Feedback repression of PPARα signaling by Let-7 microRNA

Highlights

- PPARα activation represses let-7 microRNA expression
- Let-7 microRNA promotes decay of Rnf8 mRNA and loss of RNF8 protein
- RNF8 promotes RXRα protein degradation
- Let-7-RNF8-RXRα axis controls hepatic lipid metabolism through a negative feedback loop

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In brief

Yagai et al. identify a negative feedback loop involving PPARα/RXRα control of hepatic lipid metabolism. The study demonstrates let-7 microRNA repression by PPARα activation, RNF8 mRNA and protein decay by let-7 microRNA, and RXRα protein degradation by RNF8 E3 ubiquitin ligase.
Feedback repression of PPARα signaling by Let-7 microRNA

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This study highlights a let-7-RNF8-RXR regulatory axis that modulates hepatic lipid catabolism.

SUMMARY

Peroxisome proliferator-activated receptor α (PPARα) controls hepatic lipid homeostasis and is the target of lipid-lowering fibrate drugs. PPARα activation represses expression of let-7 microRNA (miRNA), but the function of let-7 in PPARα signaling and lipid metabolism is unknown. In the current study, a hepatocyte-specific let-7b/c2 knockout (let7b/c2<sup>Hep</sup>) mouse line is generated, and these mice are found to exhibit pronounced resistance to diet-induced obesity and fatty liver. Let-7 inhibition by hepatocyte-specific let-7 sponge expression shows similar phenotypes as let7b/c2<sup>Hep</sup> mice. RNA sequencing (RNA-seq) analysis reveals that hepatic PPARα signaling is repressed in let7b/c2<sup>Hep</sup> mice. Protein expression of the obligate PPARα heterodimer partner retinoid X receptor α (RXRα) is reduced in the livers of let7b/c2<sup>Hep</sup> mice. Ring finger protein 8 (Rnf8), which is a direct target of let-7, is elevated in let7b/c2<sup>Hep</sup> mouse liver and identified as a E3 ubiquitin ligase for RXRα. This study highlights a let-7-RNF8-RXRα regulatory axis that modulates hepatic lipid catabolism.

INTRODUCTION

Let-7 microRNA (miRNA), which is one of the first miRNAs discovered, plays significant roles in embryogenesis, development, metabolism, and oncogenesis (Büssing et al., 2008; Jovanovic and Hengartner, 2006; Schickel et al., 2008; Stefan and Slack, 2008). Let-7c belongs to the let-7 family, with the mature sequence being highly similar among the family members and sharing the same target messenger RNAs (mRNAs). A previous study revealed that mature let-7c and its primary transcript, long non-coding RNA (lncRNA) AK033222 (also known as Mir99-ahg), were potently and rapidly repressed by activation of hepatic peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor that predominantly modulates lipid metabolism (Shah et al., 2007). Let-7 miRNA potentiates the decay of mRNAs and inhibits protein translation related to cell proliferation, cell differentiation, immune response, and glucose metabolism (Johnson et al., 2005; Liu et al., 2011; Mayr et al., 2007; Schulte et al., 2011; Zhu et al., 2011). Whole-body let-7 inhibition in transgenic mice expressing Lin28a or Lin28b results in resistance to hepatic steatosis and obesity (Zhu et al., 2011), and glucose metabolism is also partially improved by a global let-7 inhibitor (Frost and Olson, 2011), suggesting a role for let-7 in modulating glycolipid metabolism. However, how hepatic let-7 modulates lipid metabolism remains unknown.

In this study, PPARα activation by synthetic Wy-14,643 or endogenous ligands was found to suppress expression of the let-7 family, an effect dependent on hepatic PPARα. The biological effects of hepatic let-7 on hepatic lipid metabolism were further analyzed by use of both hepatocyte-specific let-7b/c2 knockout (let7b/c2<sup>Hep</sup>) mice and hepatocyte-specific let-7 sponge-mediated let-7 inhibition. Hepatic let-7 deficiency prevented hepatic steatosis and obesity induced by high-fat diet (HFD) feeding accompanied by inhibition of the PPARα signaling. Further analyses revealed that RXRα protein levels were decreased in let-7-7-disrupted hepatocytes. Ring finger protein 8 (Rnf8) was identified as a E3 ubiquitin ligase for RXRα, and Rnf8 mRNA was revealed as a direct target of let-7. These data demonstrate that hepatic let-7 deficiency improves hepatic steatosis during obesity by the RNF8-RXRα axis, suggesting a let-7-RNF8-RXRα axis that may act as a negative feedback loop to attenuate PPARα-mediated lipid-modulating signaling.

RESULTS

Expression of let-7 miRNA in response to PPARα activation

The Let-7 miRNA family consists of 9 mature miRNAs processed from 12 precursors and 8 primary transcripts (Table S1). Although the mature let-7-5p sequence is highly similar among...
other let-7 family members, the precursors have unique sequences in their terminal loop and 3p region. To determine the effects of PPARα activation on individual let-7 miRNAs, the hepatic levels of the let-7 miRNA precursors were measured in wild-type (Ppara<sup>+/+</sup>) and hepatocyte-specific Ppara knockout (Ppara<sup>ΔHep</sup>) mice. Wy-14,643 as a PPARα-specific agonist was used to induce hepatic PPARα activation. In response to Wy-14,643 administration, pre-let-7a-1, a-2, b, c-1, c-2, e, f-1, f-2, g, and mirR-98 were significantly decreased, whereas pre-let-7d and pre-let-7i tended to decrease without significance in wild-type mice but not in Ppara<sup>ΔHep</sup> mice (Figure 1A). The total pooled let-7 precursor abundance was decreased by half after Wy-14643 treatment in livers of Ppara<sup>+/+</sup> mice but not Ppara<sup>ΔHep</sup> mice (Figure 1B). To analyze the effect of PPARα activation by endogenous ligands, mice were fasted for 24 h, which leads to increased PPARα signaling resulting from increased endogenous metabolites that are PPARα agonists (Kersten et al., 1999). Hepatic pre-let-7a-1, b, d, f-1, and f-2 were significantly decreased in fasted wild-type mice but not in Ppara<sup>ΔHep</sup> mice (Figure 1C), a phenotype not observed in fed mice. The total pooled let-7 precursor abundance was decreased by approximately 40% after fasting (Figure 1D). These data demonstrate that PPARα activation by either a chemical agonist or endogenous fatty acid induces a decrease of let-7 expression in a hepatocyte PPARα-dependent manner.

A time course analysis of hepatic mature let-7 expression after PPARα activation further revealed that mature let-7 family’s transcripts started to decrease by 12 h after Wy-14,643 administration. All let-7 family members were significantly repressed within 24 h (Figure 1E). Previous studies revealed that RNA-binding proteins Lin28a and Lin28b were highly expressed during embryogenesis and upregulated in some cancers to selectively block the maturation of let-7. Lin28 selectively binds the terminal loop region of let-7 precursors and inhibits miRNA processing (Piskounova et al., 2008). To explore whether Lin28 plays a role in the regulation of let-7 during PPARα activation, hepatic Lin28a and Lin28b were quantified in response to Wy-14643 treatment. However, Lin28a was not changed by Wy-14643 treatment, with Lin28b undetectable in the liver (Figures S1A and S1B).

**Hepatocyte-specific let-7b/c2 knockout results in resistance to obesity**

To further analyze the physiological roles of hepatic let-7, hepatocyte-specific let-7b/c2 knockout (let7b/c2<sup>ΔHep</sup>) mice were generated by mating Alb-Cre (Yakar et al., 1999) and let-7b/c2 floxed (Madison et al., 2013) mouse lines. Let7b/c2<sup>ΔHep</sup> mice showed normal development and fertility, with hepatic Ppara<sup>+/+</sup> and hepatocyte-specific Let-7 sponge expression results in resistance to obesity

miRNA sponges are a well-recognized method to perform loss-of-function analyses of miRNAs in vivo and in vitro (Ebert and Sharp, 2010). Sponges RNA has complementary binding sites for a given miRNA and is degraded instead of the target mRNAs as a decoy, repressing miRNA activity. To further analyze the role of hepatic let-7 in obesity and hepatic steatosis, an adeno-associated virus 8 (AAV8) designated for hepatocyte-specific expression of let-7 sponge was constructed (let-7 sponge) (Figures S3A and S3B). Primary hepatocytes were transduced with AAV-EFGR, and 5 days after infection, a pronounced EGFP expression was observed, indicating successful infection of cells by AAV. Conversely, let-7 sponge AAV-infected hepatocytes exhibited very low EGFP fluorescence, suggesting that let-7 miRNA was abundantly expressed in hepatocytes (Figure 2H). EGFP DNA quantification confirmed that the infection efficiency of let-7 sponge expressing AAV was equivalent with AAV-EFGR control (Figure S3C). To inhibit let-7 activity in vivo, the recombinant AAV vectors were intravenously injected to wild-type mice followed by HFD feeding for 8 weeks. Let-7 sponge AAV-injected mice showed less body weight gains starting from 3 weeks after HFD feeding than the EGFP AAV group (Figure 2L). Lipid accumulation was decreased in let-7 sponge AAV-treated liver (Figure S3D). The let-7 sponge group did not show significant differences in lean body weight ratio and liver-body weight ratio (Figures 2J and 2L), whereas the fat-weight-to-body-weight ratio was decreased (Figure 2K). Serum and hepatic TG were also significantly decreased (Figures 2M and S3F), whereas serum and liver-body weight ratios were significantly lower (Figures 2E and 2F). Serum triglyceride (TG) and total cholesterol (TC) were also markedly decreased in let7b/c2<sup>ΔHep</sup> mice (Figures 2G and S1F). These data indicated that let7b/c2<sup>ΔHep</sup> mice are resistant to HFD-induced obesity.

In addition, serum alanine aminotransferase (ALT) was decreased in let7b/c2<sup>ΔHep</sup> mice, suggesting less hepatotoxicity from HFD feeding (Figure S1G). Although both hepatic TG and TC were significantly decreased (Figures S1H and S1I), non-esterified fatty acids (NEFAs) were increased in let7b/c2<sup>ΔHep</sup> mice (Figure S1J). These data suggested that let7b/c2<sup>ΔHep</sup> mice had lower constitutive hepatic fatty acid esterification and/or lipid synthesis, whereas fatty acid intake was not changed by the loss of hepatic let-7b/c2. In normal chow-diet-fed mice, no significant differences were found in body weight, serum TG, or hepatic TC and TG (Figures S2A, S2B, S2D, and S2E) between the two genotypes, whereas serum TC was slightly decreased in let7b/c2<sup>ΔHep</sup> mice (Figures S2C). Previous studies showed that whole-body let-7 miRNA inhibition modulated glucose metabolism (Frost and Olson, 2011; Zhu et al., 2011). To determine whether these phenotypes were derived from glucose metabolism, insulin, glucose, and pyruvate tolerance tests (ITT, GTT, and PTT, respectively) were performed and no significant differences were found between let7b/c2<sup>+/+</sup> and let7b/c2<sup>ΔHep</sup> mice (Figures S2F, S2G, and S2H). These data suggest that hepatic let7b/c2 disruption improves fatty liver and attenuates obesity during HFD feeding, whereas hepatocyte let7b/c2 disruption does not affect insulin sensitivity.
TC, hepatic TC, and NEFA did not show significant alterations (Figures S3E, S3G, and S3H). ITT and GTT did not show significant differences (Figures S3I and S3J). Consistent with the phenotype observed in let7b/c2 ΔHep mice, these data indicated that inhibition of mature let-7 activity resulted in lipid synthesis reduction and obesity resistance.

**PPARα target gene mRNAs are lower in let7b/c2 ΔHep livers**

To identify potential mechanisms contributing to the observed phenotypes mediated by hepatic let-7 deletion, RNA sequencing (RNA-seq) was carried out to analyze the gene expression profiles in livers of let7b/c2 ΔHep mice, these data indicated that inhibition of mature let-7 activity resulted in lipid synthesis reduction and obesity resistance.

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**RXRα protein is reduced by Let-7 miRNA inhibition**

Nuclear receptors, including the PPAR family and FXR, form obligate heterodimers with RXRα. Pathway analysis revealed that PPARα, β/δ, and farnesoid X receptor (FXR) pathways were robustly inhibited in the let7b/c2 ΔHep livers (Figures 3A and S1K; Table S2). These are all ligand-activated nuclear receptors that heterodimerize with RXRα. mRNA-encoding proteins involved in fatty acid oxidation, cell proliferation (Figure 3B), lipid accumulation, and glucose metabolism (Figure 3C) were all found to be decreased by hepatic let-7 depletion. Western blot analysis revealed a significant decrease in expression of PPARα target gene products, including cytochrome P450 ω-hydroxylase 4A (CYP4A); enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase (EH-HADH); hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, α subunit (HADHA); and keratin, type I cytoskeletal 23 (KRT23) in let7b/c2 ΔHep livers (Figure 3D). Several PPARα target genes were repressed even in 1-week HFD-fed livers when the body weight was not altered between let7b/c2 ΔHep and let7b/c2 ΔHep mice (Figure S2I), indicating the inhibition of PPAR signaling was independent of body weight change.
binding to their respective responsive elements in the enhancer/promoter regions of target genes (Rigano et al., 2017). Given that PPARs, β3, γ, and FXR pathways were significantly repressed, it is reasonable to speculate whether their common heterodimer RXR is regulated by let-7. No significant differences in Rara or Ppara mRNA levels were found between let7b/c2+/+ and let7b/c2ΔHep livers (Figures S4A and S4B), whereas the RXRa protein in let7b/c2ΔHep livers was significantly decreased compared with let7b/c2+/+ livers (Figure 3F). Then, freshly isolated let7b/c2+/+ and let7b/c2ΔHep primary hepatocytes were subjected to RNA and protein analyses, revealing that the RXRa protein but not the Rxa mRNA was decreased in let7b/c2ΔHep hepatocytes (Figures 3E and S4C). Consistently, forced expression of let-7 sponge resulted in a decrease in the RXRa protein but not Rxa mRNA (Figures 3G and S4D). A previous study revealed that RXRa inhibition resulted in obesity and amelioration of hepatic steatosis (Yamauchi et al., 2001). An RXRa inhibition experiment was performed in primary hepatocytes by use of the RXRa inhibitor HX-531. Lipid accumulation induced by palmitic acid treatment was repressed by HX-531 administration.
in hepatocytes (Figure S4E). Western blot analysis of RXRα in the livers of mice fed on chow or HFD and treated with vehicle or Wy-14643 demonstrated that PPARα activation resulted in lower RXRα protein levels under both chow diet and HFD (Figure S4F). These results indicate that let-7 positively modulates the RXRα protein but not Rxra mRNA levels, which may contribute to less hepatic lipid accumulation in livers of let-7-deficient mice.

**Rnf8 mRNA is a let-7 miRNA direct target**

The ubiquitin-proteasome system is a multi-step process that regulates protein stability and involves enzymes of three different classes. Ubiquitin is activated by the E1 enzyme, and then the E2 enzyme transfers ubiquitin to lysine residues in the target protein. The E3 enzyme binds with the target protein and mediates ubiquitin binding. The polyubiquitinated proteins are then degraded by the 26S proteasome (Glickman and Ciechanover, 2002). Recent studies demonstrated that Rnf8 encoded an E3 ubiquitin ligase targeting various proteins (Fritsch et al., 2014; Lee et al., 2016; Paul and Wang, 2017). Previous research revealed that RNF8 bound to RXRα in the nucleus (Takano et al., 2004). Because let7b/c2Dhep mice exhibited a decrease in the RXRα protein but not Rxra mRNA, the question arises whether the RXRα protein is degraded by the ubiquitin-proteasome system.

To determine whether Rnf8 is a direct target of let-7 miRNA, the sequence of Rnf8 and the densitometric quantification in whole-liver lysates from let7b/c2Dhep mice (F), let-7 sponge expressing AAVinfected mice (G), and pre-let-7c-1-–AAVinfected mice (H).

Figure 3. PPARα target gene expressions were repressed by RXRα protein reduction in let7b/c2Dhep and let-7 sponge AAV-transduced mice

(A) Heatmap of PPARα target genes identified by differential gene expression analysis of RNA-seq data from let7b/c2Dhep and let7b/c2Dhep livers after HFD feeding. (B and C) mRNA analysis by qRT-PCR of PPARα target genes involved in fatty acid oxidation and cell proliferation (B) and lipid accumulation and glucose metabolism (C) in HFD-fed let7b/c2Dhep and let7b/c2Dhep livers.

(D) Western blot analysis for PPARα target genes in HFD-fed let7b/c2Dhep and let7b/c2Dhep liver lysates.

(E) Western blot analysis of PPARα and RXRα protein expression in nuclear fractions isolated from let7b/c2Dhep and let7b/c2Dhep hepatocytes.

(F–H) Western blot analysis of RXRα and the densitometric quantification in whole-liver lysates from let7b/c2Dhep mice (F), let-7 sponge expressing AAVinfected mice (G), and pre-let-7c-1-–AAVinfected mice (H).

Data are presented as mean ± SEM (n = 4–5 mice per group; *p < 0.05, **p < 0.01, ***p < 0.001)
sequence, and site 2 is in the 3’ UTR (Figure 4A). Western blot analyses for the RNF8 protein were performed with an antibody verified with Rnf8-overexpressed cell lysates (Figure S4J). Western blot and qRT-PCR analyses revealed that the RNF8 protein and Rnf8 mRNA were significantly increased in let7b/c2/Hep/livers respectively (Figures 4B and 4C). In contrast, pre-let-7c-1-expressing mouse livers showed a significant decrease of the RNF8 protein (Figure 4F). The Rnf8 mRNA showed a similar tendency without statistical significance (Figure S4G), suggesting that translational inhibition by let-7 miRNA contributed to the RNF8 protein decrease more than mRNA decay. To assess whether Rnf8 mRNA is a direct target of let-7 miRNA, 3’ UTR reporter assays were performed. The wild-type Rnf8 3’ UTR including a predicted let-7 binding site was cloned into a luciferase reporter vector, and a mutant vector lacking a let-7 binding site candidate was also constructed. Using wild-type Rnf8 3’ UTR, luciferase activity was significantly decreased by co-transfection with a let-7c mimic in HepG2 cells. Conversely, luciferase activity was unchanged with the mutated vector (Figure 4G). These data indicated that Rnf8 mRNA was a direct target of let-7 miRNA.

RNF8 is a E3 ubiquitin liganse for the RXR protein

To analyze whether RNF8 is involved in RXR ubiquitination in hepatic cells, RNF8 and RXR expression vectors were co-transfected into Hepa-1c1c7 (Hepa-1) cells. The cell lysates were subjected to western blot analysis, revealing that the RXR protein was significantly decreased when RNF8 was co-transfected (Figures 4H and 4I). Rnf8 mRNA was increased by more than 4,000-fold compared with the control expression vector transfection group (Figure 4J). Co-transfection with Rnf8 had no impact on increases in Rnra mRNA (Figure 4K). These results suggested that RNF8 expression did not affect Rnra mRNA levels but decreased RXR protein levels in vitro.

To determine whether the decrease of the RXR protein was caused by the ubiquitin-proteasome pathway, Hepa-1 cells transfected with RNF8 and RXR expression vectors were treated with the proteasome inhibitor MG-132. The MG-132-treated group showed a 1.4-fold increase of the RXR protein, whereas only a small increase was noted without RNF8 (Figures 4L and S4H). Given that RNF8 is a E3 ubiquitin liganse for RXR, the RXR protein should be polyubiquitinated in Hepa-1 cells. Hepa-1 cells transfected with an RXR expression vector together with or without an RNF8 expression vector were subjected to co-immunoprecipitation (coIP) assays to detect polyubiquitinated RXR. Western blot analysis of the immunoprecipitated samples revealed that cells transfected with RXR and RNF8 expression vectors contained significantly more polyubiquitinated RXR than the non-RNF8 expressing cells (Figure 4M). K48 polyubiquitin is a polyubiquitin chain contributing to protein degradation in the ubiquitin/proteasome pathway. Western blot analysis for RNF8- and RXR-overexpressed cell lysate was performed with a K48 polyubiquitin-specific antibody, revealing that RNF8 accelerated K48 polyubiquitination of RXR (Figure S4I). Together with data in the current study, a 3-step inhibition mechanism for the PPARα/RXRα pathway in fatty liver was elucidated (Figure 4N).

**DISCUSSION**

Although whole-body let-7 inhibition in transgenic mice expressing Lin28a or Lin28b improves hepatic steatosis and obesity (Zhu et al., 2011), and a global let-7 inhibitor partially improves glucose metabolism (Frost and Olson, 2011), the role of let-7 in hepatic lipid metabolism has previously not been explored. In the current study, for the first time, a pronounced resistance to HFD-induced obesity was noted in mice lacking hepatic let-7 or in mice infected with AAV expressing a let-7 miRNA sponge. However, let-7 did not influence insulin sensitivity or glucose tolerance. Mechanistically, a let-7-RNF8-RXR axis was identified to explain the observed phenotypes in the current study. These results provide novel insights into let-7 miRNA modulation of metabolic diseases.

A striking finding of this study is that the let-7-RNF8-RXR axis modulates lipid homeostasis during obesity. When hepatic let-7b/c2 was genetically disrupted or inhibited by let-7 sponge, the mice showed resistance to HFD-induced fatty liver and obesity. RNA-seq data revealed a consistent downregulation of the target genes of several nuclear receptors, including PPARs, that all shared RXR as the heterodimer partner, leading to the hypothesis that RXRα is modulated by let-7 deficiency. Hepatic RXRα protein, but not mRNA, was decreased in the liver of hepatic let-7-deficient mice as well as in primary hepatocytes isolated from hepatic let-7-deficient mice, possibly contributing to repression of nuclear receptor pathways including PPAR signaling. The positive regulation of let-7 in RFXα protein expression was further supported by the gain-of-function data from overexpressing let-7 in both livers and cultured hepatocytes. Given that the RXR protein, but not mRNA, levels were modulated by let-7, a posttranslational modulation of RXR by let-7 was suspected. Although RNF8 is known to bind with RXRα in the nucleus (Takano et al., 2004), we found that RNF8 acted as an E3 ubiquitin liganse for RXR protein in vitro and regulated protein degradation of RXRα at the posttranslational level. To explain how RNF8 was increased in hepatic let-7-deficient livers, RNF8 protein levels were first found to be decreased in the hepatic let-7-deficient livers and increased in hepatic let-7-overexpressing livers, and then Rnf8 mRNA was further identified as an mRNA target of let-7. These data together support the existence of a let-7-RNF8-RXRα pathway in the liver.

Implicit in the present findings is that decreased RXRα expression may contribute to the phenotype of hepatic let-7-deficient mice. Although RXRα inhibition is already known to ameliorate obesity and hepatic steatosis in mice in vivo (Yamauchi et al., 2001), we further demonstrated that RXRα inhibition markedly inhibited lipid accumulation in mouse primary hepatocytes in the current study, which together support the view that RXRα inhibition contributes to the improvement of hepatic steatosis and obesity. RXRα is a versatile nuclear receptor contributing to several cellular processes ranging from cell proliferation to lipid metabolism by modulating different target genes (Evans and Mangelsdorf, 2014; Lefebvre et al., 2010). Activation of different RXR-partnered nuclear receptors yields different, or even
opposite, effects in modulating the obesity-associated metabolic diseases (Cariou et al., 2006; Gao et al., 2009; Ma et al., 2013; Spruiell et al., 2014). Because hepatic let-7 deficiency alleviated fatty liver and obesity in HFD-fed mice, the beneficial effects of RXRα inhibition might be superior to its harmful effects in restricting hepatic lipid accumulation. In this case, the decreased RXRα expression in hepatic let-7-deficient mice may contribute to the resistance to HFD-induced obesity and fatty liver. Although decreased RXRα expression by hepatic let-7 deficiency is suggested to contribute to the phenotype, we cannot rule out the possibility that hepatic let-7 deficiency improved obesity and fatty liver by other mechanisms.

A PPARα-let-7-RNF8-RXRα negative feedback loop is suggested as one potential downstream pathway after PPARα activation. In the current study, hepatocyte-specific Ppara knockout mice in combination with both chemical PPARα agonist and fasting-induced increase of endogenous PPARα ligands were used and confirmed a hepatocyte PPARα-dependent modulation of hepatic let-7 expression. Once hepatocyte PPARα was activated, expression of the hepatic let-7 family was inhibited, and the hepatic PPARα signaling pathway was reduced by the proposed let-7-RNF8-RXRα axis once hepatic let-7 was inhibited, as revealed by using hepatic let-7-deficient mice. These results suggest a possible PPARα-let-7-RNF8-RXRα negative feedback

Figure 4. RNF8 is decayed by let-7 miRNA, and RXRα protein is ubiquitinated by RNF8 E3 ubiquitin ligase
(A) Predicted let-7 miRNA binding sites in Rnf8 mRNA. (B-F) Western blot analysis and densitometric quantification of RNF8 protein (B, D, and F) and qRT-PCR of Rnf8 mRNA (C and E) in let7b/c2+/+ and let7b/c2Hep (B and C); EGFP and let-7 sponge AAV-transduced (D and E); EGFP and pre-let-7c-1 AAV-transduced (F) livers treated with HFD feeding. (G) 3' UTR reporter assays in HepG2 cells transfected with Rnf8 wild-type or mutant 3' UTR reporter constructs and a let-7c mimic expression vector. (H and I) Western blot analysis (H) and densitometric quantification (I) of RXRα expression in Rpra- and Rnf8-transfected Hepa-1 cells. (J and K) Fold change of Rnf8 (J) and Rora (K) mRNA by qRT-PCR analysis in Rpra- and Rnf8-transfected Hepa-1 cells. (L) Western blot analysis and the densitometric quantification of RXRα in Rpra- and Rnf8-transfected Hepa-1 cells treated with the proteasome inhibitor MG-132. (M) Ubiquitination assays for RXRα and Rnf8-transfected and MG-132-treated Hepa-1 cells. RXRα was immunoprecipitated and polyubiquitin detected by anti-ubiquitin antibody. RXRα expression was confirmed in whole-cell lysate as input. (N) Scheme of 3-step inhibition for PPARα/RXRα pathway that the current study demonstrates.
loop, with the detailed mechanisms underlying PPARα-activation-repressed let-7 expression still awaiting further study.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at [https://doi.org/10.1016/j.celrep.2021.109506](https://doi.org/10.1016/j.celrep.2021.109506).

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**AUTHOR CONTRIBUTIONS**

T. Yagai, T. Yan, Y.L., S.T., D.A., D.K., and C.N.B. performed the research and analyzed the data. T. Yagai, T. Yan, M.L., H.M., and F.J.G. designed and supervised the research and wrote the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**STAR METHODS**

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse monoclonal anti-CYP4A | Santa Cruz | Cat# sc-271983; RRID: AB_10715105 |
| Mouse monoclonal anti-RNF8 | Santa Cruz | Cat# sc-271462; RRID: AB_10648902 |
| Mouse monoclonal anti-Ub | Santa Cruz | Cat# sc-8017; RRID: AB_628423 |
| Mouse monoclonal HA-probe | Santa Cruz | Cat# sc-7392; RRID: AB_627809 |
| Mouse monoclonal j-Actin | Santa Cruz | Cat# sc-47778; RRID: AB_626632 |
| Rabbit polyclonal anti-EHHADH | Proteintec | Cat# 26570-1-AP; RRID: AB_2880556 |
| Rabbit polyclonal anti-HADHA | Proteintec | Cat# 10758-1-AP; RRID: AB_2115593 |
| Rabbit polyclonal anti-KRT23 | Origene | Cat# TA321839 |
| Rabbit monoclonal anti-RXRα | Abcam | Cat# ab125001; RRID: AB_10975632 |
| Rabbit polyclonal anti-Histon H3 | Abcam | Cat# ab1791; RRID: AB_302613 |
| Rabbit polyclonal anti-PPARγ | Abcam | Cat# ab126285 |
| Rabbit monoclonal anti-K48-linkage Ubiquitin | Cell Signaling Technology | Cat# 8081; RRID: AB_10859893 |
| **Bacterial and virus strains** |        |            |
| AAV8-Alb-EGFP; -let-7 sponge; -pre-let-7c-1 | This paper | N/A |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Protease inhibitor cocktail | Sigma-Aldrich | Cat# P9599 |
| Protein A Agarose | Sigma-Aldrich | Cat# P2545 |
| Wy-14,643 | APExBIO | Cat# A4305 |
| MG-132 | Sigma-Aldrich | Cat# M8699 |
| Dexamethasone | Sigma-Aldrich | Cat# D2915 |
| Insulin-Transferrin-Serum | Sigma-Aldrich | Cat# I3146 |
| Glutax-supplement | Thermo Fisher Scientific | Cat# 35050079 |
| HEPES | Thermo Fisher Scientific | Cat# 15630080 |
| Humulin R (human recombinant insulin) | Eli Lilly | N/A |
| **Critical commercial assays** |        |            |
| Taqman microRNA Assay | Thermo Fisher Scientific | Cat# 4427975 Assay ID:000377; 000378; 000379; 002286; 002406; 00382; 002282; 002221; 000577 |
| L-Type Triglyceride M | Fujifilm Wako Diagnostics | Cat# 994-02891; 990-02991 |
| Cholesterol E | Fujifilm Wako Diagnostics | Cat# 999-02601 |
| HR Series NEFA-HR(2) | Fujifilm Wako Diagnostics | Cat# 999-34691; 995-34791; 991-34891; 993-35191; 276-76491 |
| SimpleChIP Plus Enzymatic Chromatin IP kit | Cell Signaling Technology | Cat# 9005 |
| ALT assay kit | Catachem Inc | Cat# V165-12 |
| **Deposited data** |        |            |
| Raw and analyzed data | This paper | GEO: GSE165521 |
| **Experimental models: Cell Lines** |        |            |
| Human: Hep G2 cells | ATCC | Cat# HB-8065 |
| Mouse: Hepa-1c1c7 cells | ATCC | Cat# CRL-2026 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Frank J. Gonzalez (gonzalef@mail.nih.gov).

Materials availability
Plasmids generated in this study are available from the lead contact without restriction.

Data and code availability
RNA-seq data in this study have been deposited at GEO and publicly available as of the date of publication. Accession number is listed in the Key resources table. Other raw data reported in this paper will be shared by the lead contact upon request.

This paper does not report 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

Animals
All mouse studies were approved by the NCI Animal Care and Use Committee and performed in accordance with the Institute of Laboratory Animal Resources guidelines. Six-week-old male C57BL/6N mice were purchased from Charles River Laboratories. The
Ppara wild-type (Ppara+/+) and hepatocyte-specific Ppara knockout (PparaHep+) mice used in this study were described previously (Brocker et al., 2017). Hepatocyte-specific let-7b/c2 knockout (let7b/c2Hep+) were generated by mating Alb-Cre (Yakar et al., 1999) and let7-b/c2 flox (Madison et al., 2013) mouse lines. Mice were housed in a temperature (22°C) and light-controlled vivarium with free access to water and standard rodent chow food, 60% high fat diet (HFD) (S3282 from Bio-Serv) or 0.1% Wy-14,643 diet (F3254 from Bio-Serv). For gavage injection, Wy-14,643 was dissolved in 1% carboxymethyl cellulose and administered at 50 mg/kg. Mouse body composition was analyzed by MRI and Echo Medical Systems in Mouse Metabolism Core in NIDDK. All experiments were started with 7- to 8-week-old male mice.

**METHOD DETAILS**

**Quantitative RT-PCR**

Total RNA was extracted from frozen tissues using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. The purity and concentration of the total RNA were determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific). One μg of total RNA was reverse transcribed using qScript cDNA synthesis kit (Quantabio). For let-7 precursor measurement, the total RNA was treated with DNase I (Thermo Fisher scientific) before reverse transcription. Primers for let-7 miRNA precursors were designed at the stem portion of the hairpin structure as a previous study (Schmittgen et al., 2008). PerfeCTa SYBR Green Supermix (Quanta Bio) was used for SYBR green detection. For mature let-7, the total RNA was reverse-transcribed by Taqman miRNA assay (Thermo Fisher Scientific) and subjected to qRT-PCR as the manufacturer’s instructions. Actb and U6 snRNA were used as reference genes for mRNA and miRNA respectively. Polymerase reaction and the fluorescence detection were performed by QuantStudio 7 Flex real-time PCR system (Thermo Fisher Scientific).

**Hematoxylin and eosin and oil red O staining**

Freshly isolated liver tissues were embedded in OCT compound (Sakura Finetek), and rapidly frozen by liquid nitrogen. The samples were sliced and stained by Histoserve, Inc. Imaging was performed using a KEYENCE BZ-X710 microscope (Keyence).

For primary hepatocytes, 4% paraformaldehyde (Fujifilm wako chemicals), 60% oil red O solution (Muto pure chemicals) and hematoxylin (Muto pure chemicals) were used for fixation and staining respectively. Morphometric analyses were performed by ImageJ software (NIH).

**Biochemical analyses for triglyceride, total cholesterol, and non-esterified fatty acid**

Frozen liver tissues were homogenized by Precellys tissue homogenizer (Bertin Instruments) in 50 mM Tris-HCl with 5% Triton X-100 as previously described (Li et al., 2017). The lysate and serum samples were subjected to L-type Triglyceride M (Fujifilm Wako Diagnostics), Cholesterol E (Fujifilm Wako Diagnostics), and HR series NEFA-HR(2) (Fujifilm Wako Diagnostics) for measuring triglyceride (TG), total cholesterol (TC), and non-esterified fatty acid concentrations (NEFA), respectively.

**Biochemical analysis for serum alanine aminotransferase**

Serum samples were subjected to commercial ALT assay kit (Catachem) and monitored at 340 nm for 10 min with a microplate reader (BioAssay Systems).

**Insulin, Glucose, and Pyruvate tolerance test**

Intraperitoneal insulin, glucose, and pyruvate tolerance test was performed with the protocol provided from National Mouse Metabolic Phenotyping Center. One drop blood was taken via tail tip cut from 4 h-fasted mice. The blood glucose was measured by glucometer for a baseline. Then, 0.5 U/mL Humalin R (Eli Lilly), 20% Dextrose (Hospira) and sodium pyruvate (Sigma) was intraperitoneally injected to the mice at 0.5 U/kg, 1g/kg and 1g/kg, respectively. Blood glucose was measured at 15, 30, 45, 60, and 120 min after injection.

**Construction of hepatocyte-specific let-7 sponge and pre-let-7c-1 expression adeno-associated virus (AAV) vector**

The albumin promoter-driven self-complementary EGFP-AAV plasmid (pscAAV-ALBp-EGFP) described in a previous study (Kim et al., 2019) was used as a backbone vector. Single strand let-7 sponge and the complementary oligos shown in Figure S3A were purchased from Integrated DNA Technologies. The oligos were annealed and cloned into pscAAV-ALBp-EGFP plasmid with Not I and Stu I restriction enzymes. For pre-let-7c-1 expression AAV, the 470 bp mouse genomic sequence around pre-let-7c-1 was amplified using custom primers (Table S3) from a MirLet7c-1 expression plasmid (Origene). The amplified fragments were cloned into pscAAV-ALBp-EGFP plasmid with Not I, Stu I, and EcoR V restriction enzymes. The plasmids were transfected into HEK293T cells to produce AAV8 vectors by the triple transfection method then purified using polyethylene glycol precipitation followed by cesium chloride density gradient fractionation as previously described (Park et al., 2009). AAV was transduced into primary hepatocyte at 1 × 10^5 infectious unit (ifu) and one mouse at 1 × 10^11 ifu via tail vein injection respectively. For validation of infection efficiency, EGFP DNA isolated from the primary hepatocytes by QIAamp DNA Mini Kit (QIAGEN) and concentrated AAV solutions were amplified by qRT-PCR, and then analyzed by comparative Ct method.
**Primary hepatocyte isolation and culture**

Primary hepatocytes from C57BL/6N mice were isolated by a two-step perfusion method modified from a previous study (Yagai et al., 2014). Hank’s balanced salt solution (HBSS) without CaCl₂, MgCl₂ and MgSO₄ (Thermo Fisher Scientific) was used as basic solution. 25 mL HBSS with 1mM EDTA was perfused into liver via the portal vein for one mouse. Then, 25 mL HBSS containing 0.025% collagenase type I (Thermo Fisher Scientific), 0.025% collagenase type II (Thermo Fisher Scientific), 0.005% trypsin inhibitor (Thermo Fisher Scientific) and 0.075% CaCl₂·H₂O (Mallinckrodt Pharmaceuticals) was perfused. The digested liver was passed through a 70-μm cell strainer. Hepatocytes were precipitated by centrifugation at 50 × g for 2 min. The dead hepatocytes were removed by Percoll (GE Healthcare) density centrifugation at 70 × g for 10 min. Hepatocytes were cultured in collagen-coated plates (Corning) with William’s Medium E (Lonza) containing 400 ng/ml dexamethasone (Sigma-Aldrich), 1 x insulin-transferrin-selenium (Sigma-Aldrich), 1 x Glutamax (Thermo Fisher Scientific), 25 mM HEPES (Thermo Fisher Scientific), and 5% FBS (Gemini).

**RNA-seq and pathway analysis**

Total liver RNA was prepared by RNeasy plus mini kit (QIAGEN). The purity and concentration of extracted RNA were measured by HiSeq3000/4000 system (Illumina) with paired-end 150 read length. The RNA-seq datasets generated during this study are available at Gene Expression Omnibus (Accession number: GSE165521). The comprehensive gene expression profile was subjected to Ingenuity Pathway Analysis (QIAGEN).

**Ubiquitination assay for RXRα protein**

pSG5-mouse Rrxra (Leid et al., 1992) and pCMV6-mouse Rnf8 (Origene) expression plasmids were co-transfected into Hepa-1c1c7 cells (ATCC) using Lipofectamine 3000 (Thermo Fisher Scientific). pRK5-HA-Ubiquitin-WT (Addgene) were co-transfected for poly-ubiquitin type analysis. Three days after transfection, cell lysates were harvested with RIPA buffer. For ubiquitination assays, 20 μM MG-132 (Sigma-Aldrich) was added to the medium and the cells were cultured for 4 h before harvesting. For RXRα immunoprecipitation, rabbit monoclonal RXRα antibody (Abcam) was added to 400 μL cell lysate adjusted at 1 mg/ml protein concentration. The cell lysate was incubated at 4°C for overnight. The following day, protein A agarose (Sigma-Aldrich) was added and incubated for 2 h at 4°C. Then, the RXRα-ubiquitin complexes were added to Laemmli sample buffer (Bio-Rad) with 5% 2-mercaptoethanol and heated to 95°C for 5 min. The samples were subsequently analyzed by western blot.

**Western blot analysis**

Liver tissue or cell samples were lysed in RIPA buffer. 10 μg protein was added to Laemmli sample buffer (Bio-Rad) with 5% 2-mercaptoethanol at 95°C for 5 min. The samples were loaded in Criterion TGX precast gel (Bio-Rad), separated by electrophoresis, then transferred using a Trans-Blot Turbo Transfer System (Bio-Rad). The protein transferred PVDF membrane was incubated with 5% skim milk containing primary antibody overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies in 5% skim milk was incubated for two h at room temperature. After the incubation, the washed membrane was exposed to SuperSignal West Dura Extended Duration Substrate (Thermo Fisher Scientific) for luminol reaction. Chemiluminescence was imaged and quantified using a ChemiDoc MP Imaging System (Bio-Rad). Anti-RXRα (Abcam, ab125001) and anti-PPARα (Abcam, ab126285) antibodies were used for the protein detection.

**Dual luciferase reporter assay**

Let-7adf cluster EP fragments were amplified from mouse genome by the primers listed in Table S3. The insert was digested and cloned into pGL4.27 (Promega). The constructed reporter vector and phRL-TK renilla luciferase expression vector (Promega) were co-transfected into primary hepatocyte by Lipofectamine 3000 (Thermo Fisher Scientific). Three days later, the cells were lysed using the passive lysis buffer supplied in Firefly & Renilla Luciferase Single Tube Assay Kit (Biotium). Dual luciferase assay was performed as the manufacture’s protocol and Veritas Microplate Luminometer (Turner BioSystems). For the mouse Rnf8 3’UTR reporter assay, Rnf8 3’UTR wild-type (WT) and mutant (Mut) fragments in Table S1 were purchased from Integrated DNA Technologies. The UTR inserts were cloned into pmirGLO vector (Promega) for Rnf8 3’UTR WT and Mut reporters, respectively. 20 nM let-7c mimic or the scramble control (Dharmacon) was transfected into HepG2 cells by Lipofectamine 3000 (Thermo Fisher Scientific). The next day, reporter vectors were transfected using the same technique. Five days later, the cells were lysed and subjected to dual luciferase assay as same manner above.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis and graphing of the data were performed using GraphPad Prism. Data are presented as mean ± SE for biological replicates. Statistical significance is indicated by the following annotations: *p < 0.05, **p < 0.01, ***p < 0.001 in Figures 1A and 1C and *p < 0.05, **p < 0.01, ***p < 0.001 in other Figures. p values were calculated by two-sided paired t tests followed by Bonferroni analysis. Please note that statistical details are found in the figure legends.
Supplemental information

Feedback repression of PPARα signaling

by Let-7 microRNA

Tomoki Yagai, Tingting Yan, Yuhong Luo, Shogo Takahashi, Daisuke Aibara, Donghwan Kim, Chad N. Brocker, Moshe Levi, Hozumi Motohashi, and Frank J. Gonzalez
Supplemental Figure 1. Lin28 expressions in liver and phenotypic analyses of let7b/c2+/+ and let7b/c2ΔHep mice. Related to Figure 1 and 2.

(A and B) Fold change of Lin28a (A) and Lin28b (B) mRNA abundance in Wy-14,643 treated liver. N.D.: Non-Detected.

(C and D) Fold change of pre-let-7b and pre-let-7c-2 (C) and mature let-7b and let-7c (D) abundance in let7b/c2+/+ and let7b/c2ΔHep livers.

(E) Average daily food intake (kcal/mouse/day) of let7b/c2+/+ and let7b/c2ΔHep when HFD-feeding.

(F-J) Biochemical analyses for serum total cholesterol (TC) (F); alanine aminotransferase activity (ALT) (G); hepatic triglycerides (TG) (H); hepatic TC (I); and hepatic non-esterified fatty acid (NEFA) (J) in let7b/c2+/+ and let7b/c2ΔHep mice after HFD-feeding.

(K) Heat map of FXR target genes identified by differential gene expression analysis of RNA-seq data from let7b/c2+/+ and let7b/c2ΔHep livers after HFD-feeding. n = 5 mice/group. Data are presented as mean ± SEM.
Supplemental Figure 2. Biochemical assays for normal chow diet-fed let7b/c2<sup>+/+</sup> and let7b/c2<sup>ΔHep</sup> mice. Related to Figure 2.

Body weight alterations of let7b/c2<sup>+/+</sup> and let7b/c2<sup>ΔHep</sup> mice during normal chow diet-feeding. Average daily food intake (kcal/mouse/day). N = 5 mice/group. Data are presented as mean ± SEM.

(B-E) Biochemical analyses for serum TG (B); serum TC (C); Hepatic TG (D); and Hepatic TC (E) in let7b/c2<sup>+/+</sup> and let7b/c2<sup>ΔHep</sup> mice after normal chow diet-feeding.  

(F-H) ITT (F); GTT (G); and PTT (H) for normal chow diet-fed let7b/c2<sup>+/+</sup> and let7b/c2<sup>ΔHep</sup> mice.

(I) mRNA analysis by qRT-PCR of PPAR<sub>α</sub> target genes in let7b/c2<sup>+/+</sup> and let7b/c2<sup>ΔHep</sup> livers after 1-week HFD-feeding. n = 5 mice/group. Data are presented as mean ± SEM.
A 5' - G C C G C A C C A T A C A - 3'  

B Self-complementary AAV-8

C Fold change

D EGFP let-7 sponge

E Serum TC

F Hepatic TG

G Hepatic TC

H Hepatic NEFA

I ITT

J GTT
Supplemental Figure 3. let-7 sponge AAV construction and phenotypes of let-7 sponge AAV-transduced mice related to Figure 2.

(A) Sequence of let-7 sponge inserted in let-7 sponge AAV vector.
(B) Scheme of let-7 sponge expression AAV vector.
(C) qPCR analysis of GFP DNA in primary hepatocytes transduced by EGFP and let-7 sponge expressing AAV.
(D) Oil Red O staining of liver sections from wild-type mice infected with EGFP or let-7 sponge expressing AAV after being on HFD for eight weeks. Bars = 100µm
(E-H) Biochemical analyses for serum TC (D); hepatic TG (E); hepatic TC (F); and hepatic NEFA (G) concentration in EGFP and let-7 sponge AAV-infected mice after HFD-feeding.
(I and J) ITT (H); and GTT (I) for normal chow diet-fed EGFP and let-7 sponge AAV-infected mice.

Data are presented as mean ± SEM. (n = 4-5 mice per group; *p < 0.05)
Supplemental Figure 4. RXRα expressions, functions and protein ubiquitination by let-7 miRNA related to Figure 3 and 4.

(A-B) Fold change of PPARα (A); and RXRα (B) mRNA in let7b/c2+/+ and let7b/c2αHep livers fed HFD.

(C) Fold change of RXRα mRNA in let7b/c2+/+ and let7b/c2αHep primary hepatocytes.

(D) Fold change of RXRα mRNA in EGFP, pre-let-7c-1 and let-7 sponge AAV-transduced liver after HFD-feeding.

(E) Oil red O staining and the morphometric analysis for lipid accumulation in mouse primary hepatocytes treated with Palmitic acid (800 µM) and/or HX-531 (5 µM).

(F) Western blot analysis and the densitometric quantification of RXRα in 60% HFD and/or Wy-14,643-treated mouse livers.

(G) Fold change of Rnf8 mRNA in EGFP and pre-let-7c-1 AAV-transduced liver.

(H) Western blot analysis and the densitometric quantification of RXRα in Rxra transfected Hepa-1 cells treated with the proteasome inhibitor MG-132.

(I) Western blot analysis of K48 polyubiquitin chain in Rxra and Rnf8 transfected Hepa-1 cells.

(J) Western blot analysis of RNF8 protein in Rnf8 transfected Hepa-1 cells.

Data are presented as mean ± SEM. (n = 4-5 blots or fields per group; *p < 0.05, **p < 0.01, ***p < 0.001)
### Table 1. let-7 microRNA family in mice related to Figure 1

| Mature let-7 miRNA | Precursor | Primary transcript | Clustered let-7 precursor | Chromosome | miRBase ID |
|--------------------|-----------|--------------------|---------------------------|------------|------------|
| let-7a             | pre-let-7a-1 | let-7adf cluster   | pre-let-7d, pre-let-7f-1  | Chr.13     | MI0000556  |
|                   | pre-let-7a-2 | 3110039I08Rik      |                           | Chr.9      | MI0000557  |
| let-7b             | pre-let-7b  | Lincppara          | pre-let-7c-1              | Chr.15     | MI0000558  |
| let-7c             | pre-let-7c-1| 2810055G20Rik      | pre-let-7b                | Chr.16     | MI0000559  |
|                   | pre-let-7c-2| Lincppara          | pre-let-7b                | Chr.15     | MI0000560  |
| let-7d             | pre-let-7d  | let-7adf cluster   | pre-let-7a-1, pre-let-7f-1| Chr.13     | MI0000405  |
| let-7e             | pre-let-7e  | Spaca6             |                           | Chr.17     | MI0000561  |
| let-7f             | pre-let-7f-1| let-7adf cluster   | pre-let-7a-1, pre-let-7d  | Chr.13     | MI0000562  |
|                   | pre-let-7f-2| Huwe1              | pre-miR-98                | Chr.X      | MI0000563  |
| let-7g             | pre-let-7g  | Wdr82              |                           | Chr.9      | MI0000137  |
| let-7i             | pre-let-7i  | mmu-let-7i         |                           | Chr.10     | MI0000138  |
| miR-98             | pre-miR-98  | Huwe1              | pre-let-7f-2              | Chr.X      | MI0000586  |

Summary of mature let-7 miRNA, the precursors and the primary transcripts. Mature let-7a, c and f are processed from two precursors. Pre-let-7a-1, pre-let-7d and pre-let-7f-1 are processed from an identical primary transcript. Pre-let-7b and pre-let-7c-1 are also processed from an identical primary transcript.
Supplemental Table 2. Downregulated pathways in \textit{let7b/c2}^{ΔHep} mouse liver compared with \textit{let7b/c2}^{+/+} related to Figure 3

| Upstream Regulator | Expr Log Ratio | Molecule Type                          | Predicted Activation State | Activation z-score |
|--------------------|----------------|----------------------------------------|----------------------------|--------------------|
| PPARA              | 0.032          | ligand-dependent nuclear receptor       | Inhibited                 | -6.751             |
| INSIG1             | 0.13           | other                                  | Inhibited                 | -3.564             |
| Alpha catenin group| -1.333         | ligand-dependent nuclear receptor       | Inhibited                 | -3.171             |
| HOXA3              | -1.333         | transcription regulator                 | Inhibited                 | -3.134             |
| CFTR               | -1.333         | ion channel                            | Inhibited                 | -3.119             |
| PPARG              | -1.333         | ligand-dependent nuclear receptor       | Inhibited                 | -3.134             |
| NR1H4              | 0.035          | ligand-dependent nuclear receptor       | Inhibited                 | -2.721             |
| PPARD              | 0.035          | ligand-dependent nuclear receptor       | Inhibited                 | -2.619             |
| KLF15              | 0.056          | transcription regulator                 | Inhibited                 | -2.498             |
| LPL                | 1.748          | enzyme                                 | Inhibited                 | -2.449             |
| PNPLA2             | 0.618          | enzyme                                 | Inhibited                 | -2.433             |
| ABCG1              | -0.922         | transporter                             | Inhibited                 | -2.415             |
| let-7a-5p (and other miRNAs w/seed GAGGUAG) | -0.922 | mature microRNA                          | Inhibited     | -2.415             |
| ZFP36              | -0.182         | transcription regulator                 | Inhibited                 | -2.412             |
| let-7              | -0.182         | microRNA                               | Inhibited                 | -2.401             |
| ZBTB20             | 0.618          | transcription regulator                 | Inhibited                 | -2.401             |

Pathway analysis for RNA-seq data from 60% HFD-fed \textit{let7b/c2}^{+/+} and \textit{let7b/c2}^{ΔHep} mouse livers. PPAR and FXR pathways (blue labeled) were significantly repressed by \textit{let7b/c2} deletion in liver.
# Supplemental Table 3. Primers and Oligos used in this paper related to STAR Methods

## Primers for mouse Quantitative RT-PCR

| Forward (5’ to 3’) | Reverse (5’ to 3’) |
|-------------------|-------------------|
| **pre-Let-7a-1**  | TTAGGGTCAACCACCCAC |
| **pre-7a-2**      | ATCACCTTAGAAAGACAGTAGATTG |
| **pre-7b**        | TAGAGTTATCTCAAGGGAGATACTCTC |
| **pre-7c-1**      | AGATGTCCTCAAGGGAAAGAGAG |
| **pre-7c-2**      | TTTTGGCTCTGGCACCACCC |
| **pre-7d**        | GCGGCCACCTAGCAGAAAGAG |
| **pre-7e**        | TTAGGGTCATACCCCATCTTGG |
| **pre-7f**        | TTAGGGTCATACCCCATCTTGG |
| **pre-7g**        | TGAGGGTCTATGATACCACCCG |
| **pre-7i**        | GGTCGGGTTGTGACATTGC |
| **pre-miR98**     | GTGGGGTAGGGATTTTAGGCC |
| **Cyp4a14**       | CTTGACTTTCTTTCGCTGC |
| **Ehhadh**        | CCGGGTCAATGCCAAGAG |
| **Hadha**         | AGGATCCTACGCTCCAGAGG |
| **Krt23**         | CTGATTCAGCTCCGCCAAGA |
| **Aldh3a2**       | GGGTTAATGTCCTTCAAGG |
| **Cidea**         | TGGCACATACTGAGGAGAG |
| **Cidec**         | TGGCACAATCTGAGGAGAG |
| **Gpam**          | GTGTACCCGAAGGTCTCCA |
| **Pctp**          | GGCTCTCCTCGCAGATGTTT |
| **Gyk**           | TGGCCTAATGAAAGCTGGGG |
| **Gapdh**         | AGGTCGGTGTGAACGGATT |
| **Ppara**         | CACGCATGTGAAGGCTGTAA |
| **Rxra**          | GACGCCAACTACCAAGAG |
| **Rnf8**          | GACTTTCCAGGAGGAGC |
| **Egfp**          | AAGCTGACCCTGAAGTTCATCTG |

## Oligos for Let-7 sponge AAV construction (5’ to 3’)

| Let-7 sponge         | CTAGAGGATCTTGGGAGGTTGAGTCTGAGCAGTATTGATGATGAGTGGTACAGCTGTGAGTGGAGGTGTTGAGATGTCGGTGGCTGGG |
|----------------------|---------------------------------------------------------------------------------------------|
| Let-7 sponge         | TGCAGGATCTTGGGAGGTTGAGTCTGAGCAGTATTGATGATGAGTGGTACAGCTGTGAGTGGAGGTGTTGAGATGTCGGTGGCTGGG |
| Let-7 sponge         | TGCAGGATCTTGGGAGGTTGAGTCTGAGCAGTATTGATGATGAGTGGTACAGCTGTGAGTGGAGGTGTTGAGATGTCGGTGGCTGGG |
| Let-7 sponge         | TGCAGGATCTTGGGAGGTTGAGTCTGAGCAGTATTGATGATGAGTGGTACAGCTGTGAGTGGAGGTGTTGAGATGTCGGTGGCTGGG |

## Primers for pre-let-7c-1 AAV construction (5’ to 3’)

| Let-7c-1 NotI EcoRV | AAACGCGCGCCGCAATTGGAAGATGTTGCAAGG |
|---------------------|-----------------------------------|

## Oligos for Rnf8 3’UTR luciferase reporter vectors

| Rnf8 3’UTR Wild-type | AAAGCTGACGATGCCATGGAGGTTGAGAAGCCAGTGGTACAGCAGTATTGATGATGAGTGGTACAGCTGTGAGTGGAGGTGTTGAGATGTCGGTGGCTGG |
|----------------------|---------------------------------------------------------------------------------------------|
| Rnf8 3’UTR Mutant    | AAAGCTGACGATGCCATGGAGGTTGAGAAGCCAGTGGTACAGCAGTATTGATGATGAGTGGTACAGCTGTGAGTGGAGGTGTTGAGATGTCGGTGGCTGG |

## Primers and oligos used for qRT-PCR. AAV constructs and Rnf8 3’UTR reporter vectors.