1   TMEM135 is an LXR-Inducible Regulator of Peroxisomal Metabolism

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21 **Summary**

22   The liver x receptors (LXRs) are key regulators of systemic lipid metabolism. We identified

23 transmembrane protein 135 (TMEM135), a peroxisomal protein with an unknown function, as an

24 LXR target gene. The LXRs induce *TMEM135* transcription in humans via an LXR response

25 element located downstream of the putative transcriptional start site, but do not increase

26 *Tmem135* in murine cells. Functionally, knockdown of TMEM135 in hepatocytes *in vitro* and *in*

27 *vivo* results in fatty acid accumulation due to reduced peroxisomal β-oxidation, which was most

28 pronounced with elevated fatty acid loading. Mechanistically, proteomic and Western blot

29 analyses indicated that TMEM135 mediates import of peroxisome matrix proteins necessary for

30 β-oxidation and bile acid synthesis. These findings indicate that the LXRs regulate peroxisomal

31 metabolism via transcriptional control of *TMEM135*, and TMEM135 mediates an auxiliary matrix

32 protein import pathway and thus may serve as a therapeutic target for metabolic and aging

33 disorders associated with peroxisome dysfunction.

34 **Keywords:** peroxisome, liver x receptors, nuclear receptors, lipid metabolism, β-oxidation, liver

35 metabolism, steatosis, fatty acid metabolism, cholesterol, bile acid synthesis

36 **Introduction**

37   The liver x receptors (LXR) α (NR1H3) and β (NR1H2) belong to the nuclear receptor

38 superfamily and are key regulators of cholesterol and fatty acid metabolism (Hong and Tontonoz,

39 2014). Oxysterols are natural LXR ligands (Peet et al., 1998) that increase during cholesterol

40 loading. The LXRs heterodimerize with the retinoic acid receptor (RXR) and bind to LXR response

41 elements (LXRE) within the promoters of target genes (Peet et al., 1998). The LXREs belong to

42 the direct repeat 4 (DR4) class that consist of two hexanucleotide half-sites separated by a 4-

43 nucleotide spacer (Teboul et al., 1995; Willy et al., 1995). The protein products of known LXR

44 target genes affect systemic lipid metabolism. Well known examples include increasing

45 cholesterol efflux via ATP-binding cassette (ABC) sub-family A, member 1 (ABCA1) (Costet et

46 al., 2000), and sub-family G, member 1 (ABCG1) (Kennedy et al., 2001); limiting cholesterol

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47   uptake via induction of myosin regulatory light chain interacting protein (MYLIP) that targets the

48   low-density lipoprotein receptor (LDLR) for ubiquitin-mediated degradation (Zelcer et al., 2009);

49   and increased lipogenesis via induction of sterol regulatory element binding protein 1c (SREBP1c,

50   encoded by sterol regulatory element binding transcription factor 1 gene or *SREBF1*) (Repa et

51   al., 2000; Yoshikawa et al., 2001). Also, the *NR1H3* isoform of the LXRs is itself an LXR target

52   gene (Laffitte et al., 2001; Whitney et al., 2001), leading to auto-amplification of LXR actions in

53   certain species and tissues. Although many LXR target genes have been discovered, additional

54   target genes may remain unidentified.

55   We have previously investigated LXR actions within the steroidogenic corpus luteum of

56   the ovary (Seto and Bogan, 2015; Xu et al., 2018a; Xu et al., 2018b). To uncover novel LXR target

57   genes, rhesus macaque luteal cells were treated with vehicle or 1 µM T0901317 (T09, synthetic

58   LXR agonist) for 24 hours, mRNA were isolated and used for microarray analysis of gene

59   expression on the Affymetrix® Rhesus Macaque genome chip (unpublished data). Genes that

60   were differentially expressed (>2-fold change in T09 vs vehicle, $p < 0.05$ with Benjamini and

61   Hochberg correction for false discovery rate) were identified. Several known LXR target genes

62   were differentially expressed including *ABCA1*, *ABCG1*, *NR1H3*, *SREBF1*, and *MYLIP*. One gene

63   that was differentially expressed and not previously determined to be an LXR target gene was

64   transmembrane protein 135 (*TMEM135*). Therefore, the objective of the current study is to

65   determine if *TMEM135* is an LXR target gene and to identify its physiologic role in lipid

66   metabolism.

67   **Results**

68   <u>LXR Agonist Increases Expression of TMEM135 in Human Hepatocyte and Macrophage Cell</u>

69   <u>Lines</u>

70   Macrophage and hepatocyte cell lines were selected for initial studies on LXR regulation

71   of *TMEM135* transcription as these cell types play a pivotal role in LXR-mediated reverse

72   cholesterol transport from peripheral tissues to the liver (Hong and Tontonoz, 2014). The LXR

73   ligand T09 increased the mRNA expression of *TMEM135* in both HepG2 (Figure 1A) and Hep3B

74   (Figure 1B) hepatocyte cell lines, with an approximately 2-fold maximum increase in HepG2 cells

75   and a 3-fold increase in Hep3B cells. In monocyte-derived macrophages (THP-1), T09 also

76   induced *TMEM135* mRNA expression up to 4-fold (Figure 1C). The protein synthesis inhibitor

77   cycloheximide was used to determine whether the effect of T09 on *TMEM135* in THP-1 cells

78   requires synthesis of new proteins. Cycloheximide did not significantly affect basal or T09-

79   stimulated *TMEM135* mRNA expression (Figure 1D), indicating that T09 induces *TMEM135* via

80   a direct transcriptional mechanism not involving the synthesis of intermediary proteins. As

81   TMEM135 has previously been reported to be localized to mitochondria (Exil et al., 2010; Lee et

82   al., 2016), the effect of T09 on TMEM135 protein expression in mitochondria/peroxisome-

83   enriched fractions from HepG2 cells was determined. Relative concentrations of TMEM135 were

84   significantly ($p < 0.05$) increased approximately 1.8-fold following a 24-hour T09 treatment (Figure

85   1E and 1F).

86   A LXRE Downstream of the Putative Transcription Start Site Mediates LXR Agonist Induction of

87   *TMEM135*

88   A 4 kb region spanning from -3319 to +682 bp (relative to the transcriptional start site) of

89   the human *TMEM135* promoter was analyzed with MatInspector (Cartharius et al., 2005) for

90   potential LXRE binding sites. This analysis yielded three potential LXREs (Figure S1), and these

91   were arbitrarily designated LXRE1, LXRE2 and LXRE3 in order from most distal to most proximal

92   to the translation start site. Electrophoretic mobility shift assays (EMSA) were used to determine

93   if LXR/RXR heterodimers bind these LXREs. As shown in Figure 2A, NR1H3 and RXRA nuclear

94   receptors together, but not individually, caused a shift in mobility of the fluorescent LXRE1 probe

95   indicating that NR1H3/RXRA heterodimers bind the LXRE1 sequence in the *TMEM135* promoter.

96   A 200-fold molar excess of unlabeled LXRE1, LXRE2, and LXRE3 oligonucleotides eliminated

97   the appearance of the shifted fluorescent band while the same molar excess of mutant LXRE1,

98   LXRE2, and LXRE3 oligonucleotides (Figure S1B) did not prevent NR1H3/RXRA binding to the

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99   fluorescent probe; which validated the specificity of NR1H3/RXRA heterodimer binding to all 3

100   LXREs in the *TMEM135* promoter (Figure 2A). Similar results were obtained for NR1H2/RXRA

101   heterodimers (Figure 2B), indicating that both LXR isoforms specifically bind to all 3 LXRE sites

102   in the *TMEM135* promoter. The LXRE1 and LXRE2 sites have identical hexanucleotide half-sites,

103   whereas the LXRE3 site is unique (Figure S1B). To indicate if these sequence variations may

104   cause differences in relative binding affinity, a fluorescent LXRE3 probe was incubated with

105   NR1H3/RXRA heterodimers and increasing concentrations (from 0.25 to 10-fold molar excess) of

106   either LXRE1 or LXRE3 unlabeled competitor DNA. Oligonucleotides with the LXRE3 sequence

107   more effectively inhibited binding to the fluorescent probe than oligonucleotides with the LXRE1

108   sequence (Figure 2C). As a complementary approach, because the LXRE1 and LXRE3 sites

109   were fluorescently labelled with spectrally distinct fluorophores, an EMSA was performed where

110   a mixture of fluorescent LXRE1 and LXRE3 probes were incubated with increasing concentrations

111   of NR1H3/RXRA heterodimers. This approach resulted in a higher percentage of LXRE3 than

112   LXRE1 bound across a range of NR1H3/RXRA heterodimer concentrations (Figure 2D).

113   Collectively, this indicates that LXRE3 has a higher binding affinity for NR1H3/RXR heterodimers

114   than LXRE1.

115   Luciferase reporter assays were used to study transcription initiation from the *TMEM135*

116   promoter. Transfection of HepG2 cells with wild type *TMEM135* promoter/pGL 4.17 resulted in a

117   large increase in luciferase expression that was responsive to T09 as compared to the empty pGL

118   4.17 vector (Figure 2E). Furthermore, luciferase activity was further amplified by co-transfection

119   of plasmids that constitutively express NR1H3 and RXRA (Figure 2E). Next, we determined the

120   requirement of each individual LXRE to LXR agonist-induced transcription from the *TMEM135*

121   promoter. Promoters containing all combinations of wild type and mutant LXREs (Figure S1B)

122   were generated, and T09-induced luciferase activity was determined. LXR-agonist induced

123   luciferase activity was significantly ($p < 0.05$) higher in cells transfected with the wild type

124   *TMEM135* promoter as compared to the *TMEM135* promoter with all 3 LXREs mutated (Figure

125 2F). Furthermore, constructs containing the wild type LXRE3 site and mutated LXRE1 and/or

126 LXRE2 sites were not significantly different from the wild type promoter, while all constructs that

127 contained a mutated LXRE3 were not significantly different from the promoter that had all three

128 LXREs mutated (Figure 2F). This indicates that even though all 3 LXREs bind LXR/RXR

129 heterodimers, the LXRE3 site alone mediates LXR agonist-induced transcription from the

130 *TMEM135* promoter.

131   We next determined whether *Tmem135* mRNA expression was induced by T09 in mice.

132 The murine LXRE3 site showed relatively little homology with the human sequence as a total of

133 3 nucleotides within both hexanucleotide half-sites were unique in mice (Figure S2A). In mouse

134 hepatocyte (BNL 1NG A.2) and macrophage (RAW 264.7) cell lines, T09 did not significantly

135 induce *Tmem135* mRNA expression (Figure S2B) whereas it did increase the known LXR target

136 gene *Abca1* (Figure S2C). These data indicate that *Tmem135* is not an LXR target gene in mice.

137 <u>*TMEM135* is a Direct Target Gene of the LXRs</u>

138   To directly determine whether the LXRs are needed for T09-induced *TMEM135*

139 transcription, siRNA-mediated knockdown of the LXRs was performed in HepG2 cells. The

140 *NR1H2* siRNA caused an approximately 75% decrease ($p< 0.05$) in *NR1H2* mRNA expression

141 compared to the control and *NR1H3* siRNA groups (Figure 3A). The *NR1H3* siRNA resulted in an

142 approximately 70-80% decrease ($p< 0.05$) in *NR1H3* mRNA expression compared to the control

143 siRNA depending on the presence or absence of T09 (Figure 3B). The *NR1H2* siRNA by itself

144 also caused a significant reduction in T09-stimulated *NR1H3* mRNA expression and tended to

145 slightly improve *NR1H3* knockdown when co-transfected with the *NR1H3* siRNA (Figure 3B).

146 Decreased *NR1H3* mRNA expression is expected to occur following NR1H2 knockdown because

147 *NR1H3* itself is an LXR target gene (Kennedy et al., 2001; Whitney et al., 2001). Consistent with

148 *NR1H3* being an LXR target gene, T09 induced a significant increase in *NR1H3* in the control

149 siRNA group, while T09 was not as effective at inducing *NR1H3* in the *NR1H2* siRNA group

150 (Figure 3B).

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151   The known LXR target gene *ABCA1* was significantly increased by T09 in the control

152   siRNA group, and the effect of T09 was completely abolished by *NR1H2/NR1H3* siRNA co-

153   transfection (Figure 3C). Also, *NR1H3* knockdown alone significantly inhibited the T09-induced

154   increase in *ABCA1* (Figure 3C). Results for *TMEM135* were very similar to *ABCA1* with T09

155   causing a significant increase in the control siRNA group, and the effect of T09 was completely

156   blocked by *NR1H2/NR1H3* siRNA co-transfection (Figure 3D). Furthermore, *NR1H3* knockdown

157   itself significantly inhibited the T09-induced increase in *TMEM135* as compared to the control

158   siRNA (Figure 3D).

159   The LXRE3 site of the human *TMEM135* promoter lies downstream of the putative

160   transcriptional start site, but before the translation initiation site (Figure S1). To determine whether

161   LXR-mediated transcription of *TMEM135* results in an mRNA with a truncated 5' UTR, primers

162   and a probe were designed to amplify and detect a 76 base pair region encompassing the LXRE3

163   site in the 5' UTR. There was a significant ($p < 0.05$) reduction in mRNA expression of full-length

164   5' UTR transcripts caused by T09 in the control siRNA group, while this effect was blocked by

165   *NR1H2/NR1H3* siRNA co-transfection (Figure 3E). Furthermore, *NR1H2/NR1H3* co-knockdown

166   resulted in a significant ($p < 0.05$) increase in basal mRNA expression of full-length 5' UTR

167   transcripts compared to all other groups (Figure 3E). ChIP was used to determine if the reduction

168   in full-length 5' UTR transcripts is associated with increased NR1H2 and/or NR1H3 binding to the

169   LXRE3 site on the *TMEM135* gene. Monoclonal antibodies specific for each LXR isoform were

170   used for ChIP, and QPCR of the LXRE3 region on chromatin purified via ChIP indicated that T09

171   treatment caused a significant increase in binding of NR1H3 to LXRE3 in the *TMEM135* gene

172   (Figure 3F). This indicates that LXR binding to LXRE3 increases mRNA expression of *TMEM135*

173   via a transcript with a truncated 5' UTR relative to the putative mRNA sequence.

174   <u>TMEM135 Mediates Fatty Acid Metabolism and Proliferation in HepG2 Cells</u>

175   To begin unraveling the biologic function of TMEM135, a series of knockdown experiments

176   were performed. Transfection of HepG2 cells with siRNA against *TMEM135* decreased its mRNA

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177   expression by 70-90%. As TMEM135 has previously been implicated in fat metabolism (Exil et

178   al., 2010), and the LXRs are known to induce lipogenesis (Hong and Tontonoz, 2014), we first

179   determined its effect on triglycerides and mRNA expression of fatty acid oxidation and

180   lipogenesis-associated genes. Knockdown of TMEM135 significantly increased basal triglyceride

181   accumulation in HepG2 cells (Figure 4A). Furthermore, T09 significantly increased intracellular

182   triglyceride concentrations, while TMEM135 knockdown further increased triglyceride

183   accumulation in the presence of T09 (Figure 4A). An increase in triglyceride accumulation could

184   result from a decrease in fatty acid oxidation and/or an increase in lipogenesis. There were no

185   significant effects of TMEM135 knockdown on mRNA expression of the key regulator of fatty acid

186   β-oxidation, peroxisome proliferator activated receptor alpha (*PPARA*), while it tended to increase

187   the PPARA target gene carnitine palmitoyltransferase 1A (*CPT1A*) (Kersten and Stienstra, 2017)

188   (Figure 4B). It is known that the LXRs induce lipogenesis via induction of SREBP1c (*SREBF1*

189   gene) (Repa et al., 2000; Yoshikawa et al., 2001). As expected, T09 caused a significant increase

190   in *SREBF1* and the SREBP1c/LXR target gene fatty acid synthase (*FASN*) (Figure 4B), consistent

191   with the T09-induced increase in triglyceride accumulation (Figure 4A). However, TMEM135

192   knockdown significantly suppressed both basal and T09-induced *SREBF1* mRNA expression

193   (Figure 4B). This indicates that the increase in triglyceride accumulation in HepG2 cells occurred

194   despite an apparent reduction in lipogenesis. Thus, an inhibition of fatty acid oxidation seems a

195   more likely explanation for the increase in triglycerides. The tendency for an increase in *CPT1A*

196   mRNA caused by TMEM135 knockdown is not evidence against a decrease in fatty acid oxidation

197   because reduced β-oxidation would result in fatty acid accumulation, and because fatty acids are

198   the endogenous ligands for PPARA (Kersten and Stienstra, 2017), this would also cause

199   increased *CPT1A* mRNA expression. Further supporting an impairment in fatty acid β-oxidation,

200   TMEM135 knockdown significantly reduced ATP concentrations when HepG2 cells were

201   incubated in glucose-free medium (Figure 4C).

202   During these experiments it appeared that TMEM135 knockdown also inhibited replication

203   of HepG2 cells. Because enhanced β-oxidation is a hallmark of hepatocellular carcinoma (HCC)

204   (Beyoglu and Idle, 2013) and our previous experiments indicated a key role for TMEM135 in fatty

205   acid metabolism, we determined whether TMEM135 regulated proliferation of HepG2 cells.

206   Knockdown of *TMEM135* significantly ($p< 0.05$) reduced viable HepG2 cell numbers at 48 and 72

207   hours post-transfection as compared to cells transfected with the control siRNA (Figure 4D). Cell

208   cycle analysis indicated that *TMEM135* knockdown significantly ($p< 0.05$) increased the

209   percentage of HepG2 cells in the G0/G1 stage, with a corresponding significant reduction in the

210   percentage of cells in the S phase (Figure 4E). The increase in G0/G1 arrest was associated with

211   alterations in mRNA expression of tumor suppressor and cell cycle genes. There were significant

212   increases in cyclin dependent kinase inhibitor 2A (*CDKN2A*) and tumor protein p53 (*TP53*) (Figure

213   4F), which restrict passage through the G1/S checkpoint (LaPak and Burd, 2014). There were

214   also significant increases in cyclin dependent kinase 2 (*CDK2*) and cyclin E1 (*CCNE1*) (Figure

215   4F), which increase prior to passage through the G1/S checkpoint, but no change in cyclin A2

216   (*CCNA2*) (Figure 4F) which is increased in the S phase (Harper and Adams, 2001). Collectively,

217   these data indicate that TMEM135 knockdown reduces HepG2 proliferation by restricting passage

218   through the G1/S checkpoint.

219   <u>Liver-Selective TMEM135 Knockdown Reduces Peroxisomal β-Oxidation</u>

220   To determine the physiologic function of TMEM135, a siRNA knockdown experiment was

221   performed in male C57BL/6 mice. Mice received either a non-targeting control or *Tmem135*

222   siRNA and were sacrificed 4 days later in either the *ad libitum* fed state or after a 12-hour fast.

223   Because we determined that *Tmem135* is not an LXR target gene in mice (Figure S2), we used

224   fasting to induce hepatic fat accumulation. The *Tmem135* siRNA caused an approximately 60%

225   mRNA knockdown in the liver while no knockdown was observed in other tissues including

226   skeletal muscle, adipose, and heart (Figure 5A). Knockdown of TMEM135 in the liver was further

227   confirmed by Western blot and proteomic analysis of mitochondria/peroxisome-enriched fractions

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228   (Figure 5B). Fed mice injected with the *Tmem135* siRNA gained significantly less weight during

229   the 4-day treatment, while the loss in weight from fasting was similar for both siRNAs (Figure 5C).

230   There was no significant effect of the *Tmem135* siRNA on basal or fasting-induced hepatic

231   triglycerides (Figure S3A), while there was a tendency for increased hepatic non-esterified fatty

232   acid (NEFA) concentrations in TMEM135 knockdown mice in the fed state (Figure S3B). There

233   were no significant effects of TMEM135 knockdown on liver ATP or glycogen concentrations

234   (Figure S3C-D). Also, there were no significant effects of TMEM135 on serum lipids (total

235   cholesterol, HDL cholesterol, triglycerides), although there was a trend for fasting to reduce serum

236   triglycerides in control siRNA mice that was not observed in TMEM135 knockdown mice (Figure

237   S4A). There were no significant effects of TMEM135 knockdown on serum NEFA, glucose,

238   insulin, or β-hydroxybutyrate concentrations (Figure S4B-E). The mRNA expression of key genes

239   involved in fatty acid β-oxidation were determined in the liver. As expected, fasting significantly

240   induced mRNA expression of *Ppara*, *Cpt1a*, acyl-CoA dehydrogenase medium chain (*Acadm*),

241   uncoupling protein 2 (*Ucp2*), and sirtuin 3 (*Sirt3*) (Figure 5D). Similarly, knockdown of TMEM135

242   significantly increased *Acadm* and *Sirt3* in fed animals, while *Ucp2* was significantly reduced by

243   TMEM135 knockdown in fasted animals (Figure 5D). As expected with increased β-oxidative flux,

244   fasting significantly increased NADH in animals receiving the control siRNA, while TMEM135

245   knockdown significantly reduced NADH (Figure 5E) indicating an impairment in β-oxidation in

246   fasted mice. Reduced hepatic NADH during fasting could be due to reduced β-oxidation in

247   peroxisomes and/or mitochondria. However, there was a reciprocal significant increase in hepatic

248   ketone concentrations in fasted mice with TMEM135 knockdown (Figure 5F). Because

249   ketogenesis occurs exclusively in mitochondria and is intricately linked with fatty acid oxidation

250   (Newman and Verdin, 2017), this indicates that mitochondrial fatty acid β-oxidation was not

251   impaired. Thus, knockdown mice have impaired fatty acid β-oxidation in peroxisomes but not

252   mitochondria.

253   TMEM135 Knockdown Reduces Import of Peroxisome Matrix Enzymes

254   TMEM135 is a peroxisomal protein with high homology to the Tim17 family that mediate

255   translocation of proteins across mitochondrial membranes (Zarsky and Dolezal, 2016). In addition

256   to peroxisomes, TMEM135 has been reported to be localized to mitochondria (Exil et al., 2010;

257   Lee et al., 2016). Therefore, we hypothesized that TMEM135 mediates protein import into

258   peroxisomes and/or mitochondria. A mitochondria/peroxisome-enriched fraction was prepared

259   from frozen mouse livers. This fraction was validated to contain both mitochondria and

260   peroxisomes as indicated by presence of the mitochondria marker cytochrome c oxidase subunit

261   4I1 (COX4I1) and the peroxisome marker ATP binding cassette subfamily D member 3 (ABCD3,

262   also known as PMP70), while COX4I1 and ABCD3 were not detected in the cytosolic fraction

263   (Figure 6A). Furthermore, the cytosolic protein tubulin beta class I (TUBB) segregated to the

264   cytosolic fraction and was not detected in the mitochondria/peroxisome-enriched fraction.

265   Proteomic analysis of a subset of fed mice was used to provide an unbiased estimate of

266   differential protein abundance in the mitochondria/peroxisome-enriched fractions. A total of 23

267   proteins were significantly less abundant (Fisher's Exact Test, $p < 0.05$) in the

268   mitochondria/peroxisome-enriched fraction from TMEM135 knockdown livers (Figure 6B).

269   Interestingly, these 23 proteins included nearly all the matrix proteins known to be necessary for

270   peroxisomal bile acid synthesis and β-oxidation of very-long chain fatty acids, branched chain

271   fatty acids, and dicarboxylic acids (Baes and Van Veldhoven, 2016; Waterham et al., 2016)

272   (Figure 6 C-D). Analysis of ABCD3 expression by proteomic and Western blot analysis

273   demonstrated that total peroxisome content was not significantly altered by TMEM135 knockdown

274   (Figure 6E), indicating that the lower abundance of peroxisomal proteins in the proteomic data

275   was not simply due to reduced peroxisome content. Four peroxisomal proteins were selected for

276   further analysis via Western blot: acetyl-CoA acyltransferase 1 (ACAA1), acyl-CoA oxidase 1

277   (ACOX1), sterol carrier protein 2 (SCP2), and catalase (CAT). All these proteins were enriched in

278   the mitochondria/peroxisome fraction, but except for SCP2 (which had low detection sensitivity)

279   each protein was also clearly detectable in the cytosolic fraction (Figure 6F). This contrasts with

280 the peroxisome membrane protein ABCD3 which appeared to be exclusively localized to the

281 mitochondria/peroxisome fraction (Figure 6A). Collectively, detection of these proteins in the

282 cytoplasm indicates that their import into peroxisomes may be rate-limiting.

283 We next used Western blot to quantitatively determine import of ACAA1, ACOX1, SCP2,

284 and CAT into peroxisomes. Whole cell lysates (using TUBB as loading control) and

285 mitochondria/peroxisome-enriched fractions (using ABCD3 as loading control for peroxisomes)

286 were analyzed (Figure 7A). The signal for each protein was normalized to the respective loading

287 control, and the ratio of peroxisome to whole cell lysate was used to determine import. Using this

288 method, there were no significant differences between siRNAs for peroxisome import of ACOX1

289 and CAT in either fed or fasted animals (Figure 7B). However, relative ACAA1 import was

290 significantly reduced in TMEM135 knockdown mice when fasted (Figure 7B). Furthermore, SCP2

291 import was significantly lower in TMEM135 knockdown mice in both the fed and fasted state

292 (Figure 7B). Collectively, Western blot confirmed that TMEM135 mediates the import of ACAA1

293 and SCP2 into peroxisomes, and thus may mediate the import of additional proteins identified via

294 proteomics.

295 <u>TMEM135 Knockdown Inhibits Fasting-Induced β-Oxidation</u>

296 We next determined the effect of TMEM135 on markers of peroxisome function. In fasted

297 mice, TMEM135 knockdown resulted in a significant increase in total fatty acids and linoleic acid,

298 with trends ($0.05 < p < 0.1$) for increases in several other fatty acids (Figure 7C-D). In general,

299 mitochondria preferentially oxidize short and medium chain fatty acids (<C12), both mitochondria

300 and peroxisomes oxidize long chain fatty acids (C14-C18), while peroxisomes exclusively oxidize

301 very-long chain fatty acids (>C20) (Cipolla and Lodhi, 2017). While mitochondria are believed to

302 be the principal site of long chain fatty acid oxidation, peroxisomes also directly oxidize long chain

303 fatty acids in a cooperative manner with mitochondria (Noland et al., 2007). The peroxisomal

304 contribution to long chain fatty acid oxidation becomes quantitatively greater during physiologic

305 states of increased fatty acid load such as fasting, diabetes, and high fat diets (Baes and Van

306   Veldhoven, 2016). The significant increase in linoleic and total fatty acids is consistent with an

307   impairment in β-oxidation in fasted TMEM135 knockdown mice, and when considering hepatic

308   NADH and ketone concentrations (Figure 5E-F), indicates that the impairment occurred in

309   peroxisomes. Also, linoleic acid is an essential fatty acid obtained via dietary sources (Saini and

310   Keum, 2018), which indicates that TMEM135 is not altering fatty acid synthesis or desaturation.

311   With the exception of 22:6 that is derived from 18:3 (Saini and Keum, 2018), fasting itself did not

312   increase any of the very-long chain fatty acids that are exclusively oxidized by peroxisomes and

313   may explain why TMEM135 knockdown did not affect very-long chain fatty acid concentrations in

314   fasted mice. Interestingly, in fed mice TMEM135 knockdown significantly decreased 22:0 and

315   tended to decrease 24:0, indicating a slight increase in peroxisomal β-oxidation in the fed state.

316   Regarding bile acids, hepatic concentrations of cholic acid were not significantly altered by

317   TMEM135 knockdown (Figure S5). Collectively, these data indicate that TMEM135 may not be

318   obligatory to basal peroxisome function, but under physiologic conditions of elevated hepatic fatty

319   acid flux, TMEM135 may play a key role in enhancing peroxisomal function to assist the liver in

320   meeting the increased demand for β-oxidation.

321   **Discussion**

322   Peroxisomes have key catabolic functions including α and β-oxidation of fatty acids, and

323   anabolic activities including the synthesis of bile acids and ether phospholipids (Baes and Van

324   Veldhoven, 2016; Waterham et al., 2016). There are 2 main classes of peroxisome disorders: 1)

325   peroxisome biogenesis disorders, and 2) single peroxisomal enzyme deficiencies (Baes and Van

326   Veldhoven, 2016; Waterham et al., 2016). Outcomes from these disorders range from infant death

327   to mild degenerative neurosensory problems (Baes and Van Veldhoven, 2016; Waterham et al.,

328   2016). Hepatic pathologies are one of the principal features associated with peroxisome

329   disorders, which are often caused by a failure to import matrix enzymes into peroxisomes (Baes

330   and Van Veldhoven, 2016). In the current study we determined that TMEM135 is an LXR-

331   inducible protein that regulates peroxisome function by mediating an auxiliary import pathway for

332   matrix enzymes to enter peroxisomes.

333   Multiple lines of evidence support TMEM135 as being a regulator of peroxisome function.

334   When hepatic β-oxidation (both peroxisomal and mitochondrial) was stimulated by fasting,

335   TMEM135 knockdown mice had significantly lower NADH (Figure 5E) and significantly higher

336   total fatty acid and linoleic acid concentrations (Figure 7C-D), indicating an impairment in β-

337   oxidation. Meanwhile, hepatic ketones were significantly increased in knockdown mice when

338   fasted (Figure 5F), and ketogenesis occurs exclusively in mitochondria and is intricately linked to

339   β-oxidation (Newman and Verdin, 2017). Collectively, these findings indicate that β-oxidation was

340   impaired in peroxisomes, but not in mitochondria. The increase in ketones may have resulted

341   from the increased availability of fatty acids to the mitochondria and/or due to the increased

342   NAD+/NADH ratio, both of which stimulate ketogenesis (Newman and Verdin, 2017). Additionally,

343   many effects of hepatocyte-specific TMEM135 knockdown in the current study are consistent with

344   typical features of peroxisome dysfunction. Liver-specific peroxisome deficiency in mice

345   (peroxisomal biogenesis factor 5 or *Pex5* conditional knockout) results in hepatic fat accumulation

346   despite significantly reduced mRNA expression of *Srebf1* and increased PPARA target gene

347   mRNA expression, and a stunting of body growth (Peeters et al., 2011). In our study, knockdown

348   of TMEM135 caused fatty acid accumulation both *in vitro* and *in vivo.* In HepG2 cells, increased

349   triglycerides occurred despite a simultaneous significant reduction in mRNA expression of

350   *SREBF1* (Figure 4A-B), and some PPARA target genes were increased by TMEM135 knockdown

351   in fed mice (Figure 5D). Also, TMEM135 knockdown caused a significant reduction in body weight

352   gain of knockdown mice in the fed state (Figure 5C). Collectively, these data indicate that

353   TMEM135 mediates peroxisome function.

354   Mechanistically, our data indicate that TMEM135 modulates peroxisome function by

355   regulating the import of matrix enzymes into peroxisomes. This differs from the classical

356   mechanism involving peroxisomal targeting signals (PTS), which are short amino acid sequences

357   at the N or C-terminus that interact with various members of the peroxin family of proteins to

358   facilitate import into the peroxisome matrix (Haimovich et al., 2016). Among the proteins whose

359   import was quantified by Western blot in the current study ACOX1 and SCP2 contain a PTS1

360   sequence on their C-terminus that is recognized by PEX5, and ACAA1 contains a PTS2 sequence

361   at its N-terminus that interacts with PEX7 (peroxisomal biogenesis factor 7) (Haimovich et al.,

362   2016; Mizuno et al., 2013). Therefore, TMEM135 is likely not obligatory for their import, but rather

363   serves an auxiliary role. It is not known whether TMEM135-mediated protein import occurs via a

364   mechanism that involves, or occurs independently of, peroxins. Because TMEM135 is a

365   transmembrane protein with TIM17 homology (Zarsky and Dolezal, 2016), a peroxin-independent

366   mechanism seems most likely. Regardless, TMEM135-mediated protein import may be

367   necessary under conditions of high peroxisome activity when peroxin-dependent import becomes

368   saturated, which is consistent with our finding that fasting-induced hepatic β-oxidation is

369   dependent on TMEM135.

370   An auxiliary role of TMEM135 in peroxisome protein import makes it a potential therapeutic

371   target, and because it is also an LXR target gene in humans, pharmacologic modulation of the

372   LXRs may be useful for altering peroxisome metabolism. The LXRs are master regulators of

373   reverse cholesterol transport (RCT), a process whereby excess cholesterol is removed from

374   peripheral tissues and transported to the liver for elimination in bile acids (Hong and Tontonoz,

375   2014). There has been much interest in the development of LXR agonists to treat human diseases

376   including atherosclerosis, Alzheimer's disease, and other metabolic disorders (Hong and

377   Tontonoz, 2014). However, the therapeutic potential of LXR agonists has been limited because

378   they also increase hepatic lipogenesis via induction of SREBP1c (Repa et al., 2000; Yoshikawa

379   et al., 2001). Our data indicate that the LXRs can stimulate peroxisomal β-oxidation by increasing

380   transcription of *TMEM135*, which consequently may limit steatosis during LXR-induced

381   lipogenesis. It should be noted that our data indicate that *TMEM135* is an LXR target gene in

382   humans, but in mice *Tmem135* is either not induced by the LXRs or is a low-affinity target gene

15

383   (Figure S2). Thus, LXR agonist-induced steatosis may be less pronounced in human compared

384   to mice hepatocytes due to species differences in LXR regulation of *TMEM135* transcription.

385   Previous evidence of a link between the LXRs and peroxisomal β-oxidation is limited and

386   conflicting. One group reported increased hepatic peroxisomal β-oxidation in mice treated with an

387   LXR agonist through an undefined mechanism involving increased oxidative gene expression

388   (Beyer et al., 2004; Hu et al., 2005). However, another report indicates that the LXRs suppress

389   peroxisomal β-oxidation by inhibiting expression of the peroxisomal membrane transporter

390   ABCD2 (Gondcaille et al., 2014). It would be interesting to determine how LXR-mediated

391   suppression of ABCD2 and induction of TMEM135 influences overall peroxisome function.

392   Elevated TMEM135 may protect against lipid loading but may also contribute to hepatic

393   pathologies. For example, metabolomics studies have reported that elevated serum bile acids

394   and urinary bile salts represent the core metabolic phenotype in the transition from healthy liver

395   to nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, cirrhosis, and HCC (Beyoglu and

396   Idle, 2013). Additionally, elevated β-oxidation and reduced mitochondrial respiration are part of

397   the metabolic remodeling that occurs in chronic liver disease (Beyoglu and Idle, 2013). These

398   metabolic changes are consistent with high peroxisome activity as peroxisomes play a key role in

399   bile acid synthesis, they contribute to fatty acid β-oxidation, and can also indirectly alter

400   mitochondrial respiration (Baes and Van Veldhoven, 2016). In the current study TMEM135

401   knockdown reduced proliferation of the HepG2 cell line by increasing G0/G1 arrest. Also, a

402   metabolomics study found that linoleic acid is significantly reduced in human HCC tumors

403   compared to nontumor liver tissue from the same subjects (Beyoglu et al., 2013), and in our data

404   linoleic acid was significantly increased by TMEM135 knockdown *in vivo* (Figure 7C), consistent

405   with a role for TMEM135 in pathogenic β-oxidation. There is additional evidence in the literature

406   of an oncogenic role for TMEM135. It was among genes identified by transcriptomic analysis as

407   being significantly reduced in expression when proliferation of HepG2 and Huh7 cells were

408   inhibited by knockdown of the COP9 signalosome subunit 5 (Lee et al., 2011). Also, a genomic

16

409 rearrangement that results in a TMEM135-containing fusion transcript has been linked with

410 aggressive prostate cancer (Yu et al., 2014), and Cas9-mediated insertion of a suicide gene into

411 cells expressing this same fusion transcript reduces tumor burden and mortality in mouse

412 xenografts (Chen et al., 2017). Thus, TMEM135 may represent a novel target in the treatment of

413 certain cancers and chronic liver disease.

414 　　　　Previous studies to determine the biological function of TMEM135 are limited. The first

415 indication of its function came from a mouse model of mitochondrial acyl-CoA dehydrogenase

416 very long chain (ACADVL) deficiency (Exil et al., 2010). Under conditions of increased demand

417 for β-oxidation such as fasting and cold stress, ACADVL-deficient mice have reduced survival

418 due to cardiac dysfunction (Exil et al., 2010). It was discovered that TMEM135 was elevated more

419 than four-fold in the hearts of ACADVL-deficient mice that survived birth (Exil et al., 2010). The

420 ACADVL enzyme catalyzes the first step in mitochondrial β-oxidation using fatty acids larger than

421 14 carbons as substrates (McAndrew et al., 2008). Because ACADVL deficiency restricts the

422 mitochondria from directly oxidizing long chain fatty acids, data from the current study indicate

423 that the increase in TMEM135 likely promoted survival by increasing peroxisomal β-oxidation and

424 the processing of long and very-long chain fatty acids to medium chain fatty acids that were

425 subsequently oxidized in the mitochondria in an ACADVL-independent manner. Another study

426 into TMEM135 function reported that a mutation in *Tmem135* was responsible for accelerated

427 aging of the retina in a mouse model of age-related macular degeneration (Lee et al., 2016).

428 Neurodegenerative disorders are commonly associated with peroxisome dysfunction (Cipolla and

429 Lodhi, 2017), which is consistent with a *Tmem135* mutation causing accelerated aging in the

430 retina. Both of these previous studies on TMEM135 reported that it is localized to mitochondria

431 (Exil et al., 2010; Lee et al., 2016), whereas our data indicates it functions in peroxisomes. In

432 support of a peroxisomal origin of action, another previous study clearly demonstrated that

433 TMEM135 (formerly known as PMP52) is localized to peroxisomes and not mitochondria (Wiese

434 et al., 2007). Because peroxisomes and mitochondria can physically associate via the formation

17

435 of tethering complexes (Schrader et al., 2015), it seems likely that reports of TMEM135

436 association with mitochondria (Exil et al., 2010; Lee et al., 2016) were due to peroxisomes that

437 were tethered to mitochondria, and TMEM135 effects on mitochondria are indirectly mediated via

438 altered peroxisome function.

439 The critical role of peroxisomes in human health is illustrated by the spectrum of

440 peroxisome disorders; but in addition to these inborn errors of metabolism it is becoming

441 increasingly apparent that peroxisomes play key roles in other metabolic and age-related

442 diseases including cancer, neurodegenerative disorders, and diabetes (Cipolla and Lodhi, 2017).

443 This may be due to direct catabolic and anabolic actions of peroxisomes, as well as interactions

444 between peroxisomes and other organelles including mitochondria (Cipolla and Lodhi, 2017;

445 Schrader et al., 2015). Our data indicate that TMEM135 regulates peroxisome function by

446 mediating an auxiliary matrix protein import pathway. Furthermore, the effects of TMEM135 are

447 inducible as it is an LXR target gene in humans, and it is likely to be regulated by other metabolic

448 signaling pathways. In support of this, PPARA is the master regulator of β-oxidation in the liver

449 and in primary human hepatocytes *TMEM135* mRNA expression is consistently induced by

450 PPARA agonists (Kersten and Stienstra, 2017) and its promoter is bound by PPARA as

451 determined using ChIP-seq (McMullen et al., 2014), indicating it is also a PPARA target gene.

452 These findings implicate TMEM135 as a potential therapeutic target in the treatment of metabolic

453 and age-related diseases that are associated with peroxisome dysfunction.

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466   **Author Contributions**

467   Conceptualization, R.L.B. and B.J.R.; Methodology R.L.B. and B.J.R.; Investigation,

468   R.L.B., B.J.R., T.W.M., S.G., C.E.G., and Y.X.; Resources, R.L.B. and B.J.R.; Writing – Original

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471   **Declaration of Interests**

472   The authors declare no competing interests.

473   **References**

474   Baes, M., and Van Veldhoven, P.P. (2016). Hepatic dysfunction in peroxisomal disorders. Biochim
475   Biophys Acta *1863*, 956-970.
476   Beyer, T.P., Schmidt, R.J., Foxworthy, P., Zhang, Y., Dai, J., Bensch, W.R., Kauffman, R.F., Gao,
477   H., Ryan, T.P., Jiang, X.C., et al. (2004). Coadministration of a liver X receptor agonist
478   and a peroxisome proliferator activator receptor-alpha agonist in Mice: effects of nuclear
479   receptor interplay on high-density lipoprotein and triglyceride metabolism in vivo. J
480   Pharmacol Exp Ther *309*, 861-868.
481   Beyoglu, D., and Idle, J.R. (2013). The metabolomic window into hepatobiliary disease. J Hepatol
482   *59*, 842-858.
483   Beyoglu, D., Imbeaud, S., Maurhofer, O., Bioulac-Sage, P., Zucman-Rossi, J., Dufour, J.F., and
484   Idle, J.R. (2013). Tissue metabolomics of hepatocellular carcinoma: tumor energy
485   metabolism and the role of transcriptomic classification. Hepatology *58*, 229-238.
486   Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M.,
487   Bayerlein, M., and Werner, T. (2005). MatInspector and beyond: promoter analysis based
488   on transcription factor binding sites. Bioinformatics *21*, 2933-2942.
489   Chen, Z.H., Yu, Y.P., Zuo, Z.H., Nelson, J.B., Michalopoulos, G.K., Monga, S., Liu, S., Tseng, G.,
490   and Luo, J.H. (2017). Targeting genomic rearrangements in tumor cells through Cas9-
491   mediated insertion of a suicide gene. Nat Biotechnol *35*, 543-550.
492   Cipolla, C.M., and Lodhi, I.J. (2017). Peroxisomal Dysfunction in Age-Related Diseases. Trends
493   Endocrinol Metab *28*, 297-308.
494   Costet, P., Luo, Y., Wang, N., and Tall, A.R. (2000). Sterol-dependent transactivation of the ABC1
495   promoter by the liver X receptor/retinoid X receptor. J Biol Chem *275*, 28240-28245.
496   Exil, V.J., Silva Avila, D., Benedetto, A., Exil, E.A., Adams, M.R., Au, C., and Aschner, M. (2010).
497   Stressed-induced TMEM135 protein is part of a conserved genetic network involved in fat
498   storage and longevity regulation in Caenorhabditis elegans. PLoS One *5*, e14228.

Geisler, C.E., Hepler, C., Higgins, M.R., and Renquist, B.J. (2016). Hepatic adaptations to maintain metabolic homeostasis in response to fasting and refeeding in mice. Nutr Metab (Lond) *13*, 62.

Gondcaille, C., Genin, E.C., Lopez, T.E., Dias, A.M., Geillon, F., Andreoletti, P., Cherkaoui-Malki, M., Nury, T., Lizard, G., Weinhofer, I., et al. (2014). LXR antagonists induce ABCD2 expression. Biochim Biophys Acta *1841*, 259-266.

Haimovich, G., Cohen-Zontag, O., and Gerst, J.E. (2016). A role for mRNA trafficking and localized translation in peroxisome biogenesis and function? Biochim Biophys Acta *1863*, 911-921.

Harper, J.W., and Adams, P.D. (2001). Cyclin-dependent kinases. Chem Rev *101*, 2511-2526.

Hong, C., and Tontonoz, P. (2014). Liver X receptors in lipid metabolism: opportunities for drug discovery. Nat Rev Drug Discov *13*, 433-444.

Hu, T., Foxworthy, P., Siesky, A., Ficorilli, J.V., Gao, H., Li, S., Christe, M., Ryan, T., Cao, G., Eacho, P., et al. (2005). Hepatic peroxisomal fatty acid beta-oxidation is regulated by liver X receptor alpha. Endocrinology *146*, 5380-5387.

Jensen, J.T., Addis, I.B., Hennebold, J.D., and Bogan, R.L. (2017). Ovarian Lipid Metabolism Modulates Circulating Lipids in Premenopausal Women. J Clin Endocrinol Metab *102*, 3138-3145.

Kennedy, M.A., Venkateswaran, A., Tarr, P.T., Xenarios, I., Kudoh, J., Shimizu, N., and Edwards, P.A. (2001). Characterization of the human ABCG1 gene: liver X receptor activates an internal promoter that produces a novel transcript encoding an alternative form of the protein. J Biol Chem *276*, 39438-39447.

Kersten, S., and Stienstra, R. (2017). The role and regulation of the peroxisome proliferator activated receptor alpha in human liver. Biochimie *136*, 75-84.

Laffitte, B.A., Joseph, S.B., Walczak, R., Pei, L., Wilpitz, D.C., Collins, J.L., and Tontonoz, P. (2001). Autoregulation of the human liver X receptor alpha promoter. Mol Cell Biol *21*, 7558-7568.

LaPak, K.M., and Burd, C.E. (2014). The molecular balancing act of p16(INK4a) in cancer and aging. Mol Cancer Res *12*, 167-183.

Lee, W.H., Higuchi, H., Ikeda, S., Macke, E.L., Takimoto, T., Pattnaik, B.R., Liu, C., Chu, L.F., Siepka, S.M., Krentz, K.J., et al. (2016). Mouse Tmem135 mutation reveals a mechanism involving mitochondrial dynamics that leads to age-dependent retinal pathologies. Elife *5*.

Lee, Y.H., Judge, A.D., Seo, D., Kitade, M., Gomez-Quiroz, L.E., Ishikawa, T., Andersen, J.B., Kim, B.K., Marquardt, J.U., Raggi, C., et al. (2011). Molecular targeting of CSN5 in human hepatocellular carcinoma: a mechanism of therapeutic response. Oncogene *30*, 4175-4184.

Masood, A., Stark, K.D., and Salem, N., Jr. (2005). A simplified and efficient method for the analysis of fatty acid methyl esters suitable for large clinical studies. J Lipid Res *46*, 2299-2305.

McAndrew, R.P., Wang, Y., Mohsen, A.W., He, M., Vockley, J., and Kim, J.J. (2008). Structural basis for substrate fatty acyl chain specificity: crystal structure of human very-long-chain acyl-CoA dehydrogenase. J Biol Chem *283*, 9435-9443.

McMullen, P.D., Bhattacharya, S., Woods, C.G., Sun, B., Yarborough, K., Ross, S.M., Miller, M.E., McBride, M.T., LeCluyse, E.L., Clewell, R.A., et al. (2014). A map of the PPARalpha transcription regulatory network for primary human hepatocytes. Chem Biol Interact *209*, 14-24.

Mizuno, Y., Ninomiya, Y., Nakachi, Y., Iseki, M., Iwasa, H., Akita, M., Tsukui, T., Shimozawa, N., Ito, C., Toshimori, K., et al. (2013). Tysnd1 deficiency in mice interferes with the peroxisomal localization of PTS2 enzymes, causing lipid metabolic abnormalities and male infertility. PLoS Genet *9*, e1003286.

Newman, J.C., and Verdin, E. (2017). beta-Hydroxybutyrate: A Signaling Metabolite. Annu Rev Nutr *37*, 51-76.

Noland, R.C., Woodlief, T.L., Whitfield, B.R., Manning, S.M., Evans, J.R., Dudek, R.W., Lust, R.M., and Cortright, R.N. (2007). Peroxisomal-mitochondrial oxidation in a rodent model of obesity-associated insulin resistance. Am J Physiol Endocrinol Metab *293*, E986-E1001.

Peet, D.J., Janowski, B.A., and Mangelsdorf, D.J. (1998). The LXRs: a new class of oxysterol receptors. Curr Opin Genet Dev *8*, 571-575.

Peeters, A., Swinnen, J.V., Van Veldhoven, P.P., and Baes, M. (2011). Hepatosteatosis in peroxisome deficient liver despite increased beta-oxidation capacity and impaired lipogenesis. Biochimie *93*, 1828-1838.

Repa, J.J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J.M., Shimomura, I., Shan, B., Brown, M.S., Goldstein, J.L., and Mangelsdorf, D.J. (2000). Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. Genes Dev *14*, 2819-2830.

Saini, R.K., and Keum, Y.S. (2018). Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance - A review. Life Sci *203*, 255-267.

Salway, J.G. (1969). The simultaneous determination of acetoacetate and glucose in capillary blood. Clin Chim Acta *25*, 109-116.

Schrader, M., Godinho, L.F., Costello, J.L., and Islinger, M. (2015). The different facets of organelle interplay-an overview of organelle interactions. Front Cell Dev Biol *3*, 56.

Schwartz, D.M., and Wolins, N.E. (2007). A simple and rapid method to assay triacylglycerol in cells and tissues. J Lipid Res *48*, 2514-2520.

Seto, N.L., and Bogan, R.L. (2015). Decreased cholesterol uptake and increased liver x receptor-mediated cholesterol efflux pathways during prostaglandin F2 alpha-induced and spontaneous luteolysis in sheep. Biol Reprod *92*, 128.

Stolowich, N.J., Petrescu, A.D., Huang, H., Martin, G.G., Scott, A.I., and Schroeder, F. (2002). Sterol carrier protein-2: structure reveals function. Cell Mol Life Sci *59*, 193-212.

Teboul, M., Enmark, E., Li, Q., Wikstrom, A.C., Pelto-Huikko, M., and Gustafsson, J.A. (1995). OR-1, a member of the nuclear receptor superfamily that interacts with the 9-cis-retinoic acid receptor. Proc Natl Acad Sci U S A *92*, 2096-2100.

Walker, P.G. (1954). A colorimetric method for the estimation of acetoacetate. Biochem J *58*, 699-704.

Waterham, H.R., Ferdinandusse, S., and Wanders, R.J. (2016). Human disorders of peroxisome metabolism and biogenesis. Biochim Biophys Acta *1863*, 922-933.

Whitney, K.D., Watson, M.A., Goodwin, B., Galardi, C.M., Maglich, J.M., Wilson, J.G., Willson, T.M., Collins, J.L., and Kliewer, S.A. (2001). Liver X receptor (LXR) regulation of the LXRalpha gene in human macrophages. J Biol Chem *276*, 43509-43515.

Wiese, S., Gronemeyer, T., Ofman, R., Kunze, M., Grou, C.P., Almeida, J.A., Eisenacher, M., Stephan, C., Hayen, H., Schollenberger, L., et al. (2007). Proteomics characterization of mouse kidney peroxisomes by tandem mass spectrometry and protein correlation profiling. Mol Cell Proteomics *6*, 2045-2057.

Willy, P.J., Umesono, K., Ong, E.S., Evans, R.M., Heyman, R.A., and Mangelsdorf, D.J. (1995). LXR, a nuclear receptor that defines a distinct retinoid response pathway. Genes Dev *9*, 1033-1045.

Xu, Y., Hernandez-Ledezma, J.J., Hutchison, S.M., and Bogan, R.L. (2018a). The liver X receptors and sterol regulatory element binding proteins alter progesterone secretion and are regulated by human chorionic gonadotropin in human luteinized granulosa cells. Mol Cell Endocrinol *473*, 124-135.

598 Xu, Y., Hutchison, S.M., Hernandez-Ledezma, J.J., and Bogan, R.L. (2018b). Increased 27-
599   hydroxycholesterol production during luteolysis may mediate the progressive decline in
600   progesterone secretion. Mol Hum Reprod *24*, 2-13.
601 Yoshikawa, T., Shimano, H., Amemiya-Kudo, M., Yahagi, N., Hasty, A.H., Matsuzaka, T.,
602   Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., et al. (2001). Identification of liver X
603   receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein
604   1c gene promoter. Mol Cell Biol *21*, 2991-3000.
605 Yu, Y.P., Ding, Y., Chen, Z., Liu, S., Michalopoulos, A., Chen, R., Gulzar, Z.G., Yang, B., Cieply,
606   K.M., Luvison, A., et al. (2014). Novel fusion transcripts associate with progressive
607   prostate cancer. Am J Pathol *184*, 2840-2849.
608 Zarsky, V., and Dolezal, P. (2016). Evolution of the Tim17 protein family. Biol Direct *11*, 54.
609 Zelcer, N., Hong, C., Boyadjian, R., and Tontonoz, P. (2009). LXR regulates cholesterol uptake
610   through Idol-dependent ubiquitination of the LDL receptor. Science *325*, 100-104.

611 **Figure Legends**

612

613 **Figure 1: A synthetic LXR agonist induces TMEM135 expression in human hepatocyte**

614 **(HepG2, Hep3B) and monocyte-derived macrophage (THP-1) cell lines via a direct**

615 **transcriptional mechanism.** Panel A contains the results from treatment of HepG2 cells in the

616 presence or absence of the synthetic LXR agonist T09 for 6, 12, 24 and 48 hours; while panel B

617 is the effect of a 24-hour T09 treatment on *TMEM135* mRNA expression in Hep3B cells (n = 4).

618 Panel C is a time course of T09 treatment in THP-1 cells (n = 4). For panels A-C, asterisks denote

619 significant ($p < 0.05$) difference between DMSO and T09 at the indicated timepoint. Panel D is the

620 effect of the protein synthesis inhibitor cycloheximide on *TMEM135* mRNA expression in THP-1

621 cells. Cells were treated for 24 hours in a 2 × 2 factorial with T09 and cycloheximide (n = 5).

622 Columns with different letters are significantly different ($p < 0.05$). Panel E is Western blot analysis

623 of TMEM135 protein in the mitochondria/peroxisome-enriched fraction of HepG2 cells treated with

624 or without T09 for 24 hours (n = 4). The approximate molecular weights of the protein ladder are

625 indicated, as well as the TMEM135 and COX4I1 (housekeeping control) bands. Panel F is results

626 of densitometry analysis of the blot shown in Panel E, asterisk denotes significant difference ($p <$

627 0.05). For all panels, error bars indicate ± one standard error of the mean (SEM).

628 **Figure 2: The LXRs bind all three potential LXREs in the human *TMEM135* promoter with**

629 **LXRE3 mediating LXR-induced *TMEM135* transcription.** Panel A is an EMSA using LXRE1 as

630 the fluorescent probe. The nuclear receptor(s) used in the reaction are indicated beneath the

631   image, with the unlabeled competitor DNA (200-fold molar excess) shown above the image. Panel

632   B substitutes NR1H2 for NR1H3 in the binding reactions. Panel C utilizes fluorescent LXRE3 as

633   the probe. The image has been cropped to only show the bound and free fluorescent LXRE3

634   probe. The nuclear receptor(s) used in the reaction are indicated beneath the image. Increasing

635   amounts (0.25, 0.5, 1, 2, and 10-fold molar excess per competitor) of unlabeled LXRE1 or LXRE3

636   were included in some reactions as indicated above the images. In Panel D, an EMSA was

637   performed using a mixture of LXRE1 and LXRE3 probes that were labeled with spectrally-distinct

638   fluorescent dyes, as well as increasing quantities of LXR/RXR proteins. The percent of each probe

639   bound in each reaction was determined by densitometry and plotted. Panel E displays luciferase

640   activity (arbitrary units normalized to β-galactosidase) derived from cells transfected with the

641   empty vector or the *TMEM135* promoter-containing construct in the presence and absence of

642   T09. The effect of increased expression of NR1H3 and RXRA is also shown (n = 4). Panel F

643   contains the fold-increase in luciferase activity induced by T09 from the wild type *TMEM135*

644   promoter, as well as *TMEM135* promoters containing all possible combinations of mutant LXRE

645   sites (n = 4). All transfections in Panel F included *NR1H3* and *RXRA* co-transfection to increase

646   basal expression of these nuclear receptors. An X indicates point mutations were introduced into

647   the corresponding LXRE (see Figure S1), Luc = luciferase. Error bars indicate one SEM, columns

648   without common letters are significantly (p< 0.05) different. See also Figures S1 and S2.

649   **Figure 3: The LXRs are obligatory for LXR agonist-induced *TMEM135* mRNA expression,**

650   **and cause transcription of *TMEM135* with an abbreviated 5' UTR.** Panels A, B, C, and D show

651   the effects of siRNA transfection in HepG2 cells on T09-induced mRNA expression of the LXR

652   isoforms *NR1H2* and *NR1H3*, *ABCA1*, and *TMEM135*; respectively. Panel E shows the effect of

653   LXR knockdown in HepG2 cells on T09-induced expression of *TMEM135* mRNA transcripts

654   containing the full 5' UTR. Primers and probe used for QPCR in panel E amplify a 76 bp region

655   within the 5' UTR that encompasses the LXR binding site in genomic DNA. Panel F is results from

23

656   ChIP analysis. For all panels, error bars indicate one SEM, columns without common letters are

657   significantly (p< 0.05) different (n = 4).

658   **Figure 4: TMEM135 regulates fatty acid metabolism and proliferation of HepG2 cells.** Panel

659   A displays the effect of TMEM135 knockdown in the presence and absence of the lipogenic LXR

660   agonist T09 on intracellular triglyceride accumulation in HepG2 cells. Panel B is mRNA

661   expression in HepG2 cells for *TMEM135*, genes involved in fatty acid oxidation (*PPARA*, *CPT1A*),

662   and genes involved in fatty acid synthesis (*SREBF1*, *FASN*). For panels A and B, columns without

663   a common letter are significantly (p< 0.05) different (n = 4). Panel C is the effect of TMEM135

664   knockdown on ATP content (normalized to total protein content) in HepG2 cells after a 4-hour

665   incubation in glucose-free, sodium pyruvate-free media. The chart is plotted with the control

666   siRNA set to 1, and the asterisk denotes a significant difference (n = 4). Panel D is the effect of

667   TMEM135 knockdown on proliferation of HepG2 cells. Asterisks denote significant difference at

668   the corresponding timepoint (n = 4). Panel E is the effect of TMEM135 knockdown on cell cycle

669   progression in HepG2 cells, and panel F is the effect on mRNA expression of cell cycle-associated

670   genes. For panels E-F, asterisks denote significant differences due to TMEM135 knockdown (n

671   = 4).

672   **Figure 5: Liver-specific knockdown of TMEM135 inhibits peroxisomal β-oxidation.** Panel A

673   is mRNA expression of *Tmem135* in the listed tissues isolated from fed mice (n = 5). Panel B

674   displays TMEM135 protein expression in the mitochondria/peroxisome-enriched fraction from

675   livers. The right half of the image was incubated with antibody pre-absorbed with the immunizing

676   peptide. The sample contained in each lane is indicated beneath the images: MW = molecular

677   weight marker, Con = control siRNA, KD = Knockdown, *Tmem135* siRNA. The chart in panel B

678   displays the results of densitometry analysis of all 3 TMEM135 bands in the

679   mitochondria/peroxisome-enriched fraction of mice liver (fed and fasted animals pooled, n = 10

680   mice), as well as spectral counts from one peptide identified via proteomic analysis that was

681   mapped to TMEM135 (n = 3, fed animals only). In Panels A-B, asterisks denote significant

24

682   difference due to *Tmem135* siRNA. Panel C displays the change in body weight of animals from

683   the time of siRNA injection until sacrifice 4 days later (n = 5). Panel D displays hepatic mRNA

684   expression of *Tmem135* and genes involved in fatty acid oxidation, panel E displays hepatic NAD

685   concentrations, and Panel F is hepatic ketone concentrations (total ketones = β-hydroxybutyrate

686   + acetoacetate). For panels C-F, asterisks denote significant difference (p< 0.05) due to

687   *Tmem135* siRNA within feeding status, and # indicates significant difference due to fasting in

688   animals receiving control siRNA (n = 5). See also Figures S3 and S4.

689   **Figure 6: TMEM135 mediates import of peroxisome matrix enzymes.** Panel A displays the

690   relative purity of the mitochondria/peroxisome-enriched fraction used for proteomic analysis as

691   determined by Western blot analysis of controls for mitochondria (COX4I1), peroxisome (ABCD3),

692   and cytosolic (TUBB) proteins. For each image, the lanes from left to right are the molecular

693   weight marker (MW), whole cell lysate (WC), cytosolic fraction (Cyto), and the

694   mitochondria/peroxisome-enriched fraction (M/P). Panel B are proteins significantly decreased

695   (Fisher's Exact Test, p< 0.05) in the mitochondria/peroxisome-enriched fraction from TMEM135

696   knockdown livers, as well as the respective mean ± SEM of their spectral counts (n = 3, fed mice

697   only). Panel C illustrates peroxisomal β-oxidation of very-long chain fatty acids (VLCFA) and

698   dicarboxylic acids (DCA). VLCFA and DCA are transported into peroxisomes via ABCDs (ABCD

699   members 1-3). Panel D illustrates peroxisomal bile acid synthesis and β-oxidation of branched

700   chain fatty acids (BCFA). Bile acid intermediates (BAI) and BCFA are transported into

701   peroxisomes via the ABCD proteins. CA = cholic acid, CDCA = chenodeoxycholic acid, BrAcyl-

702   CoA = branched chain acyl-CoA. For panels C-D, the enzymes catalyzing each step are shown

703   in boxes, and enzymes that were significantly less abundant in the mitochondria/peroxisome-

704   enriched fraction from TMEM135 knockdown mice (Panel B) are shaded. Panel E displays relative

705   peroxisome content in the mitochondria/peroxisome-enriched fractions as determined by

706   proteomic and Western blot analysis. For proteomics, only fed mice were analyzed (n = 3). For

707   Western blot, the ABCD3:COX4I1 ratio in the mitochondria/peroxisome-enriched fraction is

708   shown (n = 5). Panel F is subcellular distribution of ACAA1, ACOX1, SCP2, and CAT. ACOX1

709   migrates in 2 separate bands in Western blot, with the smaller band being proteolytically

710   processed ACOX1 (Mizuno et al., 2013). Furthermore, SCP2 (also known as SCPx) is a 58 kDa

711   protein that contains a 45 kDa 3-ketoacyl CoA thiolase domain and a 13 kDa sterol-carrier domain

712   (Stolowich et al., 2002), which are separated by proteolytic cleavage (Mizuno et al., 2013). The

713   antibody used in the current study recognizes the full-length 58 kDa protein (indicated by arrow)

714   and the 45 kDa 3-ketoacyl CoA thiolase domain, but not the 13 kDa sterol-carrier domain. The

715   identity of the other bands in the SCP2 blot is not known.

716   **Figure 7: Reduced peroxisomal import of matrix enzymes impairs β-oxidation.** Panel A

717   contains images from Western blot analysis of whole cell and mitochondria/peroxisome-enriched

718   fractions (Mito/Perox-Enriched Fraction) in livers of fed and fasted mice. Loading controls were

719   TUBB for whole cell lysates, COX4I1 for mitochondria, and ABCD3 for peroxisomes. The siRNA

720   treatment is indicated beneath the lanes, Con = control siRNA, KD = Knockdown, *Tmem135*

721   siRNA. For SCP2, the band used for densitometry analysis is indicated by asterisks. Panel B

722   displays the results of densitometry analysis of the blots shown in Panel A. Peroxisome import

723   was determined by first normalizing each protein in the mitochondria/peroxisome-enriched

724   fraction to ABCD3 to account for differences in peroxisome content, and the ratio relative to the

725   whole cell lysate (normalized to TUBB) was used to determine peroxisome import. Panel C plots

726   concentrations of C18 fatty acids quantified by GC. In panels B-C, asterisks denote significant

727   difference (p< 0.05) within feeding status due to *Tmem135* siRNA, and # indicates significant

728   difference due to fasting in mice receiving the control siRNA (n = 5). Panel D displays the mean

729   ± SEM for all fatty acids (μg fatty acid per mg protein) quantified by GC. In the P column, asterisks

730   denote significant difference (p< 0.05) and ^ denotes trends (0.05 < p < 0.1) within feeding status

731   due to *Tmem135* siRNA. In the fasted mice control siRNA column, # indicates significant (p< 0.05)

732   difference due to fasting in control siRNA mice. See also Figure S5.

733   **STAR Methods**

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Randy L. Bogan (boganr@email.arizona.edu). Transfer of resources is subject to the acceptance of a Material Transfer Agreement as required by policy at the University of Arizona. Resources are transferred and distributed by several means according to the needs of the end-user. For other academic and non-profit researchers, the resources are typically transferred under standard Material Transfer Agreements. For requests by for-profit organizations, the resources are made available under standard non-exclusive license.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Lines

All cell lines were obtained from American Type Culture Collection (ATCC) and were cultured at 37°C, 5% CO_2 in air, in a humidified incubator. Human cells (all derived from males) included the hepatocyte lines HepG2 (ATCC® HB-8065™) and Hep 3B2.1-7 [Hep3B] (ATCC® HB-8064™), and the monocyte line THP-1 (ATCC® TIB-202™). Murine cells included the hepatocyte line (embryonic-derived) BNL 1NG A.2 (ATCC® TIB-76™) and the macrophage line (male) RAW 264.7 (ATCC® TIB-71™). Growth media for all cell lines except THP-1 was DMEM/F12 (Sigma Aldrich, Inc.) supplemented with Pen/Strep (100 units/ml penicillin and 100 µg/ml streptomycin) and 10% fetal bovine serum (FBS). THP-1 cells were incubated in RPMI media (Sigma) supplemented with 2-mercaptoethanol (0.05 mM), Pen/Strep, and 10% FBS. THP-1 cells were differentiated into macrophages by treatment with 12-myristate-13-acetate (PMA, 100 ng/ml) for 5 days prior to experimentation.

Mice

All procedures involving mice were approved by the University of Arizona IACUC. Male C57BL/6 mice, 12 weeks of age, were used. Mice were group housed until initiation of experimental procedures, at which point they were switched to individual caging.

METHOD DETAILS

27

760 Cell Treatments

761   T0901317 (Cayman Chemical, Inc.) was dissolved in DMSO and used at a 1 µM

762 concentration unless indicated otherwise. Cycloheximide (ACROS Organics, Inc.) was dissolved

763 in DMSO and used at a final concentration of 50 µg/ml. The final DMSO concentration was held

764 constant in all groups at less than 0.2% (*v:v*). For siRNA knockdown experiments, Silencer® select

765 pre-designed siRNAs (Life Technologies, Inc.) against human *NR1H2* (s14684), *NR1H3*

766 (s19568), *TMEM135* (s35201), and a negative control siRNA (catalog 4390843) were purchased

767 and transfected into HepG2 cells using Lipofectamine® RNAiMAX (Life Technologies) or TransIT-

768 siQuest® reagent (Mirus Bio, LLC.) according to manufacturer instructions. All experiments were

769 repeated 4-5 times on different days or with different passages of cells.

770 HepG2 Mitochondria/Peroxisome-Enriched Fraction Isolation

771   Cells were harvested and washed three times in 1 ml cell resuspension buffer (1 mM Tris-

772 HCl, pH 7.4, 130 mM NaCl, 5 mM KCl, 7.5 mM $MgCl_2$). Cells were then resuspended in 600 µl

773 homogenization buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 0.15 mM $MgCl_2$, plus protease and

774 phosphatase inhibitor cocktail), incubated on ice for 10 min, and homogenized for 30 strokes in a

775 glass dounce homogenizer with a tight-fitting pestle. The homogenate was mixed with 100 µl of

776 2M sucrose and centrifuged at 1,200 × g for 5 min at 4° C, and the supernatant transferred to a

777 new tube. This low speed centrifugation step was repeated two more times. Mitochondria and

778 peroxisomes were pelleted by centrifugation at 7,000 × g for 10 min at 4° C and the supernatant

779 removed. The pellet was washed once with 300 µl of wash buffer (10 mM Tris-HCl, pH 7.4, 0.15

780 mM $MgCl_2$, 250 mM sucrose), and centrifuged at 9,500 × g for 5 min. Pellets were solubilized in

781 RIPA buffer containing protease and phosphatase inhibitors, and protein concentration was

782 determined by BCA assay (Fisher Scientific) following manufacturer recommendations.

783 Electrophoretic Mobility-Shift Analysis (EMSA)

784   The DNA sequences for human *NR1H2*, *NR1H3*, and RXR α (*RXRA*) were synthesized

785 by Life Technologies and cloned into the pTarget™ mammalian expression vector (Promega

28

786   Corporation) using XhoI and KpnI restriction enzyme sites. Inserts were confirmed by DNA

787   sequence analysis. Recombinant NR1H2, NR1H3, and RXRA proteins were produced using the

788   TNT® Quick Coupled Transcription/Translation System (Promega). Fluorescent probes were

789   synthesized by Integrated DNA Technologies and corresponded to the LXRE1 sequence with

790   IRDye 700 attached to the 5' end, and probes corresponding to the LXRE3 sequence were

791   labelled with IRDye 800. Additionally, unlabeled DNA oligos corresponding to LXREs 1-3, as well

792   as mutated LXREs 1-3, were synthesized (Figure S1B). Single stranded DNA oligos were

793   annealed by mixing sense and antisense oligos in a 1:1 molar ratio, heating to 100°C for 5

794   minutes, and slowly cooling to room temperature. The EMSA binding reactions were carried out

795   for 30 min at room temperature in 10 mM Tris, 50 mM KCl, pH 7.5; 3.5 mM DTT, 0.25% Tween-

796   20, 1 μg Poly (dI.dC), 5 nM fluorescent probe (1 nM each for LXRE1/LXRE3 competition

797   experiment), and 1.5 μl of TNT lysate per protein (non-induced TNT lysate substituted in control

798   reactions). Unlabeled competitor DNA was added prior to the addition of fluorescent probe at a

799   200-fold molar excess unless indicated otherwise. Following the incubation period, EMSA

800   reactions were resolved on a 6% TBE gel (Life Technologies). Imaging and densitometry were

801   performed with a Li-COR Odyssey CLx and Image Studio version 3.1 software, respectively.

802   Reporter Assays

803   The region from -2662 to -1 bp (relative to translation start site) of the human *TMEM135*

804   promoter was synthesized by Life Technologies. Additionally, the same region of the human

805   *TMEM135* promoter was synthesized except that point mutations were introduced into each of

806   the three LXREs (Figure S1B). The normal and mutant *TMEM135* promoters were cloned into the

807   pGL4.17 vector (Promega) using SacI and XhoI restriction enzyme sites, which expresses firefly

808   luciferase from a promoter cloned into the vector. A unique PacI site was identified in the region

809   between LXRE1 and LXRE2, and a unique EcoRI site was identified in the region between LXRE2

810   and LXRE3. These restriction sites were used to generate six more *TMEM135* promoter/pGL4.17

811   constructs so that all possible combinations of wild type and mutated LXREs were obtained. All

29

812   constructs were verified using DNA sequence analysis. These reporter vectors were transfected

813   into HepG2 cells along with a β-galactosidase control plasmid (Promega) to normalize

814   transfection efficiency. In some transfections, *NR1H3*/pTarget and *RXRA*/pTarget constructs

815   were co-transfected. Vectors were transfected in a ratio of 60:20:10:10 (luciferase:β-

816   galactosidase:NR1H3:RXRA) using Lipofectamine® 3000 according to manufacturer

817   recommendations, with no-insert plasmids substituted in control transfections to keep DNA

818   concentrations constant. Each vector combination was transfected in triplicate to HepG2 cells in

819   96 well plates, and cells were incubated with DNA/Lipofectamine complexes for 24 hours at which

820   point media were changed and cells were treated for an additional 48 hours with DMSO or T09.

821   Cell lysates were prepared using mammalian protein extraction reagent (Fisher), and the lysate

822   was fractionated to quantify luciferase and β-galactosidase activity using luminescent detection

823   kits (Fisher). The ratio of luciferase to β-galactosidase activity was calculated, and the fold-change

824   in T09 vs DMSO treated cells was determined for each transfection.

825   Chromatin Immunoprecipitation (ChIP) Analysis

826   HepG2 cells were fixed for 10 min at room temperature with 1% formaldehyde. The

827   reaction was quenched by adding glycine to a 125 mM final concentration and incubating 5 min

828   at room temperature. The cells were trypsinized, harvested, and washed twice with PBS. Nuclei

829   were isolated by incubating cell pellets in 500 µl of a hypotonic buffer for 15 min on ice (20 mM

830   Tris-HCL, pH 7.4, 10 mM NaCl, 3 mM MgCL$_2$, with protease and phosphatase inhibitor cocktail

831   from Promega). Triton X-100 detergent was added to a 0.5% *v:v* final concentration to lyse the

832   cells followed by centrifugation at 1200 × g for 10 min to pellet nuclei. The supernatant was

833   removed, and the nuclei pellet washed once with 500 µl hypotonic buffer. The pellet was

834   resuspended in 500 µl of RIPA buffer with protease and phosphatase inhibitors, and nuclei were

835   sonicated in an ice-water bath using a Branson 250 digital sonifier programmed to cycle at a 10%

836   amplitude with 5 seconds on followed by 20 seconds off, and a 3-minute total sonication time that

837   was determined in preliminary time-course studies to produce chromatin of optimal length

838   (approximately 200-1000 base pairs). The sonicated lysates were centrifuged at 8,000 × g for 1

839   minute and the supernatant recovered. An aliquot of cleared lysate was treated with 25 µg RNAse

840   A and 50 µg proteinase K in a buffer containing 500 mM NaCl and heated at 65°C for 1.5 hours

841   to reverse cross-links. The DNA concentration in this aliquot was determined using a fluorometric

842   assay (Life Technologies) following manufacturer recommendations. The quantity of DNA used

843   in each ChIP was held constant within replicates (8 to 10 µg), and 10% of the input amount was

844   saved. Chromatin was diluted to a 500 µl final volume in binding/wash buffer (50 mM Tris, 150

845   mM NaCl, 1% Triton X-100) containing protease and phosphatase inhibitors, and 3 µg of antibody

846   was added per reaction. Tubes were incubated on an end-over-end rotator at 4°C overnight.

847   Antibodies used for ChIP were mouse monoclonal IgG2a including: 1) anti-NR1H3 (clone

848   PPZ0412, R&D Systems catalog PP-PPZ0412-00), 2) anti NR1H2 (clone K8917, R&D Systems

849   catalog PP-K8917-00), and 3) non-specific control IgG2a (clone MOPC-173, Abcam catalog

850   ab18413). The following day 25 µl of Protein G-conjugated Dynabeads (Life Technologies) were

851   added and incubated for 1 hour at 4°C. The beads were recovered using a magnet and washed

852   three times at 4°C for 5 minutes each wash with 1 ml of binding/wash buffer. The beads were

853   then washed once in final wash buffer (50mM Tris, 500 mM NaCl, 1% Triton X-100). Elutions

854   were performed with 130 µl elution buffer (100mM NaHCO$_3$, 500mM NaCl, 1% SDS) containing

855   25 µg RNAse A and 50 µg proteinase K, and ChIP reactions and input controls were incubated

856   on a thermal mixer at 65°C for 1.5 hours. The supernatant was recovered, and DNA purified using

857   PCR purification columns (Life Technologies) following manufacturer recommendations. Purified

858   DNA was analyzed by QPCR in a duplex reaction with primers and a Taqman probe specific for

859   the region spanning LXRE3 on the *TMEM135* gene, and a non-specific locus was simultaneously

860   quantified in the same reaction as a control for non-specific DNA carryover (Table S1). Serial

861   dilutions of purified sonicated DNA were included to verify linear amplification and PCR efficiency,

862   and Ct values from ChIP reactions and input controls were used to calculate the percentage of

31

863 DNA recovered relative to the input. Data were normalized by calculating the ratio of the

864 TMEM135 LXRE3 to the non-specific chromatin locus.

865 Semi-Quantitative Real-Time PCR

866 Cells or tissue were homogenized in Trizol reagent for isolation of mRNA and were further

867 purified over RNeasy columns according to manufacturer recommendations (Life Technologies).

868 Concentrations of RNA were quantified by spectrophotometry, and 0.2-1 µg RNA was treated with

869 DNAse I and reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription

870 Kit (Life Technologies). Following reverse transcription, cDNA were diluted to 10 ng/µl (based on

871 original RNA concentration) and used for semi-quantitative real-time PCR analysis (QPCR).

872 Primer and probe sequences used for QPCR are listed in Table S1. The QPCR assays were

873 performed on a StepOne Plus Real-Time PCR system (Life Technologies) using TaqMan® MGB

874 probe or SYBR green detection. Relative mRNA abundance was determined by extrapolation of

875 threshold (Ct) values from a standard curve of serial cDNA dilutions and normalized to the

876 housekeeping gene mitochondrial ribosomal protein S10 (*MRPS10*).

877 Cell Proliferation Assay

878 HepG2 cells were plated at 15,000 cells per well in 96-well plates and transfected with

879 siRNAs immediately. At the appropriate post-transfection time, viable cells were quantified using

880 a CyQuant® Direct Cell Proliferation assay (Life Technologies) according to manufacturer

881 recommendations, and fluorescence (excitation 495 nm, emission 527 nm) determined using a

882 Synergy H1 plate reader (BioTek Instruments, Inc.) in area scanning mode to account for uneven

883 cell distribution. The number of cells per well was estimated by extrapolation from a standard

884 curve of increasing cell numbers that was generated at the time of plating.

885 Flow Cytometry Analysis of Cell Cycle

886 Freshly harvested HepG2 cell pellets were fixed by slow addition of 1 ml ice-cold 70%

887 ethanol while vortexing and incubated at -20°C overnight. Fixed cells were harvested by

888 centrifugation at 850 × g for 5 min and resuspended in 1 ml DNA staining buffer (100 µg/ml RNAse

889   A, 50 µg/ml propidium iodide, in PBS). Cells were incubated for 30 min at 37°C, placed on ice,

890   and analyzed within one hour. A Becton Dickinson FACSCANTO II flow cytometer equipped with

891   a 488 nm argon ion laser and a 585/42 bandpass filter was used for detection of bound propidium

892   iodide. List mode data files consisting of 10,000 events were acquired and the percentage of cells

893   in G0/G1, S, and G2/M stages was determined with CellQuest PRO software (BD Biosciences).

894   <u>HepG2 ATP Quantification</u>

895   At 48 hours post-siRNA transfection, HepG2 cells were switched to serum-free, glucose-

896   free, sodium pyruvate-free DMEM media and incubated at 37°C for 4 hours. Cellular ATP

897   concentrations were determined using a homogenous luminescent ATP detection kit (Abcam

898   catalog ab113849). Additional HepG2 cells treated in parallel to those used for ATP determination

899   were lysed in RIPA buffer and a BCA protein assay (Fisher) was performed, and ATP content

900   was normalized to protein concentrations.

901   <u>Triglyceride Quantification</u>

902   HepG2 cells or frozen mouse liver were sonicated in PBS containing protease and

903   phosphatase inhibitors (Promega) in an ice-water bath using a Branson 250 digital sonifier

904   programmed to cycle at a 10% amplitude with 5 seconds on followed by 20 seconds off, and a 30

905   second total sonication time. Triglycerides were extracted using an organic solvent procedure

906   optimized for triglycerides (Schwartz and Wolins, 2007). Lysates were diluted 1:1 with deionized

907   water, and 400 µl of diluted lysate was combined in a glass tube with 1.8 ml of a 4:1

908   isopropanol:hexane mixture. Samples were vortexed for 1 minute and incubated at room

909   temperature for 30 min. Next, 500 µl of a 1:1 hexane:diethyl ether solution was added, the

910   samples were vortexed as before, and incubated at room temperature for 1 minute. One ml of

911   deionized water was added, samples were vortexed as before, and incubated at room

912   temperature for 20 minutes to allow phase separation. The organic phase was transferred to a

913   new glass tube and dried under a gentle stream of nitrogen in a water bath at 30°C. Dried extracts

914   were dissolved in 200 µl assay buffer (50 mM potassium phosphate, pH 7.0, 0.01% Triton X-100)

915   by 5 total cycles of vortexing for 1 minute and incubating at room temperature for 3 minutes.

916   Extracts (50 µl/well) were transferred to a clear 96 well plate, and 250 µl of triglyceride detection

917   reagent (Pointe Scientific, Inc., catalog T7532) was added. Triglyceride standards (Pointe

918   Scientific) diluted in assay buffer were also run with each assay. Plates were incubated at 37°C

919   for 30 minutes with vigorous shaking, and absorbance at 500 nm was determined with a Synergy

920   H1 plate reader. The protein concentration in sonicated lysates was determined using a BCA

921   protein assay, and triglyceride content was normalized to protein content.

922   <u>In Vivo siRNA Delivery and Tissue Harvest</u>

923   Ambion® In Vivo pre-designed siRNA (Life Technologies) against *Tmem135* (s91285) or

924   *in vivo* negative control #1 siRNA (catalog 4459405) were complexed with Invivofectamine® 3.0

925   reagent (Life Technologies) according to manufacturer recommendations. The siRNA complexes

926   were delivered via a 200 µl tail-vein injection at a final siRNA concentration of 1 mg/kg body

927   weight. Mice were weighed prior to siRNA injection and again before euthanization. Mice were

928   euthanized 4 days after siRNA injection at 4 hours after lights on, with or without a preceding 12

929   hour fast. Serum, liver, heart, adipose, and skeletal muscle were collected and snap frozen.

930   Tissues were powdered while frozen and stored at -80° C until used.

931   <u>Serum Analytes</u>

932   Total cholesterol, HDL cholesterol, and triglyceride concentrations in serum were

933   determined as we have described previously (Jensen et al., 2017). Briefly, the HDL fraction of

934   serum was isolated by mixing serum 1:1 with 15% polyethylene glycol, centrifuging at 2,000 × g

935   for 10 minutes at room temperature, and harvesting the HDL-containing supernatant. The

936   cholesterol concentrations in whole serum and the HDL fraction were determined by a

937   fluorometric enzyme assay consisting of cholesterol esterase (0.3 units/ml), cholesterol oxidase

938   (1 unit/ml), horse radish peroxidase (1 unit/ml), and Amplex UltraRed® (50 µM, Life Technologies)

939   in assay buffer (0.1 M potassium phosphate, pH 7.0, 50 mM NaCl, 5 mM cholic acid, 0.1% Triton

940   X-100). The cholesterol assay was incubated at 37°C for 30 minutes, and fluorescence measured

941 in a Synergy H1 plate reader at 555 nm excitation and 585 nm emission wavelengths. Triglyceride

942 concentrations in whole serum were determined using the same assay described earlier for

943 HepG2 and mouse liver samples. Commercial enzymatic assays were used to quantify serum

944 concentrations of β-hydroxybutyrate (BHB) (Cayman Chemical, catalog 700190), non-esterified

945 fatty acids (NEFA) (Catachem Inc., catalog C514-0A), glucose (Pointe Scientific, Inc., catalog

946 G7521), and a commercial ELISA was used to quantify serum insulin (Mercodia AB, catalog 10-

947 1247) according to manufacturer recommendations.

948 <u>Liver Analytes</u>

949 Commercial kits were used for the extraction and quantification of liver NAD+ and NADH

950 (Abcam Inc., catalog ab176723), and glycogen (BioAssay Systems, catalog E2GN), according to

951 manufacturer recommendations. Concentrations of ATP and NEFAs in liver were determined as

952 we have described elsewhere (Geisler et al., 2016). Briefly, for ATP determination frozen

953 powdered liver was homogenized in ATP releasing agent (Sigma Chemical Co., catalog FLSAR).

954 ATP was quantified with a luminescent ATP Determination Kit (Life Technologies, catalog

955 A22066) and normalized to tissue weight. For NEFAs, powdered liver was sonicated in PBS as

956 described earlier, and 100 µl lysate was mixed with 1 ml of 100% ethanol. Samples were vortexed

957 for 10 minutes, centrifuged at 16,000 × g for 5 minutes, and the supernatant recovered. A

958 commercial colorimetric assay for NEFAs (Catachem Inc., catalog C514-0A) was used and

959 standards were diluted in ethanol, and NEFA concentrations were expressed on an equal protein

960 basis.

961 To quantify ketones (BHB and acetoacetate), powdered liver was sonicated in PBS,

962 acidified with 10% (*w:v*) trichloroacetic acid, centrifuged to remove proteins, and the supernatant

963 was recovered and neutralized. Concentrations of BHB in the extract were determined using the

964 same enzymatic assay described for serum. Concentrations of acetoacetate were determined

965 using a modified assay based on methods described previously (Salway, 1969; Walker, 1954).

966 Briefly, samples and standards were divided with one portion subjected to boiling (100° C for 5

967   minutes) to degrade acetoacetate, with the remaining portion kept on ice. A three-fold volume of

968   freshly prepared diazo reagent (Walker, 1954) was then added to all samples and incubated at

969   room temperature in the dark for 30 minutes. The reaction was stopped by adding a 20% volume

970   of 2N NaOH, and absorbance was measured at 480 nm. The absorbance from the boiled sample

971   was subtracted from the respective non-boiled sample, and acetoacetate concentrations were

972   calculated from the resultant standard curve. Concentrations of cholic acid in liver were quantified

973   using a competitive enzyme immunoassay according to manufacturer recommendations (Cell

974   Biolabs, Inc., catalog MET-5007).

975   <u>Liver Subcellular Fractionation</u>

976   Powdered liver (approximately 50 mg) was homogenized for 40 strokes in a glass dounce

977   homogenizer with a tight-fitting pestle using 600 μl of cold fractionation buffer (10 mM Tris-HCl,

978   pH 7.4, 1 mM EGTA, 200 mM sucrose, protease and phosphatase inhibitor cocktail) on ice. The

979   homogenate was transferred to a new tube, and the dounce homogenizer was washed with an

980   additional 600 μl of fractionation buffer and transferred to the same tube. The homogenate was

981   centrifuged at 600 × g for 10 minutes at 4°C to pellet unbroken cells and nuclei, and the

982   supernatant transferred to a new tube. This low speed spin was repeated once more.

983   Mitochondria and peroxisomes were pelleted by centrifugation at 7,000 × g for 10 minutes, and

984   the supernatant (cytosolic fraction) was transferred to a new tube. The pellet was washed with

985   300 μl fractionation buffer and pelleted as before. The pellet was resuspended in 500 μl of

986   fractionation buffer and a 30 μl aliquot was pelleted and solubilized in RIPA buffer for a BCA

987   protein assay. Following protein assay mitochondria/peroxisome fractions were partitioned into

988   30 μg (protein content) aliquots followed by centrifugation and removal of the supernatant, and

989   the pellets were frozen at -80°C.

990   <u>Proteomic Analyses</u>

991   The mitochondria/peroxisome-enriched fraction from a subset of fed control and

992   TMEM135 knockdown mice was solubilized in RIPA buffer, proteins were precipitated with

36

993 acetone, and proteins were sent to the Arizona Proteomics Consortium for mass spectrometry

994 identification of proteins. Proteins were digested with trypsin and equal quantities of protein (500

995 ng) were loaded. Samples were analyzed on a Thermo Q Exactive Plus Orbitrap mass

996 spectrometer. Peptide identification in the resultant tandem mass spectra was performed using

997 Proteome Discoverer Software version 1.3.0.339 scanning with the SEQUEST algorithm against

998 the mouse proteome database (Mouse_unitprotkb_proteome_2016_0720_cont.fasta). Data

999 analysis was performed using Scaffold version 4.8.1, with a peptide identification threshold of

1000 95.0% and a minimum protein identification threshold of 99.9% and 2 unique peptides.

1001 Western Blot

1002 All primary antibodies were obtained from Abcam unless otherwise indicated. Catalog

1003 numbers and final concentrations or dilutions of each antibody were: rabbit polyclonal anti-ACAA1

1004 (catalog ab154091, 1 µg/ml), rabbit monoclonal anti-ACOX1 (catalog ab184032, 1:2500 dilution),

1005 rabbit monoclonal anti-CAT (catalog ab209211, 1:2000 dilution), rabbit monoclonal anti-SCP2

1006 (catalog ab140126, 1:2500 dilution), rabbit polyclonal anti-TMEM135 (catalog ab167096, 1

1007 µg/ml), rabbit monoclonal anti-COX4I1 (Li-COR Inc., catalog 926-42214, 1:1000 dilution), mouse

1008 monoclonal anti-COX4I1 (catalog 14744, 0.25 µg/ml), mouse monoclonal anti-TUBB (Sigma

1009 Aldrich catalog T8328, 0.5 µg/ml), and rabbit polyclonal anti-PMP70 (catalog ab3421, 0.5 µg/ml).

1010 Proteins (12.5 µg/well) were resolved on 4-12% Bis-Tris gels (Life Technologies) and

1011 subsequently transferred to nitrocellulose membranes. The membranes were blocked with 5%

1012 non-fat dry milk (NFDM) (*w:v*) in TBS with 0.1% (*v:v*) Tween (TBST) for 1 hour at room temp. All

1013 primary antibodies were diluted in TBST with 1% NFDM and incubated with the membranes on a

1014 rocking platform at 4° C overnight. The membranes were washed 4x with TBST, and IRDye®

1015 680RD or 800CW-conjugated secondary antibodies (LI-COR Biosciences, Inc.) were diluted

1016 1:4000 in TBST + 1% NFDM and incubated with membranes for 1 hour at room temp. The

1017 membranes were washed as before. Imaging and densitometry were performed with a Li-COR

1018 Odyssey CLx and Image Studio version 3.1 software, respectively.

1019   Gas Chromatography (GC)

1020   Lysates were prepared by sonicating frozen powdered liver in PBS as described earlier.

1021   Insoluble material was pelleted by centrifugation at 600 × g for 5 minutes. A BCA protein assay

1022   was performed on the cleared lysate. A one-step transesterification reaction was performed

1023   (Masood et al., 2005). Briefly, 1.7 ml of acidic methanol (1.6 ml methanol and 100 µl acetyl

1024   chloride) was added to borosilicate glass tubes with Teflon-lined caps. Butylated hydroxytoluene

1025   (BHT, 50 µg/ml) was added to methanol to prevent fatty acid oxidation. Tridecanoic acid (13:0,

1026   Cayman Chemical) was used as an internal standard because preliminary studies determined

1027   that it was not present in liver lysates. Tridecanoic acid was diluted in methanol/BHT to 100 µg/ml,

1028   and 100 µl was added to all samples and standards. Authentic standards for the following fatty

1029   acids were obtained from Cayman Chemical: 12:0, 14:0, 16:0, 16:1, 18:0, 18:1 cis(n9), 18:2

1030   cis(n6), 20:3 cis(n6), 20:4 (n6), 22:0, 22:6 (n3), 24:0, 24:1, and 26:0. Pooled mixtures containing

1031   increasing concentrations of these fatty acids were prepared and dissolved in chloroform. 100 µl

1032   of authentic standards were added to standard tubes, and 100 µl of chloroform was added to

1033   unknown tubes. Next, 100 µl PBS was added to standard tubes, and 800 µg (protein content) of

1034   lysate diluted to a 100 µl volume with PBS was added to unknown tubes. The tubes were tightly

1035   capped and vortexed for 1 minute, and then incubated in a heating block at 100°C for one hour.

1036   The tubes were cooled to room temperature, 750 µl hexane was added and the tubes were

1037   vortexed for 30 seconds, and the upper organic layer was transferred to a GC sample vial. The

1038   hexane extraction was repeated once more. Hexane extracts were evaporated under a gentle

1039   stream of nitrogen to dryness and dissolved in 50 µl hexane.

1040   GC was performed on an Agilent Technologies 6890N GC equipped with a Varian CP-Sil

1041   88 column for fatty acid methyl ester analysis (100 m × 0.25 mm inner diameter × 0.2 µm film

1042   thickness) and a flame ionization detector. Inlet temperature was 250° C, and 1 µl of each

1043   standard or unknown was manually injected at a split ratio of 7.5:1. Helium was used as carrier

1044   gas at a 1.0 ml/minute constant flow. The oven was programmed for an initial temperature of 80°

38

1045 C with a 4° C/min ramp to 220° C, a 5-minute hold, then a 4° C/min ramp to 240° C followed by a

1046 10-minute hold. The detector was set at 270°C using air (450 ml/minute) and hydrogen (40

1047 ml/minute), and nitrogen was used as make-up gas (10 ml/minute constant flow). The sampling

1048 frequency was 20 Hz.

1049   OpenLAB CDS ChemStation Edition software version C.01.06 (Agilent Technologies) was

1050 used to analyze GC data. Retention times of authentic standards were matched with

1051 corresponding peaks in unknown samples. For each fatty acid, the peak area for standards and

1052 unknowns was divided by the internal standard peak area. The normalized peak area from

1053 increasing concentrations of authentic standards was plotted, and the R^2 for the resultant standard

1054 curves were > 0.995 in all cases. All fatty acids except 12:0 and 26:0 were identified in the

1055 unknowns, and fatty acid concentrations were determined by extrapolating the normalized peak

1056 area from the respective standard curve. Concentrations were expressed on an equal protein

1057 basis.

1058 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1059   Statistical analyses were performed using Stata Version 14 and all differences were

1060 considered significant at p< 0.05. Information on the sample size, mean and error estimates, and

1061 indicators of statistical significance are presented in the figures and their corresponding legends.

1062 For immortalized cell lines, the total sample size (n) corresponds to cells on a different passage

1063 number and/or experiments performed on different days. Time course data in immortalized cell

1064 lines were analyzed by mixed effects regression analysis to account for repeated measures.

1065 Treatments were coded 0 or 1, and hours post-treatment and treatment × time interaction were

1066 included as fixed effects in the regression model with experimental replicate as the random effect.

1067 Differences between treatments at each timepoint were determined from the treatment × time

1068 interaction coefficient. For all other comparisons involving more than 2 groups, mixed effects

1069 regression analysis was performed using treatment as the fixed effect and experimental replicate

1070 as the random effect to account for repeated measures. Pairwise differences between treatments

39

1071   were then determined using the Bonferroni multiple comparison method to control for the type 1

1072   error rate. For comparisons involving only 2 groups, a paired t-test was performed.

1073   For all mice data, the sample size refers to the number of mice. Regression analysis was

1074   used for mice outcomes except where indicated otherwise. Samples were coded 0 or 1 for fasting,

1075   TMEM135 knockdown fed mice, and TMEM135 knockdown fasted mice. The regression model

1076   utilized fasting, TMEM135 knockdown in the fed state, and TMEM135 knockdown in the fasted

1077   state, as predictors. The fasting coefficient indicated the difference due to fasting in control mice

1078   only, while the TMEM135 knockdown coefficients indicated the difference between control mice

1079   and TMEM135 knockdown mice within the fed state or fasted state. Differences were considered

1080   significant at $p < 0.05$, with p values ≥ 0.05 and < 0.10 considered trends. Only fed mice were

1081   used in proteomic analysis. Because the proteomic data does not follow a normal distribution due

1082   to some proteins not being detected in all samples, significant differences in proteomic data were

1083   determined from weighted spectra using the non-parametric Fisher's Exact test ($p < 0.05$)

1084   contained within the Scaffold version 4.8.1 software. The list of proteins that were significantly

1085   reduced in knockdown livers was further trimmed by hand to exclude proteins that were not

1086   detected in all the samples from mice that received the control siRNA.

Figure 1

Figure 2

A   *NR1H2* Knockdown

B   *NR1H3* Knockdown

C   *ABCA1* mRNA

D   *TMEM135* mRNA

E   *TMEM135* mRNA with Full 5' UTR

F   LXR Binding to *TMEM135* Promoter
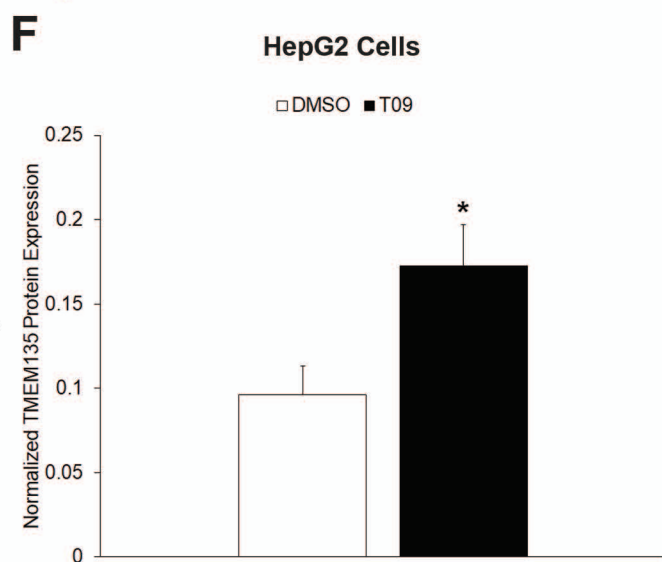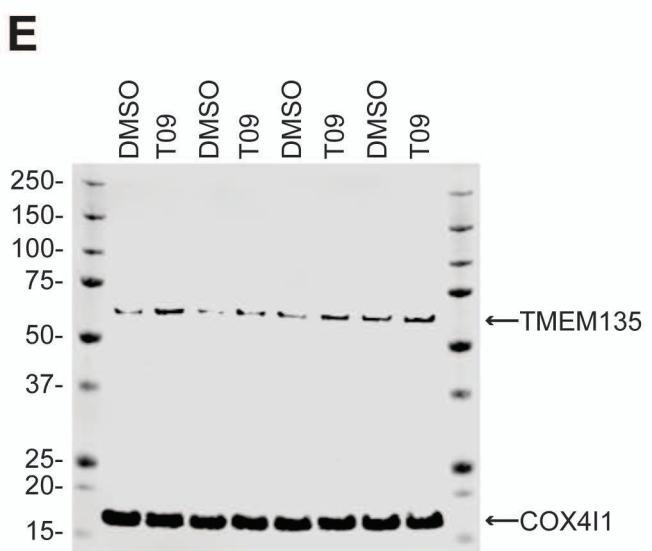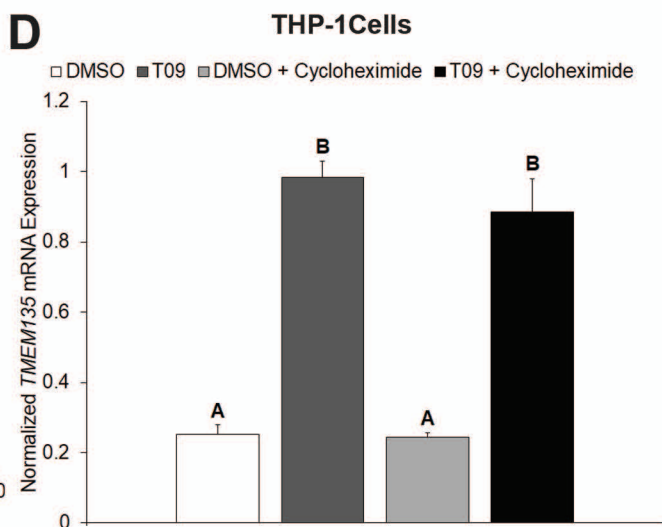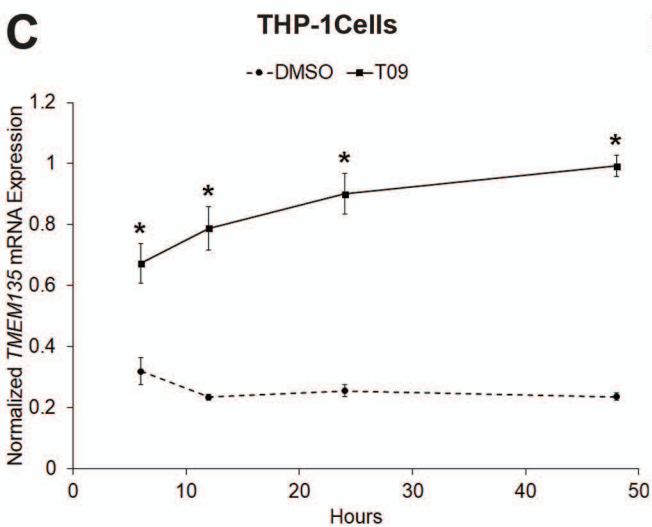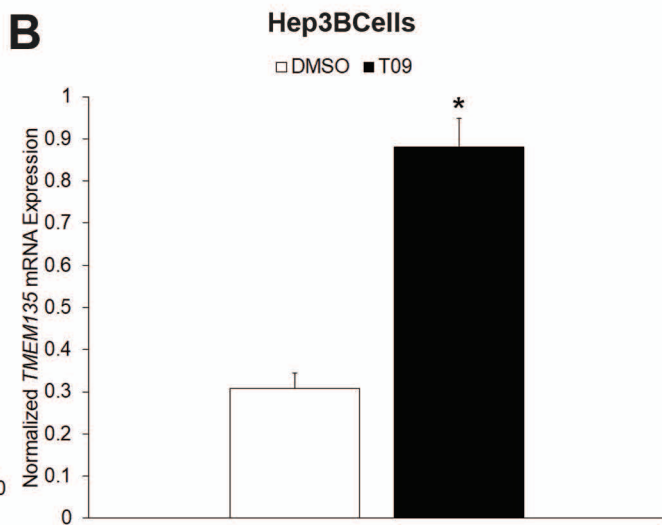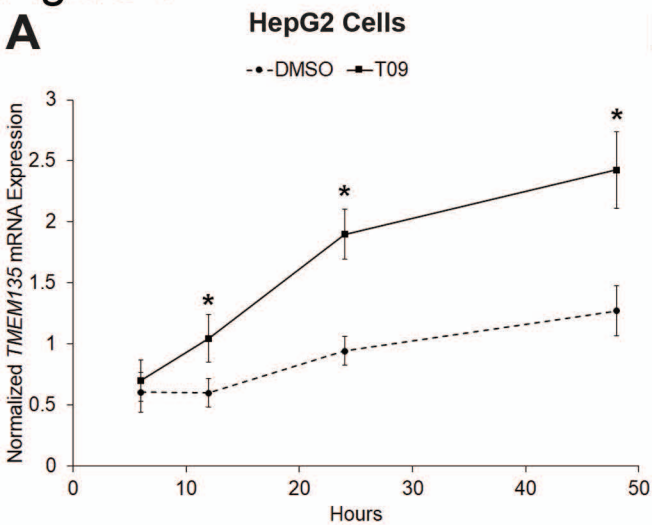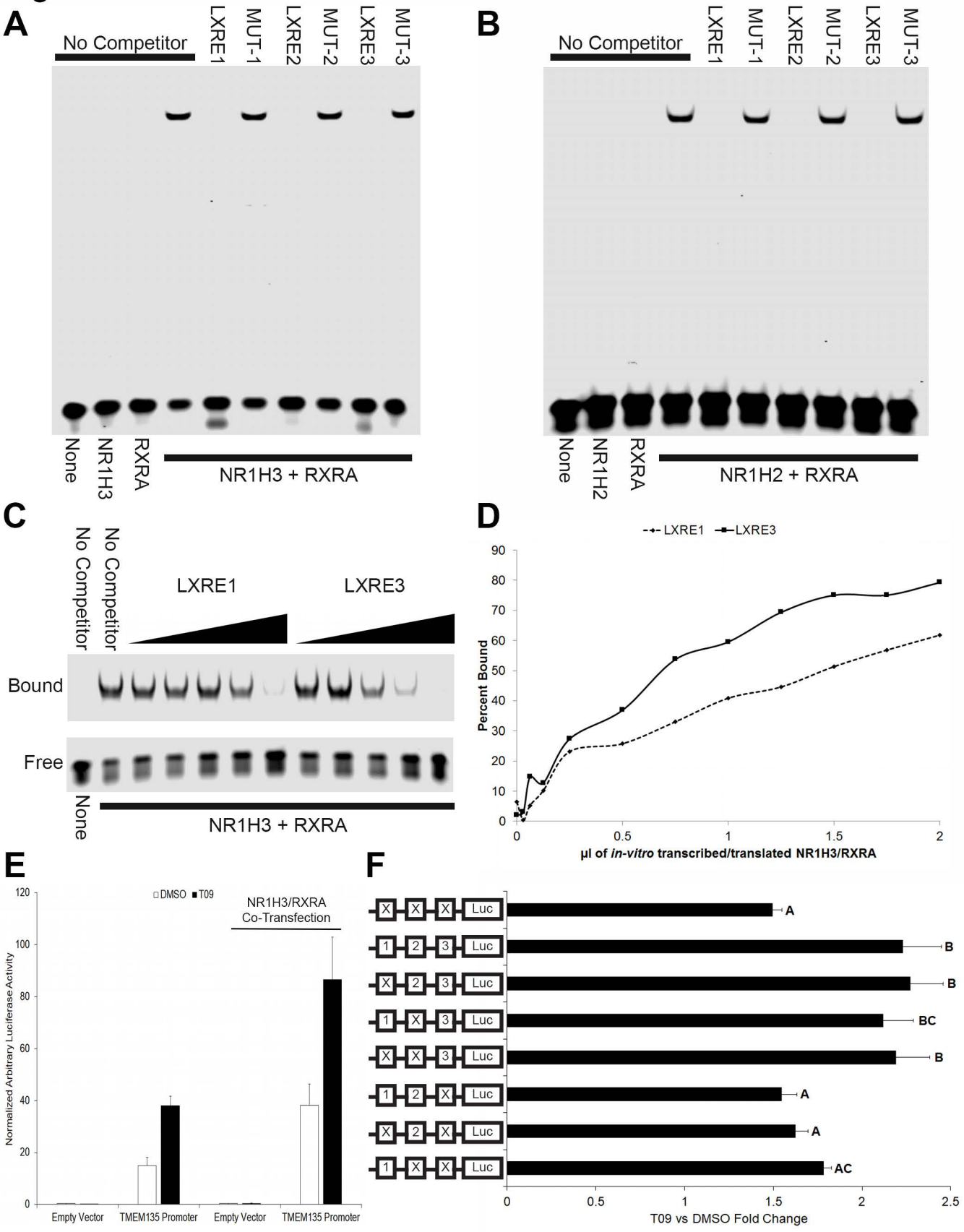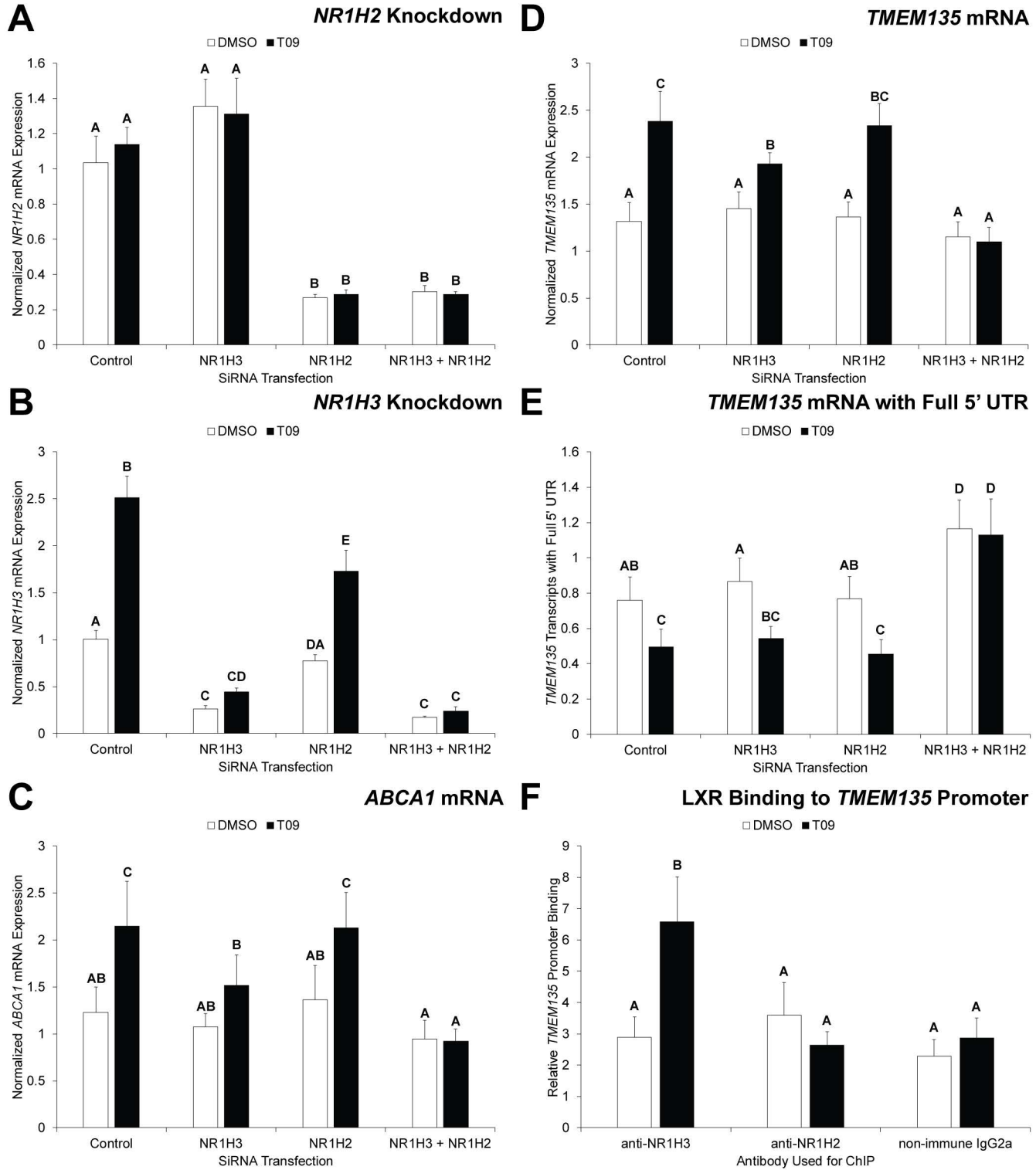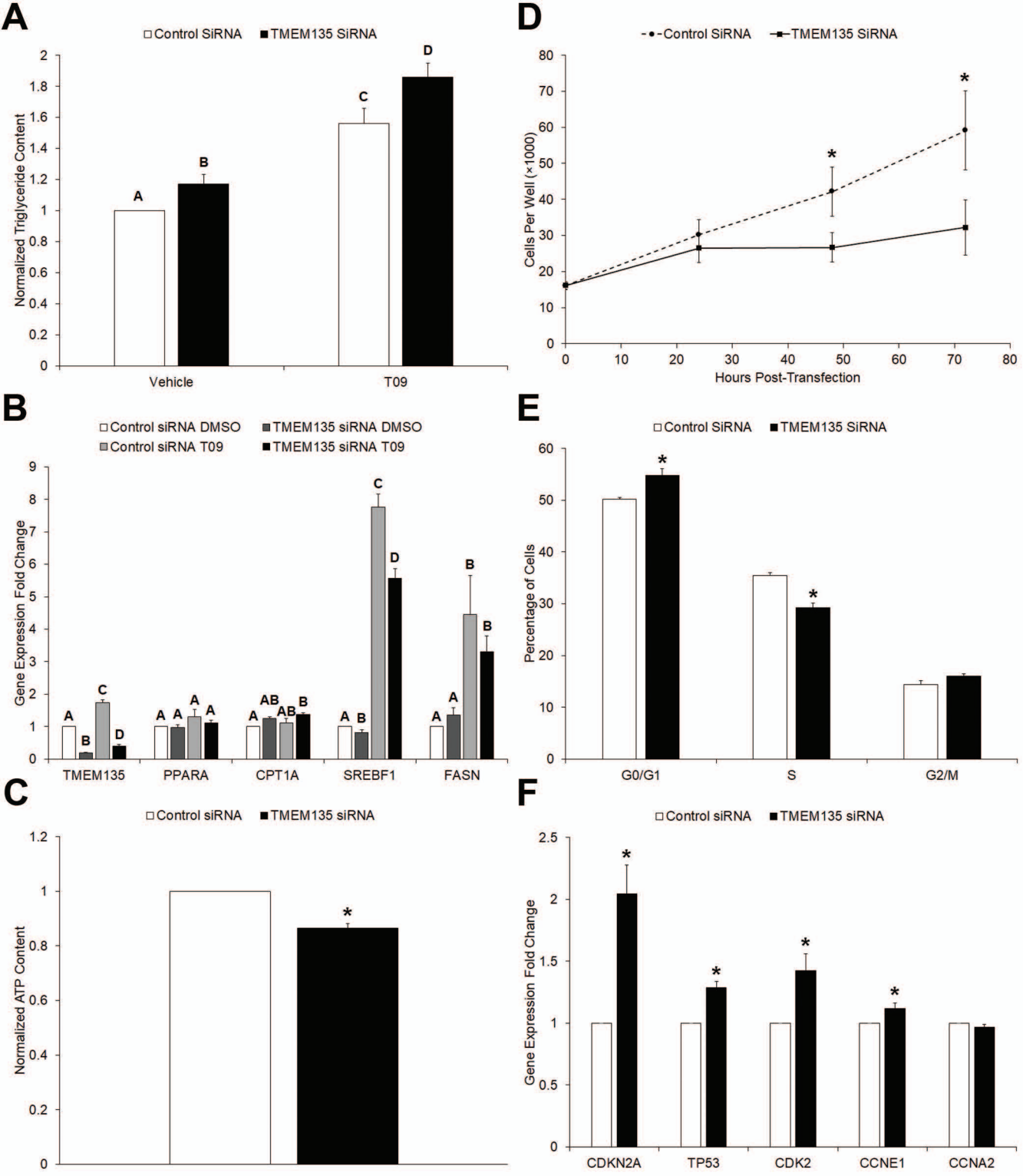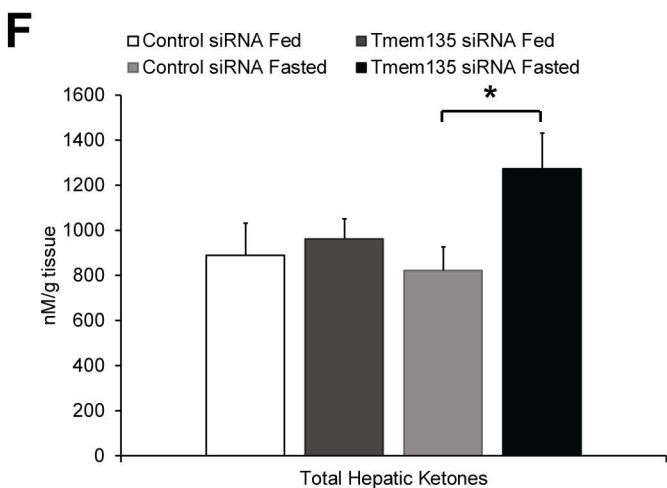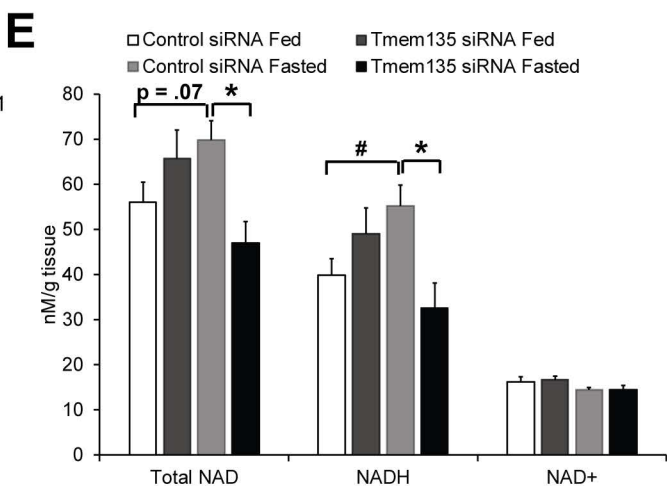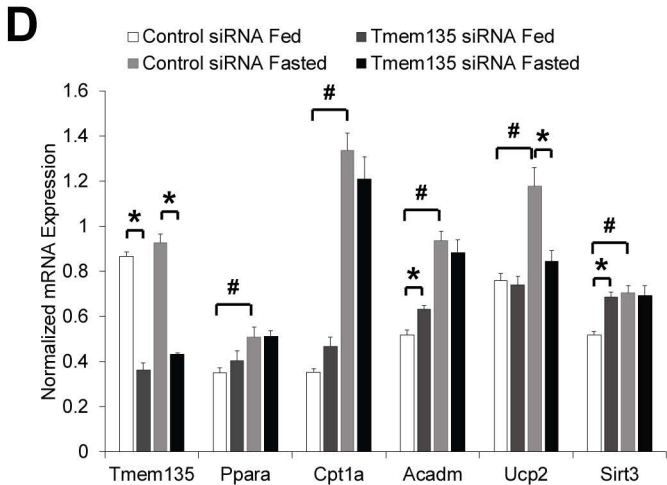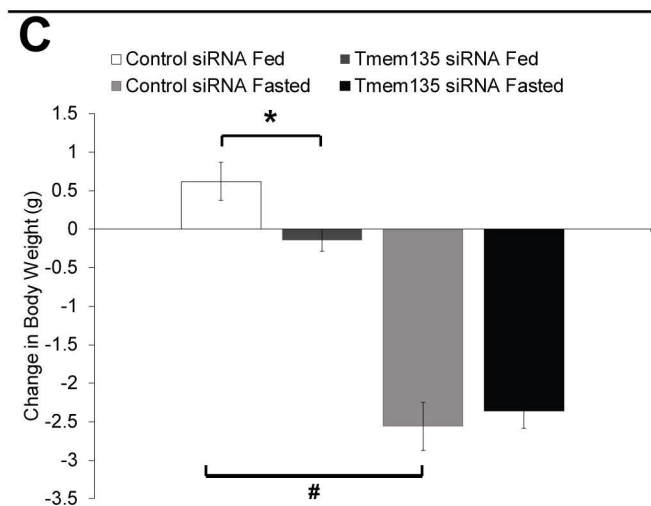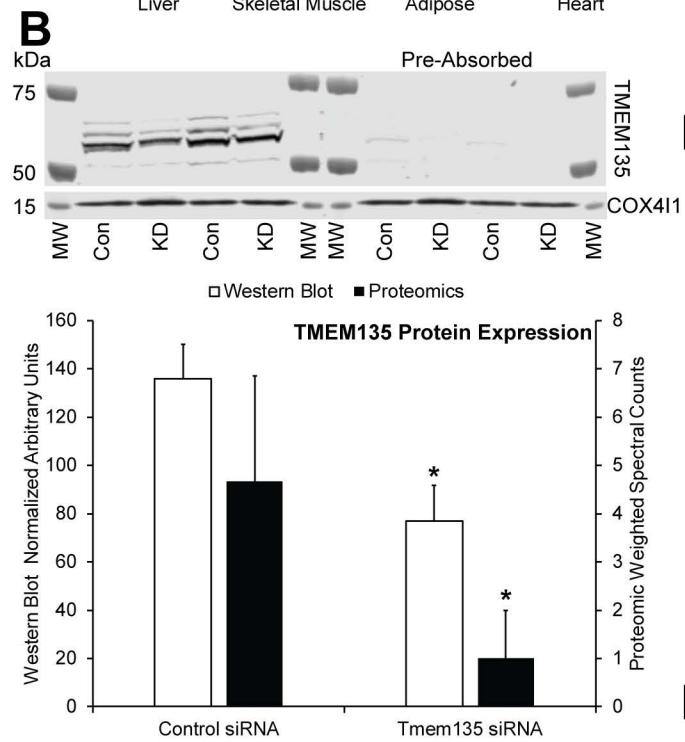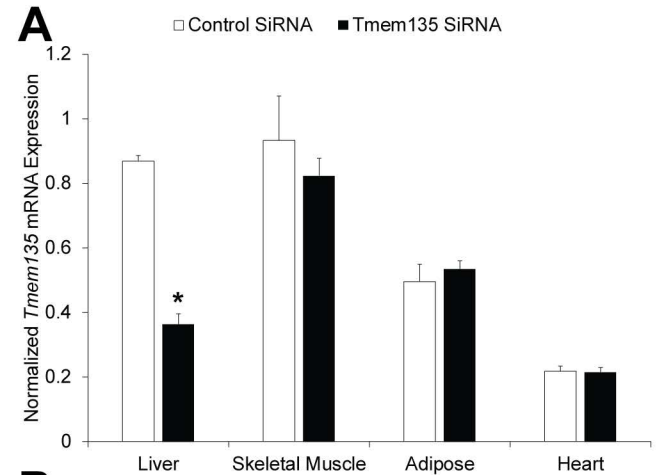
Figure 3

Figure 4

Figure 5
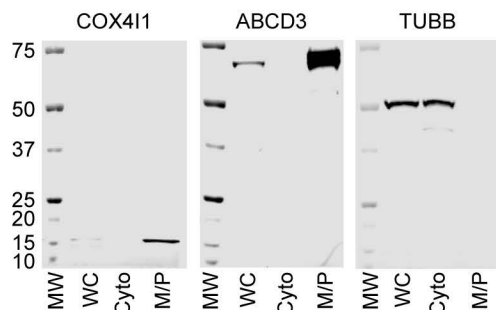
B — Weighted Spectral Counts ± SEM

Protein Symbol	Control siRNA	Tmem135 siRNA
CPS1	110 ± 16.3	84.7 ± 34.1
CAT	125 ± 7.6	76 ± 14
SCP2	128 ± 13.1	69.7 ± 35.3
HSD17B4	66 ± 8.5	46.7 ± 9.5
CES1	61.3 ± 11.8	43.3 ± 7.2
NUDT7	28 ± 8.2	17 ± 17
ACOX1	27 ± 6.7	13 ± 10.5
PC	24.3 ± 3.8	15.7 ± 4.7
ACOX2	22 ± 4.4	13.3 ± 11.9
CES3	17.7 ± 3.7	10.7 ± 6.1
GLUD1	21 ± 2.3	13 ± 3.1
DHRS4	23.3 ± 3.4	11.3 ± 6.4
HACL1	20.7 ± 3.7	9.7 ± 5.7
COMT	17.7 ± 5.8	8.3 ± 8.3
MTTP	15 ± 4.7	7.7 ± 4.3
PSAP	14 ± 4.5	5.3 ± 3.2
ACAA1	35 ± 6.5	9.3 ± 9.3
ALDH3A2	11 ± 2.9	5.3 ± 3.5
ALDH6A1	11 ± 2.9	5.7 ± 3.5
AMACR	10 ± 2.6	4 ± 4
SUCLA2	9 ± 1.7	3.3 ± 2.4
ACADL	5 ± 0.6	1.7 ± 0.9
SLCO1A1	3.7 ± 1.2	1 ± 1

Figure 6

A

Fed Mice | Fasted Mice

Whole Cell Lysate | Mito/Perox-Enriched Fraction | Whole Cell Lysate | Mito/Perox-Enriched Fraction

ACAA1
ACOX1
SCP2
CAT
Controls: TUBB | COX4I1 (Mitochondria) / ABCD3 (Peroxisomes)

Con KD Con KD Con KD Con KD Con KD

B

□ Control siRNA Fed   ■ Tmem135 siRNA Fed
▨ Control siRNA Fasted   ■ Tmem135 siRNA Fasted

Relative Peroxisome Import

ACAA1   ACOX1 70 kDa   ACOX1 48 kDa   SCP2   CAT

C

□ Control siRNA Fed   ■ Tmem135 siRNA Fed
▨ Control siRNA Fasted   ■ Tmem135 siRNA Fasted

µg Fatty Acid per mg protein

Stearic Acid 18:0   Oleic Acid 18:1 (p = .076)   Linoleic Acid 18:2

D

	Fatty Acids					
	Fed Mice			Fasted Mice		
	Control siRNA	*Tmem135 siRNA*	*P*	*Control siRNA*	*Tmem135 siRNA*	*P*
14:0	0.32 ± 0.06	0.29 ± 0.03		0.73 ± 0.12 #	0.85 ± 0.18	
16:0	48.9 ± 3.6	45.7 ± 2.2		70.2 ± 4.4 #	83.1 ± 7.5	^
16:1	2.4 ± 0.5	2.2 ± 0.2		3.5 ± 0.7	5.3 ± 1.0	^
18:0	31.3 ± 1.3	29.4 ± 1.2		31.3 ± 1.6	33.2 ± 0.4	
18:1	22.9 ± 3.4	22.0 ± 1.9		41.1 ± 4.8 #	56.7 ± 9.9	^
18:2	27.1 ± 2.0	28.5 ± 1.9		58.1 ± 5.1 #	78.0 ± 10.0	*
20:3	2.2 ± 0.2	2.1 ± 0.2		1.1 ± 0.1 #	1.6 ± 0.1	^
20:4	21.0 ± 1.6	19.2 ± 1.4		20.8 ± 1.1	23.4 ± 0.7	
22:0	1.7 ± 0.1	1.4 ± 0.1	*	1.4 ± 0.1	1.6 ± 0.1	
22:6	13.0 ± 1.2	11.7 ± 1.0		17.1 ± 1.1 #	19.0 ± 0.9	
24:0	1.01 ± 0.03	0.83 ± 0.05	^	0.95 ± 0.08	1.01 ± 0.05	
24:1	0.87 ± 0.04	0.87 ± 0.05		1.0 ± 0.1	1.19 ± 0.06	^
Total	172.5 ± 12.9	164.1 ± 7.7		247.4 ± 14.2 #	304.8 ± 28.3	*

Figure 7