

Supplementary Figures

Figure S1

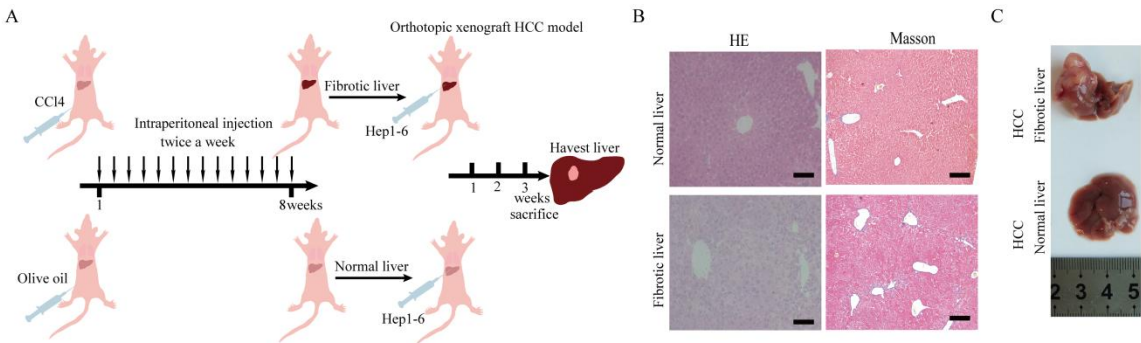


Figure S1 Liver cirrhosis promotes the progression of liver cancer

A Schematic diagram of an orthotopic xenograft hepatocellular carcinoma (HCC) model in mice with liver fibrosis.

B Representative HE and MASSON staining images of livers from cirrhotic and control mice ($n = 1$ mouse per group for histology; 4 mice per group in total). Scale bar: 100 μ m.

C Representative images of the liver from mouse orthotopic xenograft HCC mode ($n=3$).

Figure S2

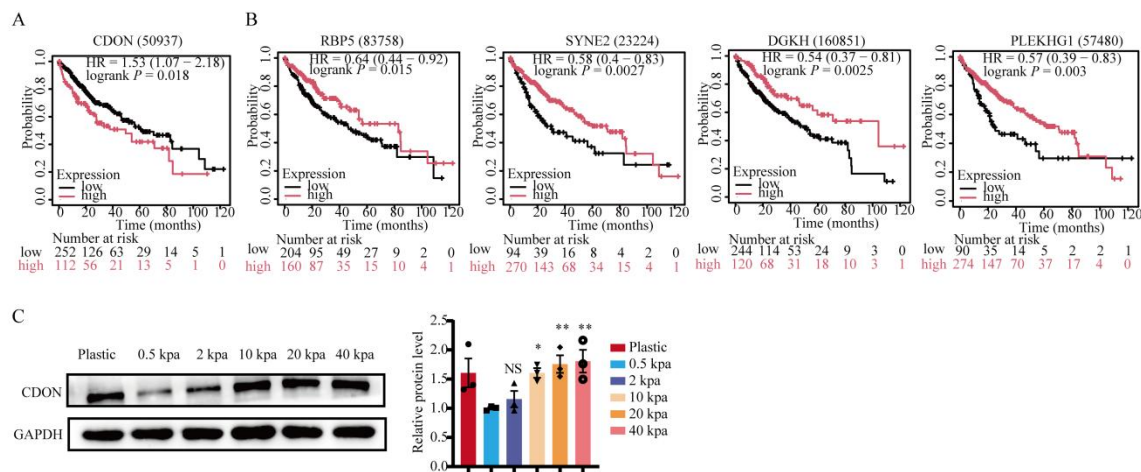


Figure S2 CDON clinical relevance and protein levels

A. Kaplan-Meier (KM) curve of CDON

B. KM curves of molecules (RBP5, SYNE2, DGKH, PLEKHG1)

C Detection of CDON protein levels in different stiffness ECM (tissue culture plastic (TCP, GPa range, representing a supra-physiological stiff reference or positive extreme control), 0.48 ± 0.16 kPa, 2.01 ± 0.75 kPa, 10.61 kPa, 19.66 ± 1.19 kPa, and 40.40 ± 2.39 kPa) by Western blot. The positive extreme control does not participate in the comparison ($n=3$).

Figure S3

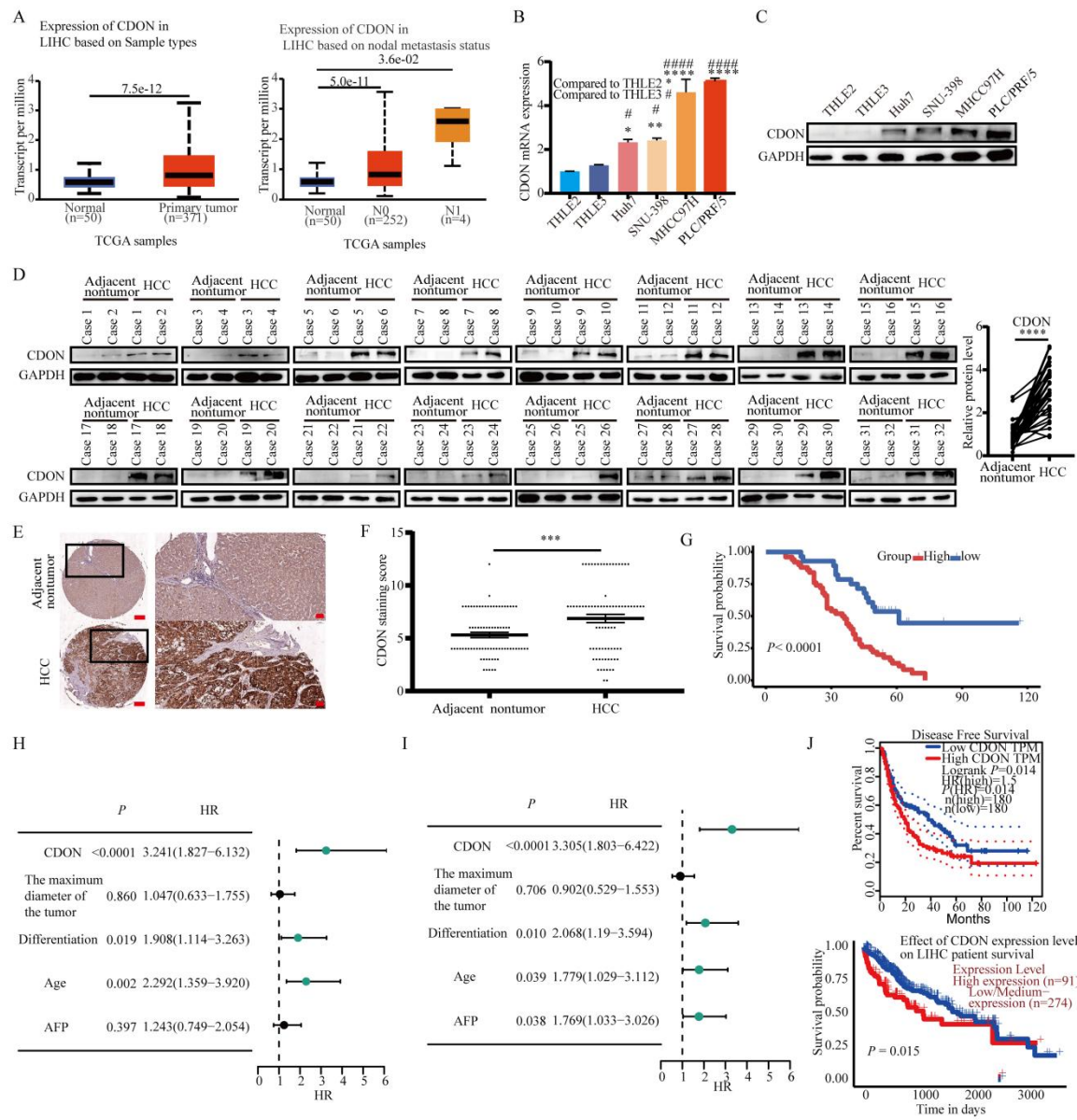


Figure S3 CDON is highly expressed in HCC and serves as an independent prognostic

factor.

A. Expression of CDON in normal individuals and HCC patients, as well as its expression in HCC patients at different stages in The Cancer Genome Atlas (TCGA) data.

B - C. Detection of CDON mRNA and protein levels in normal hepatocytes (THLE2 and THLE2) and HCC cells (Huh7, SNU - 398, MHCC97H, PLC/PRF/5) by quantitative real - time polymerase chain reaction (qRT - PCR) and Western blotting.

D. Detection of CDON protein levels in HCC patients by Western blotting ($n=32$).

E. Detection of CDON protein levels in HCC patients by immunohistochemical staining. Scale bar: 200 μm (left) ,50 μm (right).

F. Immunohistochemical score statistics graph ($n=78$).

G The KM curve was plotted by <https://www.bioinformatics.com.cn>, an online platform for data analysis and visualization.

H Forest Plot of univariate Cox regression analysis of CDON and clinical indicators.

I Forest Plot of multivariate Cox regression analysis.

J Generation of KM and Disease Free Survival curves using GEPIA (<http://gepia.cancer-pku.cn/>) and Kaplan - Meier plotter (<https://kmplot.com/analysis/>), respectively.

Figure S4

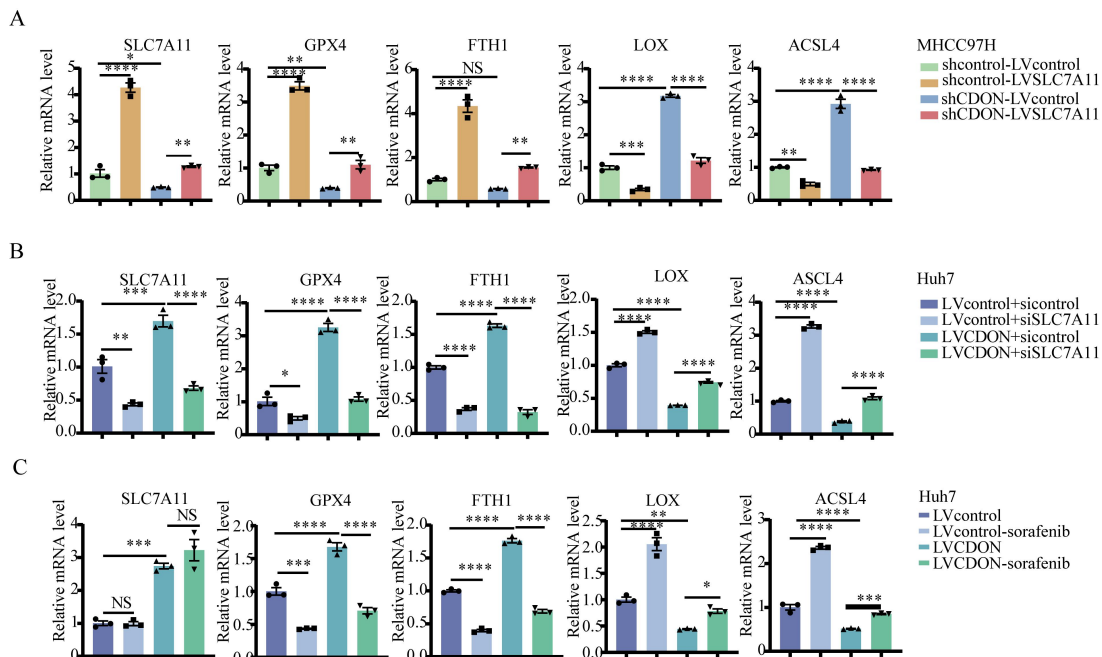


Figure S4 CDON and SLC7A11 or sorafenib jointly regulate ferroptosis in HCC.

A-B. qRT-PCR detection of the expression of ferroptosis-related molecules (SLC7A11, GPX4, FTH1, LOX, ACSL4) in HCC cells under the combined regulation of CDON and SLC7A1 ($n = 3$).

C. qRT-PCR detection of the expression of ferroptosis-related molecules in HCC cells under the combined regulation of CDON and sorafenib ($n = 3$).

Figure S5

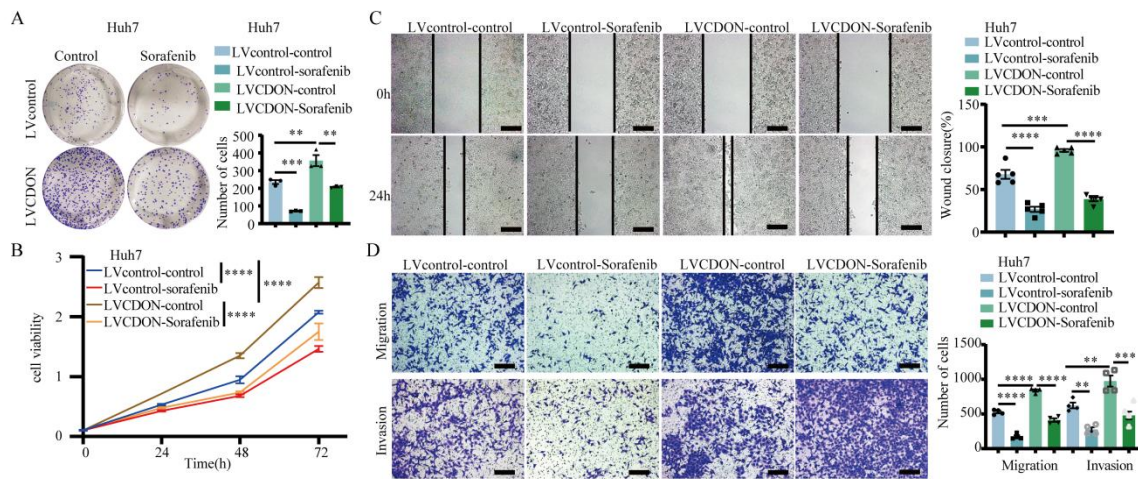


Figure S5 CDON promotes the resistance of HCC to sorafenib

A-B. Colony formation and CCK-8 assays were used to detect the proliferation ability of Huh7-LVCDON cells treated with Sorafenib (5 μ M) for 24 hours ($n = 3$).

C A Wound healing assay was used to detect the migration ability in the indicated cells. Scale bar: 100 μ m ($n = 5$).

D The migratory and invasive capacity of the indicated cells was analyzed by Transwell assay. Scale bar: 100 μ m ($n = 4$).

Figure S6

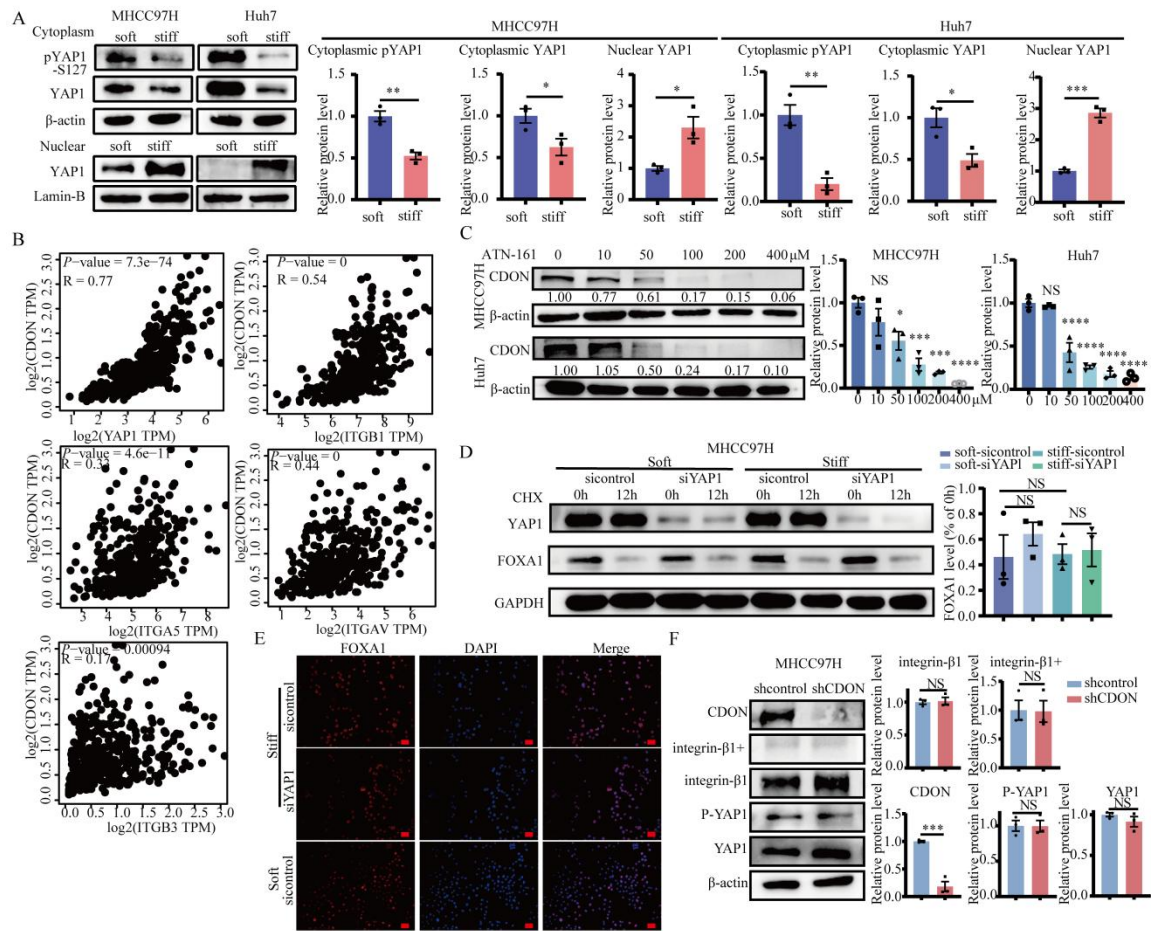


Figure S6 ECM stiffness regulates CDON expression through the integrins/YAP1-FOXA1 axis in HCC cells.

A MHCC97H and Huh7 cells were cultured on soft and stiff ECM conditions. Western blot was used to detect the protein levels of YAP1 and p-YAP1 in the cytoplasm and nucleus of MHCC97H and Huh7 cells ($n = 3$).

B Correlation analysis diagram of CDON and key molecules involved in the signal transduction of ECM stiffness, including ITGB1 (integrin β 1), ITGB3 (integrin β 3), ITGA5 (integrin α -5), ITGAV (integrin α -v), and YAP1 by GEPIA (<http://gepia.cancer-pku.cn/>).

C Western blot detected the expression of CDON after HCC cells were treated with

ATN-161 (0, 10, 50, 100, 200, 400 μ M) ($n = 3$).

D MHCC97H cells were cultured on soft and stiff ECM conditions. Then, they were transfected with siYAP1. Western blot was used to detect the expression of YAP1 and FOXA1 in the treated cells after treatment with protein synthesis inhibitor, CHX (10 μ M, 12h) ($n = 3$).

E Immunofluorescence assay for the expression of FOXA1 in the indicated HCC cells. Scale bar: 50 μ m.

F Western blot detected the expression of YAP1, p-YAP1, integrins, and their active forms(Integrin β 1+) ($n = 3$).

Figure S7

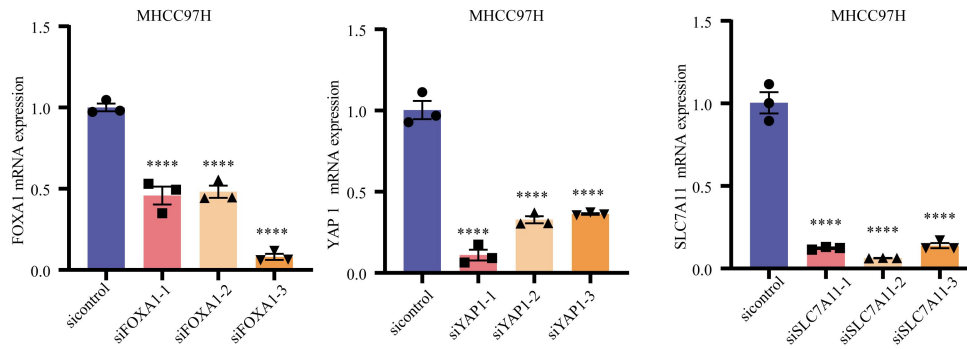


Figure S7 The qRT-PCR detection diagram shows the knockdown efficiency of YAP1, FOXA1, and SLC7A11 ($n = 3$).

Supplementary Tables

Table S1 Cox regression analysis

Variable	Univariate COX analysis		Multivariate COX analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
CDON (High vs. Low)	3.241 (1.827-6.132)	0.00003 ****	3.305 (1.803-6.422)	0.00007 ****
The maximum diameter of the tumor (>5cm vs. ≤5cm)	1.047 (0.6331-1.755)	0.860	0.9016(0.5287-1.553)	0.706
Differentiation (Poor vs. Well/Mod)	1.908 (1.114-3.263)	0.019*	2.068 (1.190-3.594)	0.010*
Age (>52.5(median) vs. ≤52.5)	2.292 (1.359-3.920)	0.002*	1.779(1.029-3.112)	0.039*
AFP (>20 vs. ≤ 20 ng/mL)	1.243 (0.7486-2.054)	0.397	1.769 (1.033-3.026)	0.038*

Table S2 Research Resource Identifiers (RRIDs) of the cell line

Cell line name	Research Resource Identifiers (RRIDs)
MHCC97H	CVCL_4972
PLC/PRF/5	CVCL_0485
SNU-398	CVCL_0077
Huh 7	CVCL_0336
THLE2	CVCL_3803
THLE3	CVCL_3804
HEK-293T	CVCL_0063
Hepa 1-6	CVCL_0327

Table S3 Knockdown shRNA/siRNA sequence list

	Sequences (5'→3')	
The siRNA of the negative control shcontrol	sense	UUCUCCGAACGUGUCACGUTT
	antisense	ACGUGACACGUUCGGAGAATT
		TTCTCCGAACGTGTCACGT
SiYAP1-1		GGUCAGAGAUACUUCUUAUAAU
SiYAP1-2		GCUCAGAUCUUCUUAACA
SiYAP1-3		GGUGAUACUAUCAACCAAAGC
SiFOXA1-1		CCGGCAACAUGUUCGAGAA
SiFOXA1-2		CCAGCGACUGGAACAGCUAG
SiFOXA1-3		CGCUGAGCGAGAUUCUACCA
SiSLC7A11-1		CCUGUCACUAUUUGGAGCUUU
SiSLC7A11-2		CCUGCGUAUUAUCUCUUUAUU
SiSLC7A11-3		CCCUGGAGUUAUGCAGCUAAU
ShCDON-1		GCTTCTGAATATCCTGTCAAA
ShCDON-2		GCGATACTCAGATCATGCTAA
ShCDON-3		GCCTTCAAAGTCGAATATAAA

Table S4 Expected modulus of elasticity after polymerization of relative acrylamide and bis-acrylamide concentrations.

Acrylamide %	Bisacrylamide %	40% acrylamide (mL)	2% bisacrylamide (mL)	Water (mL)	Young ' S modulus, E ± SD (kPa)
3	0.06	0.75	0.3	8.95	0.48 ± 0.16
4	0.1	1	0.5	8.5	2.01 ± 0.75
10	0.1	2.5	0.5	7	10.61
8	0.264	2	1.32	6.68	19.66 ± 1.19
8	0.48	2	2.4	5.6	40.40 ± 2.39

Table S5 List of Antibodies

Antibody name	Source and dilution ratio
anti-CDON	Proteintech,17634-1-AP,1:1000
anti-HMGB1	Proteintech,10829-1-AP,1:6000
anti-p53	Proteintech,10442-1-AP,1:10000
anti-SLC7A11	ABclonal, A13685,1:3000
anti-YAP1	ABclonal, A21216,1:20000
anti-P-YAP1-S127	ABclonal, AP0489,1:1000
anti-Lamin B 1	Proteintech,12987-1-AP,1:20000
anti-FOXA1	Proteintech,20411-1-AP,1:3000
anti- β -actin	ABclonal, AC026,1:100000
anti-GAPDH	ABclonal, A19056,1:100000
anti-Flag	ABclonal,AE092, 1: 200(IP), 1:400(IF)
anti-GST	ABclonal, AE077,1:4000
anti-LOX	Zenbio, R381849,1:1000
anti-FTH1	ABclonal, A19544,1:4000
anti-ACSL4	ABclonal, A20414,1:1000
anti-GPX4	Proteintech,67763-1-lg,1:1000(IHC),1:2000(WB)
anti-CD31	Abcam, ab182981,1:2000
anti-CD68	Abcam, ab303565,1:1000
anti-F4/80	CST,70076T,1:200
anti-ki67	ABclonal, A20018,1:1000
anti-Histone H3	ABclonal, AC070, 1:10000
anti- Integrin β 1	Zenbio, R24729, 1:4000
anti-Hust-4	Sigma-Aldrich, MAB2079Z, 1:500
anti-CDON	GeneTex, GTX105422, 1:200
anti-FOXA1	CST, #53528, 1:50(ChIP), 1:400(IF)
Anti-Ubiquitin	CST, #3936, 1:1000

anti-Cleaved caspase-3	HUABIO,ET1602-47,1:1000
anti-LC3B	HUABIO,ET1701-65,1:2000
anti-P62	Proteintech, 18420-1-AP,1:10000
anti-Phospho-MLKL(S358)	HUABIO,HA724063,1:5000
anti-MLKL	HUABIO,ET1601-25,1:5000
anti-RIPK3	HUABIO,HA722183,1:2000

Table S6 Primer sequence list

The name of the primer		sequence (5'->3')
Primers for real-time PCR		
(HUMAN)GAPDH	forward primer	TCATTTCTGCTGGTATGACAACGA
(HUMAN)GAPDH	reverse primer	GTCTTACTCCTTGGAGGCC
(HUMAN)FTH1	forward primer	CTTCCTTCAGGATATCAAGAAACC
(HUMAN)FTH1	reverse primer	TCCAAATGTAATGCACACTCC
(HUMAN)SLC7A11	forward primer	GGTCCATTACCAGCTTTTGTACG
(HUMAN)SLC7A11	reverse primer	AATGTAGCGTCCAAATGCCAG
(HUMAN) GPX4	forward primer	GTGGATGAAGATCCAACCC
(HUMAN) GPX4	reverse primer	TTGTCGATGAGGAAGTTGG
(HUMAN) ACSL4	forward primer	CATCCCTGGAGCAGATACTCT
(HUMAN) ACSL4	reverse primer	TCACTTAGGATTTCCCTGGTCC
(HUMAN)LOX	forward primer	CAAGGGACATCAGATTTCTTACC
(HUMAN)LOX	reverse primer	CCATACTGTGGTAATGTTGATGAC
(HUMAN)P53	forward primer	GAGGTTGGCTCTGACTGTACC
(HUMAN)P53	reverse primer	TCCGTCCCAGTAGATTACCAC
(HUMAN)β-actin	forward primer	CCTTCCTGGGCATGGAGTC
(HUMAN)β-actin	reverse primer	TGATCTTCATTGTGCTGGGTG
(HUMAN)FOXA1	forward primer	GCAATACTCGCCTTACGGCT
(HUMAN)FOXA1	reverse primer	TACACACCTTGGTAGTACGCC
(HUMAN)YAP1	forward primer	TGTCCCAGATGAACGTCAC
(HUMAN)YAP1	reverse primer	CAGGAAGAGGACCTGAAGC
(HUMAN)CDON	forward primer	GAGTTCAGACTTGGCACCT
(HUMAN)CDON	reverse primer	CAATGCAGTACTACAGGTCCA

Table S7 Primer sequence list

Primers used for ChIP		sequence (5'→3')
distant region	forward primer	TTGGGAGAGGGACAGAGGAG
distant region	reverse primer	TGGGCTTCAACAAGTGAGCA
binding site 1	forward primer	GTGTTACACAGCTCCCTG
binding site 1	reverse primer	TGCAGTCCCAAGCTTCAAAGA
binding site 2	forward primer	TGAAGCTTGGGACTGCACAT
binding site 2	reverse primer	TGCCATTTGAATTCATATGCCT
binding site 3	forward primer	GGCACTGTGAGATCTGGAGG
binding site 3	reverse primer	ACCACACCCAGCCAGAAAAA
binding site 4	forward primer	TGAGCCAAGATCGCACCATT
binding site 4	reverse primer	ATGCCAGTCCTCCCTTACCT
binding site 5	forward primer	TGAGTGGCAGGTGCTTTTCA
binding site 5	reverse primer	AACTCAGGGGCTAGGAGAGG
binding site 6	forward primer	GGTGGTGGGGGAATCAGATG
binding site 6	reverse primer	CCCCTCGCCTCTACCTAAGT
human CTGF	forward primer	TTCTGTGAGCTGGAGTGTGC
human CTGF	reverse primer	GCCAATGAGCTGAATGGAGT

Table S8 Online analysis websites used in this study

website name	Website address
Kaplan-Meier Plotter	https://kmplot.com/analysis/
GEPIA	http://gepia.cancer-pku.cn/index.html
UALCAN	https://ualcan.path.uab.edu/
UCSC	https://genome.ucsc.edu/
Cistrome DB	http://cistrome.org/db/#/
STRING	https://cn.string-db.org/cgi/input?sessionId=bCgNvE5EcK1I&input_page_show_search=on
JASPAR	https://jaspar.elixir.no/

Supplementary Methodology

Colony formation assay and Cell Counting Kit-8 (CCK-8) assay

The cells were treated and then inoculated into 6-well plates (1×10^3 cells per well) in a 37°C, 5% CO₂ incubator for 2 weeks. Then, the culture medium was removed, and the cells were fixed with 4% polyformaldehyde. After staining with 0.5% crystal violet, the

cells were photographed.

We inoculated the cells with different treatments into 96-well plates (3×10^3 cells per well), and used the CCK-8 (BMU106, Abbkine) according to its instructions to measure the absorbance at 450 nm using a microplate reader for the examination.

Transwell assays

Transwell assays were conducted using an 8- μ m aperture chamber (Promoter Biotechnology, Wuhan, China) to detect cell migration and invasion. 12% FBS-containing DMEM was added to the bottom chamber, while the top chamber was coated with Matrigel (60 μ l, diluted 1:5 with DMEM, ABW) for the invasion assay. 4×10^4 (migration experiment) and 8×10^4 (invasion experiment) cells were suspended in serum-free DMEM and added to the top chamber and cultured at 37°C with 5% CO₂ for 24 h and 48 h, respectively. The cells were fixed in 4% paraformaldehyde for 20 min, stained with 0.5% crystal violet for 20 min. The cells that failed to penetrate the pores were removed with a cotton swab, and then images were captured using an Olympus microscope (randomly selecting at least 4 areas for imaging).

Immunohistochemical Staining (IHC)

Sections of the paraffin were baked in the oven at 65 °C for 2h and then followed by dewaxing with an environmentally friendly dewaxing solution and a series of ethanol gradients (100–70%). The antigen recovery was performed using sodium citrate buffer, followed by high-temperature microwave treatment for 15 minutes and cooling at room temperature. Next, follow the instructions of the mouse SP detection kit (ZSGB-BIO, Beijing, China, SP-9002) and the DAB immunohistochemical detection kit (Biosci, Wuhan, China) for the operation. The primary antibody used in Table S5. IHC scores were performed by two independent observers who were blinded to the clinical outcome. The percentage of positive cells was evaluated on a scale of 0 to 4: 0 (negative), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), or 4 (76%-100%). The immunostaining intensity

was scored on a scale of 0 to 3: 0(negative), 1 (weak), 2 (medium), or 3 (strong). The final IHC scores (0~12) were calculated by multiplying the above two scores. The optimal cut-off value for CDON expression was determined by X-tile based on overall survival (OS), which categorized patients into high- and low-expression groups. Each case was ultimately considered“low” if the final score ranged from 0~4, and“high” if the final score ranged from 5~12.

Quantitative Real-Time polymerase chain reaction (qRT-PCR),

Total RNA was extracted from liver cell lines and tissues using TRIzol reagent (Invitrogen). Next, reverse transcription polymerase chain reaction (RT-PCR) was performed using SuperMix to synthesize complementary DNA. qRT-PCR for mRNA expression levels was carried out using SYBR qPCR Master Mix. SYBR qPCR Master Mix and SuperMix were obtained from Vazyme (Nanjing, China). The primers used for real-time PCR can be found in Table S6.

Extraction of cytoplasmic and nuclear proteins

Use the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, P0027) to separate the cytoplasmic and nuclear proteins, and then proceed with the subsequent experiments.

Bioinformatic analysis

This study utilized the online websites listed in Table S8.