therefore, whether the combined reduction in Lcn2 mRNA expression and hydroxymethylation upon α5 integrin depletion leads to liver diseases needs to be further explored.

Although claudin 1 expression was found to be epigenetically regulated by DNA methylation, no alteration in the hydroxymethylation level in Claudin 1 promoters was found, possibly due to the inconsistency of DNA hydroxymethylation levels between individual mice. Interestingly, two loci in the intergenic regions of the Claudin 1 gene were hypohydroxymethylated in Itgα5-sgRNA mice compared with control mice. Whether the intergenic epigenetic changes regulated by signaling pathways result in changes in gene transcription requires further investigation in the future. These results indicate that the roles of α5 integrin-SRC signaling in regulating hepatocyte TJs are at least partially attributable to DNA ShmC and hypohydroxymethylation of Claudin 1 or other polarity-related genes.

Finally, we showed that the lower expression of α5 integrin in hepatocytes in patients with liver cirrhosis is associated with matrix deposition in the space of Disse. More importantly, the lower expression of α5 integrin in hepatocytes was accompanied by lower protein levels of claudin 1 and DNA ShmC level in hepatocytes in patients with liver cirrhosis. Therefore, we believe that the decrease in α5 integrin, claudin 1, and ShmC levels in hepatocytes may be caused by the deposition of ECM.

In summary, our study provides new insights into the regulation of claudin 1 expression and hepatic TJ maintenance by α5 integrin-SRC signaling through associated epigenetic modifications, which offers therapeutic promise for liver diseases caused by abnormal expression of claudin 1 and the destruction of hepatic TJs.

Limitations of the study

The hepatic polarity in patients with cirrhosis is disturbed. In this study, we found that the expression of α5 integrin and claudin 1 was reduced in hepatocytes with excessive extracellular matrix deposition in patients with cirrhosis, but the molecular mechanisms and downstream signaling pathways are not fully understood. In future studies, we hope to discover the molecular pathways by which the extracellular matrix-integrin signaling regulates the hepatic tight junctions in patients with cirrhosis, and thus uncover targets for the treatment of liver cirrhosis.

STAR METHODS

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QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105611.

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AUTHOR CONTRIBUTIONS
YM was responsible for the conception and design, execution of the experiments, collection and assembly of data, and data analysis and writing the article. WZ was responsible for collecting the information on patients and data analysis. WL was responsible for administrative support, and collection and assembly of data. XL was responsible for immunohistochemistry staining and technical support. YL was responsible for cell protein extraction. XH was responsible for siRNA transfection. PW was responsible for collecting the information of patients and data analysis. HZ was responsible for conception and design, data analysis and interpretation, writing the article, funding acquisition, and final approval of the article. All authors read and approved the final article.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit anti-Claudin 1 | Thermo Fisher Scientific | Cat#71-7800; RRID: AB_2533997 |
| Rabbit anti-Integrin α5 | Abcam | Cat#ab150361; RRID: AB_2631309 |
| Rabbit anti-ZO-1     | Thermo Fisher Scientific | Cat#ab214728; RRID: AB_2797407 |
| Rabbit anti-S-hmC    | Abcam | Cat#ab214727; RRID: AB_2802117 |
| Rabbit anti-5-mC     | Abcam | Cat#ab214727; RRID: AB_2802117 |
| Rabbit anti-Src      | Cell Signaling Technology | Cat#2108; RRID: AB_331137 |
| Rabbit anti-phospho-Src (Tyr416) | Cell Signaling Technology | Cat#2101; RRID: AB_331697 |
| Rabbit anti-TET1     | Abcam | Cat#ab191698; RRID: AB_2858250 |
| Rabbit anti-GAPDH    | Cell Signaling Technology | Cat#5174; RRID: AB_10622025 |
| Alexa Fluor® 488 Conjugate (Goat Anti-Rabbit IgG) | Cell Signaling Technology | Cat#4412S |
| IRDye 800CW Goat anti-rabbit (H + L) | Abcam | Cat#ab216773 |
| **Biological samples** |        |            |
| human adipose stem cells | Li et al., 2014 | N/A |
| human adipose tissues | Li et al., 2014 | N/A |
| human umbilical vein endothelial cells | Guo et al., 2017 | N/A |
| human fetal hepatic progenitor cells | Zhang et al., 2014 | N/A |
| human liver tissues | this paper | N/A |
| fetal bovine serum | Invitrogen | Cat#10100147 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Gelatin | Sigma-Aldrich | Cat#G-2500 |
| Glutaraldehyde | Sigma-Aldrich | Cat#G7651 |
| Ethanolamine | Sigma-Aldrich | Cat#411000 |
| Triton X-100 | Sigma-Aldrich | Cat#V900502 |
| Ammonium hydroxide | Modern Oriental Fine Chemistry | Cat#82503 |
| Albumin fraction V | Sigma-Aldrich | Cat#9048-46-5 |
| Insulin-transferrin-selenium | Thermo Fisher Scientific | Cat#41400 |
| Hepatocyte growth factor | Peprotech | Cat#100-39 |
| Oncostatin M | Peprotech | Cat#300-10 |
| Dexamethasone | Sigma-Aldrich | Cat#D8893 |
| Paraformaldehyde | Solarbio | Cat#P1110 |
| **Critical commercial assays** |        |            |
| RNeasy Mini Kit | Qiagen | Cat#74106 |
| High-Capacity RNA-to-cDNA™ Kit | Applied Biosystems | Cat#4387406 |
| QIAamp DNA Mini Kit | Qiagen | Cat#34361 |
| PowerUp™ SYBR® Green Master Mix | Applied Biosystems | Cat#A25742 |
| BCA protein assay | Thermo Fisher Scientific | Cat#23227 |
| **Deposited data** |        |            |
| RNA sequencing | this paper | GSE159053 |
| hMeDIP sequencing | this paper | GSE162409 |
| Original images of western blot | this paper | https://doi.org/10.17632/vyk2c7dz8f1 |
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Experimental Models: Cell Lines** | | |
| L02 | Pituo Biological Technology, Shanghai, China | Cat#PT-H1315 |
| **Experimental models: Organisms/strains** | | |
| Itga5-sgRNA or control mice | this paper | N/A |
| **Oligonucleotides** | | |
| Itga5 sgRNA F: caccgAAGTCCCCGTCGAC GTCTGGGCG; R: aaacCGCCCAGACG TCGACGGGGACTTc | this paper | N/A |
| **Software and algorithms** | | |
| GraphPad Prism | GraphPad Software | Version 7.0 |
| ImageJ | NIH | https://imagej.nih.gov/ij/ |
| Primer Premier 5 | Premier Biosoft | http://www.premierbiosoft.com/primersdesign/ |
| **Other** | | |
| Dharma FECT 4 | Dharmacon | Cat#T-2004-02 |
| DyLight™ 594 Phalloidin | Cell Signaling Technology | Cat#12877 |
| Haematoxylin-eosin stain kit | Solarbio | Cat#G1120 |
| Masson trichrome stain kit | Solarbio | Cat#G1340 |
| Sirius Red staining buffer | Solarbio | Cat#G1472 |
| DAB Kit | ZSGB-BIO | Cat#PV-8000 |
| DAPI | Cell Signaling Technology | Cat#D9564 |
| SDS-PAGE | Thermo Fisher Scientific | Cat#NP0301BOX |
| PVDF membrane | Merck Millipore | Cat#IPVH000010 |
| TBST | Solarbio | Cat#T1081 |
| DMEM/F12 | Invitrogen | Cat#12400-024 |
| William’s E Medium | Invitrogen | Cat#A12176-01 |
| RIPA tissue/cell Extraction Reagent | Solarbio | Cat#R0010 |

### RESOURCE AVAILABILITY

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Haiyan Zhang (culture@ccmu.edu.cn).

#### Materials availability

This study did not generate new unique reagents.

#### Date and code availability

- The RNA sequencing data and hMeDIP sequencing data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. Original western blot images have been deposited at Mendeley and are publicly available as of the date of publication. The DOI is listed in the key resources table.
This study did not generate original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human liver tissues, cell lines and animals

Human liver samples were obtained with informed patient consent and under the approval of the Ethics Committee of Capital Medical University (Beijing, China). Age and gender of human patients are provided in the Table S1. hASC-HLCs, hFHPC-HLCs and HUVECs were established by our laboratory; L02 cell lines were purchased from Pituo Biological Technology, Shanghai, China. BALB/c mice (male; 18–20 g; 4- to 6-week-old, specific pathogen free) were obtained and housed in the Laboratory Animal Center of Capital Medical University. All animal studies were conducted in accordance with the Animal Care and the Ethics Committee of Capital Medical University.

METHOD DETAILS

Human samples

Human liver samples including health or cholestatic cirrhosis were obtained from individuals who had undergone liver surgery (Table S1). Human liver tissues were obtained with informed patient consent and under the approval of the Ethics Committee of Capital Medical University (Beijing, China) for this project. All procedures involving human tissues and cells collection were reviewed and approved by the ethics committee of Capital Medical University and adhered to the principles of the Declaration of Helsinki.

Cell culture and differentiation

Hepatocyte-like cells (HLCs) derived from human fetal hepatic progenitor cells (hFHPCs) or human adipose stem cells (hASCs) as previously described. The hepatocyte cell lines L02 cells were purchased from Shanghai Pituo Biological Technology Co., Ltd (Shanghai, China).

The HLCs were harvested and cultured on endothelial cell-derived matrix at a density of 75,000/cm² with MEM/NEAA (Invitrogen, Grand Island, NY), supplemented with 0.5 mg/mL albumin fraction V (BSA; Sigma-Aldrich, St. Louis, MO, USA), 1% insulin-transferrin-selenium (ITS; Sigma-Aldrich), 20 ng/mL hepatocyte growth factor (HGF; Peprotech, Rocky Hill, NJ, USA), 10 ng/mL oncostatin M (OSM; Peprotech) plus 10⁻⁶ M dexamethasone (DEX; Sigma-Aldrich). L02 cells were cultured on endothelial cell-derived matrix with DMEM/F12 (Invitrogen) supplemented with 10% foetal bovine serum (Invitrogen). After a 24-h incubation, the mediums were changed to William’s E Medium (Invitrogen).

Preparation of endothelial cell-derived matrix

Endothelial cell-derived matrix derived from primary human umbilical vein endothelial cells was prepared as previously described.

Animals

Hepatocyte-specific ablation of α5 integrin in mice (Itga5-sgRNA) were generated using clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system. Briefly, four pairs of single-guide RNA (sgRNA), that target the upstream and downstream coding sequence regions of Itga5 exon 7 were designed for target-based screening using CRISPR design tool (http://crispr.mit.edu) and for high on-target efficiency and low off-targeting score. The sequence of sgRNA using in the study were F: caccgATCCCCGTCGACGTCTGGGCG; R: aaacCGCCCAGACGTAGCCGGGGACTTc was inserted into pUCA (Luc) vector. The Itga5-sgRNA or control mice were obtained respectively by administered target sgRNA-pCas9 or control sgRNA-pCas9 packaged using the adeno-associated virus serotype 8 (AAV8) vector with liver-specific promoter-1 (LP1) promoter at a dose of 1 x 10¹¹ vector genomes (vgs) via tail vein injection per mouse by BRL MEDICINE Company (Shanghai, China). 18–20 g male mice were used in this study. Mice were sacrificed at 4 weeks after injection. The liver tissues were harvested and the properties were determined. All animal work was performed in accordance with the Animal Care and the Ethics Committee of Capital Medical University.
siRNA transfection
HLCs and L02 cells were plated in antibiotic-free basal medium 24 h prior to transfection. The siRNA transfection was performed following the manufacturer’s protocol as previously described. Briefly, ON-TARGET SMARTpool siRNAs directed against human α5 integrin (L-008003-00-0005, Dharmacon, Lafayette, LA, USA), human SRC (L-003175-00-0005, Dharmacon) or non-targeting siRNAs (D-001810-10-05, Dharmacon) were mixed with Transfection DharmaFECT 4 (Dharmacon). After a 20-min incubation at room temperature, the complexes were added to the cells at a final siRNA concentration of 50 nM. The medium was replenished with fresh medium for 24 h post-transfection. Experiments were performed 48–72 h after transfection.

RNA sequencing and data analysis
Total RNA of liver tissues was harvested from Itga5-sgRNA and control mice using the TRIzol (Invitrogen) reagents and quantified by the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Approximately 1-2 μg of total RNA isolated from were processed with a Ribo-Zero Magnetic Gold kit (Epicentre Biotechnologies, USA) to reduce ribosomal RNA abundance. Sequencing libraries were prepared using the KAPA Stranded RNA-Seq Library Prep Kit (Illumina, CA, USA), the library quality was identified by an Agilent 2100 Bioanalyzer. Quantitative PCR was performed to quantify the sequencing libraries, and the mixed different sample libraries were sequenced using an Illumina HiSeq 4000 platform by KangChen Biotech Company (Shanghai, China).

Image processing and base recognition were performed using Solexa pipeline software (version 1.8). The output sequencing reads quality was analysed using FastQC software (version 0.11.5). The RNA sequencing reads were aligned to the mice reference genome mm10 using HISAT2 (version 2.1.0) with default parameters. The normalized expression value, fragments per kilobase of transcript per million mapped reads (FPKM), was calculated for each gene using StringTie (version 1.3.1c). FPKM at the gene level and transcript level were calculated using Ballgown (version 2.8.4). Differentially expressed genes with a fold change ≥1.5, p value ≤0.05 and FPKM ≥0.5 were screened and retained in groups.

Quantitative RT-PCR
Quantitative RT-PCR was performed as previously described. Total cellular RNA was extracted from 2.0×10^5 cells with the RNeasy Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions. For PCR analysis, 1 μg of RNA was reverse-transcribed to cDNA using Superscript III reverse transcriptase and random hexamer primers (Invitrogen). Real-time PCR analysis was performed on a Thermo Fisher Scientific applied Biosystems QuantStudio 5 system (Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Master Mix (Applied Biosystems). The reaction consisted of 10 μL of SYBR Green PCR Master Mix, 1 μL of a 5 μM mix of forward and reverse primers, 8 μL of water, and 1 μL of template cDNA in a total volume of 20 μL. Cycling was performed using QuantStudio™5 Real-Time PCR thermal cycler (Applied Biosystems, USA). The relative expression of each gene was normalized against 18S rRNA. The data are presented as the mean ± s.d. values. The primers used are listed in supplemental materials Table S2.

Immunofluorescence staining
Immunofluorescence analysis was performed as previously described. Briefly, the cells or frozen mouse tissue sections were fixed with 4% paraformaldehyde for 20 min at room temperature, followed by permeabilization with 0.3% Triton X-100 in PBS for 5 min. For immunofluorescence staining of 5hmC and 5mC, the cells or frozen mouse tissue sections were treated with 2 N HCl for 30 min at 37°C to denature the DNA to a single strand. The cells were rinsed and blocked with 10% goat serum (Zsgb-Bio, Beijing, China) for 60 min at room temperature. The cells were then incubated with the primary antibodies, which are listed in Table S3, at 4°C overnight. Following three 5-min washes in PBS with gentle agitation, an Alexa Fluor-conjugated secondary antibody (Invitrogen) (Table S3) at 1:500 was added, and the samples were incubated for 30 min at 37°C. The nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). Images were captured using a Leica TCS SP8 STED confocal laser scanning microscope (objective lenses: HC PL APO 63×/1.40 oil CS; detectors: 1 sensitive Hybrid GaAsP, 2 PMT and a motorized xyz-stage; Lasers: UV Lasers, AR Particle Lasers, White Laser Systems, STED Depletion Lasers; Leica, Wetzlar, Germany). Relative intensities of staining were quantitative assessed using Image J software (National Institutes of Health, Bethesda, MD, USA).
Haematoxylin-eosin, masson and immunohistochemical staining

For histological preparation, the liver tissues were fixed with 10% formalin for 24 h at room temperature, followed by dehydration with graded alcohols, and embedding in paraffin. The embedded tissues were then consecutively sliced into 5 μm thick sections, followed by rehydration and routinely stained with haematoxylin-eosin (HE) or Masson trichrome staining kit (Solarbio, Beijing, China). For quantifying the distance of the sinusoidal space using ImageJ software, open the picture, use the straight line tool to draw the ruler line segment, click Analyze, and Set Scale, enter the length of the known line segment in the Known Distance Box, and enter the unit in the Unit Of Length, and click OK. Use the straight line tool to draw the distance to be measured, click Analyze, and then Measure.

For immunohistochemistry, after rehydrated, the sections were heated to recover antigens, and incubated in 3% H2O2 for 10 min at room temperature. Subsequently, the samples were incubated with primary antibodies, which are listed in Table S3, at 4°C overnight. Following three 5-min washes in PBS, a tagged secondary antibody (Zsgb-Bio, Beijing, China), with goat anti-mouse or goat anti-rabbit was added for 20 min at room temperature. The DAB colour reagent kit (Zsgb-Bio, Beijing, China) was used for colour development, the positive cells showed a brown colour. Then the slices were dehydrated and mounted with neutral balsam. Images were captured under a fluorescence microscope (DM3000, Leica, Germany). The relative density of α5 integrin and claudin 1 in hepatocytes in healthy control and patients with cholestatic cirrhosis was analysed using ImageJ software. First, convert the picture to an 8-bit grayscale picture. Then select Uncalibrate OD to convert 8-bit grayscale to OD values. Set Measurements and click Threshold to adjust the threshold and select the positive signals in hepatocytes, click Analyze, and then Measure.

Transmission electron microscopy analysis

The ultra-structural analysis was performed using transmission electron microscopy (TEM) as previously described. The samples were examined using a HT7700 transmission electron microscope (Hitachi, Tokyo, Japan).

Western blotting

Cells were lysed on ice using RIPA tissue/cell Extraction Reagent (Solarbio). The samples were normalized for protein concentration using the BCA protein assay (Invitrogen). Each sample (15–20 μg) was analysed using 10% SDS-PAGE (Invitrogen) and transferred to a PVDF membrane (Merck Millipore, Darmstadt, Germany). The membranes were blocked in 5% NFDM in 1×TBST, and were incubated overnight at 4°C with the specific primary antibodies (Table S3). Glyceraldehyde-phosphate dehydrogenase (GAPDH) were used as internal reference. The membranes were washed with 1×TBST and incubated with IRDye conjugated secondary antibodies (Table S3) for 1 h at room temperature. The membranes were scanned with the Odyssey detection system (Li-COR, Lincoln, NE, USA). Relative densities of proteins were quantitatively assessed using Image J software. The data are presented as the mean ± s.d. values.

Dot blotting

Total DNA from liver tissues were obtained using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Spot 2 μL denatured DNA sample on NC membrane. Dry the membrane, sandwich the cool-dried membrane in filter paper, and bake at 80°C for 2 h. The membranes were then blocked in 5% non-fat dry milk in 1×TBST, and were incubated overnight at 4°C with the specific primary antibodies (Table S3). The membranes were washed with 1×TBST and incubated with IRDye conjugated secondary antibodies (Table S3) for 1 h at room temperature. The membranes were scanned with the Odyssey detection system (Li-COR). Relative densities of DNAs were quantitatively assessed using Image J. The data are presented as the mean ± s.d.

hMeDIP-PCR

hMeDIP assays combined with qPCR were used to quantitatively assess the demethylation status. Genomic DNA was extracted and randomly sheared to an average length of 0.3-1.0 kb by sonication (Diagenode, Denville, NJ, USA). The DNA fragments were used as the starting material. The DNA was denatured for 10 min at 95°C and immunoprecipitated overnight at 4°C with 8 μg of antibody (Eurogentec, BEL, EUR) against 5-hydroxymethylcytosine. After incubation with 60 mL of mouse anti-IgG magnetic beads (BioLabs S1430S) for 2 h at 4°C, the mixture was washed with 1 mL of cold WB1, WB2, and WB3 buffer. Purified DNA was analyzed by qPCR on QuantStudio™ 5 Real-time PCR System (Applied Biosystems).
Reactions were performed in 9-μL volumes containing 1 μL of the DNA template, 5 μL of 2× Master Mix (Arraystar, MD, USA) and 0.5 μL of each primer (claudin 1: F: 5’ TTATCTCATCTTGGCCTCTTG-3’, R: 5’ TCCACTCTGCTGCTCTG-3’; Lcn2: F: 5’TTCTTCCCCAAAGTAACCTGGA-3’, R: 5’ACTGCAACCTCCTTGTCATCT-3’). The relative methylation changes were determined by measuring the amount of immunoprecipitated DNA after normalization compared with the input DNA (MeDIP/ Input%).

**hMeDIP sequencing**

The hMeDIP sequencing was performed by KangChen Bio-tech, Shanghai, China. Briefly, DNA samples were fragmented to a size range of ~200-1 000 bp with a Diagenode BioRuptor (Diagenode). About 1 μg of fragmented DNA was ligated to Illumina’s genomic adapters with Genomic DNA Sample Kit (FC-102-1002, Illumina), following the manufacturer’s instructions. Around 300–900 bp ligated DNA fragments were further denatured in 95°C into single strand DNA then immunoprecipitated by anti 5hmC antibody (Diagenode). The enriched DNA was amplified by PCR and purified by agarose gel. The completed libraries were quantified by an Agilent 2100 Bioanalyzer. The DNA fragments in well mixed libraries were denatured with 0.1 M NaOH to generate single-stranded DNA molecules for amplification, loaded onto channels of the flow cell at 8 pM concentrations, and amplified in situ using HiSeq3000/4000 PE Cluster Kit (PE-410-1001, Illumina). Sequencing was carried out by running 300 cycles useHiSeq4000 SBS Kit (FC-410-1003, Illumina).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

At least three independent determinations of each parameter were compared among the treatment groups by unpaired two-tailed Student’s t test using Graphpad prism 8 software. Data presented as the mean ± SD. The differences were considered significant if p < 0.05.