SUPPORTING INFORMATION related to

miR-33 controls the expression of biliary transporters, and mediates statin- and diet-induced hepatotoxicity

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SUPPORTING FIGURE LEGENDS

Figure S1. Expression profile of selected hepatic genes following treatment with anti-miR-33 oligonucleotides. C57BL/6 mice (n=5) were injected with saline, or scrambled or anti-miR-33 oligonucleotides (5 mpk i.v., for 3 consecutive days), fed chow for 7 days, and then fasted overnight before sacrifice. Relative hepatic mRNA expression of genes involved in bile metabolism and of two previously described miR-33 targets were determined by real time qPCR. Data are shown as mean ± SD; ** $P < 0.01$

Figure S2. Bile acid secretion and synthesis are decreased in cells overexpressing miR-33. HuH7 cells were seeded in 24-well plates and transduced with empty (open bars) or miR-33 (closed bars) adenovirus. The next morning, cells were pulsed in media supplemented with 0.2% BSA and cyclodextran:cholesterol spiked with [^{14}C]-cholesterol (prepared as described in Christian *et al.*, 1997). The final specific activity in the media was 2 μCi/mL. After 6 h, cells were washed in PBS and chased in fresh media supplemented with 0.2% BSA for 24 or 48 h. Chloroform- and water-soluble radiolabeled sterols (cholesterol and bile acids, respectively) were separated as described in Methods for the RCT experiment. Figure shows data from 8 replicas per condition. ** $P < 0.01$ miR-33 *vs.* empty; ¶ 48 h *vs.* 24 h.

Figure S3. Hepatic expression of selected genes involved in lipid metabolism in mice overexpressing miR-33.

A. C57BL/6 mice (n=5) were transduced with empty or miR-33 encoding adenovirus vectors and then switched to a lithogenic diet, as described in *Fig. 3*. The relative abundance of selected genes was determined by real time qPCR.

B. Potential miR-33 response element in the 3'UTR of mouse and human *SREBP-1*. Asterisks denote complementarity to miR-33.

C. The 3'UTR of mouse *Srebp-1*, or the potential murine miR-33 responsive element shown in panel B were cloned downstream of a luciferase reporter. The effect of miR-33 overexpression on Luc activity was assayed as described in *Fig. 2*. Negative control (empty luc vector) and a positive control (reporter containing a 100% match to miR-33) were also included in these experiments.

D. Nuclear extracts were prepared from the livers of the same mice (as described in Sheng et al, 1995). Pieces from 2 livers were pooled to generate each nuclear extract sample. Fifty μg of total protein were separated by SDS-PAGE and probed with antibodies against the mature SREBP1, and β-actin.

E. C57BL/6 mice (n=5) were transduced with empty or miR-33 encoding adenovirus vectors and kept on a standard chow diet. The relative abundance of selected genes was determined by real time qPCR. Data are shown as mean ± SD; nd, not detected; * $P < 0.05$; ** $P < 0.01$; nd, not detected.

Figure S4. Analysis of mouse and human *ABCG5* and *ABCG8* as potential direct targets of miR-33.

A. Human HepG2 and Huh7 hepatoma cells were transduced with empty or miR-33- encoding adenovirus, and the expression of *ABCG5* and *ABCG8* was measured 48 h later by real time qPCR.

B. The 3'UTR of mouse and human ABCG5 and ABCG8 were amplified from genomic DNA and cloned downstream of a luciferase reporter. The effect of miR-33 overexpression on Luc activity was assayed as described in *Fig. 2*. Negative control (empty luc vector) and a positive control (reporter containing a 100% match to miR-33) were also included in these experiments.

C. Human Huh7 hepatoma cells were transduced with empty or miR-33-encoding adenovirus, and then incubated in the presence or absence of agonists for LXR:RXR [T0901317:9-*cis*-retinoic acid; 1μmol/L each] for 48 h. The protein levels of ABCA1 and ABCG5 were measured by Western blot using 50 μg of total protein extract.

D. Analysis of the 3'UTR of human and mouse *ABCG5* and *ABCG8*. Sequences in red and underlined are partially complementary to the seed sequence of miR-33. Sequences in italics and underlined are polyA signals. Data are shown as mean ± SD; * $P < 0.05$; ** $P < 0.01$.

Figure S5. Simvastatin and lithogenic diet induce liver toxicity.

A. C57BL/6 mice (n=4) were gavaged with simvastatin and kept on chow for a week. The expression of miR-33 was normalized to that of U6 snRNA

B. C57BL/6 mice (n=4) were gavaged with simvastatin and fed a lithogenic diet as described in *Fig. 6*. The expression of miR-33 was normalized to that of U6 snRNA.

C. Daily body weight change (n=6).

D. Daily average food consumption (n=6).

E. Lipidomics analysis of hepatic contents (n=3) was performed by ESI-M. Individual molecular species were quantified by comparisons to the internal standards as described in Methods.

F. Lipidomics analysis of plasma (n=3).

Data are shown as mean ± SD.

Figure S6. Silencing miR-33 rescues the liver damage induced by simvastatin and lithogenic diet. C57BL/6 mice (n=12) were injected with scrambled or anti-miR-33 oligonucleotides, and subsequently dosed with simvastatin and fed a lithogenic diet, as described in *Fig. 7*.

A. Relative expression of miR-33, normalized to U6 snRNA (n=4).

B. Lipidomics analysis of hepatic contents (n=3) was performed by ESI-MS. Individual molecular species were quantified by comparisons to the internal standards as described in Methods.

C. Lipidomics analysis of plasma (n=3).

D. Relative expression of detoxifying genes in the same mice (n=5 for each group of scrambled; n=10 for the anti-miR-33 group). The expression of *Oatp4* and *Car* was significantly higher in the antisense group, compared to both scrambled groups. The expression of most transcripts, with the exception of *Cyp3a11* and *Mrp4,* was significantly decreased in the moribund group.

Data are shown as mean ± SD.

SUPPORTING FIGURES

Figure S1

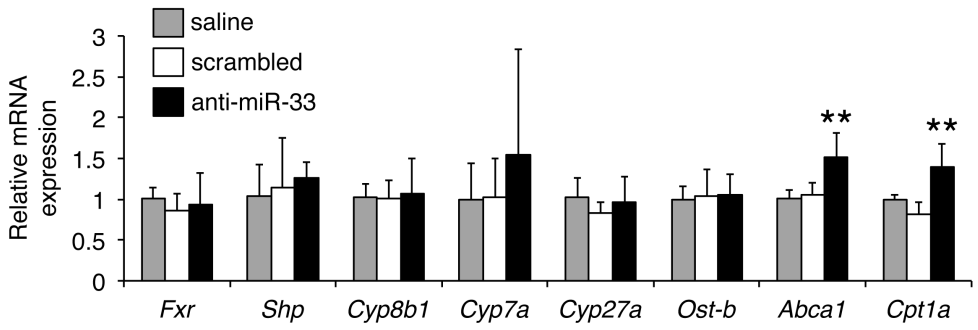
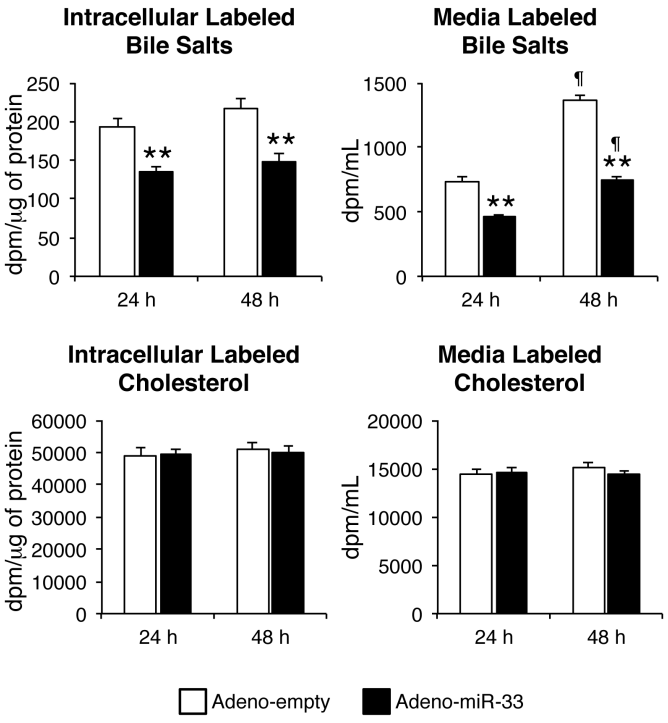


Figure S2


Intracellular Labeled Bile Salts
dpm/μg of protein
250
200
150
100
50
0
**
**
24 h
48 h
Media Labeled Bile Salts
dpm/mL
1500
1000
500
0
¶
¶
**
**
24 h
48 h
Intracellular Labeled Cholesterol
dpm/μg of protein
60000
50000
40000
30000
20000
10000
0
24 h
48 h
Media Labeled Cholesterol
dpm/mL
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10000
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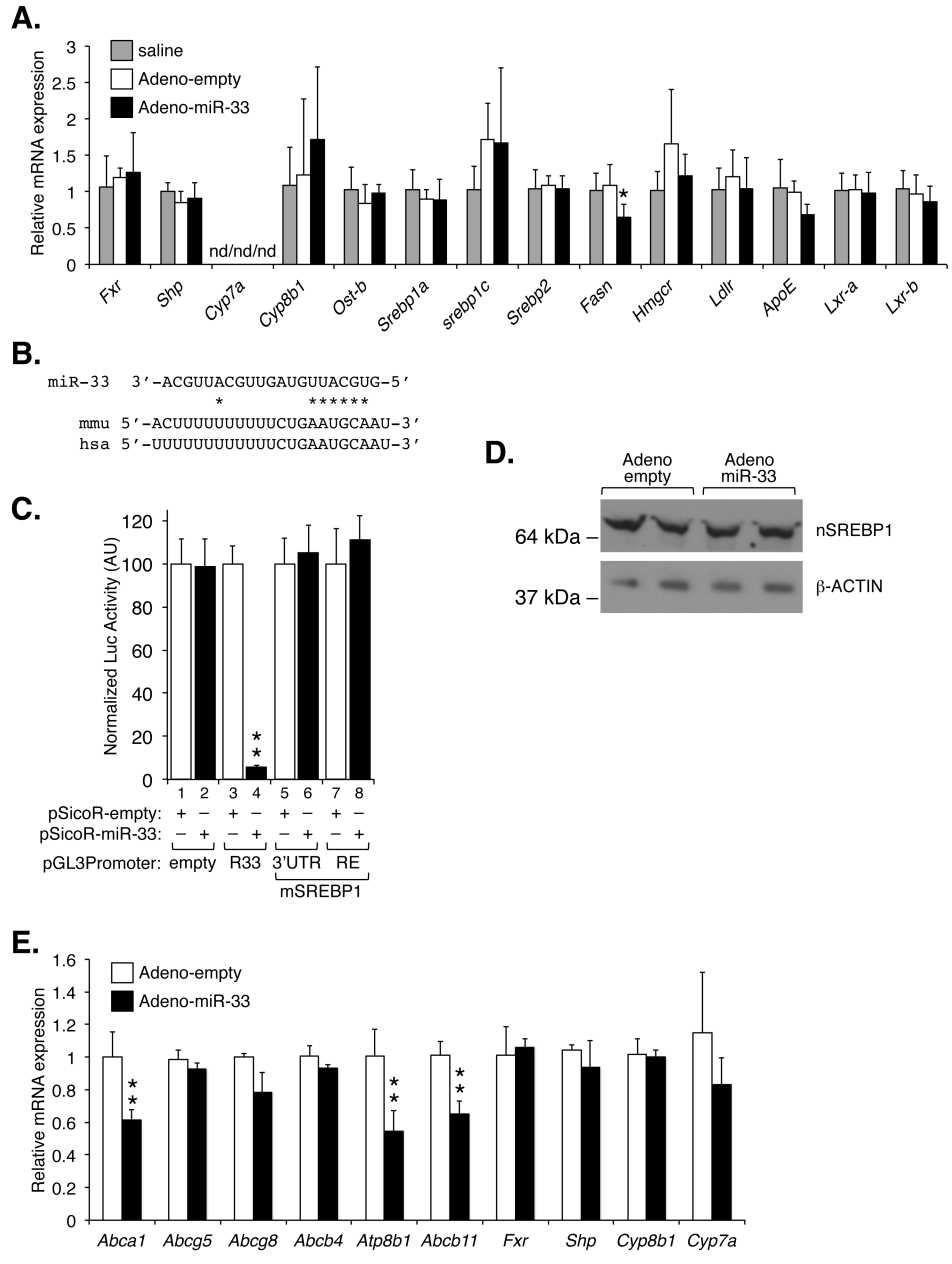
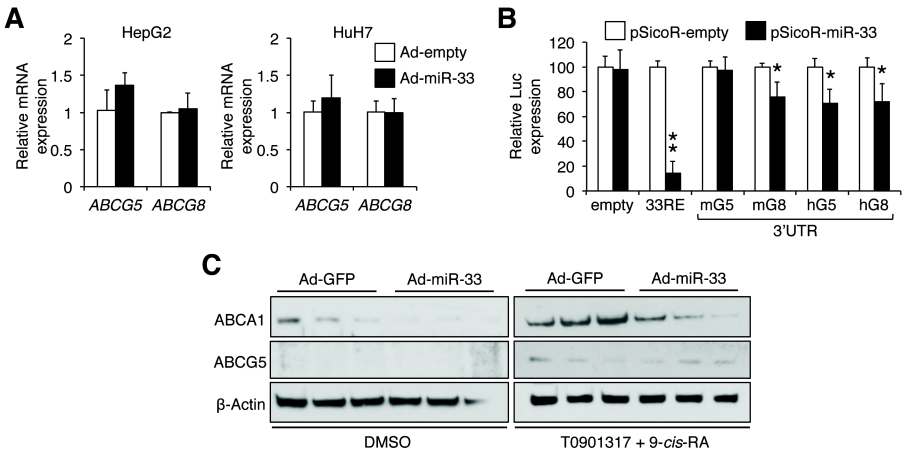
0
24 h
48 h
Adeno-empty
Adeno-miR-33

Figure S3


A.
saline
Adeno-empty
Adeno-miR-33
Relative mRNA expression
nd/nd/nd
Fxr
Shp
Cyp7a
Cyp8b1
Ost-b
Srebp1a
srebp1c
Srebp2
Fasn
Hmgcr
Ldlr
ApoE
Lxr-a
Lxr-b
B.
miR-33 3'-ACGUUACGUUGAUGUUACGUG-5'
mmu 5'-ACUUUUUUUUUUCUGAAUGCAAU-3'
hsa 5'-UUUUUUUUUUUUCUGAAUGCAAU-3'
C.
Normalized Luc Activity (AU)
pSicoR-empty:
pSicoR-miR-33:
pGL3Promoter: empty R33 3'UTR RE
mSREBP1
D.
Adeno empty
Adeno miR-33
64 kDa –
37 kDa –
nSREBP1
β-ACTIN
E.
Adeno-empty
Adeno-miR-33
Relative mRNA expression
Abca1
Abcg5
Abcg8
Abcb4
Atp8b1
Abcb11
Fxr
Shp
Cyp8b1
Cyp7a

S

Figure S4



D

```
                    miR-33: 3'-ACGUUACGUUGAUGUUACGUG-5'
                                                ||||||
          seed target: 5'-NNNNNNNNNNNNNCAAUGCAN-3'
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hsa *ABCG5* 3'UTR:

aagccauggcuggga**aAAUGgA**agugaagcugccgacugugcaugacugcucugaacgucugaaaugagagugccauguauuucuuucuugacaggacaucucaagucuuuuaaccau
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gauuuuacugagg*auuuaaauaaa*aaaccguaggaauggcucaacaguga

mmu *Abcg5* 3'UTR:

guuaagaugacaggcaggaaaggguuaauggcagcacgcccacuguggagcacagagaaguacugcuacaaccaucaggauuccaucugcgacccuugugucugacccuugugc
uauccggagccccaagggcaacgagaacucacagcccucugcuauuccagcuugugggggcaauguggugcuuggacauuugacugaacuggucca**uAAUGuA***aauaauaauaaau*uc
auaaaccuacaggacauua

hsa *ABCG8* 3'UTR:

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mmu *Abcg8* 3'UTR:

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Figure S5

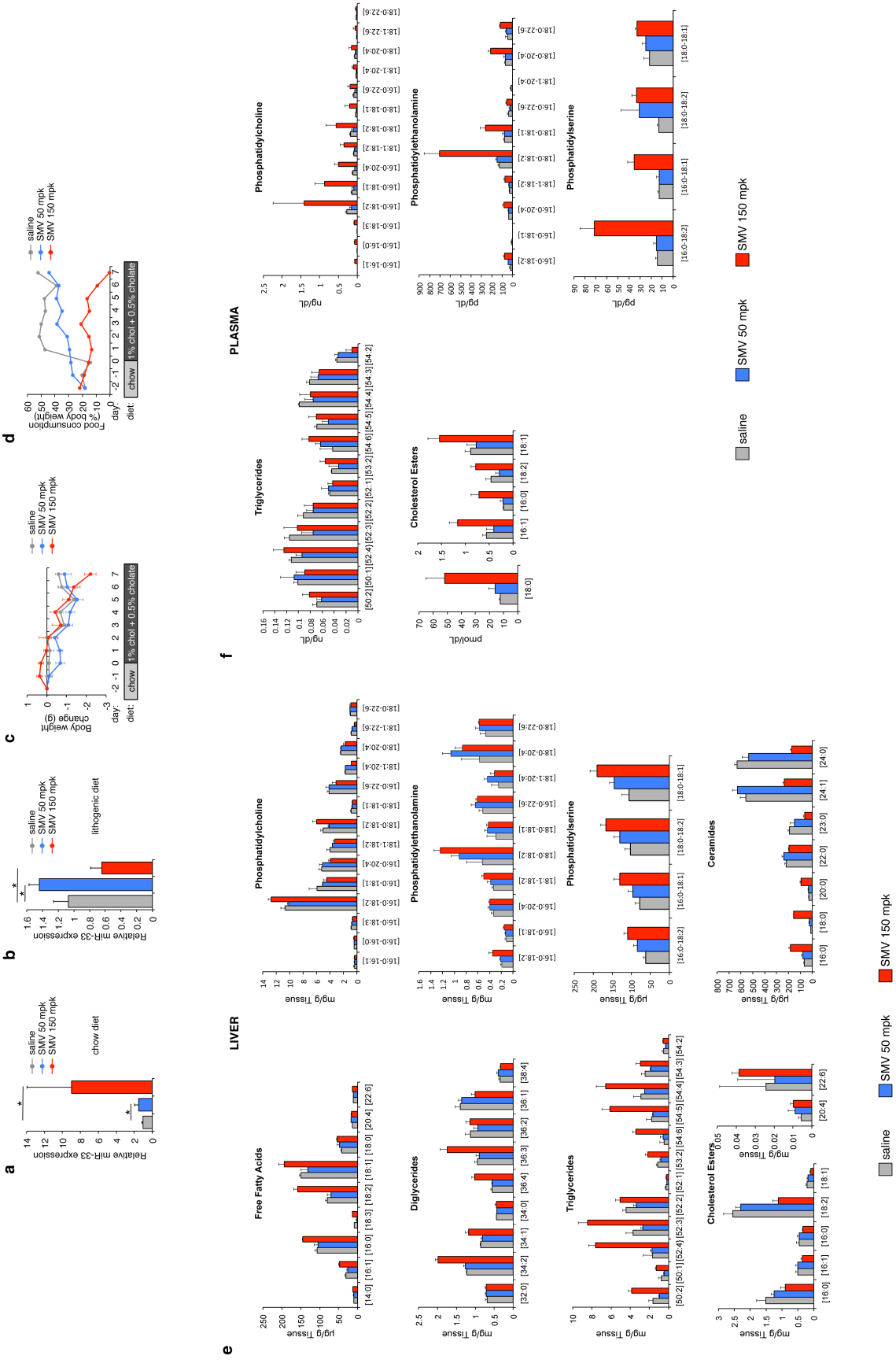
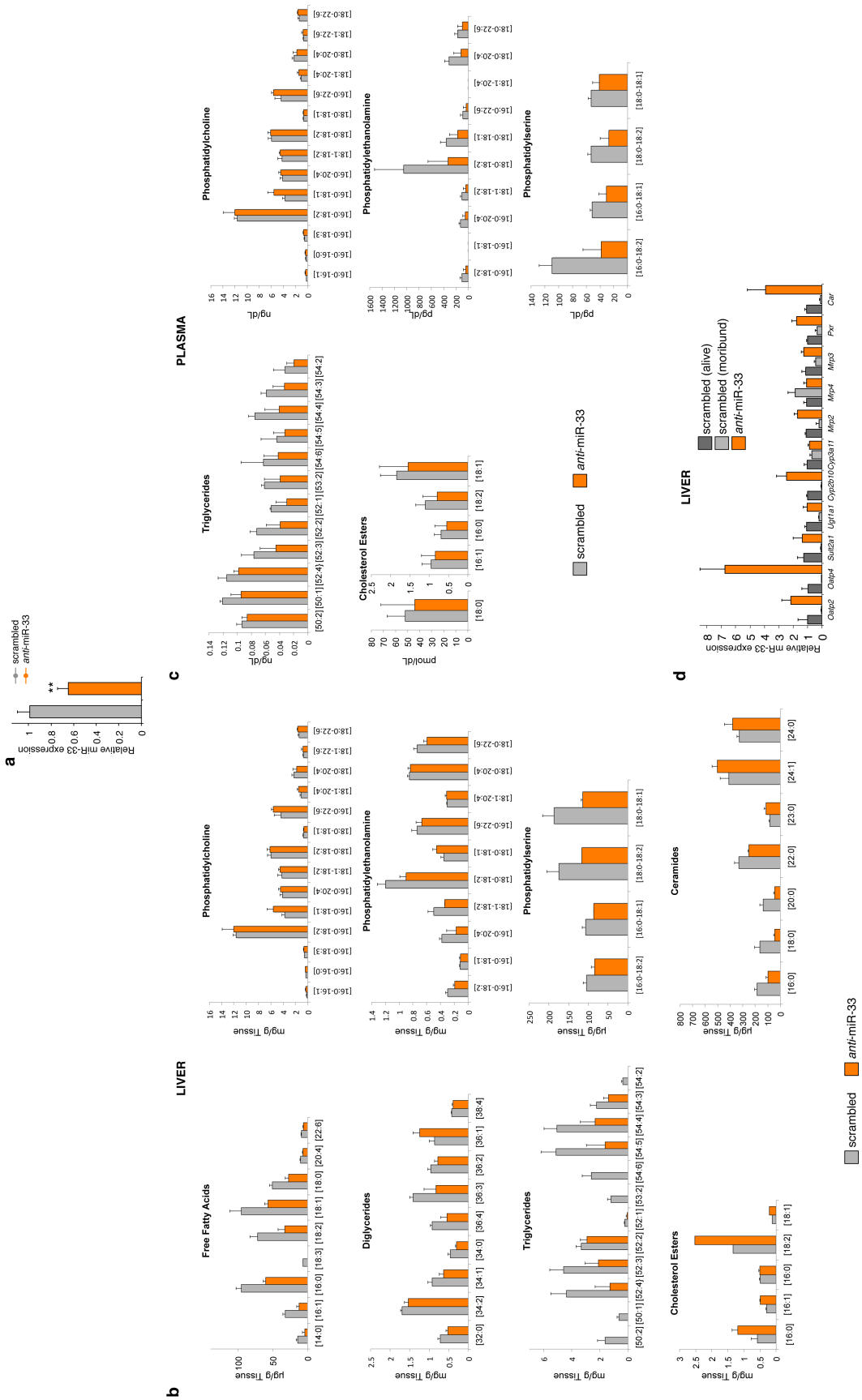

a
b
c
d
e
f
saline
SMV 50 mpk
SMV 150 mpk
chow diet
lithogenic diet
Relative miR-33 expression
Body weight change (g)
Food consumption (% body weight)
day:
diet:
chow
1% chol + 0.5% cholate
LIVER
PLASMA
Free Fatty Acids
Diglycerides
Triglycerides
Cholesterol Esters
Phosphatidylcholine
Phosphatidylethanolamine
Phosphatidylserine
Ceramides
Cholesterol Esters
μg/g Tissue
mg/g Tissue
ng/dL
pg/dL
pmol/dL

Figure S6


a
Relative miR-33 expression
scrambled
anti-miR-33
**
b
LIVER
Free Fatty Acids
Diglycerides
Triglycerides
Cholesterol Esters
Phosphatidylcholine
Phosphatidylethanolamine
Phosphatidylserine
Ceramides
c
PLASMA
Phosphatidylcholine
Phosphatidylethanolamine
Phosphatidylserine
Triglycerides
Cholesterol Esters
d
LIVER
scrambled (alive)
scrambled (moribund)
anti-miR-33
Relative miR-33 expression

SUPPORTING REFERENCES

Christian AE, Haynes MP, Phillips MC, Rothblat GH (1997) Use of cyclodextrins for manipulating cellular cholesterol content. J Lipid Res 38:2264-2272.

Sheng Z, Otani H, Brown MS, Goldstein JL (1995) Independent regulation of sterol regulatory element-binding proteins 1 and 2 in hamster liver. Proc. Natl. Acad. Sci. USA 92: 935-938.