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Supplemental Information

Circadian Clock Regulation of Hepatic Lipid Metabolism by Modulation of m^{6}A mRNA Methylation

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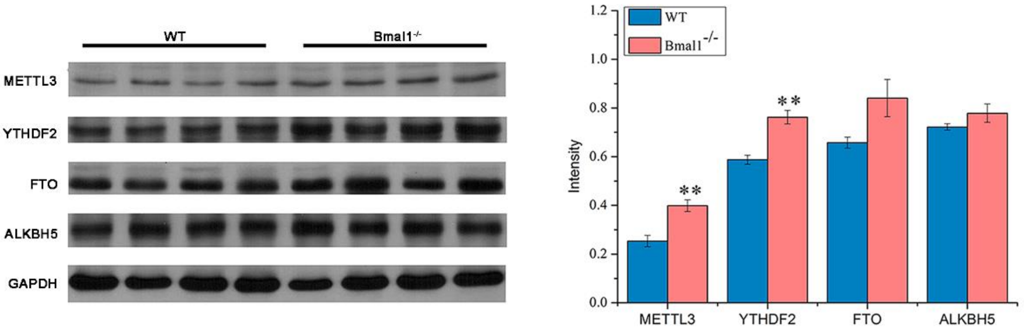

Figure S1. Liver-specific Bmal1$^{f/f}$-AlbCre-knockout affects m^{6}A mRNA methylation in mice. Related to Figure 1. Western blotting was used to measure METTL3, YTHDF2, ALKBH5, and FTO protein levels in the liver of WT and *Bmal1*$^{-/-}$ mice at ZT0 (*n* = 4 per group). WT: Bmal$^{f/f}$; *Bmal1*$^{-/-}$: Bmal$^{f/f}$-AlbCre. All data are representative of three independent experiments. Data are represented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ by two-tailed *t*-test.

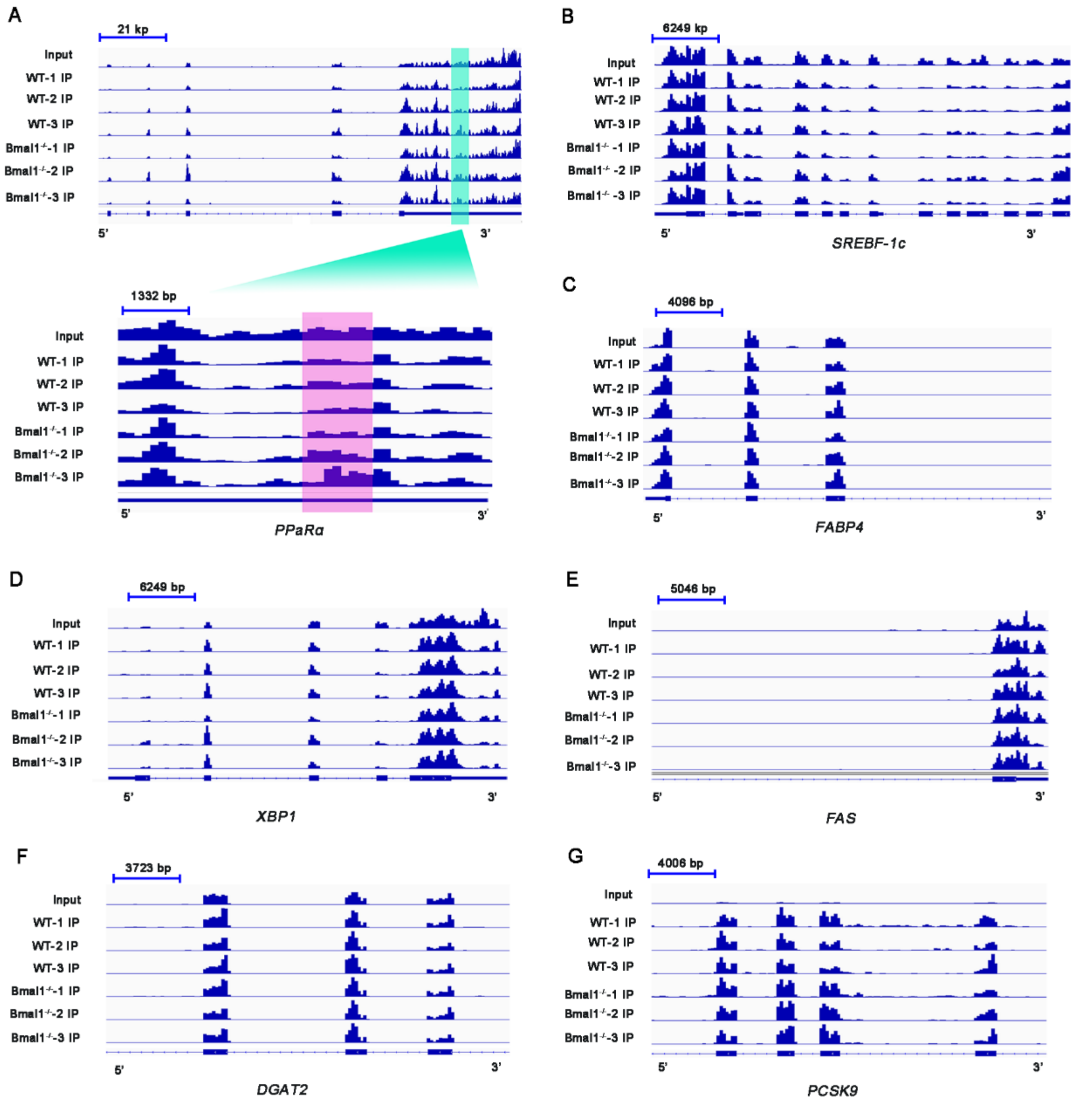

Figure S2. Liver-specific Bmal1$^{f/f}$-AlbCre-knockout changes *PPaRα* mRNA methylation in mice. Related to Figure 2. (**A-G**) Integrative genomics viewer (IGV) plots showing a increase in m^{6}A methylated peaks for *PPaRα* mRNA, but no change for other lipid metabolism genes among the input samples, WT IP, and *Bmal1*$^{-/-}$ IP. Blue boxes represent exons (n = 3 per group). WT: Bmal$^{f/f}$; *Bmal1*$^{-/-}$: Bmal$^{f/f}$-AlbCre.

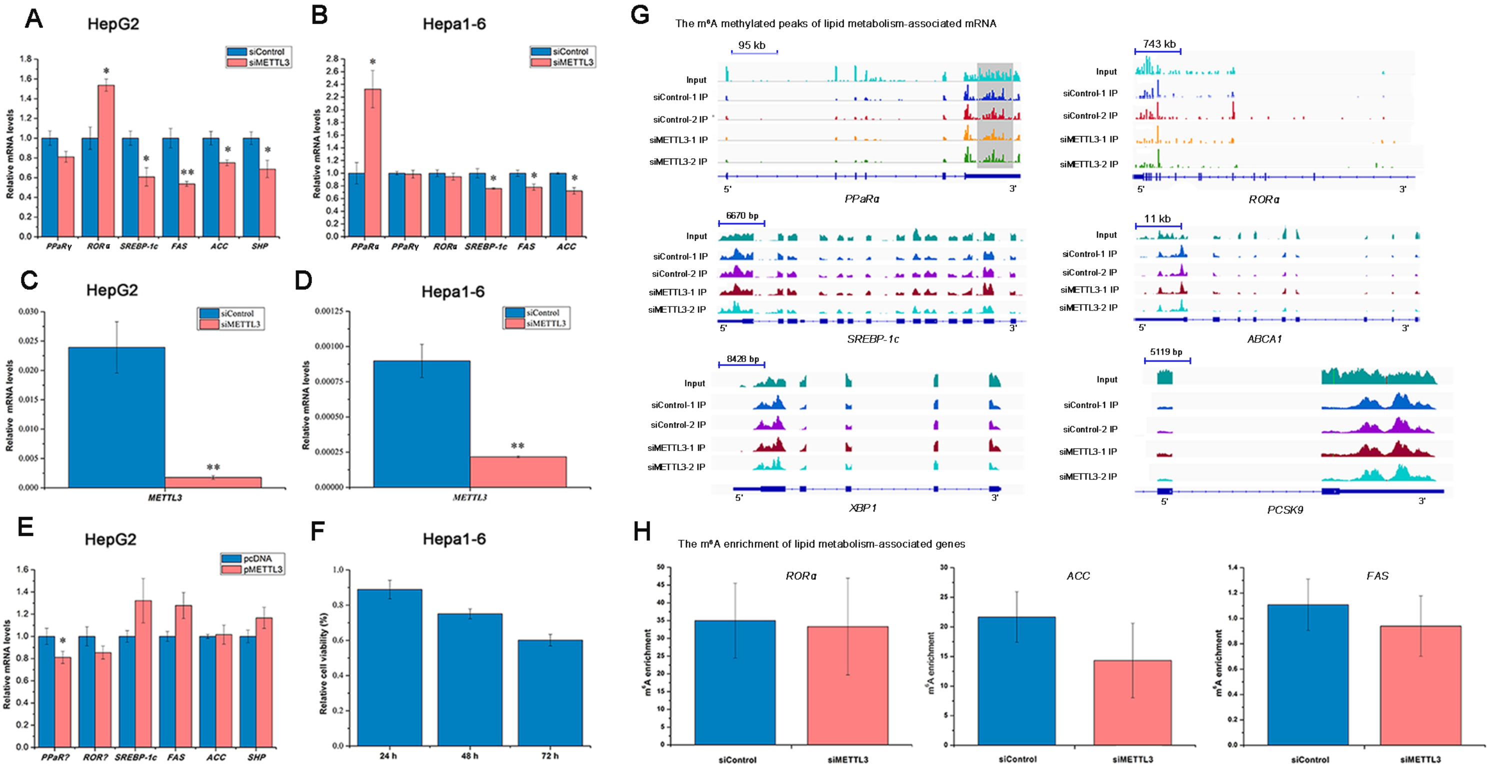
A
HepG2
siControl
siMETTL3
Relative mRNA levels
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
*
*
**
*
*
PPaRγ
RORα
SREBP-1c
FAS
ACC
SHP
B
Hepa1-6
siControl
siMETTL3
Relative mRNA levels
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
*
*
*
*
PPaRα
PPaRγ
RORα
SREBP-1c
FAS
ACC
C
HepG2
siControl
siMETTL3
Relative mRNA levels
0.000
0.005
0.010
0.015
0.020
0.025
0.030
**
METTL3
D
Hepa1-6
siControl
siMETTL3
Relative mRNA levels
0.00000
0.00025
0.00050
0.00075
0.00100
0.00125
**
METTL3
E
HepG2
pcDNA
pMETTL3
Relative mRNA levels
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
*
PPaR?
ROR?
SREBP-1c
FAS
ACC
SHP
F
Hepa1-6
Relative cell viability (%)
0.0
0.2
0.4
0.6
0.8
1.0
24 h
48 h
72 h
G
The m6A methylated peaks of lipid metabolism-associated mRNA
95 kb
Input
siControl-1 IP
siControl-2 IP
siMETTL3-1 IP
siMETTL3-2 IP
5'
3'
PPaRα
743 kb
Input
siControl-1 IP
siControl-2 IP
siMETTL3-1 IP
siMETTL3-2 IP
5'
3'
RORα
6670 bp
Input
siControl-1 IP
siControl-2 IP
siMETTL3-1 IP
siMETTL3-2 IP
5'
3'
SREBP-1c
11 kb
Input
siControl-1 IP
siControl-2 IP
siMETTL3-1 IP
siMETTL3-2 IP
5'
3'
ABCA1
8428 bp
Input
siControl-1 IP
siControl-2 IP
siMETTL3-1 IP
siMETTL3-2 IP
5'
3'
XBP1
5119 bp
Input
siControl-1 IP
siControl-2 IP
siMETTL3-1 IP
siMETTL3-2 IP
5'
3'
PCSK9
H
The m6A enrichment of lipid metabolism-associated genes
RORα
m6A enrichment
0
5
10
15
20
25
30
35
40
45
50
siControl
siMETTL3
ACC
m6A enrichment
0
5
10
15
20
25
30
siControl
siMETTL3
FAS
m6A enrichment
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
siControl
siMETTL3

Figure S3. Lipid metabolism gene expression, m^{6}A methylated peaks and enrichment following knockdown or overexpression of *METTL3.* Related to Figure 3. (**A**) Knockdown of METTL3 changed mRNA expression of genes associated with lipid metabolism. Expression of *ROR*α mRNA was significantly increased, however, expression of *SREBP-1c*, *Fas*, *ACC*, *SHP* mRNA were significantly decreased following siMETTL3 versus siControl in HepG2 cells (*n* = 3). (**B**) METTL3 knockdown significantly up-regulated expression of *PPaR*α mRNA and decreased expression of *SREBP-1c*, *Fas*, *ACC* mRNA in Hepa1-6 cells (*n* = 3). (**C-D**) Expression of *METTL3* mRNA in HepG2 (C) and Hepa1-6 (D) cells after transfection of siMETTL3 versus siControl, respectively. The mRNA expression of *METTL3* in HepG2 cells is significantly higher than in Hepa1-6 cells (*n* = 3). (**E**) Lipid metabolism genes expression following overexpression of METTL3 in HepG2 cells. Difference of *ROR*α, *SREBP-1c*, *Fas*, *ACC*, *SHP* mRNA were not observed by overexpression of METTL3, with the exception of decreased *PPaRγ* mRNA (*n* = 3). (**F**) Knockdown of METTL3 in Hepa1-6 cells reduced the relative cell viability determined by MTT at 24, 48, and 72 h after transfection (*n* = 6). (**G**) Integrative genomics viewer (IGV) plots showing increase of *PPaRα* (A) in m^{6}A methylated peaks, but no difference for, *RORα* (B)*,SREBP-1c* (C)*, ABCA1* (D)*, XBP1* (E)*,* and *PCSK9* (F) mRNA among the siControl IP and siMETTL3 IP. Blue boxes represent exons and blue lines represent introns. *n* = 2. (**H**) The m^{6}A enrichment of *RORα, ACC,* and *FAS* mRNA in HepG2 cells at 48 h post-transfection (*n* = 3). Data are representative of three independent experiments (**A-F, H**). Data are represented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ by two-tailed *t*-test.

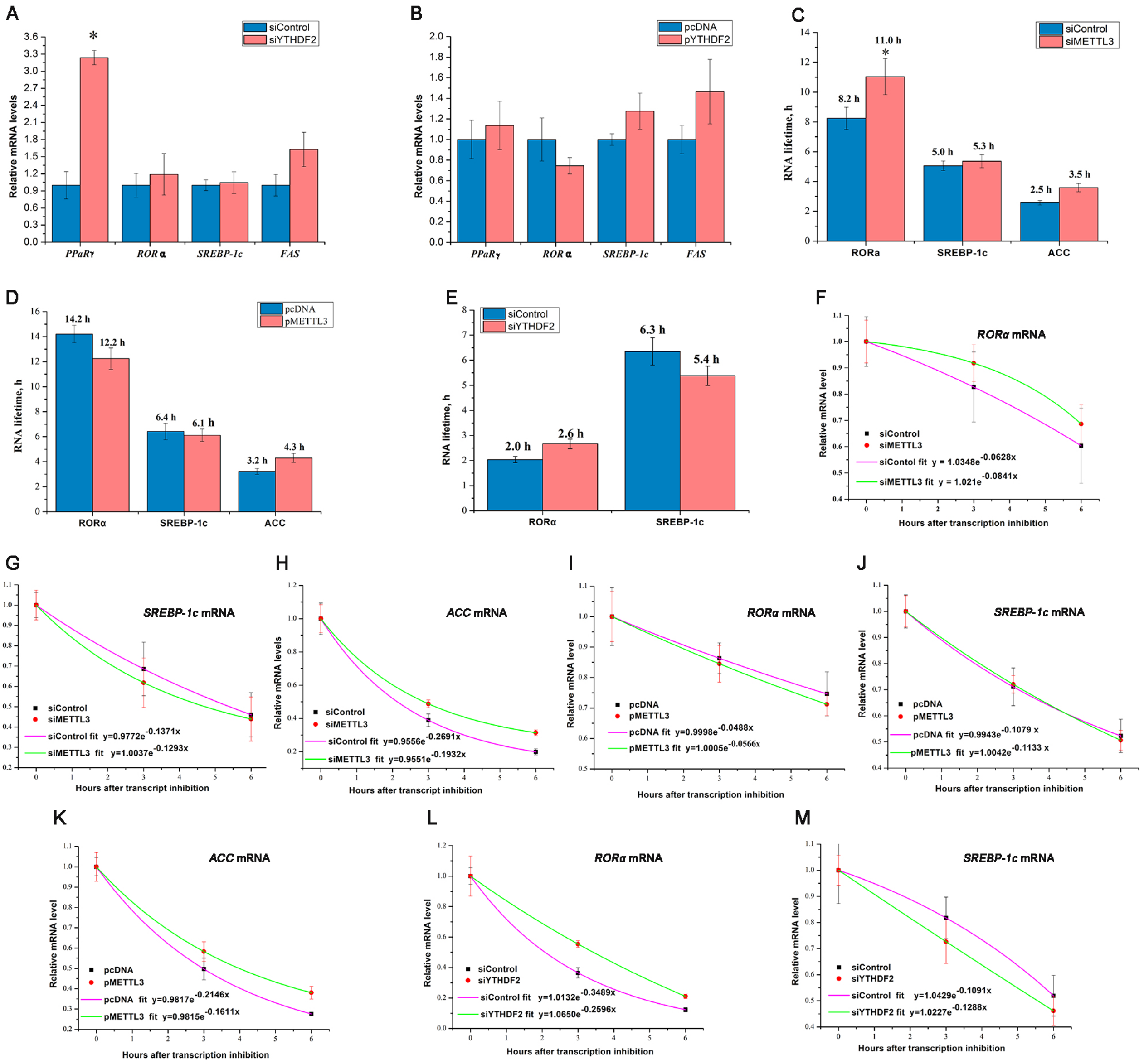
A
Relative mRNA levels
0.0 0.3 0.6 0.9 1.2 1.5 1.8 2.1 2.4 2.7 3.0 3.3 3.6
siControl
siYTHDF2
*
PPaRγ
RORα
SREBP-1c
FAS
B
Relative mRNA levels
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0
pcDNA
pYTHDF2
PPaRγ
RORα
SREBP-1c
FAS
C
RNA lifetime, h
0 2 4 6 8 10 12 14
siControl
siMETTL3
8.2 h
11.0 h
*
5.0 h
5.3 h
2.5 h
3.5 h
RORa
SREBP-1c
ACC
D
RNA lifetime, h
0 2 4 6 8 10 12 14 16
pcDNA
pMETTL3
14.2 h
12.2 h
6.4 h
6.1 h
3.2 h
4.3 h
RORα
SREBP-1c
ACC
E
RNA lifetime, h
0 1 2 3 4 5 6 7 8
siControl
siYTHDF2
2.0 h
2.6 h
6.3 h
5.4 h
RORα
SREBP-1c
F
RORα mRNA
Relative mRNA level
0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1
siControl
siMETTL3
siContol fit y = 1.0348e^-0.0628x
siMETTL3 fit y = 1.021e^-0.0841x
0 1 2 3 4 5 6
Hours after transcription inhibition
G
SREBP-1c mRNA
Relative mRNA level
0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1
siControl
siMETTL3
siControl fit y=0.9772e^-0.1371x
siMETTL3 fit y=1.0037e^-0.1293x
0 1 2 3 4 5 6
Hours after transcript inhibition
H
ACC mRNA
Relative mRNA levels
0.2 0.4 0.6 0.8 1.0 1.2
siControl
siMETTL3
siControl fit y=0.9556e^-0.2691x
siMETTL3 fit y=0.9551e^-0.1932x
0 1 2 3 4 5 6
Hours after transcript inhibition
I
RORα mRNA
Relative mRNA level
0.5 0.6 0.7 0.8 0.9 1.0 1.1
pcDNA
pMETTL3
pcDNA fit y=0.9998e^-0.0488x
pMETTL3 fit y=1.0005e^-0.0566x
0 1 2 3 4 5 6
Hours after transcription inhibition
J
SREBP-1c mRNA
Relative mRNA level
0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1
pcDNA
pMETTL3
pcDNA fit y=0.9943e^-0.1079 x
pMETTL3 fit y=1.0042e^-0.1133 x
0 1 2 3 4 5 6
Hours after transcription inhibition
K
ACC mRNA
Relative mRNA level
0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1
pcDNA
pMETTL3
pcDNA fit y=0.9817e^-0.2146x
pMETTL3 fit y=0.9815e^-0.1611x
0 1 2 3 4 5 6
Hours after transcription inhibition
L
RORα mRNA
Relative mRNA level
0.0 0.2 0.4 0.6 0.8 1.0 1.2
siControl
siYTHDF2
siControl fit y=1.0132e^-0.3489x
siYTHDF2 fit y=1.0650e^-0.2596x
0 1 2 3 4 5 6
Hours after transcription inhibition
M
SREBP-1c mRNA
Relative mRNA level
0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1
siControl
siYTHDF2
siControl fit y=1.0429e^-0.1091x
siYTHDF2 fit y=1.0227e^-0.1288x
0 1 2 3 4 5 6
Hours after transcription inhibition

Figure S4 Lifetimes of nascent mRNAs in HepG2 cells. Related to Figure 4. (**A**) Effect of YTHDF2 knockdown on mRNA expression of genes associated with lipid metabolism in HepG2 cells. YTHDF2 knockdown did not affect *ROR*α, *SREBP-1c*, and *Fas* mRNA expression, but increased expression of *PPaRγ* mRNA in HepG2 cells ($n = 3$). (**B**) Expression of lipid metabolism genes in the sample of pYTHDF2 versus pcDNA in HepG2 cells. We did not observe any differences in *PPaRγ*, *RORα*, *SREBP-1c*, *and Fas* mRNA expression between pYTHDF2 and pcDNA ($n = 3$). (**C-E**) Lifetime of *RORα*, *SREBP-1c*, and *ACC* mRNA in samples following knockdown of *METTL3* (**C**)*,* overexpression of *METTL3* (**D**)*,* and knockdown of *YTHDF2* (**E**) in HepG2 cells, respectively. HepG2 cells were treated with actinomycin for 0 h, 3 h, and 6 h before collection. Knockdown of METTL3 significantly increased the lifetime of *PPaRα* and *RORα* mRNA, but had no impact on the lifetime of *SREBP-1c* and *ACC* mRNA in HepG2 cells. No differences were observed for the lifetime of *RORα*, *SREBP-1c*, and *ACC* mRNA following knockdown of YTHDF2, or overexpression of METTL3 and YTHDF2 ($n = 3$). (**F-M**) Decay of *RORα*, *SREBP-1c*, and *ACC* mRNA in samples following knockdown of *METTL3,* overexpression of *METTL3,* or knockdown of *YTHDF2* in HepG2 cells, respectively ($n = 3$). All data are representative of three independent experiments. Data are represented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ by two-tailed *t*-test.

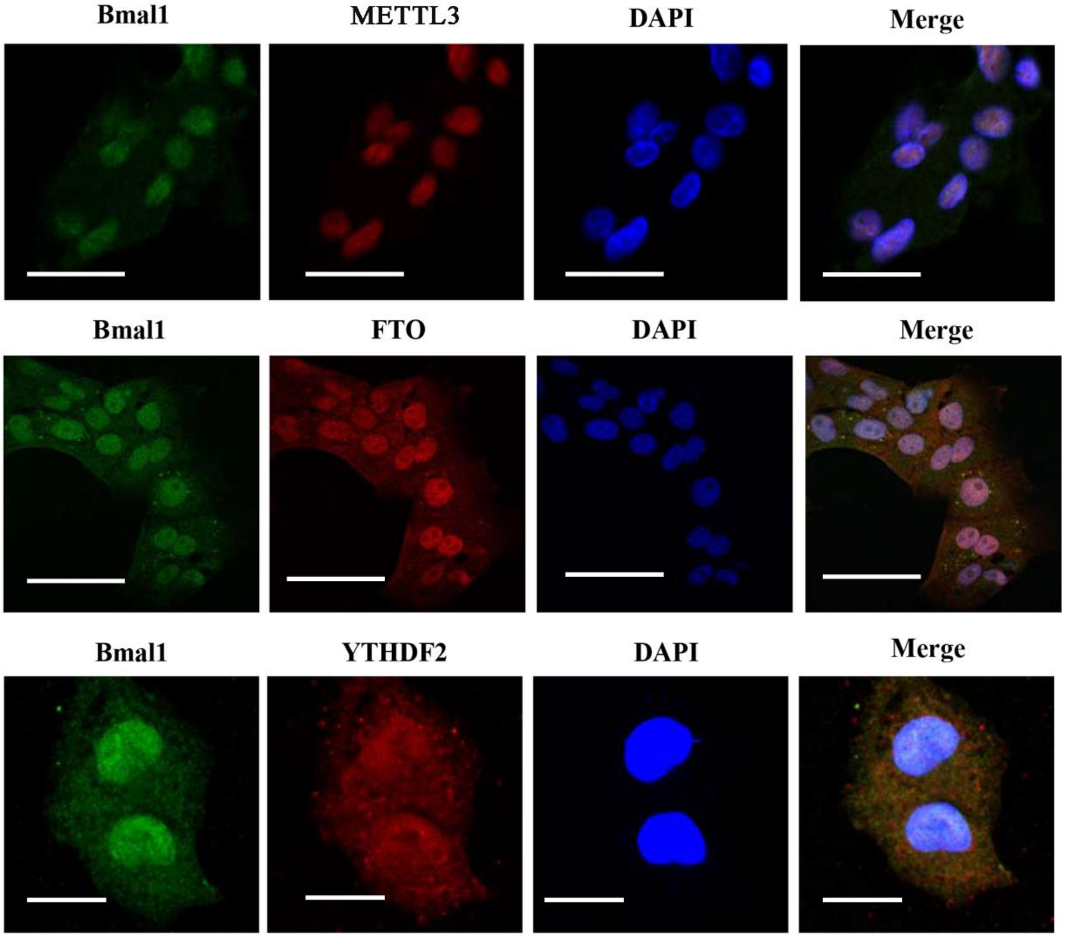

Figure S5. The distribution of METTL3, YTHDF2, FTO, and BMAL1 protein. Related to Figure 6. The distribution of METTL3, YTHDF2, FTO, and BMAL1 proteins were examined by fluorescence immunostaining in HepG2 cells, indicating that METTL3, FTO, and BMAL1 were localized to the nucleus. Conversely, YTHDF2 was localized in both the nucleus and cytoplasm. Scale bar, 70 μm. All data are representative of three independent experiments.

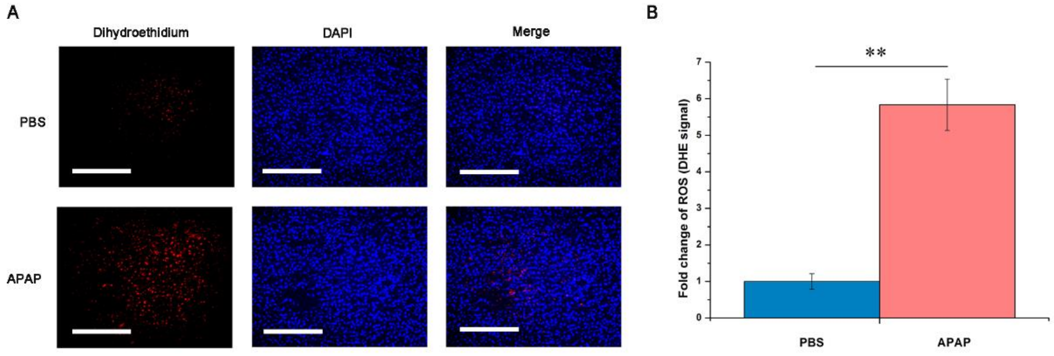

Figure S6. Detection of ROS in mouse liver by dihydroethidium. Related to Figure 6. (**A**) Representative images of ROS detection from dihydroethidium (DHE) stained liver sections from WT mice after acetaminophen (APAP) or PBS injection. Scale bar, 160 µm. (**B**) Quantification of ROS formation was achieved by measuring mean grey values of DHE signal per cell using ImageJ software, $n = 3$ per treatment. All data are representative of three independent experiments. Data are represented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ by two-tailed *t*-test.

Table S1. RNA methylation gene and m^{6}A abundance CircWave cosinor statistical output. Related to Figure 1

Genotype	Gene	F-statistic	P-value	R^2
M^{6}A writer, eraser, reader genes				
WT	*METTL3*	NA	NA	0
WT	*METTL14*	12.9429	0.00017	0.52952
WT	*FTO*	10.4670	0.00059	0.47649
WT	*ALKBH5*	7.55569	0.00300	0.39651
WT	*YTHDF2*	5.47771	0.01133	0.32264
WT	m^{6}A	8.11851	0.00408	0.51980
Bmal1$^{-/-}$	*METTL3*	3.47356	0.04545	0.20465
Bmal1$^{-/-}$	*METTL14*	9.43082	0.00083	0.42044
Bmal1$^{-/-}$	*FTO*	5.28065	0.01160	0.28118
Bmal1$^{-/-}$	*ALKBH5*	NA	NA	0
Bmal1$^{-/-}$	*YTHDF2*	NA	NA	0
Bmal1$^{-/-}$	m^{6}A	NA	NA	0
Lipid metabolism genes				
WT	*PPaRα*	26.92400	9X10-7	0.70071
WT	*PPARγ*	5.88272	0.00864	0.33842
WT	*SHP*	6.38064	0.00625	0.35685
WT	*RORα*	6.58642	0.00134	0.55645
WT	*ACC*	NA	NA	0
WT	*FAS*	NA	NA	0
WT	*FABP4*	NA	NA	0
WT	*FABP1*	5.98018	0.0081	0.34211
WT	*SREBP-1c*	5.18988	0.0138	0.31096
Bmal1$^{-/-}$	*PPARα*	10.8668	0.00034	0.44597
Bmal1$^{-/-}$	*PPARγ*	5.28796	0.01154	0.28145
Bmal1$^{-/-}$	*SHP*	5.30759	0.01138	0.2822
Bmal1$^{-/-}$	*RORα*	NA	NA	0
Bmal1$^{-/-}$	*ACC*	3.6833	0.03851	0.21435
Bmal1$^{-/-}$	*FAS*	18.9886	7.1X10-6	0.58447
Bmal1$^{-/-}$	*FABP4*	NA	NA	0
Bmal1$^{-/-}$	*FABP1*	NA	NA	0
Bmal1$^{-/-}$	*SREBP-1c*	NA	NA	0

NA: Not available, No significant co-sinor pattern detected

Table S2. Primers for qRT-PCR. Related to KEY RESOURCES TABLE.

Gene	Forward	Reverse	Accession Number
Mouse			
METTL3	AGCAGAGCAAGAGACGAATTATC	GGTGGAAAGAGTCGATCAGCA	AF135789
METTL14	CTGAGAGTGCGGATAGCATTG	GAGCAGATGTATCATAGGAAGCC	NM_201638.2
ALKBHF5	CGCGGTCATCAACGACTACC	ATGGGCTTGAACTGGAACTTG	XM_021176347.1
YTHDF2	GAGCAGAGACCAAAAGGTCAAG	CTGTGGGCTCAAGTAAGGTTC	XM_021160169.1
FTO	TTCATGCTGGATGACCTCAATG	GCCAACTGACAGCGTTCTAAG	NM_011936.2
PPARα	TGCAAACTTGGACTTGAACG	AGGAGGACAGCATCGTGAAG	XM_021183376.1
PPARd	CAAGTGGGGTCAGTCATGGAA	GCTGGAAGGAAGCGTGTGTT	U01665
PPARγ	CTGACAGGACTGTGTGAC	TCTGTGTCAACCATGGTAAT	XM_021164280.1
RORα	ACGCCCACCTACAACATCTC	TGCCCATCCATATAGGTGCT	XM_021171874.1
SREBP-1c	GGAGCCATGGATTGCACATT	GGCCCGGGAAGTCACTGT	XM_006532716.2
ACC	GCCTCCGTCAGCTCAGATAC	ATGTGAAAGGCCAAACCATC	XM_021178352.1
FAS	ATCCCAGCACTTCTTGATGG	CCGAAGCCAAATGAGTTGAT	XM_021177126.1
FABP1	CATCCAGAAAGGGAAGGACA	CTTCCCTTCATGCACGATTT	NM_017399.4
FABP4	CTTTGCCACAAGGAAAGTGG	TCCCCATTTACGCTGATGAT	XM_006530048.3
FATP4	ACTGTTCTCCAAGCTAGTGCT	GATGAAGACCCGGATGAAACG	NM_011989.4
SHP	TGGGTCCCAAGGAGTATGC	GCTCCAAGACTTCACACAGTG	NM_011850.3
GAPDH	GGCAAATTCAACGGCACAGT	AGATGGTGATGGGCTTCCC	NM_001289726.1
HPRT1	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG	NM_013556.2
Human			
METTL3	CAAGCTGCACTTCAGACGAA	GCTTGGCGTGTGGTCTTT	NM_019852.4
Continued			

METTL14	AGAAACTTGCAGGGCTTCCT	TCTTCTTCATATGGCAAATTTTCTT	NM_020961.3
ALKBH5	CGGCGAAGGCTACACTTACG	CCACCAGCTTTTGGATCACCA	NM_017758.3
YTHDF1	ACCTGTCCAGCTATTACCCG	TGGTGAGGTATGGAATCGGAG	NM_017798.3
YTHDF2	CCTTAGGTGGAGCCATGATTG	TCTGTGCTACCCAACTTCAGT	NM_001172828.1
FTO	ACTTGGCTCCCTTATCTGACC	GTGCAGTGTGAGAAAGGCTT	NM_001080432.2
PPARα	GGATGCTGGTAGCGTATGGA	GGACGATCTCCACAGCAAAT	NM_005036.4
PPARd	TCTACAATGCCTACCTGAAAAACTTC	ACAATGTCTCGATGTCGTGGATC	NM_001171820.1
RORα	ACTCCTGTCCTCGTCAGAAGA	CATCCCTACGGCAAGGCATTT	NM_002943.3
SREBP-1c	CGCCACCTGCCCCTCTCCTTCC	TGCCCTGCCACCTATCCTCTCACG	NM_004599.3
ACC	ATGTCTGGCTTGCACCTAGTA	CCCCAAAGCGAGTAACAAATTCT	NM_198836.2
FAS	TCGTGGGCTACAGCATGGT	GCCCTCTGAAGTCGAAGAAGAA	NM_004104.4
FABP4	ACTGGGCCAGGAATTTGACG	CTCGTGGAAGTGACGCCTT	NM_001442.2
FATP4	GTGAAGGCAAAGGTGCGAC	CGGAAGGTCCAGTGGGTATC	NM_005094.3
SHP	GTGCCCAGCATACTCAAGAAG	TGGGGTCTGTCTGGCAGTT	NM_021969.2
GAPDH	CGACCACTTTGTCAAGCTCA	AGGGGAGATTCAGTGTGGTG	NM_001289746.1
HPRT1	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	NM_000194.2