

1 **Circadian gene Clock regulates mitochondrial morphology and**
2 **functions by posttranscriptional way**

3 Lirong Xu¹, Qianyun Cheng¹, Bingxuan Hua³, Tingting Cai¹, Jiaxin Lin¹, Gongsheng
4 Yuan¹, Zuoqin Yan³, Xiaobo Li¹, Ning Sun¹, Chao Lu^{1,2*} Correspondence:
5 luchao@shmu.edu.cn, Ruizhe Qian^{1,2*} Correspondence: rzqian@shmu.edu.cn

6 ¹ Department of Physiology and Pathophysiology, School of Basic Medical Sciences,
7 Fudan University, Shanghai 200032, China

8 ² Basic Research Institute for Aging and Medicine, School of Basic Medical Sciences,
9 Fudan University, Shanghai 200032, China

10 ³ Department of Orthopedics, Zhongshan Hospital, Fudan University, Shanghai
11 200032, China

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45 **Abstract**

46 Many daily activities are under the control of circadian clock, including nutrition
47 metabolism and energy generation. Mitochondria, as the core factories of oxidizing
48 substrates and producing ATP, undergo changes in quantity and morphology to adapt
49 to the demand for energy. It has been demonstrated that mitochondrial gene
50 expression, dynamics and functions are all affected by circadian clock. Here, we
51 demonstrated that circadian gene Clock affects the number, architecture and function
52 of mitochondria via posttranscriptional regulation of Drp1. Clock^{Δ19} leads to
53 fragmented mitochondria accompanied with the loss of membrane potential, excessive
54 ROS accumulation and decreased mitochondrial respiration and ATP generation.
55 Clock^{Δ19} mice exhibit disordered lipid metabolism and evident nonalcoholic fatty liver
56 disease (NAFLD), which are rescued by treatment with the mitochondrial fission
57 inhibitor Mdivi-1. These results suggest a strong relationship between Clock,
58 mitochondrial dynamics and metabolic diseases and provide a new perspective on
59 disordered circadian clock and related diseases.

60 **Key words:** Circadian clock/ Mitochondria/ Post-transcriptional regulation/ Drp1/
61 Metabolism.

62 **Introduction**

63 Circadian clock orchestrates many daily activities of living beings including
64 sleep-wake cycle, food intake, digestion and hormone secretion. The accurate
65 operation of circadian system relies on the transcription-translation feedback loop
66 formed by core clock genes including Clock, Bmal1, Per, Cry, Rev-erb and
67 downstream circadian clock-controlled genes (CCGs)(Takahashi, 2015). Among these,
68 CLOCK, together with its heterodimer, BMAL1, are at the core, acting as positive
69 transcription factors in the feedback loop. Unlike mice missing Bmal1, Clock^{-/-} mice
70 appear relatively normal because of the existence of its paralog, Npas2(DeBruyne et
71 al, 2007). Clock^{Δ19} was reported to be the most noteworthy mutation in the Clock
72 gene, and it is accompanied by altered activity, food intake periods and apparent
73 metabolic disorders(Turek et al, 2005), especially in terms of lipid metabolism.
74 Clock^{Δ19} mice suffer from serious obesity and hyperlipidemia, while the Bmal1^{-/-} mice
75 are seriously underweight(Bunger et al, 2000; Lefta et al, 2012). These differences
76 indicate the possibility of independent regulatory roles of Clock and Bmal1, but the
77 underlying mechanism is still under investigation.

78 Increasingly, studies are suggesting that many genes involved in metabolism are
79 periodic, including genes associated with glucose and lipid metabolism(Menet et al,
80 2012; Neufeld-Cohen et al, 2016). Mitochondria, as the core factories oxidizing
81 nutrient substrates and generating energy, are also under the control of circadian clock.
82 First, quantitative proteomics and transcriptomics identified a predominant daily
83 phase for mitochondrial mRNA and protein accumulation, including carriers involved
84 in pyruvate and fatty acid translocation, enzymes mediating key mitochondrial
85 metabolism and components of the mitochondrial respiration chain(Neufeld-Cohen et

86 al, 2016). Second, circadian clock can also regulate the activities of many
87 mitochondrial enzymes by affecting posttranslational modifications, such as the
88 acetylation of respiration complex I, by adjusting NAD⁺/NADH levels and SIRT6
89 activities (Brown, 2016; Cela et al, 2016). In addition, mitochondria are not static
90 organelles; they experience periodic fusion and fission events (Liesa & Shirihai, 2013).
91 It was found that *Bmal1*^{-/-} mice have enlarged and dysfunctional mitochondria that are
92 less responsive to metabolic input (Jacobi et al, 2015). Additionally, mitochondrial
93 functions and dynamics in *Per1/2*^{-/-} mice are disordered (Schmitt et al, 2018). However,
94 no direct CLOCK regulation of mitochondrial dynamics has been reported.

95 *In vivo* and *in vitro* experiments have both illustrated that altered mitochondrial
96 dynamics give rise to metabolic block and abnormal quantity of ROS, resulting in
97 metabolic diseases (Liesa & Shirihai, 2013; Lopez-Lluch, 2017; Yoon et al, 2006).
98 Mitochondrial dynamics disorders are common in metabolic diseases. Mitochondria
99 in pancreatic β -cells of patients with type 2 diabetes tend to be swollen and
100 dysfunctional (Yoon et al, 2011). In turn, the inhibition of mitochondrial fission in
101 β -cells results in decreased glucose-stimulated insulin secretion (Yoon et al, 2011).
102 Similar phenomena have also been found in patients and animals with
103 hyperglycemia (Yoon et al, 2011). Recently, Moshi Song reported a close relationship
104 between mitochondrial dynamics and heart disease, wherein conditional deletion of
105 *Drp1* in the heart evoked dilated cardiomyopathy, while double ablation of *Mfn1* and
106 *Mfn2* led to eccentric hypertrophy (Song et al, 2015). Nevertheless, there are many
107 relationships that need to be clarified between circadian clock, mitochondrial
108 dynamics and diseases.

109 In this study, we provide evidence that *Clock* controls the number, morphology,
110 and functions of mitochondria by post-transcriptionally regulating mitochondrial
111 dynamics. Disordered dynamics in *Clock* ^{Δ 19} mice led to abnormal mitochondrial
112 architecture and mitochondrial dysfunction, which in turn led to the development of
113 metabolic diseases. Some metabolic disorders in *Clock* ^{Δ 19} mice can be rescued by
114 drugs that regulate mitochondrial dynamics, which suggests that *Clock*-controlled
115 mitochondrial dynamics are crucial for healthy metabolism and could provide a new
116 perspective on disordered circadian rhythms and related diseases.

117 **Results**

118 ***Clock* ^{Δ 19} mice present morphological changes in their mitochondria**

119 To balance energy supply and demand, mitochondria continually undergo changes in
120 amount and architecture. To assess whether these mitochondrial changes were under
121 the control of *Clock*, we used *Clock* ^{Δ 19} mice from the Jackson lab. First, the rhythmic
122 activity in WT mice disappeared in *Clock* ^{Δ 19} mice and tended to be disordered (Figure
123 S1A). In addition, *Clock* ^{Δ 19} mice gained weight faster than the same-age WT mice,
124 with worse tolerance of insulin (Figure S1B-C). All of these suggested that *Clock* ^{Δ 19}
125 mice experience disordered lipid and glucose metabolism. To explore mitochondrial
126 alterations in *Clock* ^{Δ 19} mice, WT and *Clock* ^{Δ 19} livers collected at ZT0 and ZT12 were
127 visualized by electron microscope. Mitochondria in *Clock* ^{Δ 19} mice liver were
128 fragmented accompanied with disordered inner membrane structure (Figure 1A).

129 Primary hepatocytes were further isolated to validate the mitochondrial changes in
130 $Clock^{\Delta 19}$ mice. First, the hepatocytes of $Clock^{\Delta 19}$ mice presented an accumulation of
131 vacuoles caused by endoplasmic reticulum swelling (Figure 1B, black arrows), which
132 underlay cellular swelling that resulted in mitochondrial dysfunction and the lack of
133 ATP generation. In addition, abundant lipid droplets revealed the lipid metabolism
134 disorders in $Clock^{\Delta 19}$ mice (Figure 1B, white arrows) and suggested the existence of
135 fatty liver disease in $Clock^{\Delta 19}$ mice. Moreover, the mitochondrial matrix was deeply
136 stained, making the cristae structure appear unclear (Figure 1B, M labeled),
137 suggesting pH changes in $Clock^{\Delta 19}$ mitochondria. Furthermore, we calculated the
138 distribution of mitochondrial surface and found most of the WT mitochondrial surface
139 was concentrated between 0.9 and 1.1 μm^2 , while the mitochondria of $Clock^{\Delta 19}$ mice
140 were approximately 0.1-0.3 μm^2 (Figure 1C), indicating that the mitochondria in
141 $Clock^{\Delta 19}$ mice are smaller, with a shorter diameter. We then infected the primary
142 hepatocytes with ad-Cox8a-GFP virus. In WT livers, the mitochondria were point
143 stained, while the point form was destroyed in the $Clock^{\Delta 19}$ liver and tended to be
144 fragmented (Figure 1D). We then used ATP6 encoded by mitochondria as a typical
145 marker to observe mitochondrial form. The spots representing ATP6 were more
146 specific and stronger in the WT liver compared with the diffused distribution in the
147 $Clock^{\Delta 19}$ liver (Figure 1E), demonstrating the destruction of mitochondrial
148 architecture in $Clock^{\Delta 19}$ mice.

149 **The mitochondrial morphological changes in $Clock^{\Delta 19}$ mice are accompanied by** 150 **dysfunction**

151 We wondered if the abnormal mitochondria morphology could cause mitochondrial
152 malfunction in $Clock^{\Delta 19}$ mice. ROS (reactive oxygen species) were measured by
153 fluorochrome tracing, and there was a large increase in ROS in $Clock^{\Delta 19}$ primary
154 hepatocytes compared with WT (Figure 2A and 2C). Mitochondria take in NADH
155 produced from nutrient oxidation and generate ATP. During this procedure, the
156 membrane potential ($\Delta\Psi_m$) is formed, which is essential for mitochondria to provide
157 energy. Here, JC-1 staining showed that $Clock^{\Delta 19}$ was associated with a decrease in
158 the membrane potential (Figure 2B and 2C), which also indicated that mitochondrial
159 function is abnormal in $Clock^{\Delta 19}$ hepatocytes.

160 Mitochondria oxidize carbohydrates and lipids to generate ATP by oxidative
161 phosphorylation. The intact mitochondria electron transport chain is critical for its
162 ability to supply energy. It was reported that the activity of complex I in mitochondrial
163 electron transport chain is Bmal1-dependent (Cela et al, 2016). To assess the impact of
164 $Clock^{\Delta 19}$ on mitochondrial complexes, we determined the electron flow of primary
165 hepatocytes by seahorse assay. The results showed that mitochondrial respiration
166 driven by succinate (complex II) and ascorbate/TMPD (complex IV) were clearly
167 decreased in $Clock^{\Delta 19}$ mice compared to the WT controls (Figure 2D). We then
168 measured the ATP concentration in primary hepatocytes to evaluate the general
169 function of mitochondria. We found that $Clock^{\Delta 19}$ primary hepatocytes had a large
170 decline in the concentration of ATP compared with WT primary hepatocytes (Figure
171 2E). The exhaustive swimming assay was then applied to evaluate the ATP supply and
172 physical power in the mice. Compared to the WT mice, $Clock^{\Delta 19}$ mice were more

173 easily exhausted, revealing ATP generation and utilization dysfunctions (Figure 2F).
174 The seahorse assay was performed to further examine mitochondrial respiration.
175 Mitochondria isolated from Clock^{Δ19} mouse livers showed lower reactions to
176 inhibitors (Figure 2G). Both of the OCR reflecting basal respiration and ATP synthesis
177 were significantly reduced in Clock^{Δ19} (Figure 2H). Moreover, we measured the
178 mitochondrial respiration of primary hepatocytes, and decreased reactions to
179 inhibitors was also evident (Figure 2I). Both basal respiration and ATP synthesis
180 appeared to be decreased in hepatocytes (Figure 2J). In addition, mitochondrial
181 respiration but not nonmitochondrial respiration showed an obvious decrease in
182 Clock^{Δ19} mice (Figure 2J). Furthermore, the decreased spare capacity in Clock^{Δ19}
183 mice indicated a weak rapid adaption to metabolic changes (Figure 2J). Collectively,
184 these findings revealed that the morphological changes in Clock^{Δ19} mitochondria are
185 associated with dysfunction in mitochondrial respiration and ATP generation.

186 **Alterations in the expression of mitochondrial-related genes in Clock^{Δ19} mice**

187 Unlike other organelles, mitochondria have an independent genome, although it only
188 codes for 13 proteins and several RNAs. Mitochondrial structural components and
189 biological functions mostly rely on the nuclear genome (Peralta et al, 2012; Scarpulla,
190 2008). To investigate the mechanism underlying mitochondrial dysfunction in
191 Clock^{Δ19} mice, we examined the mRNA expression levels of several mitochondria
192 function-related genes in livers collected every 4 h for 24 hrs. Mrps24 and Mrpl50,
193 which are encoded by the nuclear genome and participate in mitochondrial translation,
194 had decreased expression levels in Clock^{Δ19} mice compared to WT mice (Figure S2A).
195 The luciferase reporter assay indicated that the transcriptions of Mrps24 and Mrpl50
196 are regulated by CLOCK and its heterodimer, BMAL1 (Figure S2B). On the other
197 hand, genes encoded by the mitochondrial genome, including ND1 and ATP6, were
198 not significantly different between WT and Clock^{Δ19} mice, except for an upregulation
199 at ZT0 (Figure 3A). The results of the luciferase reporter assay showed that the
200 transcription of the mitochondrial D-loop was not controlled by CLOCK or BMAL1
201 (Figure 3B). However, the levels of mitochondria-specific proteins including ATP5a,
202 ATP6, ND1 and COX4 were all elevated in the Clock^{Δ19} mice total cell lysate but not
203 in mitochondrial lysate (Figure 3C), indicating that Clock mutation does not affect the
204 expression capacity of a single mitochondria but rather the quantity of mitochondria.

205 We next sought to determine the molecular mechanisms underlying excessive
206 mitochondrial fission in Clock^{Δ19} mice. We used RT-PCR to detect the mRNA
207 expression levels of genes involved in mitochondrial dynamics. The results showed
208 that, except for a slight elevation of Mfn1 in Clock^{Δ19} mice, there were no significant
209 differences in other fusion-related genes between Clock^{Δ19} and WT mice. Meanwhile,
210 Drp1 and Fis1, the primary mitochondrial fission genes had increased expression
211 levels in Clock^{Δ19} mouse livers compared with WT mouse livers. Clearly, Bnip3, a
212 main mitophagy gene, which was reported to be rhythmically expressed, decreased
213 and failed to cycle in Clock^{Δ19} mouse livers (Figure 3D). Moreover, the expression
214 levels of fusion proteins (Opa1, Mfn1 and Mfn2) were reduced in Clock^{Δ19} mouse
215 livers, although not as significantly as Bnip3 (Figure 3E). In contrast, the expression
216 levels of fission proteins (Drp1 and Fis1) were increased in Clock^{Δ19} mice compared

217 to WT mice (Figure 3E). Phosphorylation of DRP1 is important for its GTPase
218 activity, which then influences mitochondrial fission. Phosphorylation at s622 (s616
219 in humans) increases fission, while phosphorylation at s643 (s637 in humans) plays a
220 negative role in mitochondrial fragmentation (Chang & Blackstone, 2007; Chang &
221 Blackstone, 2010). However, in our research, we did not find any significant
222 differences in DRP1 phosphorylation between WT and Clock^{Δ19} mice (Figure 3E).

223 **Excessive mitochondrial fission in Clock^{Δ19} mice is due to posttranscriptional** 224 **regulation of Drp1 by CLOCK**

225 To further investigate the changes in protein expression, we used immunofluorescence
226 to study the alterations in the expression of FIS1 and DRP1; the staining intensity
227 showed similar trends with the Western blot (Figure 4A). By analyzing ChIP-Seq data,
228 we found no significant accumulation of CLOCK on the promoter region of
229 Drp1 (Annayev et al, 2014), indicating that Drp1 is probably regulated by Clock in a
230 posttranscriptional manner. Moreover, the increase of DRP1 in Clock^{Δ19} mice was
231 eliminated by the translational inhibitor cycloheximide but not by actinomycin D,
232 which is a transcriptional inhibitor (Figure 4B), suggesting that the accumulation of
233 DRP1 in Clock^{Δ19} mice is not attributable to slower protein degradation but rather to
234 more protein generation or less mRNA degradation. To compare the degradation rate
235 of Drp1 mRNA in WT and Clock^{Δ19} mice, we measured the changes in relative mRNA
236 expression levels over time after the addition of actinomycin D. Slower degradation
237 of Drp1 in Clock^{Δ19} mice verified the possibility of posttranscriptional regulation of
238 Drp1 by Clock (Figure 4C).

239 It has been reported that CLOCK can act as an mRNA splicer together with other
240 splicing factors (Yang et al, 2018). We performed RIP assay to detect whether CLOCK
241 can bind to Drp1 mRNA and further affect its stability. The RIP results showed that
242 there was a significant accumulation of CLOCK on Drp1 mRNA relative to the
243 negative control IgG in the AML12 hepatocyte cell line (Figure 4D-E). Moreover, the
244 binding was still significant in Clock^{Δ19} mouse livers (Figure 4F), suggesting that the
245 19th exon of Clock is not critical for mRNA binding but may play a role in interacting
246 with other mRNA binding factors or affect its degradation-mediating activity.
247 Moreover, by predicting the Drp1 mRNA-protein binding sites, we designed several
248 primers to search for the specific CLOCK binding sites. It was shown that there were
249 significant accumulations of CLOCK at 2042-2246 bp, 2653-2831 bp and 2905-3026
250 bp of Drp1 mRNA (Figure 4G), which are all near or in its 3'UTR region. In brief,
251 these findings suggest that CLOCK can bind to Drp1 mRNA 3' UTR region and
252 further affect its stability. Less Drp1 degradation in Clock^{Δ19} mice led to abnormal
253 mitochondrial dynamics and dysfunction.

254 **Mdivi-1 rescued mitochondrial morphological and functional changes.**

255 Considering the excessive fission and the severe dysfunction of the mitochondria of
256 Clock^{Δ19} mice, we wondered whether inhibiting fission would rescue these
257 mitochondrial changes. Mdivi-1 is an efficient mitochondrial fission repressor that
258 works by inhibiting the GTPase activity of Drp1. Incubation with Mdivi-1 for 12 or
259 24 hours both strengthened the fluorescence intensity and recovered the excessively
260 fragmented mitochondria to its granular or even tubular structures in Clock^{Δ19}

261 hepatocytes but not in WT hepatocytes (Figure 5A), which indicates that excessive
262 fission in $Clock^{\Delta 19}$ is mainly due to abnormal Drp1 regulation. Moreover, the addition
263 of Mdivi-1 substantially reduced ROS accumulation (Figure 5B) and restored the
264 decreased membrane potential in $Clock^{\Delta 19}$ mice comparing with the control (Figure
265 5C). Measurement of ATP showed an obvious restoration of mitochondrial ATP
266 generation by Mdivi-1 in $Clock^{\Delta 19}$ primary hepatocytes but not in the WT (Figure
267 5D).

268 Considering the favorable effect of Mdivi-1 on hepatocytes, we then explored its
269 effect on mouse mitochondrial function and related metabolism by intraperitoneal
270 injection. The obvious downregulation of the main mitochondrial proteins (ATP5a,
271 ND1, ATP6) in liver tissue demonstrated the inhibition of mitochondrial fission by
272 Mdivi-1 compared with DMSO (Figure 5E). Moreover, decreased ROS accumulation
273 and increased mitochondrial membrane potential both indicated a positive effect of
274 Mdivi-1 on $Clock^{\Delta 19}$ mouse mitochondrial function by injecting (Figure S3A-B). In
275 addition, the evident increase in the concentration of ATP and the recovery of
276 mitochondrial respiration also serve as direct evidence for the therapeutic effect of
277 Mdivi-1 on mitochondrial dysfunction (Figure 5F-G).

278 **Intraperitoneal injection of Mdivi-1 recovered hyperlipidemia and nonalcoholic** 279 **fatty liver disease in $Clock^{\Delta 19}$ mice.**

280 After two weeks of injection, serum samples were collected from the treated and
281 control mice, and IPGTT, blood glucose test and lipid test were conducted. There was
282 no significant difference in serum glucose levels between groups, but there was some
283 recovery on the IPTGG in the Mdivi-1-treated group (Figure 6A-B). As shown in
284 Figure 6B, Mdivi-1-treated mice exhibited stronger tolerance than the untreated and
285 DMSO-treated $Clock^{\Delta 19}$ mice. Their blood glucose levels were slightly elevated at
286 15 min after glucose injection, when the glucose levels in the control groups reached
287 their peak. These findings suggested that Mdivi-1 treatment may directly or indirectly
288 affect glucose metabolism. In addition, the effect of Mdivi-1 on serum lipid levels is
289 more complicated; there was a slight downregulation of triglyceride (TG) and density
290 lipoprotein cholesterol (LDL-C) levels in the Mdivi-1-treated group compared with
291 the DMSO control group (Figure 6C). The effect of Mdivi-1 was most evident in
292 increasing the level of HDL-C, implying a protective function of Mdivi-1 against
293 hyperlipidemia and related diseases (Figure 6C). However, there was no significant
294 glucose or lipid content improvement in WT mice receiving Mdivi-1, but a slight
295 increase in TG (Figure S4A-B), indicating that Mdivi-1 has no or possibly even a
296 negative effect on well-balanced mitochondria. We then used oil-red staining to
297 confirm the rescue effect of Mdivi-1 on fatty liver diseases. Frozen section of livers
298 showed an excellent therapeutic effect of Mdivi-1 in $Clock^{\Delta 19}$ mouse livers, which
299 suffered from serious lipid droplets deposition (Figure 6D); however, there was no
300 significant difference seen in WT livers.

301 **Discussion**

302 In our study, we demonstrated that circadian gene *Clock* plays an important role in
303 mitochondrial morphology and function by posttranscriptional regulation of Drp1.

304 Furthermore, mitochondrial architecture, membrane potential, ROS production and
305 respiration tend to be abnormal in *Clock*^{Δ19} mice due to the excessive mitochondrial
306 fragmentation.

307 Circadian clock plays critical roles in maintaining mitochondrial functions.
308 Either the accumulation of many mitochondrial proteins or the related functional
309 features such as oxygen consumption, membrane potential, ATP generation and
310 mitochondrial respiration are all rhythmic(Schmitt et al, 2018). The mitochondrial
311 circadian oscillations are abolished in *Bmal1*^{-/-} and *Per1/2*^{-/-} mice (11,12). In our study,
312 we provide evidence that the mutation of *Clock* also results in disordered
313 mitochondrial functions. Decreased Δφm, abundant ROS accumulation, and
314 decreased ATP concentration and mitochondrial respiration in *Clock*^{Δ19} verified the
315 role of *Clock* in mitochondria. It was reported that the activity of mitochondrial
316 respiration complex I oscillates and peaks at ZT12 mainly because of the
317 Nampt-NAD-SIRT6 pathway(Cela et al, 2016). Here, we used the seahorse assay and
318 determined that the activities of mitochondrial respiration complexes II and IV are
319 both decreased in *Clock* mutant mice, which indicates that there are other mechanisms
320 of circadian clock regulation of mitochondrial respiration. Indeed, it has been reported
321 that several mitochondrial complex components including NDUFA2, NDUFB5, and
322 NDUFC1 in complex I; COX4I1, COX6A1, and COX7A2 in complex IV; and
323 ATP5G2 and ATP5L in complex V are all transcriptionally regulated by circadian
324 clock elements(Schmitt et al, 2018). In addition, except for the regulation of
325 acetylation by SIRT6, recent global acetylome analyses have demonstrated that the
326 acetylation of many mitochondrial proteins is rhythmic. Moreover, some acetylation
327 is altered or abolished in *Clock*^{-/-} mice(Masri et al, 2013), indicating that CLOCK
328 plays an independent role in mitochondrial regulation as an acetyltransferase.

329 Recent studies have demonstrated that circadian factors *Bmal1* and *Per1/2* both
330 play roles in the regulation of mitochondrial morphology and affect the related
331 functions. *Bmal1*^{-/-} mitochondria tend to be enlarged and accompanied by elevated
332 ROS levels and mitochondrial dysfunction(Jacobi et al, 2015). *Per1/2* is involved in
333 the regulation of mitochondrial dynamics by affecting the rhythmic activity of
334 DRP1(Schmitt et al, 2018). In our study, *Clock*^{Δ19} mice mitochondria were
335 fragmented with smaller diameters and greater quantity in the same area when
336 compared with the WT. It has been reported that mitochondrial dynamics are
337 regulated by circadian genes, mostly through their transcriptional regulation of
338 mitochondrial dynamics genes or energy sensors(Jacobi et al, 2015; Schmitt et al,
339 2018). In this study, we demonstrated that the core mitochondrial fission mediator,
340 DRP1, is under direct posttranscriptional regulation of CLOCK. CLOCK facilitates
341 the degradation of *Drp1* mRNA by binding to its 3'UTR region. In *Clock*^{Δ19} mice,
342 decreased DRP1 degradation leads to DRP1 accumulation and then induces abnormal
343 mitochondrial fission. The fact that CLOCK-*Drp1* mRNA binding is still evident in
344 *Clock*^{Δ19} mice suggests that the 19th exon of CLOCK is not involved in its binding to
345 mRNA but rather in some other processes. It is well known that *Clock*^{Δ19} nearly loses
346 its transcriptional activity, although its interaction with BMAL1 is still

347 evident(Gekakis et al, 1998). The underlying mechanism is still under investigation;
348 we speculate that the 19th exon of CLOCK may play a similar role in the regulation of
349 CLOCK's mRNA regulating activity. In addition, yeast two-hybrid of CLOCK (1-580)
350 and the 19th exon deletion mutation of CLOCK proved that CLOCK can interact with
351 other proteins except for BMAL1 and that the 19th exon of CLOCK is important in the
352 CLOCK-CIPC complex formation(Gekakis et al, 1998; Hou et al, 2017).

353 In our study, we determined the mRNA degradation-mediating function of
354 CLOCK for the first time, which is one of the critical posttranscriptional regulation
355 processes. Indeed, except for their transcriptional regulatory roles, circadian clock
356 factors can also engage in posttranscriptional regulation to generate circadian
357 biological processes. As soon as transcription is initiated, mRNA modification starts;
358 alternative splicing(McGlincy et al, 2012; Yang et al, 2018) is regulated by circadian
359 clock, and disruption of splicing would also lead to disordered circadian rhythm by
360 influencing mRNA translocation or stability(Green, 2017). After being synthesized,
361 mRNAs translocate into the cytosol and are translated into proteins. It has been
362 reported that mRNA translocation(Chen et al, 2008) and translation are both
363 circadian-regulated processes(Green, 2017) and that the core circadian factor,
364 BMAL1, also participates in translation by interacting with several transcriptional
365 factors, including transcription initiation factors, elongation factors and ribosome
366 subunits(Lipton et al, 2015). Moreover, mRNA and protein stability(Wang et al, 2018)
367 and posttranslational modifications are also regulated by circadian clock. For example,
368 CLOCK is not only a transcription factor but also an acetyltransferase(Doi et al, 2006).
369 Except for the previously reported substrates such as histone and BMAL1(Doi et al,
370 2006; Hirayama et al, 2007), more substrates have been found, such as ASS1(Lin et al,
371 2017), which is an important enzyme in the urea cycle. The mRNA
372 degradation-mediating function of CLOCK uncovered in our study supplements the
373 general understanding of the posttranscriptional regulatory functions of the circadian
374 clock.

375 Due to its vital role in the regulation of processes essential for daily life,
376 disordered circadian rhythm and mutant circadian genes both lead to serious diseases.
377 Atherosclerosis, aging, prostate cancer and metabolic disease are all circadian-related
378 diseases. Abnormal activity, food intake and metabolic rates were revealed when
379 Clock^{Δ19} mutation was first identified. As a result, Clock mutant mice gained weight
380 faster than WT mice when fed regular or high-fat diets (Turek et al, 2005)
381 (Adamovich et al, 2014; Aviram et al, 2016). In our study, Clock^{Δ19} mice had evident
382 hyperlipidemia and NAFLD, suggesting they suffered from lipid metabolism
383 disorders. It has been reported that mitochondria tend to be round and swollen in
384 NAFLD patients, and excess ROS production is thought to be the main cause of the
385 pathological progression. The accumulation of ROS may be attributed to excessive
386 mitochondria fission resulted from fat accumulation(Galloway & Yoon, 2013).
387 Abundant ROS accumulation, excessive fission and the rescuing effect of Mdivi-1 on
388 NAFLD in Clock^{Δ19} mice verified that abnormal mitochondrial dynamics in Clock^{Δ19}
389 mice is a cause of hyperlipidemia and NAFLD. In vitro addition of Mdivi-1 caused a
390 rapid reversible dose-dependent formation of net-like mitochondria. In vivo

391 application of Mdivi-1 has been shown to protect cardiomyocytes and kidney, neuro
392 and retinal cells following ischemia/reperfusion (Brooks et al, 2009; Ong et al, 2010),
393 functioning as a protector against acute injury. In addition, it also plays a long-term
394 therapeutic role in heart failure(Givvimani et al, 2012) and cardiac hypertrophy. In our
395 study, in addition to its roles in decreasing ROS production and recovering
396 mitochondrial membrane potential and respiration, we provide a new role for Mdivi-1
397 in curing hyperlipidemia and fatty liver disease. In addition to the direct regulation
398 of lipid metabolism-related genes by Clock, here we offer a new perspective wherein
399 Clock affects lipid metabolism and related diseases by regulating mitochondrial
400 dynamics.

401 In brief, we propose that Clock plays an important role in the regulation of
402 mitochondrial dynamics and related functions by the posttranscriptional regulation of
403 Drp1 (Figure 6E). The findings in our study provide a new perspective on the
404 circadian clock, mitochondria and metabolism, which might contribute to the
405 understanding and generate new ideas for clinical application.

406 **Materials and methods**

407 **Animal studies**

408 The Clock^{Δ19} mice were purchased from the Jackson Laboratory and had been bred in
409 the Model Animal Research Center of Nanjing University. Same aged C57BL/6J mice
410 were also purchased from Model Animal Research Center of Nanjing University.
411 Mice were fed a chow diet and raised in a clean room with 12 h light and 12 h dark
412 cycles (Lights on at 8:00am and lights off at 20:00pm). All animal experiments were
413 conducted strictly in accordance with the National Institutes of Health Guide for the
414 Care and Use of Laboratory Animals and were approved by the Animal Care and Use
415 Committee of Shanghai Medical College, Fudan University.

416 For tissue collection, mice were sacrificed at the age of 8-12 weeks. Their tissues
417 were harvested every 4 h for 24 hour and then stored at -80°C or in 4%
418 paraformaldehyde.

419 For metabolic index detection, mice at the age of 10 weeks with an approximate
420 weight of 23–25 grams were divided into blank control group, vehicle control and
421 Mdivi1-treated group. After 2 weeks of treatment, the animals were first tested with
422 the intraperitoneal glucose tolerance test (IPGTT) and then euthanized. Their sera
423 were collected, and the levels of blood glucose and lipids were measured. For IPGTT
424 measurement, mice were fasted overnight for approximately 14 hours with free access
425 to water. After the measurement of their fasting blood glucose levels, the mice were
426 intraperitoneally injected with 20% glucose dissolved in 0.9% NaCl (1 g/kg). The
427 blood glucose levels were then measured at 15 min, 60 min and 120 min after
428 injection. Blood samples were obtained from the tail veins, the glucose levels were
429 measured by glucose meter (Abbott, America). For the intraperitoneal insulin
430 tolerance test (IPITT) measurement, mice were fed overnight. After measurement of
431 their postprandial blood glucose levels, the mice were intraperitoneally injected with 1
432 U/kg insulin. The subsequent measurement of blood glucose levels was similar with
433 IPGTT.

434 **Cell lines**

435 293T cells were purchased from the Cell Bank Type Culture Collection of the Chinese
436 Academy of Sciences. It was cultured in DMEM supplemented with 10% fetal bovine
437 serum (FBS), 10 U/mL penicillin and 100 mg/mL streptomycin. The cells were
438 cultured in a humidified CO₂ incubator at 37°C.

439 AML12 was purchased from the Cell Bank Type Culture Collection of the
440 Chinese Academy of Sciences. AML12 was cultured in DMEM F12 supplemented
441 with 10% FBS, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, 40
442 ng/ml dexamethasone, 10 U/mL penicillin and 100 mg/mL streptomycin in a
443 humidified CO₂ incubator at 37°C.

444 **Mdivi-1 treatment**

445 The Drp1 GTPase activity inhibitor Mdivi-1 was purchased from Topscience, China
446 (T1907) and dissolved in DMSO to create a stock concentration of 10 mg/ml. 50
447 mg/kg Mdivi-1 was given to mice by intraperitoneal injection every two days for 2
448 weeks. The vehicle group received the same quantity of DMSO.

449 **Exhaustive swimming assay**

450 Eight-week old WT and Clock^{Δ19} mice were put in a swimming box with a water
451 depth of 20 cm and temperature of 25°C. During this process, the mice were forced to
452 keep swimming by stirring the surrounding water. Mice were saved when they could
453 no longer keep their nose above the water and started inhaling. Their swimming times
454 were then recorded.

455 **Isolation of primary hepatocyte and liver mitochondria**

456 Primary hepatocytes were isolated by perfusion of D-hanks and collagenase IV
457 through the postcava to the portal vein. The liver was transferred to DMEM after
458 perfusion, disintegrated by tweezers and filtered through a 70-μm cell strainer (BD
459 Bioscience). Then, 90% percent percoll solution was used to separate the activated
460 hepatocytes from the dead ones. The isolated hepatocytes were then cultured in
461 DMEM with 10% FBS with the addition of penicillin-streptomycin solution.

462 Liver mitochondria were isolated in MSHE buffer (70 mM sucrose, 210 mM
463 mannitol, 5 mM HEPES, 1 mM EGTA, and 0.5% fatty acid free BSA. pH 7.2). A
464 piece of liver tissue was first homogenized in ~10-fold volume of cold MSHE buffer
465 and centrifuged at 800 g for 10 min to remove the tissue fragments. The supernatant
466 was centrifuged again at 8000 g for 10 min to obtain the crude mitochondria. The
467 pellet was then resuspended in 100 μl of MSHE buffer, and the protein quantities were
468 measured by BCA kit.

469 **Electron and confocal microscopy**

470 Mitochondria number and diameter of liver and primary hepatocytes were analyzed
471 by electron microscope. To visually study the mitochondrial dynamics, primary
472 hepatocytes were transfected with Ad-cox8a-GFP/RFP for 24 hours and then
473 photographed under a confocal microscope.

474 **Mitochondrial respiration assessment**

475 For isolated mitochondria coupling and electron flow analysis, an XF24 Seahorse
476 analyzer was used. Twenty micrograms of isolated mitochondria were plated on an
477 assay plate in the initiation buffer (70 mM sucrose, 220 mM mannitol, 10 mM
478 KH₂PO₄, 5 mM MgCl₂, 2 mM HEPES, 1.0 mM EGTA and 0.2% fatty acid -free BSA,

479 pH 7.2) plus substrates. For the coupling assay, 10 mM succinate and 2 μ M rotenone
480 were used. For the electron flow assay, 10 mM pyruvate, 2 mM malate and 4 μ M
481 FCCP were used. The plate was then centrifuged at 2000 g for 20 min at 4°C. After
482 centrifugation, the mitochondria were viewed briefly under the microscope and
483 transfer to a 37°C incubator for 10 min to warm. The plate was then transferred to the
484 analyzer, and the experiment was initiated. The injections were as follows: for the
485 coupling assay, 40 mM ADP, 25 μ g/ml oligomycin, 40 μ M FCCP and 40 μ M
486 antimycin were injected, while for the electron flow assay, 20 μ M rotenone, 100 mM
487 succinate, 40 μ M antimycin and 100 mM ascorbate plus 1 mM TMPD were injected.

488 For the primary hepatocyte coupling assay, 25,000 cells/well were plated on a
489 plate in advance in DMEM overnight to allow attachment. On the day of the
490 experiment, the culture media was exchanged for basal medium (Sigma, D5030) with
491 the addition of 10 mM pyruvate. The injections were as follows: 5 mg/ml oligomycin,
492 5 mM FCCP and 5 mM rotenone.

493 **Assessment of mitochondrial membrane potential, ROS production.**

494 WT and Clock ^{Δ 19} primary hepatocytes were cultured in DMEM overnight to adhere
495 and stained with a specific reagent. To measure the mitochondrial membrane potential,
496 after the removal of the culture medium and washing with PBS, the cells were stained
497 with 5 μ g/ml JC-1 (Beyotime Biotechnology, China, C2005) at 37°C for 30 min. Then,
498 they were washed with PBS 3 times before imaging. Similarly, the total cellular ROS
499 were traced by 10 μ M DCFH-DA fluorescence probe (Beyotime Biotechnology,
500 China, S0033).

501 **Luciferase reporter assay**

502 For the luciferase reporter assay, mouse Mrps24, Mrpl50 promoter and the
503 mitochondrial D-loop were cloned into pGL3-Basic vector and fused with firefly
504 luciferase. 293T cells were seeded and grown to ~80% confluency overnight and then
505 transfected with the fusion plasmids with or without Clock and Bmal1 (3 replicates).
506 After 24 h of transfection, the cells were harvested, and the luciferase activity was
507 determined with a luminometer.

508 **Gene and protein expression measurement**

509 Real-time PCR and western blot were used to measure the relative expression of
510 mitochondrial dynamic genes and other genes. The primers used are listed in Table S1.
511 The antibodies used are listed in Table S2.

512 **RNA immunoprecipitation**

513 First, 10⁷ Aml12 cells (or a piece of liver) were crosslinked with UV, harvested in
514 cold PBS and resuspended in 1 ml of RIP buffer [150 mM KCl, 25 mM Tris pH 7.4, 5
515 mM EDTA, 5 mM DTT, 5% NP40, 1 mM PMSF, 100 U/ml SUPERase• In™ RNase
516 Inhibitor (Thermo Fisher)]. The cells (liver tissue) were then mechanically sheared
517 using a Dounce homogenizer with 15–20 strokes (with a tissue homogenate) and
518 briefly sonicated (OFF:30 s; ON:30 s for 13 mins). After incubation on ice for 10 min,
519 the lysate was centrifuged at 12000 rpm for 10 min and split into three fractions of 45
520 μ l each and two of 450 μ l each (for Input, Mock and IP). Three micrograms of
521 anti-CLOCK (Abcam, ab3517), or anti-IgG (Abcam, ab172730) antibody was added
522 to the supernatant and incubated for 4 hours at 4°C with gentle rotation. Then, 40 μ l

523 of protein A/G beads were added and incubated for another 2 hours at 4°C with gentle
524 rotation. The beads were pelleted at 2,500 rpm for 30 s. The supernatant was removed,
525 and the beads were washed with RIP buffer 5 times. The beads were then resuspended
526 with 100 µl of RIP buffer, and 10% were analyzed by Western blot with
527 CLOCK-specific antibody. The remaining 90% were then digested with proteinase K
528 and DNase I. The coprecipitated RNAs were further isolated by Trizol reagent.
529 RT-PCR was then performed with specific primers.

530 **Statistical analysis**

531 Data are presented as the means ± SEM. Statistical comparisons were conducted with
532 unpaired Student's t-tests or one-way ANOVA, as appropriate, and $p < 0.05$ was
533 considered statistically significant.

534

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546 G; Writing – Original Draft, Xu L; Writing – Review & Editing, Xu L, Lu C and Qian
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549

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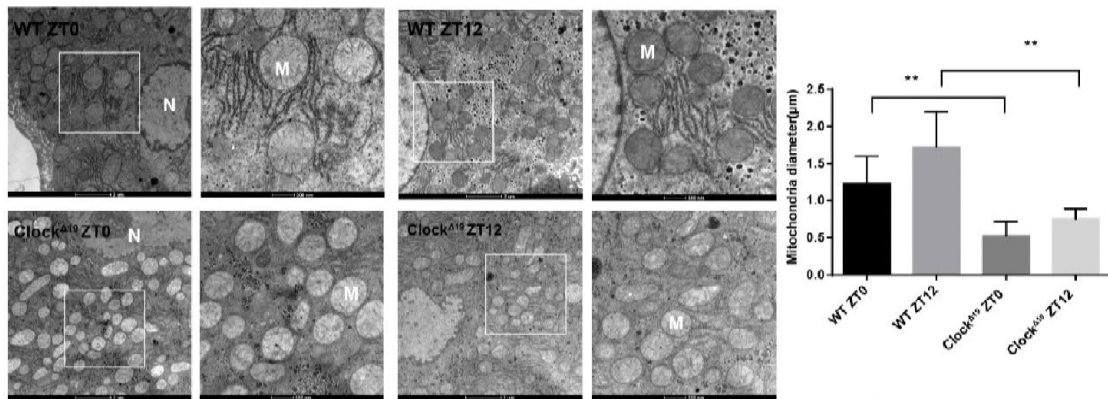
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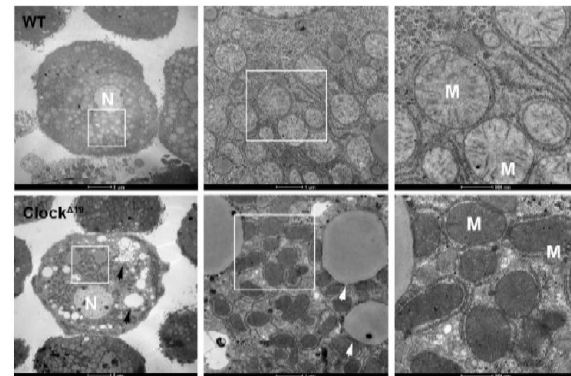
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Figure 1

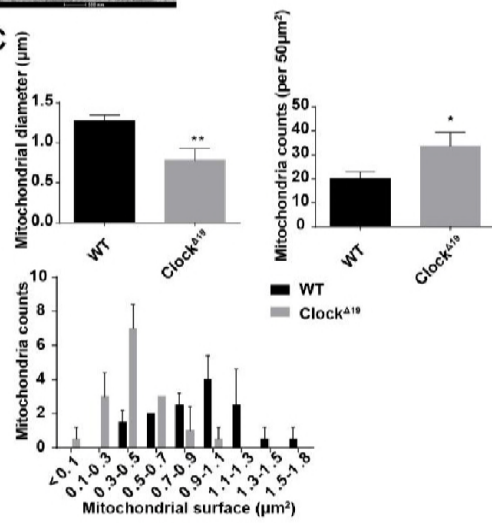
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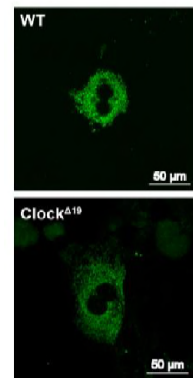
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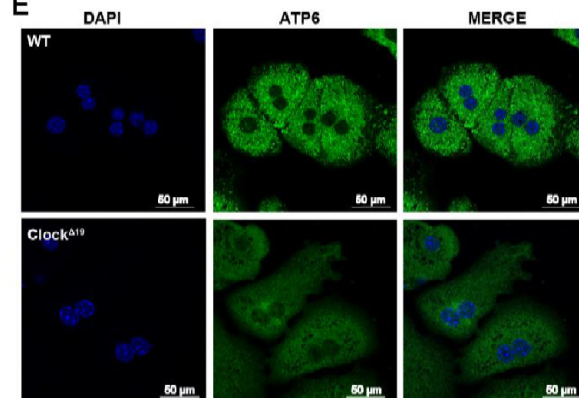
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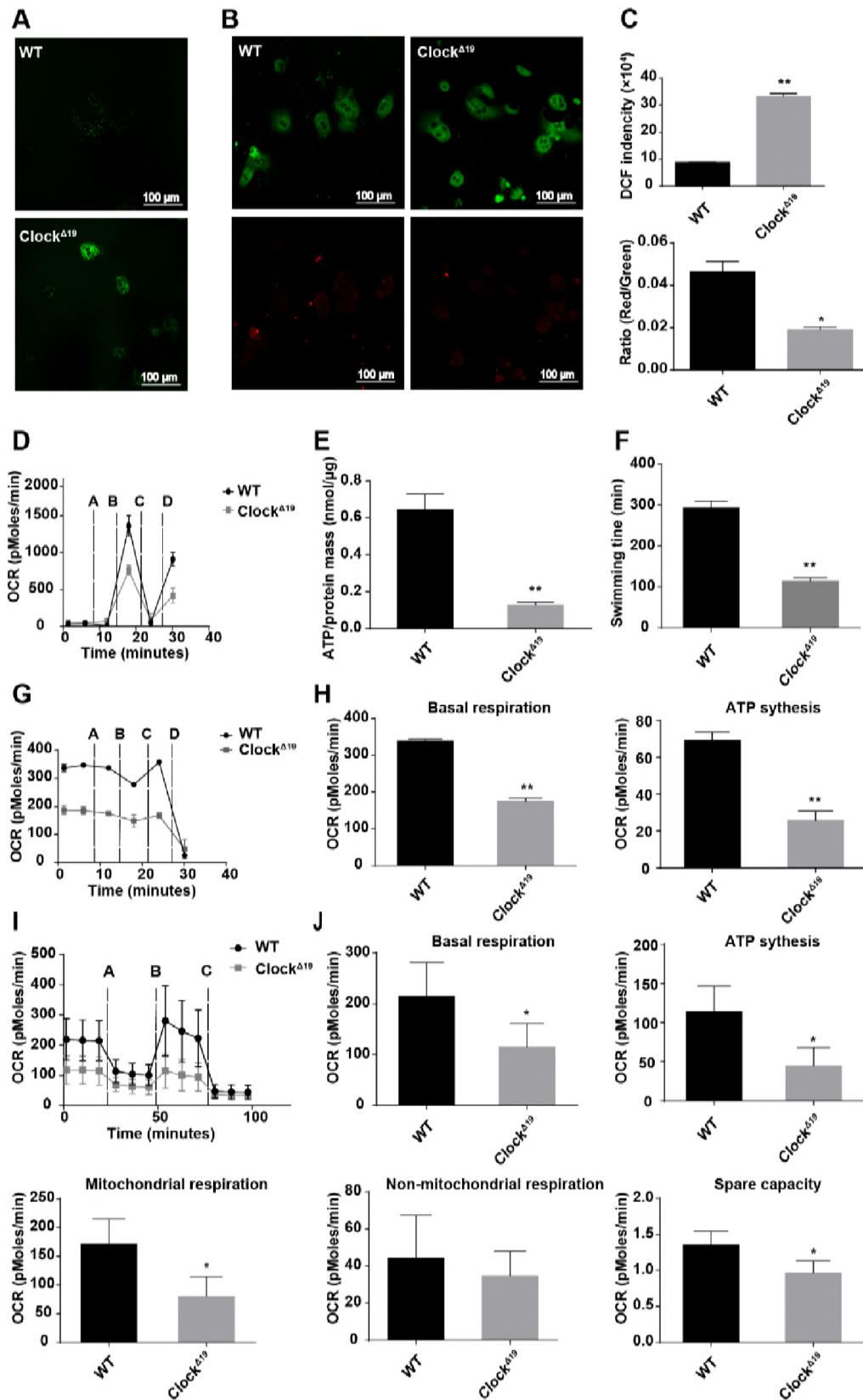
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685 **Figure 1 Clock^{Δ19} mice present morphological changes in mitochondria**

- 686 (A) EM images of WT and Clock^{Δ19} mice livers collected at ZT0 and ZT12.
687 Mitochondrial diameter was calculated based on the EM images. Data presented
688 as the mean ± SEM. **p < 0.01 vs WT ZT0; **p < 0.01 vs WT ZT12 (N: Nuclear;
689 M: Mitochondria).
- 690 (B) Representative EM images of primary hepatocytes separated from WT and
691 Clock^{Δ19} mice (Black arrows: Vacuoles; White arrows: Lipid droplets).
- 692 (C) Mitochondrial diameter and size distributions calculated from EM images in 50
693 μm². Data presented as the mean ± SEM. **p < 0.01 vs WT, *p < 0.05 vs WT.
- 694 (D) Confocal images of the primary hepatocellular mitochondria of WT and Clock^{Δ19}
695 mice. The mitochondria were tagged with ad-COX8a-GFP virus.
- 696 (E) Immunofluorescence images of WT and Clock^{Δ19} primary hepatocytes. Green:
697 ATP6 stained with FITC; Blue: nucleus stained with DAPI.
698

Figure 2

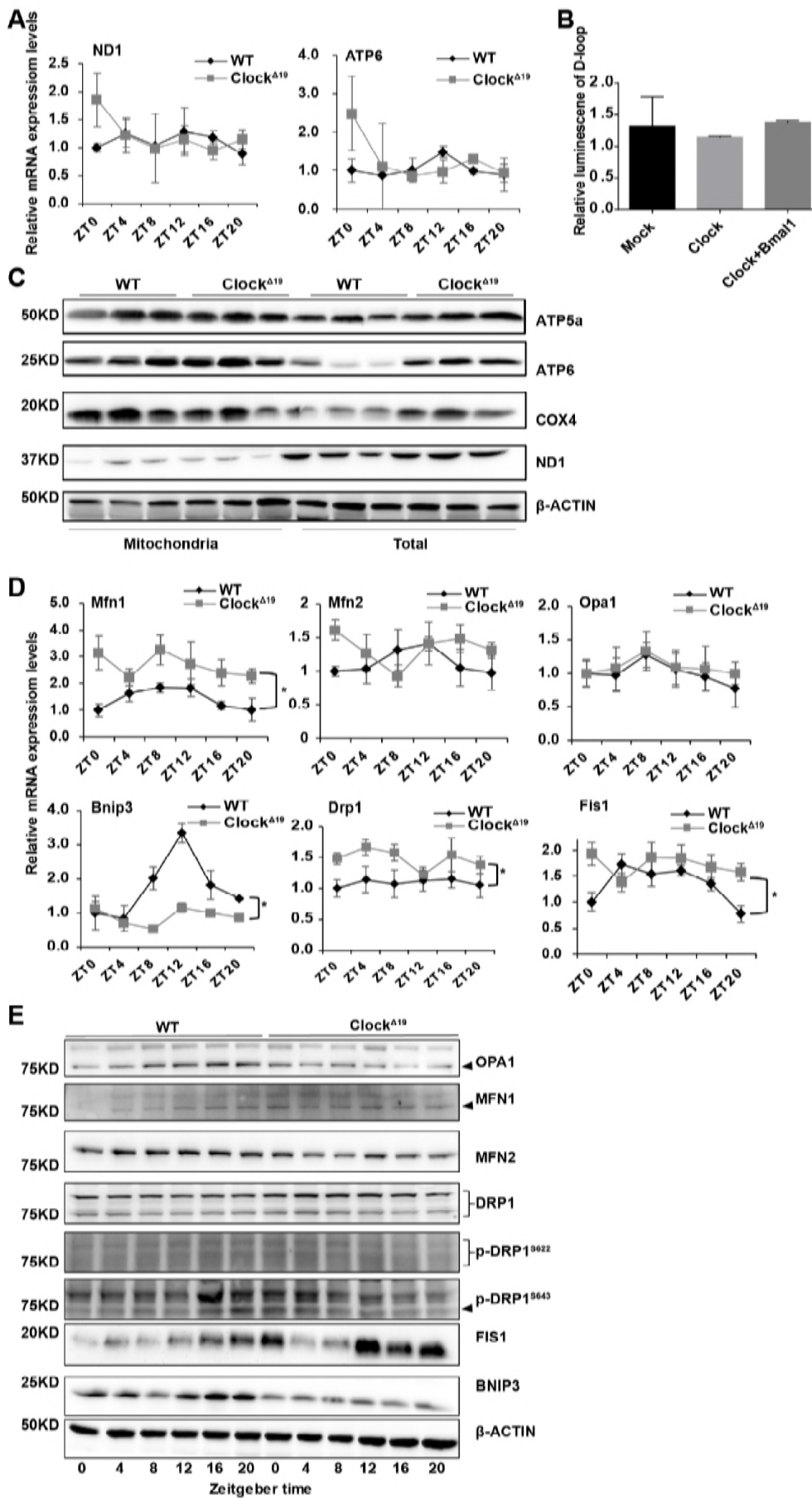


699

700 **Figure 2** The morphological changes of *Clock*^{Δ19} mice are accompanied with
 701 mitochondrial respiration and ATP generation dysfunction

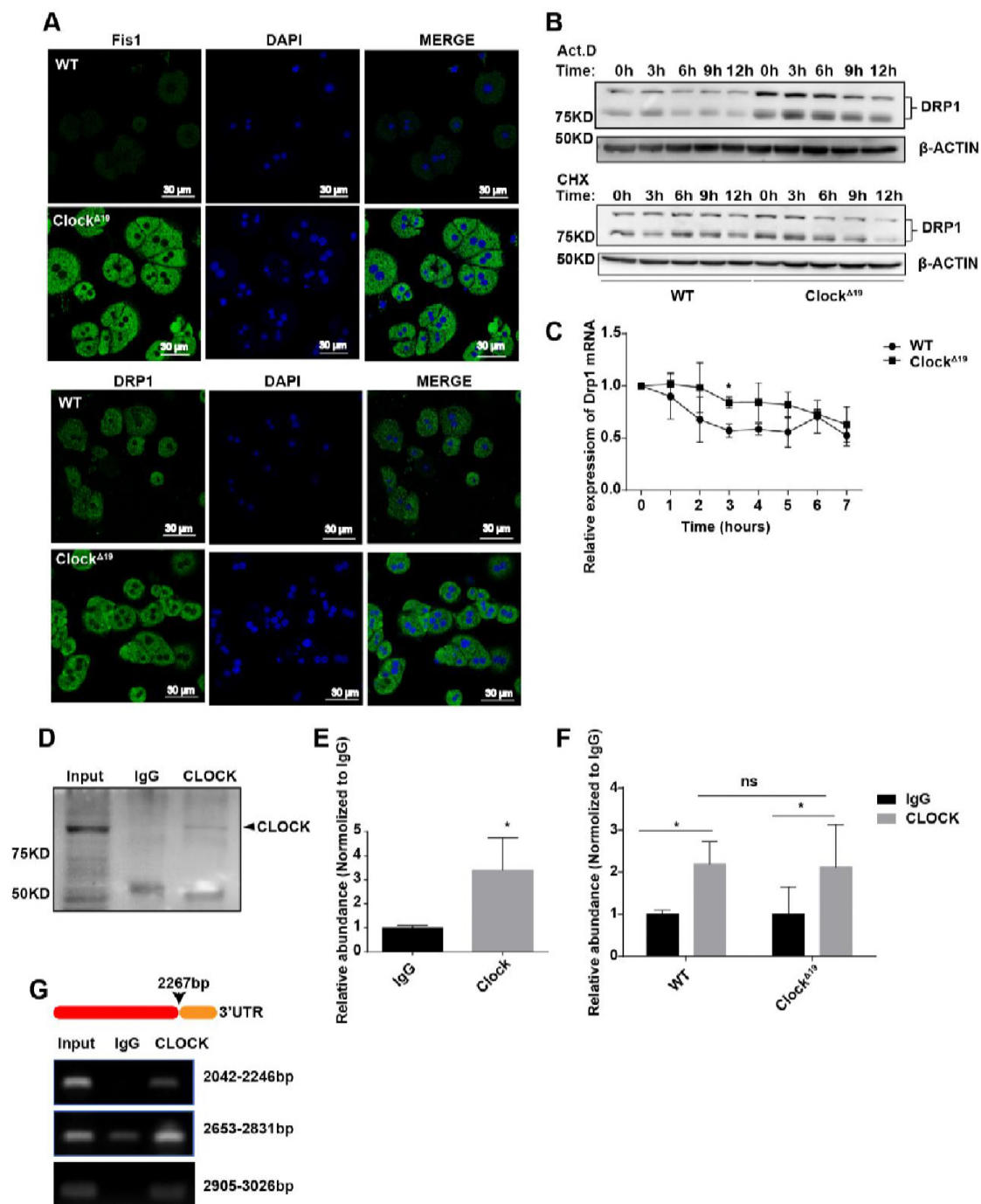
- 702 (A) ROS measurement images of WT and Clock^{Δ19} primary hepatocytes stained by
703 DCFH-DA.
- 704 (B) JC-1 staining of WT and Clock^{Δ19} primary hepatocytes, mitochondrial membrane
705 potential measured by the relative fluorescence density of the red/green ratio.
- 706 (C) Upper panel: Statistical histogram of ROS measurement, data presented as the
707 mean ± SEM. **p<0.01 vs WT. Lower panel: Statistical histogram of JC-1
708 staining, data presented as the mean ± SEM. *p < 0.05 vs WT.
- 709 (D) Electron flow assay of mitochondria separated from WT and Clock^{Δ19} livers.
710 Injections for A-D refer to rotenone, succinate, antimycin A and ascorbate/TMPD,
711 respectively.
- 712 (E) ATP generation capability presented by the ratio of ATP concentration/total
713 protein mass in WT and Clock^{Δ19} primary hepatocytes. Data presented as the
714 mean ± SEM. **p<0.01 vs WT.
- 715 (F) Exhaustive swimming assay conducted in WT and Clock^{Δ19} mice. Their
716 swimming time were recorded and represent their physical power. Data presented
717 as the mean ± SEM. **p<0.01 vs WT.
- 718 (G) Coupling assay in mitochondria isolated from WT and Clock^{Δ19} livers; the
719 injections from A to D refer to ADP, oligomycin, FCCP and antimycin A,
720 respectively.
- 721 (H) Statistical histogram of OCR represents the basal respiration and ATP synthesis
722 determined based on the coupling assay data. Data presented as the mean ± SEM.
723 **p<0.01 vs WT.
- 724 (I) Coupling assay in primary hepatocytes. The injections from A to C refer to
725 oligomycin, FCCP and antimycin A/rotenone, respectively.
- 726 (J) Statistical histogram of OCR represents basal respiration, ATP synthesis,
727 mitochondrial respiration, nonmitochondrial respiration and spare capacity
728 determined based on the coupling assay data. Data are presented as the mean ±
729 SEM. *p < 0.05 vs WT.

Figure 3



- 731 **Figure 3 Expression of mitochondria-related genes altered in Clock^{Δ19} mice**
732 (A) Diurnal mRNA expression of mitochondrial encoded genes in WT and Clock^{Δ19}
733 livers measured by real-time PCR. Data presented as the mean ± SEM.
734 (B) Luciferase reporter assay conducted in 293T cells with the mitochondrial D-loop,
735 or together with Clock and Bmal1. Data presented as the mean ± SEM.
736 (C) Western blot measurement of the protein expression levels of
737 mitochondria-specific proteins in liver mitochondrial lysates and total lysates.
738 Liver samples were collected at ZT12. (Zeitgeber time ZT0: lights on; ZT12:
739 lights off).
740 (D) Diurnal mRNA expression of genes involved in the regulation of mitochondrial
741 dynamics in WT and Clock^{Δ19} livers measured by real-time PCR. Data presented
742 as the mean ± SEM. *p < 0.05 vs WT.
743 (E) Western blot analysis of liver mitochondrial dynamics-related proteins. The liver
744 tissues were collected every 4 h for 24 hrs.
745

Figure 4



746

747 **Figure 4 Excessive mitochondrial fission in $Clock^{\Delta 19}$ is due to posttranscriptional**
 748 **regulation of Drp1 by CLOCK**

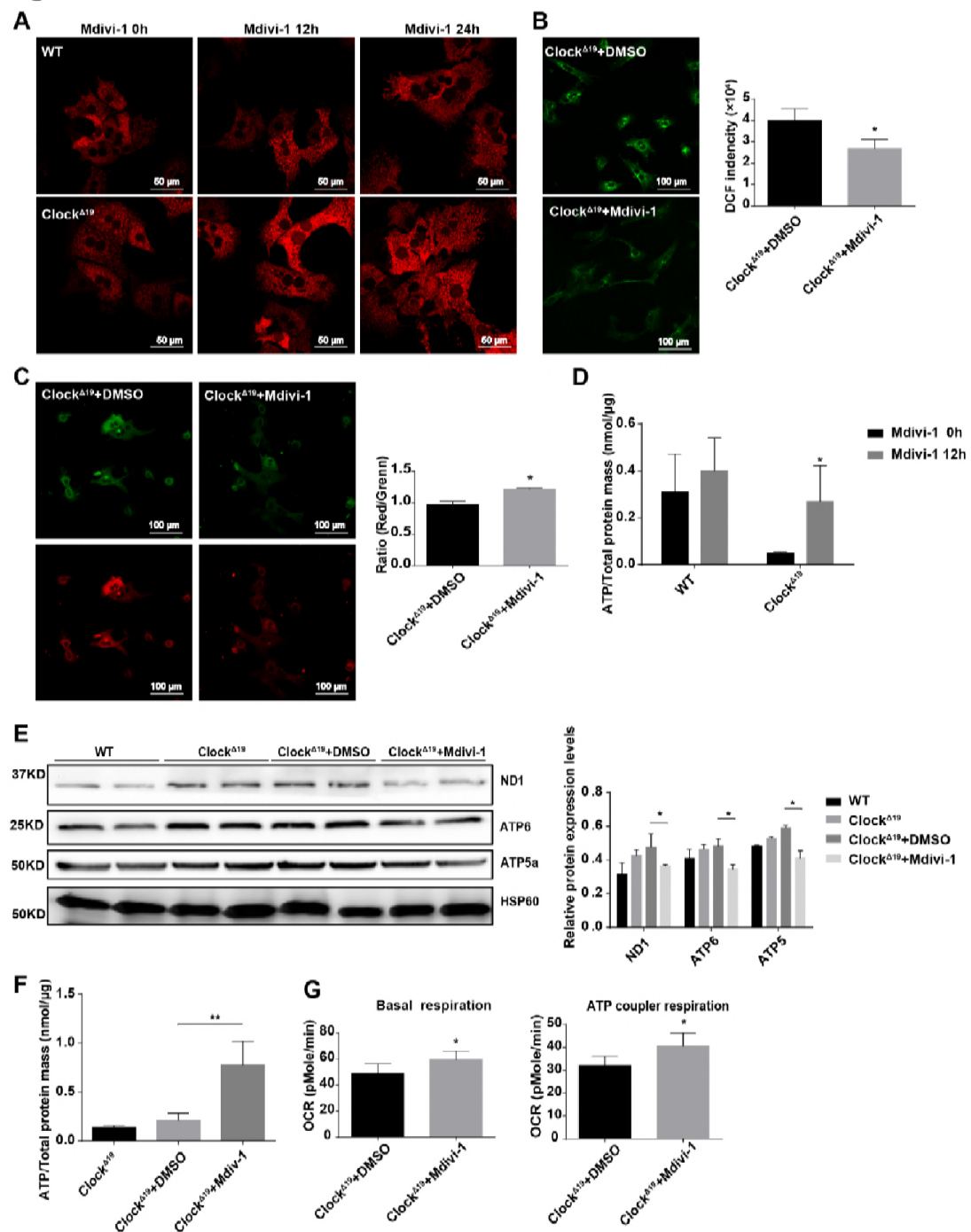
749 (A) Immunofluorescence images of WT and $Clock^{\Delta 19}$ primary hepatocytes. Green:
 750 FIS1/DRP1 stained with FITC; Blue: nucleus stained with DAPI.

751 (B) Protein expression of DRP1 in WT and $Clock^{\Delta 19}$ hepatocytes after treatment with
 752 5 μ g/ml Act.D or 50 μ g/ml CHX for 0, 3, 6, 9 and 12 hours.

753 (C) Relative mRNA expression level of Drp1 in WT and $Clock^{\Delta 19}$ hepatocytes after
 754 treatment with 5 μ g/ml Act.D for 0, 1, 2, 3, 4, 5, 6 and 7 hours. Data presented as
 755 the mean \pm SEM. * p <0.05 vs WT.

- 756 (D) Western blot detection of CLOCK after the RIP assay.
- 757 (E) RIP analysis of AML12 lysate with IgG or anti-CLOCK antibodies. Precipitated
758 mRNA was detected by RT-PCR. Data presented as the mean \pm SEM. * $p < 0.05$ vs
759 IgG.
- 760 (F) RIP analysis of WT and Clock ^{Δ 19} liver lysate with IgG or anti-CLOCK antibodies.
761 Precipitated mRNA was detected by RT-PCR. Data presented as the mean \pm SEM.
762 * $p < 0.05$ vs IgG.
- 763 (G) CLOCK binding sites on Drp1 mRNA checked by PCR with specific primers and
764 DNA gel analysis.
- 765

Figure 5



766

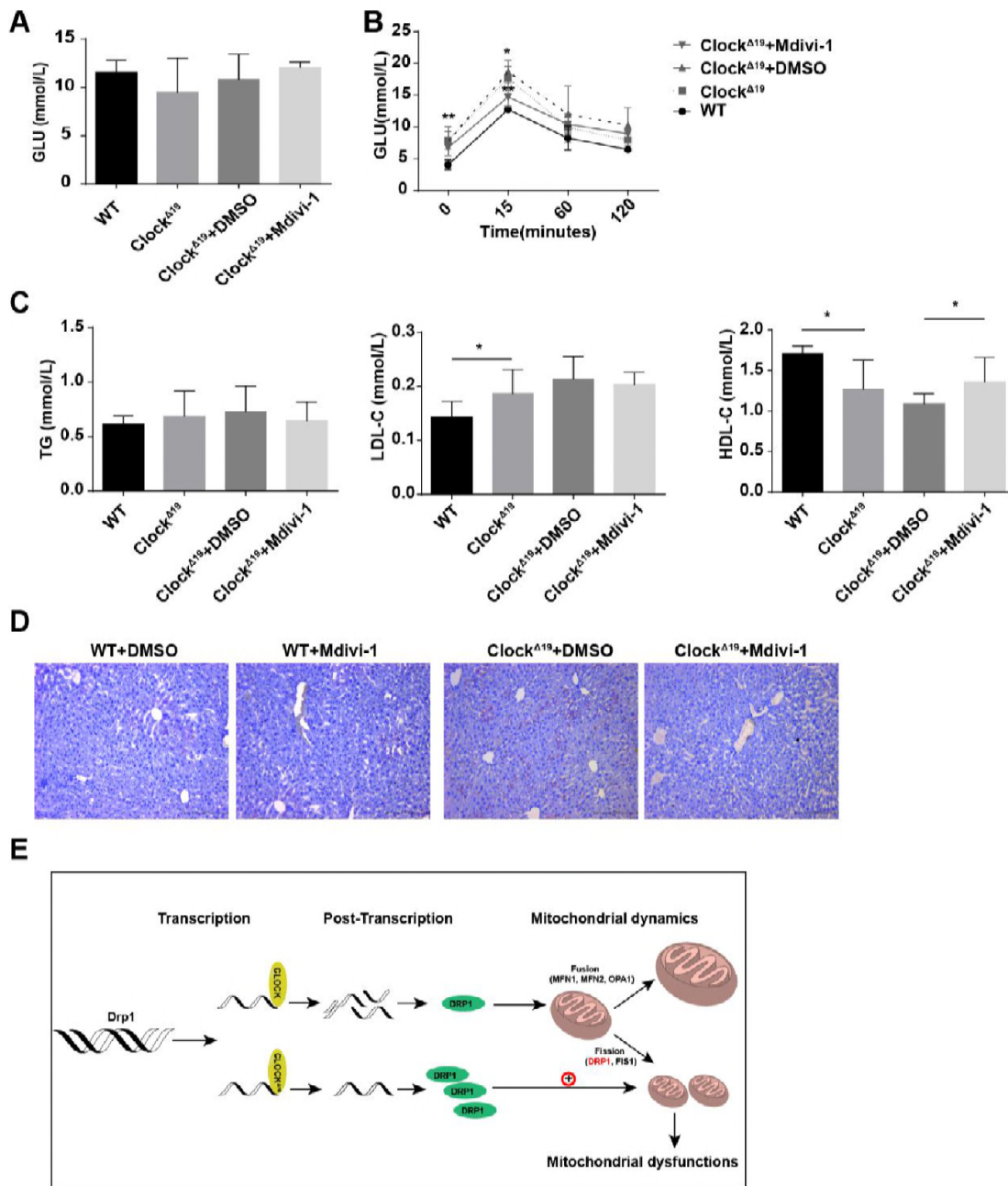
767 **Figure 5 Mdivi-1 rescued mitochondrial morphology and functional changes**

768 (A) Representative confocal images of the mitochondrial network in WT and Clock Δ^{19}
 769 primary hepatocytes with the addition of Mdivi-1 for 0, 12 and 24 hours.
 770 Mitochondria were tagged with ad-COX8a-RFP virus.

771 (B) Total ROS production of Clock Δ^{19} primary hepatocytes with addition of DMSO or
 772 Mdivi-1, as measured by DCFH-DA staining. Right panel: statistical histogram of
 773 fluorescence intensity. Data presented as the mean \pm SEM. * $p < 0.05$ vs

- 774 Clock^{Δ19}+DMSO.
- 775 (C) Mitochondrial membrane potential of Clock^{Δ19} primary hepatocytes with the
776 addition of DMSO or Mdivi-1 stained with JC-1. Right panel: statistical histogram
777 of red/green ratio. Data presented as the mean ± SEM. *p < 0.05 vs
778 Clock^{Δ19}+DMSO.
- 779 (D) ATP concentrations in WT and Clock^{Δ19} primary hepatocytes treated with Mdivi-1
780 for 0 and 12 hours. Data presented as the mean ± SEM. *p < 0.05 vs
781 Clock^{Δ19}+Mdivi-1-0 h.
- 782 (E) Western blotting of the mitochondrial proteins of WT and Clock^{Δ19} mice livers
783 injected with DMSO and Mdivi-1. Right panel: statistical histogram of relative
784 protein expression levels. Data presented as the mean ± SEM. *p < 0.05 vs
785 Clock^{Δ19}+DMSO.
- 786 (F) ATP production capability measurement of Clock^{Δ19} primary hepatocytes after
787 treatment with DMSO or Mdivi-1. Data presented as the mean ± SEM. **p < 0.01
788 vs Clock^{Δ19}+DMSO.
- 789 (G) Statistical histogram of OCR represents basal respiration and ATP synthesis based
790 on the coupling assay of the treated mice. Data presented as the mean ± SEM. *p
791 < 0.05 vs Clock^{Δ19}+DMSO.
- 792

Figure 6



793

794 **Figure 6 Intraperitoneal injection of Mdivi-1 rescued hyperlipidemia and**
 795 **nonalcoholic fatty liver disease in Clock^{Δ19} mice.**

796 (A) Fasting blood glucose levels in Clock^{Δ19} mice measured after overnight fasting
 797 (n=7). Data presented as the mean ± SEM.

798 (B) IPGTT of WT and Clock^{Δ19} mice measured after overnight fasting (n=7). Data
 799 presented as the mean ± SEM. *p < 0.05, Clock^{Δ19}+Mdivi-1 vs Clock^{Δ19}+DMSO;
 800 **p < 0.01 Clock^{Δ19} vs WT.

801 (C) TG, LDL-C and HDL-C in WT, Clock^{Δ19} and treated mice (n=7). Data presented
 802 as the mean ± SEM. *p < 0.05, Clock^{Δ19} vs WT, Clock^{Δ19}+Mdivi-1 vs
 803 Clock^{Δ19}+DMSO.

804 (D) Oil-red staining of WT and Clock^{Δ19} liver frozen sections to measure lipid
805 deposition.

806 (E) A schematic diagram illustrating the role of CLOCK in mitochondrial dynamics.

807