m^{6}A-mediated gluconeogenic enzyme PCK1 upregulation protects against hepatic ischemia-reperfusion injury

Shanshan Yu[1, 2, #], Xiao Liu[3, #], Yan Xu[1, 2, 4, #, *], Lijie Pan[2], Yihan Zhang[3], Yanli Li[1], Shuai Dong[1], Dan Tu[3], Yuetong Sun[1], Yiwang Zhang[5], Zhuowei Zhou[1], Xiaoqi Liang[2], Yiju Huang[2], Jiajie Chu[2], Silin Tu[1], Chang Liu[1], Huaxin Chen[1], Wenjie Chen[1, 2, 4, *], Mian Ge[3, *], and Qi Zhang[1, 2, 4, *]

Supplementary Materials and Methods

Liver biochemical measurement

To evaluate the liver function or hepatocyte injury, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) levels of serum or cellular supernatant were measured using Hitachi 7020 automatic biochemical analyzer (Hitachi, Tokyo, Japan).

Hematoxylin & Eosin (H&E) and immunohistochemical staining

Liver samples were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned to 5 μm slides for H&E and immunohistochemical staining. After routine dewaxing, rehydration, and antigen retrieval, the sections were then incubated with primary antibodies against PCK1 (1:400, Proteintech, 66862-1-Ig), Ly6G (1:500, Servicebio, GB11229-100), or F4/80 (1:500, Servicebio, GB113373-100) at 4 ℃ overnight. Secondary antibodies against mouse IgG (1:100, Cell Signaling Technology, 7076) or rabbit IgG (1:100, Cell Signaling Technology, 7074) were then incubated with the sections at room temperature after 3 washes with PBS containing 0.02% Triton^{TM} X-100 (Sigma-Aldrich, T8787). The images were viewed and captured by an optical microscope (Nikon, Japan).

Periodic acid-Shiff (PAS) staining

Glycogen storage in liver tissues was detected using a PAS staining kit (Beyotime,

C0142M) according to the manufacturer's instructions. Briefly, liver sections were subjected to deparaffinization and rehydration, following incubation in 1% periodic acid for 10 minutes at room temperature, rinsed in water for 5 minutes, and placed in Schiff's reagent for 60 minutes at 37 ℃. Finally, the sections were counterstained with hematoxylin (Baso, BA4041) and checked with a microscope (Nikon, Japan).

PCK1 activity assay

PCK1 activity in human or mouse liver tissues was detected using a phosphoenolpyruvate carboxylase kinase kit (NanJing JianCheng Bioengineering Institute, A131-1-1) following the manufacturer's instruction. In brief, 20 mg of liver tissues were homogenized with 200 μL ice-old extracting buffer. After centrifuging for 10 minutes at 8000 g at 4 ℃, the supernatant was collected, and then the protein level was quantified using the bicinchoninic acid (BCA) method, and PCK activity was measured. Finally, the PCK activity was normalized to the protein concentration for each sample.

Plasmid construction and virus transduction

To achieve METTL3 knockdown in HepG2 cells, short hairpin RNA (shRNA) was used. Sequences targeting human METTL3 (sh*METTL3*) or firefly luciferase (sh*Luc*, used as a non-targeting negative control) were cloned into the pLKO.1 lentiviral vector (Addgene, 10878). As for PCK1 overexpression, full-length PCK1 CDS amplified from human cDNA template was cloned into the pKD-EF1 lentiviral vector. Primers used for plasmid construction were listed in *Supplementary Table 1*, and all constructs were confirmed by Sanger sequencing before further use.

For the lentivirus package, 293T cells were seeded into 6 cm dish and expanded to 90% confluence. 336 μL pre-warmed Opti-MEM (Invitrogen, 31985070), 4 μg target plasmid, 4 μg packaging plasmid (psPAX2: pMD2.G = 2:1), and 36 μL PEI (1 μg/mL) (Polysciences, 23966) were mixed and incubated for 12 minutes at room temperature before gently added to each well. The medium was changed 11-14 hours later. 48 hours after transfection, the lentivirus-containing supernatants were collected and centrifuged

at 3600 rpm for 8 minutes. HepG2 cells were infected with lentivirus-containing supernatant supplemented with 1 μg/mL polybrene (Sigma, H9268) at approximately 30% confluence, and the medium was changed 6-8 hours later. Infected cells were selected 48 hours after infection with 2 μg/mL puromycin (Thermo Scientific, A1113803) for 2 consecutive days.

Western blot

Cell pellets or tissues were homogenized in RIPA cell lysis buffer freshly supplemented with protease inhibitor (Roche, 04693132001) and phosphatase inhibitor (Roche, 04906837001). The extracted proteins were applied to 10% SDS-PAGE gel separation and transferred onto nitrocellulose (NC) membranes. The membranes were incubated with primary antibodies at 4 ℃ overnight and then incubated with secondary antibodies at room temperature for 1 hour after 3 washes with TBST. The blots were developed and detected by Chemidoc Imaging System (Biorad®) using Immobilon ECL Ultra Western HRP Substrate (Millipore, WBULS500). Image J software was used to quantify the protein expression, and ACTB served as a loading control. Antibodies used in western blotting are presented in *Supplementary Table 5*.

Quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated from tissues or cells using TRIzol reagent (Invitrogen, 15596026), and cDNA was synthesized using HiScript III One-Step RT-PCR Kit (Vazyme, R323-01) according to the manufacturer's instructions. RT-qPCR was performed in triplicates on Light Cycler 480 II (Roche) with ChamQ Universal SYBR qPCR Master Mix (Vazyme, Q711-03). The relative mRNA levels were normalized against *GAPDH* mRNA levels. Primers used for RT-qPCR were listed in *Supplementary Table 1*.

Cell counting kit 8 (CCK8) assay

CCK8 (APExBIO, K1018-10000t) was used to measure the cell viability of HepG2 cells or mouse primary hepatocytes according to the manufacturer's instructions. In

brief, the CCK8 solution was added directly into the culture plate in a volume of 1/10 of the whole culture medium and reacted in a routine culture environment for 1-4 hours. The absorbance at 450 nm was tested by Microplate Reader (TECON, Switzerland), and cell viability was calculated.

Propidium iodide (PI) staining

PI staining was performed on HepG2 cells or mouse primary hepatocytes to evaluate cell death. Cell nuclei were counterstained with Hoechst 33342 (Beyotime, C1022). The cells were stained with 10 μg/mL PI (Beyotime, ST511) for 15 minutes at room temperature. After washing twice with PBS, cells were stained with 10 μg/mL Hoechst 33342 for 10 minutes at room temperature. Images were captured by fluorescence microscope (Zeiss, Axio Observer Z1) and analyzed by Image J software.

Lactate assay

The lactate concentration in the culture supernatant or mouse serum was measured with a lactic acid assay kit (NanJing JianCheng Bioengineering Institute, A019-2-1) according to the manufacturer's instructions. Briefly, 10 μL of samples were added into 500 μL of enzyme working solution. Then, 100 μL of chromogenic agent was added into the previous mix and incubated in a 37 ℃ water bath for exactly 10 minutes. The reaction was terminated with 1 mL of stop solution, and the OD values at 530 nm were detected using a Microplate Reader (TECON, Switzerland).

LC-MS/MS for m^6A detection and quantification

LC-MS/MS quantification for mRNA m^6A modification of mouse liver tissues was conducted by Wuhan Metware Co., Ltd, as previously described(1). Briefly, total RNA was extracted, and Poly(A) mRNAs were purified two rounds using GenEluteTM mRNA Miniprep Kit (Sigma, MRN10-1KT). 1μg purified mRNA was sufficiently digested to nucleosides with S1 nuclease, phosphodiesterase, and alkaline phosphatase at 37 °C, then extracted by chloroform to get prepared solution samples. The samples

were analyzed using a UPLC-ESI-MS/MS system (UPLC, ExionLCTM AD, https://sciex.com.cn/; MS, Applied Biosystems 6500 Triple Quadrupole, https://sciex.com.cn/). The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS. Linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired on a triple quadrupole-linear ion trap mass spectrometer (QTRAP) equipped with an ESI Turbo Ion-Spray interface, then operated in a positive ionmode and controlled by Analyst 1.6.3 software (Sciex). RNA modifications were analyzed using scheduled multiple reaction monitoring (MRM). Data acquisitions were performed using Analyst 1.6.3 software (Sciex). RNA modification contents were detected by MetWare (http://www.metware.cn/) based on the AB Sciex QTRAP 6500 LC-MS/MS platform.

Nucleus/cytoplasm fractionation

Primary hepatocytes from control and *Mettl3* cKO mice were challenged by 6 hours of hypoxia and subsequent reperfusion for 3 hours (H6R3) and collected for nucleus/cytoplasm fractionation. Separation of cytoplasmic and nuclear fraction was performed using NE-PER™ Nuclear Cytoplasmic Extraction Reagent kit (Thermo Scientific ,78835) according to the manufacturer's instruction. Briefly, 1 * 10^6 primary hepatocytes were collected and washed twice with ice-cold PBS. 200 μL ice-cold Cytoplasmic Extraction Reagent I was added to the cell pellets. After a violent vortex for 15 seconds, the lysates were incubated on ice for 15 minutes. Then, 11 μL Cytoplasmic Extraction Reagent II was added into the lysates with a subsequent incubation on ice for 1 minute. After another violent vortex for 10 seconds, the lysates were centrifuged at 13200 rpm for 5 minutes at 4 ℃. The supernatant containing the cytoplasmic fraction and the sediment containing the nuclear fraction were resuspended in 1 mL TRIzol for RNA isolation, respectively. Successful separation of cytoplasmic and nuclear fraction was confirmed by the RT-qPCR result that *Gapdh* was predominantly detected in the cytoplasmic fraction and *Neat1* was mainly detected in the nuclear fraction.

Reference

1. **Chang J-S**, **Lin Z-X**, Liu Y-J, Yang S-M, Zhang Y, Yu X-Y. Ultra performance liquid chromatography-tandem mass spectrometry assay for the quantification of RNA and DNA methylation. J. Pharm. Biomed. Anal. 2021;197:113969.

Author names in bold designate shared co-first authorship.

Supplementary Table 1. Primers

1. Primers for genotyping

Name	**Sequences**
Mettl3-F1	5'-AGAGGAGGAGAAGGTGGCAGAG-3'
Mettl3-R1	5'-CCTTTCATTCACATGGCAGCAC-3'
Mettl3-F2	5'-AGGCTCACTTGCAAGTAAAAGGAAG-3'
Mettl3-R2	5'-AGGCCTATAATCCTAGCACTG-3'
Alb-CreERT-F	5'-TCTCCCCACCTCTAGCCCAAAGAAA-3'
Alb-CreERT-R	5'-AGGCAGAGGACTGTATTGATCAGTC-3'

2. Primers for RT-qPCR (mouse)

Name	**Sequences**
mouse *Pck1*-RT-F	5'-AGGAGGTGGAGGAGATCGAC-3'
mouse *Pck1*-RT-R	5'-TTCTCTGTTTCAGGGCTCGG-3'
mouse *Mettl3*-RT-F	5'-ATGAAACCTATGCCCCTCCC-3'
mouse *Mettl3*-RT-R	5'-TCAGGCTTTTGCTTCACCAGG-3'
mouse *Wtap*-RT-F	5'-GCAAGAGTGCACCACTCAAA-3'
mouse *Wtap* -RT-R	5'-CATTTTGGGCTTGTTCCAGT-3'
mouse *Gapdh*-RT-F	5'-CATGGCCTTCCGTGTTCCT-3'
mouse *Gapdh*-RT-R	5'-GCCTGCTTCACCACCTTCT-3'
mouse *Il6*-RT-F	5'-TACCACTTCACAAGTCGGAGGC-3'
mouse *Il6*-RT-R	5'-CTGCAAGTGCATCATCGTTGTTC-3'

mouse *Tnfa*-RT-F	5'-AGCCGATGGGTTGTACCTTG-3'
mouse *Tnfa* -RT-R	5'-ATAGCAAATCGGCTGACGGT-3'
mouse *Il1b*-RT-F	5'-TGGACCTTCCAGGATGAGGACA-3'
mouse *Il1b*--RT-R	5'-GTTCATCTCGGAGCCTGTAGTG-3'
mouse *Ccl2*-RT-F	5'-TACAAGAGGATCACCAGCAGC -3'
mouse *Ccl2* -RT-R	5'-ACCTTAGGGCAGATGCAGTT-3'
mouse *Cxcl1*-RT-F	5'-TGCACCCAAACCGAAGTC-3'
mouse *Cxcl1* -RT-R	5'-GTCAGAAGCCAGCGTTCACC-3'
mouse *Cxcl2*-RT-F	5'-GCGCCCAGACAGAAGTCATA-3'
mouse *Cxcl2*-RT-R	5'-CAGTTAGCCTTGCCTTTGTTCA-3'
mouse *G6pc* -RT-F	5'-GCCTTCTATGTCCTCTTTCCC-3'
mouse *G6pc* -RT-R	5'-GCGTTGTCCAAACAGAATCC-3'
mouse *Fbp1*-RT-F	5'-ATGGATTGTGGTG TCAACTG-3'
mouse *Fbp1*-RT-R	5'-CTCATTAAGGCTGTAGATGTTACC-3'

3. Primers for RT-qPCR (human)

Name	**Sequences**
Human *PCK1*-RT-F	5'-GAGAAAGCGTTCAATGCCAG-3'
Human *PCK1*-RT-R	5'-ATGCCGATCTTTGACAGAGG-3'
Human *METTL3*-RT-F	5'-GAAGCAGCTGGACTCTCTGC-3'
Human *METTL3*-RT-R	5'-ACGGAAGGTTGGAGACAATG-3'
Human *WTAP*-RT-F	5'-TCTGCACGCAGGGAAAA-3'

Human *WTAP*-RT-R	5'-TTGATCGCTGGGTCTACCA-3'
Human *GAPDH*-RT-F	5'-GTTCTTCATGGAGCAGCACGTG-3'
Human *GAPDH*-RT-R	5'-CTGGTAGAGGATCTTCCGCATC-3'
Human *FBP1*-RT-F	5'-GCAGTCAAAGCCATCTCTTCGG-3'
Human *FBP1*-RT-R	5'-TAACCAGGTCGTTGGAGAGGAC-3'
Human *G6PC*-RT-F	5'-GCTGTGATTGGAGACTGGCTCA-3'
Human *G6PC*-RT-R	5'-GTCCAGTCTCACAGGTTACAGG-3'

4. Primers for MeRIP-qPCR

Name	**Sequences**
mouse *Pck1*-positive-m^{6}A-RT-F	5'-AGGAGGTGGAGGAGATCGAC-3'
mouse *Pck1*-positive-m^{6}A-RT-R	5'-TTCTCTGTTTCAGGGCTCGG-3'

5. Primers for TRC lentiviral vectors of shRNA and overexpression construction

Name	**Sequences**
sh*METTL3*-1-F	5'-CCGGCGTCAGTATCTTGGGCAAGTTCTCGAGAAC TTGCCCAAGATACTGACGTTTTTG-3'
sh*METTL3*-1-R	5'-AATTCAAAAA CGTCAGTATCTTGGGCAAGTT CTC GAG AACTTGCCCAAGATACTGACG-3'
sh*METTL3*-2-F	5'-CCGGGCCAAGGAACAATCCATTGTTCTCGAGAA CAATGGATTGTTCCTTGGCTTTTTG-3'
sh*METTL3*-2-R	5'-AATTCAAAAA GCCAAGGAACAATCCATTGTT CT

	CGAG AACAATGGATTGTTCCTTGGC-3'
PCK1 overexpression-F	5'-GGTTTAAACTACGGGATCCATGCCTCCTCAGC TGC-3'
PCK1 overexpression-R	5'-CGTCCTTGTAGTCGAATTCCATCTGGCTTATTC TTTGCTTCAAG-3'

Supplementary Table 2. Summary of clinical characteristics of high *PCK1* and low *PCK1* group patients included in this study

	High *PCK1*	**Low *PCK1***	***p***
n	28	28	
Male (%)	23 (82.14)	23 (82.14)	1
Age (y) (median [IQR])	45.50 [37.75, 51.25]	51.00 [40.75, 55.00]	0.163
MELD-Na (median [IQR])	25.50 [14.50, 33.25]	21.00 [10.75, 30.75]	0.485
Cirrhosis (%)	25(89.29)	25 (89.29)	1
Hepatic malignancy (%)	6 (21.43)	7 (25.00)	1
Hypertension history (%)	4 (14.29)	3 (10.71)	1
Diabetes mellitus history (%)	7 (25.00)	4 (14.29)	0.501
Smoking history (%)	4 (14.29)	5 (17.86)	1
Alcohol abuse history (%)	5 (17.86)	4 (14.29)	1
Surgical history (%)	16 (57.14)	12 (42.86)	0.423
Steatosis NASH_grade (%)			0.087
0	22 (78.57)	15 (53.57)	
1	5 (17.86)	11 (39.29)	
2	0 (0.00)	2 (7.14)	
3	1 (3.57)	0 (0.00)	
Donor type (%)			
DBD	27 (96.43)	27 (96.43)	1
DCD	1 (3.57)	1 (3.57)	

ABO incompatibility (%)	2 (7.14)	0 (0.00)	0.471
Surgery time (min) (median [IQR])	430.50 [389.75, 494.25]	405.50 [375.00, 474.25]	0.359
Anesthesia time (min) (median [IQR])	522.50 [476.25, 585.00]	515.00 [452.00, 580.00]	0.831
Anhepatic phase (min) (mean [SEM])	48.14 (1.84)	46.75 (1.96)	0.607
Cold ischemic time (min) (median [IQR])	346.50 [289.50, 399.25]	363.50 [283.50, 431.00]	0.623

Supplementary Table 3. Detailed clinical characteristics of patients included in this study

Supplementary Table 3 was affiliated as an individual .xlsx file.

Supplementary Table 4. Predicted m^{6}A sites based on *PCK1* transcript sequence

Human *PCK1* transcript (length: 4320 nt)

	Position	Sequence context	Score (binary)	Score (knn)	Score (spectrum)	Score (combined)	Decision
1	1149 (CDS)	CTTTTTCGGTGTCGCTCCTGGGACTTCAGTGAAGACCAACCCCAA	0.651	0.731	0.448	0.573	m^{6}A site (Moderate confidence)
2	2706 (3'UTR)	TTTGAGCTACCAAGAGGAGAGAACTCTGATTAAATATCTTGTGAT	0.644	0.303	0.434	0.543	m^{6}A site (Low confidence)
3	2940 (3'UTR)	ATGGTGAGGCTTATGTGGCAGGACTGGGAGGGTTTAATGCATGCT	0.672	0.573	0.443	0.575	m^{6}A site (Moderate confidence)

Mouse *Pck1* transcript (length: 2637 nt)

	Position	Sequence context	Score (binary)	Score (knn)	Score (spectrum)	Score (combined)	Decision
1	502 (CDS)	GGCCGCTGGATGTCGGAAGAGGACTTTGAGAAAGCATTCAACGCC	0.71	0.888	0.382	0.588	m^{6}A site (Moderate confidence)
2	617 (CDS)	CGCTGGCCAAGATTGGTATTGAACTGACAGACTCGCCCTATGTGG	0.628	0.794	0.495	0.583	m^{6}A site (Moderate confidence)
3	625 (CDS)	AAGATTGGTATTGAACTGACAGACTCGCCCTATGTGGTGGCCAGC	0.573	0.474	0.54	0.555	m^{6}A site (Low confidence)
4	2617 (3'UTR)	TGGGAAAAAAATTACAAAATAAACTTTTATAGAAAAAGTAGAT--	0.665	0.791	0.587	0.64	m^{6}A site (High confidence)

Supplementary Table 5. Antibodies

Antibodies	**Source**	**Identifier**
m^6A (N6-methyladenosine) antibody	Synaptic Systems	Cat# 202003
anti-METTL3 antibody, Rabbit mAb	Abcam	Cat# ab195352
mouse IgG	Beyotime	Cat# A7028
anti-mouse IgG HRP-linked antibody	Cell Signaling Technology	Cat# 7076
anti-rabbit IgG HRP-linked antibody	Cell Signaling Technology	Cat# 7074
anti-Ly6G antibody, Rabbit	Servicebio	Cat# GB11229-100
anti-F4/80 antibody, Rabbit	Servicebio	Cat# GB113373-100
anti-β-actin antibody, Rabbit	Cell Signaling Technology	Cat# 4970
anti-PCK1 antibody	Proteintech	Cat# 66862-1-Ig
anti-WTAP antibody	Cell Signaling Technology	Cat# 60188-1-Ig
anti-FBP1 antibody	Proteintech	Cat# 12842-1-AP
anti-G6PC antibody	Proteintech	Cat# 22169-1-AP

Supplementary Table 6. Reagents

Reagents	**Source**	**Identifier**
ChamQ Universal SYBR qPCR Master Mix	Vazyme	Q711-03
ClonExpress II One-Step Cloning Kit	Vazyme	C112
Collagenase IV	Sigma-Aldrich	C5138
Complete Protease Inhibitor	Roche	04693132001
Dako Real™ Kit	Dako	K5007
Direct Mouse Genotyping kit	ApexBio Technology	K1025
DMEM-high glucose medium	Thermo Scientific	C11995500BT
DMEM-no glucose medium	Gibco	11966-025
Opti-MEM serum-reducing medium	Invitrogen	31985070
EDTA antigen retrieval buffer	ZSGB-BIO	ZLI-9072
Fetal bovine serum	PAN	P30-3302
GenElute™ mRNA Miniprep Kit	Sigma-Aldrich	MRN10
Periodic acid-Schiff (PAS) staining kit	Beyotime	C0142M
Phosphoenolpyruvate carboxylase kinase kit	NanJing JianCheng Bioengineering Institute	A131-1-1
3-mercaptopropionic acid	Sigma-Aldrich	107-96-0

Lactic acid assay kit	NanJing JianCheng Bioengineering Institute	A019-2-1
NE-PER™ Nuclear Cytoplasmic Extraction Reagent kit	Thermo Scientific	78835
Hematoxylin	Baso	BA4041
Hoechst 33342	Beyotime	C1022
Immonilon ECL Ultra Western HRP Substrate	Millipore	WBULS0500
Polyethylenimine (PEI)	Polysciences	23966
Olive oil	MACKLIN	O815211
Penicillin/streptomycin	KeyGEN Biotech	KGY0023
Phosphatase inhibitor	Roche	04906831001
Polybrene	Sigma-Aldrich	H9268
HiScript III One-Step RT-PCR Kit	Vazyme	R323-01
Propidium iodide	Beyotime	C3019S
Protein A beads	Thermo Scientific	100-02D
Protein G beads	Thermo Scientific	100-04D
Proteinase K	Thermo Scientific	AM2546
Puromycin	Thermo Scientific	A1113803
RNasin® Ribonuclease Inhibitor-plus	Promega	N2611
Stbl3 *E. coli*	TransGen Biotech	CD521-01

Triton™ X-100	Sigma-Aldrich	T8787
TRIzol reagent	Invitrogen	15596026
Tween-20	Sigma-Aldrich	P1379
Tamoxifen	Sigma-Aldrich	T5648
Type I Collagen, rat tail	Invitrogen	A10483-01
William's E medium	Gibco	12551032
Plasmids		
pLKO.1 Vector	Addgene	10878