1   **Mitofusin 1 is required for the oocyte-granulosa cell communication that**

2   **regulates oogenesis**

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SUMMARY

Mitochondrial function, largely regulated by the dynamics of this organelle, is inextricably linked to oocyte health. While the proteins that modulate mitochondrial fusion, Mitofusin 1 (MFN1) and 2 (MFN2), are required for embryogenesis, their role in oocyte development remains unclear. Here we show that the oocyte-specific deletion of *Mfn1*, but not *Mfn2*, results in a complete loss of oocyte growth and ovulation due to a block in folliculogenesis at the preantral-to-antral follicle transition. We pinpoint the loss of oocyte ovulation to disrupted oocyte-somatic cell communication – *Mfn1*-null oocytes are deficient for the production of the important somatic cell signaling factor GDF9. Unexpectedly, the double loss of *Mfn1* and *Mfn2* mitigates the effects on oocyte growth and ovulation, which is explained by a partial rescue of oocyte-somatic cell communication and folliculogenesis. Together, this work demonstrates that mitochondrial function influences communication of oocyte with follicular somatic cells and suggests that the balanced expression of modulators of mitochondrial dynamics is critical for proper oocyte development.

Keywords: folliculogenesis, oocyte, mitochondria, mtDNA, fusion, mitofusin, MFN1, MFN2, GDF9.

39 **INTRODUCTION**

40 Mitochondria play a key role in oocyte development (Chiaratti et al., 2018),

41 termed oogenesis. The biogenesis of these organelles is heavily increased

42 during oocyte growth (Jansen and De Boer, 1998) and likely accounts for the

43 energetic needs of the oocyte and the early embryo (Collado-Fernandez et

44 al., 2012). Furthermore, redistribution of mitochondria through the oocyte

45 cytoplasm is fundamental to supply the energetic demands of key events such

46 as meiotic progression and chromosome segregation (Coticchio et al., 2014;

47 Udagawa et al., 2014; Wakai et al., 2014; Yu et al., 2010). In support of this,

48 mitochondrial abnormalities in oocytes are tightly associated with disrupted

49 meiotic spindle, embryonic developmental arrest, and sterility in humans and

50 animals (Van Blerkom et al., 1995; Fragouli and Wells, 2015; Johnson et al.,

51 2007; Reynier et al., 2001; Santos et al., 2006; Wai et al., 2010).

52 Interestingly, in oocytes mitochondria are more numerous, smaller, and

53 rounder in appearance than in somatic cells (Chiaratti et al., 2018; Motta et

54 al., 2000; Wassarman and Josefowicz, 1978). These morphological

55 characteristics are determined by mitochondrial dynamics, the opposing

56 processes of fusion and fission that regulate organelle activity, transport and

57 degradation (Mishra and Chan, 2014; Schrepfer and Scorrano, 2016); cells

58 lacking mitochondrial fusion have a severe defect in respiratory capacity and

59 mitochondrial DNA (mtDNA) instability (Chen et al., 2003, 2005, 2010).

60 Mitochondrial fusion is orchestrated by two dynamin-related GTPases –

61 Mitofusins 1 (MFN1) and 2 (MFN2) on the mitochondrial outer membrane

62 (Mishra and Chan, 2014; Schrepfer and Scorrano, 2016).

63   Given the unique architecture of the mitochondrial network in oocytes, it is

64   likely that *Mfn1* and *Mfn2* are more tightly regulated in the female germline

65   (Liu et al., 2016a; Wakai et al., 2014; Zhang et al., 2016; Zhao et al., 2015).

66   Indeed, while the ablation of fission leads to mitochondrial elongation in

67   oocytes (Udagawa et al., 2014), the overexpression of *Mfn1* and *Mfn2* does

68   not lead to mitochondrial elongation (Wakai et al., 2014), suggesting

69   maintaining a fragmented population is critical for the oocyte. Moreover, mice

70   with oocyte-specific deficiency for mitochondrial fission are infertile (Udagawa

71   et al., 2014), whereas overexpression of fusion genes causes marked

72   mitochondrial aggregation and damages oocyte viability (Wakai et al., 2014).

73   Although there is a clear link between mitochondrial dynamics and oocyte

74   fertility (Liu et al., 2016b; Udagawa et al., 2014; Wakai et al., 2014; Yu et al.,

75   2010; Zhang et al., 2016), many questions remain: why do mitochondrial

76   populations in the female gamete have such a unique morphology relative to

77   somatic cells? How do these changes in shape relate to the role of

78   mitochondria in oocyte development? Is the essential role of pro-fusion

79   proteins MFN1 and MFN2 in early embryogenesis (Chen et al., 2003) linked

80   to oocyte development? To tackle these questions and specifically address

81   the role of mitofusins in the female gamete, we generated oocyte-specific

82   conditional knockouts (cKO) of *Mfn1*, *Mfn2,* and both *Mfn1* & *Mfn2* (*Mfn1&2*)

83   in mice. We found that the deletion of *Mfn1* alone, but not *Mfn2*, resulted in a

84   complete loss of oocyte growth and a block in folliculogenesis at the preantral-

85   to-antral follicle transition. Further characterization of *Mfn1*-cKO oocytes

86   revealed impaired communication between the oocyte and its companion

87   somatic cells. Unexpectedly, these defects were mitigated in mice with double

88   loss of *Mfn1* and *Mfn2* in oocytes, which was explained by a partial rescue of

89   oocyte-somatic cell communication and folliculogenesis. These results

90   demonstrate that *Mfn1*, but not *Mfn2*, is required for oocyte formation and

91   ovulation, and that the balanced expression of *Mfn1* and *Mfn2* is a key

92   determinant for oocyte-follicular cell communication.

93

94   **RESULTS AND DISCUSSION**

95   ***Mfn1 deletion impairs oocyte development, which is partially rescued by***

96   ***the double loss of Mfn1 and Mfn2***

97   Oocyte development relies on i) a period of growth in which prophase I-

98   arrested oocytes amass essential molecules and organelles needed later

99   during oogenesis and embryogenesis; and, ii) a period of maturation

100   characterized by the breakdown of the nuclear membrane – known as

101   germinal vesicle (GV) – and progression of meiosis to the metaphase-II stage

102   (Clarke, 2017). These stages of oocyte development take place inside ovarian

103   follicles as somatic cells (e.g., granulosa and cumulus cells) provide an

104   adequate environment for the oocyte until its ovation (Su et al., 2009).

105   To address the role of MFN1 and MFN2 in oocyte development, *Mfn1*^{loxP}

106   and/or *Mfn2*^{loxP} males (Chen et al., 2007, 2010) were crossed to Zp3-Cre

107   females to achieve homozygous deletion of these genes specifically in

108   growing oocytes (de Vries et al., 2000). Conditional knockout females were

109   first verified for the oocyte-specific loss of *Mfn1* and/or *Mfn2*. As expected,

110   these transcripts were absent in oocytes (Figure 1A), but not in the

111   surrounding cumulus cells (Figure S1A). Moreover, mitochondrial architecture

112   in cumulus cells remained unaltered across groups (Figure S1B). Next, we

113   assessed the impact of the knockouts on fertility by mating these females with

114   WT males. Surprisingly, the deletion of *Mfn1* led to sterility in *Mfn1*-cKO and

115   *Mfn1&2*-cKO females (Figure 1B), despite normal mating behavior and

116   vaginal plug formation. In contrast, the number of pups born to *Mfn2*-cKO

117   females was not affected (Figure 1B). In addition, females with heterozygous

118   knockout oocytes (Zp3-Cre:*Mfn1*$^{+/-}$, Zp3-Cre:*Mfn2*$^{+/-}$, Zp3-Cre:*Mfn1*$^{+/loxP}$ and

119   Zp3-Cre:*Mfn2*$^{+/loxP}$) demonstrated full viability and fertility, and pups were born

120   at the expected (de Vries et al., 2000) Mendelian frequencies (data not

121   shown).

122   Given that *Mfn1* is not required in the mouse proper for normal development

123   (Chen et al., 2007), we considered that females lacking *Mfn1* and *Mfn1&2*

124   were infertile due to an MFN1 deficiency in oocytes. In line with this, no

125   ovulated oocytes were recovered from *Mfn1*-cKO mice (Figure 1C). However,

126   the deletion of both *Mfn1* and *Mfn2* recovered the loss of ovulation: ~9

127   oocytes were recovered per *Mfn1&2*-cKO mice in comparison with ~14

128   oocytes from WT mice (Figure 1C).

129   To understand the reason for this difference, we assessed in ovulated oocytes

130   the rate of first polar body (PB1) extrusion, a readout for meiotic progression

131   to the metaphase-II stage. Only 3.1% of the oocytes that were ovulated by

132   *Mfn1&2*-cKO mice contained PB1 (Figure 1D), while 61.4% of them were

133   arrested at the GV stage. This finding was confirmed by in vitro maturation of

134   GV oocytes (Figures 1E and 1F), indicating that *Mfn1&2*-cKO females were

135   infertile due to ovulation of unviable oocytes. Mating of superovulated

136   *Mfn1&2*-cKO females with WT males also confirmed this as the ovulated

137   oocytes were not fertilized (data not show). The consequence of *Mfn1*

138   ablation in the oocyte also extended to ovary development; in *Mfn1*-cKO mice

139   the ovary was ~62% smaller relative to WT, and ~60% relative to *Mfn1&2*

140   cKO (Figures 1F and 1H). Moreover, immature GV oocytes retrieved from

141   *Mfn1*-cKO ovaries were smaller and lacked companion cumulus cells when

142   compared with *Mfn1&2*-cKO oocytes (Figures 1I and 1J).

143   Together, these data establish that *Mfn1* is essential for oocyte growth and

144   ovulation, and that this essentiality is diminished in the absence of *Mfn2*.

145

146   ***Folliculogenesis is blocked at the preantral-to-antral follicle transition in***

147   ***Mfn1*-null oocytes**

148   We hypothesized that failed ovulation (Figure 1C) and the smaller size of

149   *Mfn1*-cKO ovaries and oocytes (Figures 1H and 1J) were a consequence of

150   defective folliculogenesis (Udagawa et al., 2014). With activation of oocyte

151   development, follicular cells start to differentiate and proliferate rapidly

152   following a well-established pattern of development (Figure 2A) (Clarke,

153   2017). To address our hypothesis, ovaries were evaluated by histology and

154   their follicles classified according to developmental stage as types 2, 3a, 3b,

155   4, 5a and 5b and 6 (Pedersen and Peters, 1968). In comparison with the WT

156   group, *Mfn1*-cKO ovaries harbored a greater number of preantral follicles

157   (types 2, 3a, 3b and 5a; Figure 2B). The number of antral type 6 follicles,

158   however, was not increased in *Mfn1*-cKO ovaries (Figure 2B); instead, these

159   were smaller and less developed than most antral follicles in WT ovaries

160   (Figure 2C). This impact on antral follicle development is in agreement with

161   recombination of loxP sites occurring in our system in follicles types 3b and 4

162   (de Vries et al., 2000). Key steps of folliculogenesis following these stages are

163   the massive proliferation of granulosa cells and the formation of an antrum

164   that subdivides granulosa cells into two populations: the mural granulosa and

165   the cumulus cells (Figure 2A) (Clarke, 2017).

166   To further characterize the differences in antral follicles between WT and

167   *Mfn1*-cKO mice, we subdivided type 6 follicles into four subcategories: 6a, 6b,

168   6c and 6d (Figures 2A and S2A), ranging from poorly-developed (6a and 6b –

169   abnormal) to well-developed antral follicles (6c and 6d – normal). In WT mice,

170   a low percentage of follicles was classified as abnormal (Figures 2D, S2B and

171   S2C), while in *Mfn1*-cKO mice 64.0% were classified as abnormal (Figures

172   2D, S2B and S2C). Interestingly, the *Mfn1&2*-cKO mice presented an

173   intermediate phenotype with 43.0% classified as abnormal (Figures 2D, S2B

174   and S2C).

175   Following ovulation, residual follicular cells differentiate into a gland, termed

176   corpus luteum, that is mainly involved with progesterone production during

177   early pregnancy. Consistent with a failure of *Mfn1*-cKO follicles to ovulate,

178   *Mfn1*-cKO, but not *Mfn1&2*-cKO, ovaries completely lacked corpus luteum

179   relative to the WT control (Figure 2E). Together, these results show that the

180   loss of *Mfn1* in oocytes results in a block at the preantral-to-antral follicle

181   transition, and that the combined loss of *Mfn1* and *Mfn2* mitigates this block in

182   follicular development.

183

184   ***Impaired folliculogenesis in Mfn1-deficient oocytes is associated with***

185   ***abnormal mitochondrial architecture and function***

186   MFN1 and MFN2 are key regulators of mitochondrial health (Chen et al.,

187   2003, 2005, 2007, 2010; Mishra and Chan, 2014; Papanicolaou et al., 2012;

188   Schneeberger et al., 2013; Schrepfer and Scorrano, 2016; Sebastian et al.,

189   2012), which in turn is linked with oocyte development (Van Blerkom et al.,

190   1995; Fragouli and Wells, 2015; Johnson et al., 2007; Reynier et al., 2001;

191   Santos et al., 2006). Given the differences observed, we hypothesized there

192   might be differences specific to the loss of *Mfn1* that were mitigated by the

193   double loss of *Mfn1* and *Mfn2*. Indeed, ultrastructure analysis of *Mfn1*-cKO

194   oocytes revealed pronounced defects in mitochondria including: a) a greater

195   number of less electron dense organelles; b) a decreased number of cristae;

196   and, c) an increased vesiculation of the inner membrane (Figures 3A-3C and

197   S3A). These mitochondrial abnormalities were present, albeit, to a lower

198   extent in *Mfn1&2*-cKO than in *Mfn1*-cKO oocytes (Figures 3A-3C and S3A),

199   suggesting that defective mitochondrial architecture is linked with the block of

200   *Mfn1* cKO on follicle development.

201   Deficient fusion has been shown to lead to alterations in nucleoid

202   heterogeneity and mtDNA instability and depletion (Chen et al., 2003, 2005,

203   2007, 2010). In turn, mtDNA copy number has been linked with oocyte quality

204   in mice and humans (Fragouli and Wells, 2015; Reynier et al., 2001; Santos

205   et al., 2006; Wai et al., 2010). We therefore next assessed levels of mtDNA,

206   the regulation of which is intimately tied with mitochondrial function. To

207   investigate whether the folliculogenesis block in *Mfn1*-cKO oocytes was

208   associated with changes in mtDNA stability, we measured mtDNA copy

209   number in immature GV oocytes. *Mfn1*-cKO oocytes had a ~5-fold reduction

210   in mtDNA copy number relative to the WT control (Figure 3D). In addition, a

211   comparison of *Mfn1*-cKO oocytes from preantral and antral follicles revealed

212   that the content of mtDNA dropped with follicular development (Figures 3E

213   and S3B). The loss of mtDNA was mitigated in *Mfn1&2*-cKO oocytes which

214   had only a mild depletion (~2-fold reduction) relative to the WT control (Figure

215   3D). These data support a key role of *Mfn1* for mtDNA synthesis and/or

216   stability in oocytes. Furthermore, our findings demonstrate that the double

217   deletion of *Mfn1* and *Mfn2* mitigates the loss of mtDNA in oocytes.

218   To further characterize the impact of *Mfn1* deletion on mitochondrial

219   physiology and identify defects specific to the loss of Mfn1, but not *Mfn1&2*,

220   we stained GV oocytes with TMRE to assess mitochondrial membrane

221   potential ($\Delta\Psi$m). Consistent with the observed defects in mitochondrial

222   architecture, oocytes lacking *Mfn1* and *Mfn1&2* displayed a similar drop of

223   ~40% in the $\Delta\Psi$m (Figures 3F). Additionally, a large percentage of these

224   oocytes presented mitochondrial aggregates (Figures 3G, 3H and S3C).

225   However, the content of oxidized flavin adenine dinucleotide (FAD) was less

226   altered in *Mfn1&2*-cKO oocytes in comparison with those lacking only *Mfn1*

227   (Figures 3I). Moreover, *Mfn1&2*-cKO and WT oocytes contained similar levels

228   of ATP, which was decreased by ~10 fold in the *Mfn1*-cKO group (Figure 3J).

229   Overall, the mitochondrial defects were milder in *Mfn1*-cKO oocytes,

230   indicating that the rescue effect of combined loss of *Mfn1* and *Mfn2* extended

231   to mitochondrial health. These results link abnormal mitochondrial

232   architecture, mtDNA depletion, increased levels of FAD, and lower levels of

233   ATP with impaired folliculogenesis in *Mfn1*-cKO oocytes.

234

235   ***The loss of Mfn1 dysregulates oocyte-somatic cell communication***

236   The evident loss of cumulus cells only in oocytes with *Mfn1* single deletion

237   (Figure 1I) hinted at a role for balanced expression of MFN1 and MFN2 in

238   proliferation of follicular somatic cells. This hypothesis is in agreement with a

239   milder effect of *Mfn1*&*2* cKO on antral follicle development and ATP storage in

240   oocytes (Figures 2D and 3J). Moreover, *Mfn1*-cKO ovaries presented a more

241   altered pattern of expression (Figures 4A and 4B) of genes implicated in

242   granulosa cell proliferation and follicle development (Knight and Glister,

243   2006). The interaction of the oocyte with its companion somatic cells is

244   mediated by oocyte-derived factors that stimulate proliferation and

245   differentiation of these cells (Clarke, 2017). This communication is critical for

246   oocyte development as, among other roles, companion somatic cells

247   physically and metabolically interact with the oocyte to supply it with energetic

248   molecules including ATP (Su et al., 2009; Sugiura et al., 2007). To provide

249   additional evidence that the single *Mfn1* deletion impaired cellular

250   proliferation, mice were injected for 2 h with BrdU and assessed as for the

251   percentage of BrdU-positive follicular cells. Accordingly, BrdU incorporation

252   was more sharply impaired by loss of *Mfn1* than the dual loss of *Mfn1* and

253   *Mfn2* (Figures 4C and S4A).

254   Next, we investigated whether impaired oocyte-somatic cell communication

255   could explain the deficient proliferation of follicular cells in *Mfn1*-cKO ovaries.

256   GDF9 is the most well-known oocyte-derived factor responsible for mediating

257   interaction with follicular somatic cells (Clarke, 2017; Su et al., 2009). For

258   instance, GDF9-deficient mice are infertile due to a block in folliculogenesis

259   secondary to failed growth and differentiation of follicular somatic cells (Dong

260   et al., 1996; Elvin et al., 1999). In accordance with our hypothesis, GDF9 was

261   found to be downregulated in both *Mfn1*-cKO and *Mfn1*&*2*-cKO oocytes

262   (Figures 4D and S4B). Additionally, *Mfn1*-cKO, but not *Mfn1*&*2*-cKO, oocytes

263   expressed lower levels of *Bmp15* and *Fgf8b* in comparison with WT (Figure

264   4E). BMP15 and FGF8B are additional oocyte-derived factors involved mainly

265   with the metabolic regulation of surrounding somatic cells (Su et al., 2009;

266   Sugiura et al., 2007). Therefore, these findings indicate that *Mfn1* cKO

267   disrupts communication of the oocyte with follicular somatic cells.

268   To further confirm that impaired oocyte-somatic cell interaction prevented the

269   growth of *Mfn1*-cKO oocytes, we took advantage of an in vitro system of

270   Granulosa-Oocyte Complex (GOC) culture. In this system, GOCs are derived

271   from preantral secondary follicles and kept in culture for 7 days to develop to

272   a stage comparable with oocytes from antral follicles (O'Brien et al., 2003).

273   First, we used this system to validate our in vivo findings (Figure 1J) that

274   *Mfn1*-cKO oocytes are reduced in growth. Whereas WT oocytes grew from

275   55.2 to 66.9 μm, *Mfn1*-cKO oocytes grew only from 55.3 to 57.8 μm (Figure

276   4F). To determine if exogenous addition of GDF9 rescued the growth of *Mfn1*-

277   cKO oocytes that lack GDF9 expression, we cultured GOCs in the presence

278   of recombinant GDF9 (rGDF9). Supplementation with rGDF9 was sufficient to

279   partially rescue growth in *Mfn1*-cKO oocytes (Figure 4G).

280   Finally, *Mfn1*-cKO GOC were co-cultured with either WT or *Mfn1*-cKO oocytes

281   to confirm the inability of *Mfn1*-null oocytes to modulate granulosa cell gene

282   expression (Sugiura et al., 2007; Udagawa et al., 2014). Indeed, co-culture of

283   *Mfn1*-cKO GOCs with WT oocytes upregulated *Fst*, *Inhba* and *Inhbb*, and

284   downregulated *Kitl* (Figure 4H). Importantly, *Fst* and *Kitl* were, respectively,

285   downregulated and upregulated in *Mfn1*-cKO ovaries (Figures 4A and 4B),

286   providing evidence that co-culture with WT oocytes retrieved an expression

287   pattern compatible with follicle development (Knight and Glister, 2006;

288   Udagawa et al., 2014). Together, these data support that single deletion of

289   *Mfn1* in growing oocytes impairs signaling to surrounding somatic cells,

290   thereby preventing oocyte growth and follicular cell development.

291

Conclusions

293   Our present results support three main conclusions: a) the oocyte relies on

294   *Mfn1*, but not *Mfn2*, for growth and ovulation; b) the effect of *Mfn1* deletion is

295   explained by disruption of oocyte-somatic cell communication and follicular

296   cell proliferation; and, c) loss of *Mfn2* alleviates the impact on intercellular

297   communication and follicular cell proliferation, mitigating the defects of *Mfn1*-

298   null oocytes.

299   Herein we investigate the role of pro-fusion proteins MFN1 and MFN2 in the

300   female germline during a phase of massive oocyte growth, when essential

301   molecules and organelles are amassed to support further oocyte and embryo

302   development (Clarke, 2017; Collado-Fernandez et al., 2012). In agreement

303   with the hypothesis of upregulated fission in oocytes (Chiaratti et al., 2018;

304   Udagawa et al., 2014; Wakai et al., 2014), ablation of pro-fusion genes do not

305   enhance mitochondrial fragmentation. Yet, oocytes do express both

306   mitofusins and ablation of *Mfn1* heavily impairs mitochondrial architecture,

307   function and transport. These present findings corroborate previous reports

308   (Chen et al., 2005; Tang, 2015; Udagawa et al., 2014; Wakai et al., 2014;

309   Zhang et al., 2016) and unmask a key role of MFN1 in oocytes in comparison

310   with MFN2. Previous reports have shown that mitofusins operate redundantly

311   and non-redundantly in several tissues including placenta, neurons,

312   hepatocyte, cardiomyocytes and skeletal muscle (Chen et al., 2007, 2010;

313 Dietrich et al., 2013; Papanicolaou et al., 2012; Schneeberger et al., 2013;

314 Sebastian et al., 2012). In comparison, our present findings highlight the

315 importance of distinct, non-redundant roles of MFN1 and MFN2 in the growing

316 oocyte.

317 Rather than a direct consequence of mitochondrial loss of function, the impact

318 of MFN1 deficiency in the oocyte is explained by disruption of oocyte-somatic

319 cell communication. This conclusion is supported by previous reports showing

320 cumulus cells provide the oocyte with energetic molecules (Su et al., 2009;

321 Sugiura et al., 2007), enabling oocyte development even when mitochondria

322 is dysfunctional (Collado-Fernandez et al., 2012; Johnson et al., 2007).

323 Moreover, loss of both MFN1 and MFN2, which alleviates the impact on

324 intercellular communication and follicular cell proliferation, mitigates the

325 mitochondrial defects and enables oocyte growth and ovulation. Taking into

326 account the mitohormesis theory (Yun and Finkel, 2014), these findings might

327 be explained by a possible mitochondrial stress induced by loss of *Mfn1*,

328 which in turn could have activated a signaling pathway leading to disrupted

329 oocyte-somatic cell communication. However, the link between loss of *Mfn1*

330 and disrupted oocyte-somatic cell communication remains an open question.

331 In brief, this work reframes the role of mitochondria in oocyte biology by

332 identifying a specific role for a mitochondrial protein in intercellular signaling

333 during oocyte development. Also, it suggests that the balanced expression of

334 *Mfn1* and *Mfn2* is determinant for the growing oocyte. Future work should

335 dissect how mitochondria interfere with oocyte-somatic cell communication.

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350 **AUTHOR CONTRIBUTIONS**

351 Conceptualization, F.V.M and M.R.C; Methodology, F.V.M., F.E.G.G., M.M.S.,

352 and M.R.C.; Investigation, K.F.C., B.M.G., T.S.M., A.F.Z., C.H.M., F.H.C.S,

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357 **DECLARATION OF INTERESTS**

358 The authors declare no competing interests.

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FIGURE TITLES AND LEGENDS

Figure 1. *Mfn1 deletion impairs oocyte development, which is partially rescued by the double loss of Mfn1 and Mfn2.*

(A) Immature germinal-vesicle (GV) oocytes from 5-week-old mice were evaluated by qPCR to confirm the oocyte-specific loss of *Mfn1* and *Mfn2*. n = 5 samples/group.

(B) Fertility test assay showing number of pups per plugged female. Note that deletion of *Mfn1* and *Mfn1&2* led to sterility. n = 20-52 matings/group.

(C and D) Number of ovulated oocytes retrieved from oviducts of eCG- and hCG-primed mice (C). Rate of first polar body (PB1) in ovulated oocytes (D).

(E and F) Rate of GV break down (GVBD; E) and PB1 extrusion (F) in oocytes matured in vitro.

(G and H) Ovary morphology (G) and weight (H) in 5-week-old mice. n = 10-12 ovaries/group.

(I) Cumulus-Oocyte Complexes (COCs) at the GV stage were retrieved from ovaries of eCG-primed mice aged 5 weeks (left). COCs were denuded of cumulus cells to denote oocytes (right). Note that most *Mfn1*-cKO COCs completely lacked surrounding cumulus cells (arrows), whereas grossly normal COCs were obtained from WT, *Mfn2*-cKO and *Mfn1&2*-cKO mice.

(J) Diameter of immature GV oocytes from 5-week-old mice. Note the smaller size of *Mfn1*-cKO oocytes. n = 74-178 oocytes/group.

In panels A, B and C, median (line inside the box), upper and lower quartiles (delimited by the box) and 1.5 times the interquartile range (whiskers). In panels H and J, mean $\pm$ SEM.

n.a. = not analyzed; r.u. = relative units to the WT control.

*P < 0.05. **P < 0.01. ***P < 0.0001.

See also Figure S1.

Figure 2. *Folliculogenesis is blocked at the preantral-to-antral follicle transition in Mfn1-cKO oocytes.*

(A) Cartoon illustrating follicle development as proposed by Pedersen and Peters (1968). Cre recombinase is expressed in Zp3-Cre mice starting in follicle types 3b and 4.

(B-D) Histological evaluation of ovaries from 3-week-old mice and follicular classification as types 2, 3a, 3b, 4, 5a, 5b and 6 (B). Follicles classified as type 6 represent the sum of types 6a, 6b, 6c and 6d. Types 1, 7 and 8 were not considered as these were mostly absent in 3-week-old mice. Representative histological micrographs of ovaries (C). Arrows and arrowheads indicate, respectively, normal and abnormal type 6 follicles. Percentage of abnormal (types 6a and 6b – black bar) and normal (types 6c and 6d – striped bar) follicles (D). n = 6 ovaries/group.

(E) Number of corpus luteum determined by histological analysis of ovaries from 8-week-old mice. n = 3-4 mice/group.

In panels B and E, mean ± SEM. In panel D, median (line inside the box), upper and lower quartiles (delimited by the box) and 1.5 times the interquartile range (whiskers).

r.u. = relative units to the WT control.

*P < 0.05. **P < 0.01. ***P < 0.0001.

See also Figure S2.

Figure 3. *Impaired folliculogenesis in Mfn1-deficient oocytes is associated with abnormal mitochondrial architecture and function*.

(A) Representative transmission electron microscopy micrographs of oocytes. Note that mitochondrial morphology is abnormal in *Mfn1*-cKO oocytes with evidence of organelle swelling, vesiculation of the inner membrane, decreased number of cristae and lighter matrix. Arrows indicate mitochondria whereas arrowheads denote inner mitochondrial membranes and internal vesicles.

(B) Percentage of mitochondria with normal (striped bar) and abnormal (black bar) ultrastructure characteristics in oocytes. Note that *Mfn1*-cKO oocytes depict an increased incidence of mitochondria with abnormalities. n = 53-78 samples/group.

(C) Major and minor mitochondrial diameters in oocytes. n = 173-415 samples/group.

(D and E) Number of mtDNA copies in immature germinal-vesicle (GV) oocytes from 5-week-old mice primed with eCG. Note that *Mfn1* loss impaired mtDNA accumulation more sharply than *Mfn1&2* cKO (D). Number of mtDNA copies in oocytes from preantral (12-day-old mice) and antral (32-day-old mice) follicles (E).

(F) Relative mitochondrial membrane potential ($\Delta\Psi$m) in immature GV oocytes estimated using TMRE. n = 89-229 samples/group.

(G and H) Representative confocal microscopy micrographs (G) of immature GV oocytes stained with MitoTracker CMXRos (mitochondria – Mito) and ERTracker (endoplasmic reticulum – ER). ER staining is presented as a cytoplasmic reference. Percentage of GV oocytes with mitochondrial aggregation (H). Note

the higher level of mitochondrial aggregation in *Mfn1*-cKO and *Mfn1&2*-cKO oocytes (arrows). DIC = differential interference contrast.

(I) Relative levels of NAD(P)H and FAD in GV oocytes. n = 289-794 samples/group.

(J) ATP content in immature GV oocytes. n = 15-21 samples/group.

In panels C, median (line inside the box), upper and lower quartiles (delimited by the box) and 1.5 times the interquartile range (whiskers). In panels D, E, F, I and J, mean $\pm$ SEM.

*P < 0.05. **P < 0.01. ***P < 0.0001.

See also Figure S3.

Figure 4. *The loss of Mfn1 dysregulates oocyte-somatic cell communication*.

(A and B) Five-week-old ovaries were used to determine the levels of transcripts positively (A) and negatively (B) associated with follicle development. n = 8 ovaries/group.

(C) Percentage of follicle cells positive for BrdU staining in ovaries from 8-week-old mice.

(D) Evaluation by immunoblot of the level of GDF9 in germinal-vesicle (GV) oocytes. TUBA1A was used as sample input. Values above blots indicate the level of GDF9 normalized by TUBA1A (in relation to the WT control).

(E) Transcriptional levels of *Bmp15* and *Fgf8b* in immature GV oocytes from 5-week-old mice. n = 5 samples/group.

(F and G) Oocyte diameter before (n = 18-21/group) and after (n = 58-84/group) culture of Granulosa-Oocyte Complexes (GOCs) in vitro (F). Oocyte diameter

after culture of *Mfn1*-cKO GOCs with (n = 43) or without (n = 28) recombinant GDF9 (rGDF9) (G).

(H) Coculture of *Mfn1*-cKO GOCs with WT and *Mfn1*-cKO GV oocytes (freed of cumulus cells) from 5-week-old mice. After culture, GOCs were used for evaluation of gene expression.

In panels A, B, C and E, median (line inside the box), upper and lower quartiles (delimited by the box) and 1.5 times the interquartile range (whiskers). In panels F, G and H, mean ± SEM.

*P < 0.05. **P < 0.01. ***P < 0.0001.

r.u. = relative units to the WT control. In (H), expressed in relation to *Mfn1*-cKO GOCs cocultured with *Mfn1*-cKO oocytes.

See also Figure S4.

EXPERIMENTAL PROCEDURES

Mouse breeding and fertility test

All experiments were performed in compliance with the regulations and policies of the National Council for Control of Animal Experimentation (CONCEA, Brazil) and were approved by the Animal Care and Use Committee at Universidade de São Paulo (USP) and Universidade Federal de São Carlos (UFSCar). Mfn^{loxP} conditional mice (Chen et al., 2007) were obtained from the Mutant Mouse Regional Resource Centers (RRID: MMRRC Cat# 029901-UCD, RRID:MMRRC_029901-UCD and MMRRC Cat# 029902-UCD, RRID:MMRRC_029902-UCD) whereas Zp3-Cre transgenic mice (de Vries et al., 2000) were supplied by the Jackson Laboratory (RRID: IMSR Cat# JAX:003651, RRID:IMSR_JAX:003651). Before use, Zp3-Cre mice were mated

with heteroplasmic C57BL/6N females containing mtDNA of NZB/BINJ and C57BL/6N origin (Machado et al., 2015).

Wild-type (Zp3-Cre:$Mfn1^{+/+}$:$Mfn2^{+/+}$), $Mfn1$-cKO (Zp3-Cre:$Mfn1^{loxP/-}$:$Mfn2^{+/+}$) and $Mfn2$-cKO (Zp3-Cre:$Mfn1^{+/+}$:$Mfn2^{loxP/-}$) mice were obtained by mating heteroplasmic Zp3-Cre females to $Mfn^{loxP/loxP}$ males, followed by mating of Zp3-Cre:$Mfn^{+/loxP}$ males to Zp3-Cre:$Mfn^{+/loxP}$ females. $Mfn1\&2$-cKO (Zp3-Cre:$Mfn1^{loxP/-}$:$Mfn2^{loxP/-}$) mice were similarly obtained, except that $Mfn1^{loxP/loxP}$:$Mfn2^{loxP/loxP}$ males were used. This breeding scheme ensures more effective recombination of loxP sites. Unless otherwise stated, all experiments were conducted using mice that aged 3-5 weeks.

Mice had fertility evaluated by mating 7-week-old females for up to one year with WT males. Only females with copulation plugs were considered during the fertility test.

Mouse genotyping

Ear biopsies were digested for 3 h at 55°C using a solution containing 50 mM KCl, 10 mM Tris-Cl 2 mM MgCl$_2$, 0.1 mg/ml gelatin, 0.45% Igepal CA-630, 0.45% Tween 20 and 100 µg/ml proteinase K (ThermoFisher Scientific). After digestion, samples were incubated at 95°C for 10 min, centrifuged at 10,000 x g for 5 min and the supernatant used for genotyping (Machado et al., 2015). $Mfn1$ and $Mfn2$ were genotyped by PCR as suggested by the MMRRC, with few modifications. The presence of the Zp3-Cre transgene was determined by quantitative PCR (qPCR) based on the protocol provided by the Jackson Laboratory.

Collection and in vitro culture of cells

Cumulus-Oocyte Complexes (COCs) and denuded oocytes were obtained as previously described (Nagy et al., 2003). Briefly, to obtain ovulated oocytes, females aged 3-5 weeks received an intraperitoneal injection of 5 I.U. of equine chorionic gonadotropin (eCG; Folligon, MSD Animal Health) and 5 I.U. of human chorionic gonadotropin (hCG; Chorulon, MSD Animal Health), given 46-47 h apart. Oviducts were removed from the mice 12-14 h after the hCG injection, and the ovulated oocytes retrieved in FHM medium (Nagy et al., 2003) by puncturing the ampulla with a 30G needle. These oocytes were denuded of surrounding cumulus cells by gentle pipetting in 0.3% hyaluronidase (Sigma-Aldrich) diluted in FHM (Nagy et al., 2003). Ovulated oocytes were then inspected for the presence of the first polar body (PB1) under a stereomicroscope (SMZ 745, Nikon).

To obtain COCs containing fully-grown immature oocytes at the germinal-vesicle (GV) stage, mice aged 3-5 weeks were injected with 5 I.U. of eCG and the ovaries removed after 44-46 h of injection. COCs were retrieved by puncturing the ovarian antral follicles in FHM medium (Nagy et al., 2003). Alternatively, Granulosa cell-Oocyte Complexes (GOCs) were obtained from 12-day-old females by incubating their ovaries in HEPES-buffered α-MEM (ThermoFisher Scientific) with 10 µg/ml type I collagenase and 10 µg/ml DNase for 30 min at 37°C (O'Brien et al., 2003). The ovary fragments were gently pipetted every 3 min to disrupt the tissue. COCs and GOCs had surrounding somatic cells removed by passing several times through a mouth-controlled glass pipette.

Immature GV oocytes were in vitro matured under mineral oil in 90-µl droplets of KSOM medium (Nagy et al., 2003) supplemented with 10% fetal bovine serum (ThermoFisher Scientific), 100 u/ml penicillin (ThermoFisher Scientific) and 100 u/ml streptomycin (ThermoFisher Scientific). A humidified incubator maintained at 37°C and containing 5% CO_2 in air was used. To prevent GV breakdown (GVBD), oocytes were isolated in the presence of 100 µM IBMX (Sigma-Aldrich). Then, oocytes were cultured in IBMX-free medium and evaluated as for the GVBD (judged by absence of GV) and PB1 extrusion following 3 and 16 h of IBMX washout, respectively (Udagawa et al., 2014; Wakai et al., 2014).

The coculture experiment was performed as reported previously (Udagawa et al., 2014), with few modifications. Briefly, 10 GOCs were cultured under mineral oil for 24 h together with 15 denuded oocytes (from COCs) in 40-µl droplets of KSOM medium supplemented with 100 µM IBMX. A humidified incubator maintained at 37°C and containing 5% CO_2 in air was used.

In vitro growth of oocytes was performed based on a previous report (O'Brien et al., 2003). Briefly, ~15 GOCs were cultured onto type I and III collagen 3.0-µm inserts (Corning) in 24-well plates with 750 µl of $NaHCO_3$-buffered α-MEM supplemented with 1x ITS (Sigma-Aldrich), 10 µM cilostamide (Sigma-Aldrich), 10 mIU/ml FSH (Sigma-Aldrich) and, when applicable, 100 ng/ml of rGDF9 (R&D Systems). Two-thirds of the medium was replaced on the third day of culture. GOCs were cultured for 7 days in a humidified incubator maintained at 37°C and containing 5% CO_2 in air.

When applicable, oocyte diameter was estimated under an inverted microscope (Ti-S, Nikon) using the NIS-Elements Basic Research software (Nikon).

Histological and immunofluorescence analyses

Ovaries from 21- to 23-day-old females had the number of follicles evaluated as reported previously (Pedersen and Peters, 1968), with few modifications. Briefly, fixed ovaries were dehydrated, clarified, diaphonized, embedded in paraffin and sectioned at 3 μm thickness. Sections were stained with periodic acid and hematoxylin, and analyzed using an optical microscope. Follicles in every third section throughout the ovary were counted and classified as proposed by Perdersen and Peter (1968). Only follicles in which the section included the GV of the oocyte were considered. Ovaries from 8-week-old females were similarly processed to determine the number of corpus luteum. Follicular cell proliferation was assessed as previously reported (Udagawa et al., 2014), with few modifications. Briefly, 8-week-old females were injected with 3 mg BrdU (Sigma-Aldrich) 2 h prior to ovary collection. Ovaries were then fixed for 4 h in 4% paraformaldehyde, cryoprotected in a series of sucrose solutions (10-20%) in PBS at room temperature, embedded in OCT compound (Sakura Finetek) and frozen in cold acetone. Sectioned samples of 5-μm thickness were then thoroughly washed with PBS, fixed for 15 min in 2% paraformaldehyde and, incubated for 20 min with 10 mM sodium citrate and 0.05% Tween at 95°C. Unspecific staining was then blocked by incubation with 10% BSA in PBS for 30 min. Afterwards, slides were incubated overnight with mouse anti-BrdU (50-fold dilution; Sigma-Aldrich) primary antibody, thoroughly washed and incubated for 1 h with Alexa Fluor 594-tagged anti-mouse (800-fold dilution; ThermoFisher Scientific) secondary antibody. Cell nuclei were counterstained with 10 μg/ml DAPI (Sigma-Aldrich). The following wavelengths for excitation and emission,

respectively, were considered: 591 nm and 614 nm (Alexa Fluor 594), and, 358 nm and 461 nm (DAPI). The percentage of replicating follicle cells (BrdU positive) in relation to DAPI-positive cells was determined by epifluorescence using the Ti-S microscope and the NIS-Elements Basic Research software. Only cells inside the follicle were considered during the analysis.

Mitochondrial evaluation

Oocytes were incubated for 30 min at 37°C with 100 nM MitoTracker CMXRos (ThermoFisher Scientific) and 1 µM ERTracker (ThermoFisher Scientific) diluted in FHM for analysis of mitochondria and endoplasmic reticulum (ER) distribution, respectively. Similarly, oocytes were stained with 0.1 µM TMRE (ThermoFisher Scientific) in FHM for determination of mitochondrial membrane potential ($\Delta\Psi m$). Oocytes treated with 10 µM CCCP for 30 min were used as negative control during $\Delta\Psi m$ evaluation. Oocytes were evaluated by confocal microscopy (LSM 780, Zeiss) considering the following wavelengths for excitation and emission, respectively: 579 nm and 599 nm (MitoTracker CMXRos), 374 nm and 430 nm (ERTracker), and, 485 nm and 535 nm (TMRE). Since the knockouts led to mitochondrial aggregation, which could potentially bias $\Delta\Psi m$ evaluation, oocytes were also analyzed by epifluorescence microscopy.

Oocyte autofluorescence, which is representative of the amount of oxidized flavin adenine dinucleotide (FAD) and reduced nicotinamide adenine dinucleotide (phosphate) (NAD[P]H), were determined using green (excitation = 465-495 nm; emission = 515-555 nm) and blue (excitation = 340-380 nm; emission = 435-485 nm) filters, respectively (Zeng et al., 2014).

Images were analyzed using the NIS-Elements Basic Research software or the ZEN lite (Zeiss). To control for inter-assay variation, oocytes analyzed in a specific experimental routine had fluorescence normalized by the mean fluorescence of WT oocytes analyzed in the same routine.

ATP quantification

Oocytes used for ATP quantification were placed individually into 0.2 ml tubes in 1 µl PBS and kept at -80°C until use. Oocytes treated with 20 µM oligomycin for 1 h were used as negative control. The ATP content was measured based on the luciferin-luciferase reaction using the FLASC kit (Sigma-Aldrich), following manufacturer's recommendations.

Transmission electron microscopy

Ovaries used for transmission electron microscopy were obtained from 5-week-old mice after 44 h of eCG injection. These were fixed for 4 h at 4°C in 2% paraformaldehyde and 2% glutaraldehyde in 0.01 M cacodylate buffer (pH 7.3) with 3 mM CaCl. Following, ovaries were post-fixed for 1 h at 4°C with 2% osmium tetroxide and dehydrated in a grade series of ethanol. Samples were embedded in epoxy resin (Polysciences Inc.), cut into ultra-thin sections, mounted on 300-mesh copper grids and contrasted with uranyl acetate and lead citrate (Udagawa et al., 2014; Wakai et al., 2014). The sections were examined in a JEOL JEM – 100CXII transmission electron microscopy (Jeol Inc.). Images were analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

Evaluation of mtDNA copy number

The number of mtDNA copies in oocytes was determined by qPCR as described previously (Machado et al., 2015). Briefly, a 736-bp fragment encompassing nucleotides 3,455 to 4,190 of mtDNA was amplified using primers MT12 and MT13 (Table S1) and cloned into a pCR2.1-Topo-TA (ThermoFisher Scientific). Concentration of the resulting plasmid DNA was estimated based on UV spectroscopy and store at 0.2×10^9 copies/µl at -80°C. This plasmid DNA was used to prepare a standard curve containing 0.2×10^7, 0.2×10^6, 0.2×10^5, 0.2×10^4 and 0.2×10^3 copies/µl. A fresh dilution of this standard curve was always prepared before each qPCR reaction.

Individual oocytes collected in 1 µl of PBS with 0.01% BSA were digested for 3 h at 55°C in 4 µl of digestion solution (as described for mouse genotyping, except that 100 mg/ml proteinase K was used). After digestion, samples were incubated at 95°C for 10 min, centrifuged at 10,000 x g for 5 min and 5 µl of the supernatant diluted with 45 µl of ultrapure H_2O (ThermoFisher Scientific). The standard curve and samples were always amplified in parallel using the 7500 Fast Real-Time PCR System with the following cycling conditions: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s and 62°C for 1 min. Each reaction consisted of 15 µl containing 200 nM of primers MT14 and MT15 (Table S1), 1x Power SYBR Green Master Mix and 5 µl of sample or standard-curve DNA. Samples were analyzed in triplicates and averaged for calculation of mtDNA copy number using the 7500 Fast Real-Time PCR Software (ThermoFisher Scientific).

Evaluation of gene expression

Groups of 30 GV oocytes and 10 GOCs were placed into 0.2 ml tubes along with 10 µl PBS containing 1 U/µl RNase inhibitor (RNase OUT; ThermoFisher Scientific). Samples were snap frozen in liquid nitrogen and kept at -80°C until use. As reported previously (Macabelli et al., 2014), total RNA was extracted using TRIzol reagent (ThermoFisher Scientific), treated with DNase I (ThermoFisher Scientific) and reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit (ThermoFisher Scientific). Gene transcripts were determined on the 7500 Fast Real-Time PCR as described previously (Macabelli et al., 2014), with few modifications. Briefly, each qPCR reaction consisted of 15 µl containing 1 µl of forward and reverse primers (200 nM each), 7.5 µl SYBR Green PCR Master Mix, 5 µl sample cDNA (20- to 77-fold dilution) and 1.5 µl ultrapure H_2O. A list of all primers used is presented at Table S1. These were designed using the PrimerBLAST software (National Institutes of Health) and confirmed regarding specificity of the amplified fragment by dissociation curve and gel electrophoresis.

All samples amplified for a specific target were run in duplicate in the same plate using the following cycling conditions: 95°C for 15 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Sample dilutions were determined based on standard curves generated for each gene-specific cDNA using five four-fold serial dilutions of cDNAs. These standard curves were also used for analysis of amplification efficiency (Livak and Schmittgen, 2001). Because all assays showed high amplification efficiency (~100%), data were linearized according to Livak and Schmittgen (Livak and Schmittgen, 2001). Samples that failed to amplify had cycle threshold-(CT) values regarded as equal to 45. The

geometric average of CT values of two reference genes (*Ppia* and *Hprt*) was used to normalize gene expression data (Macabelli et al., 2014).

In case of ovaries, these were placed individually into cryo-tubes and snap frozen in liquid nitrogen. Ovaries were then macerated and subjected to total RNA extraction, followed by reverse transcription using 1 µg of total RNA and the High-Capacity cDNA Reverse Transcription kit. Gene transcripts were determined as described for oocytes, except that a 321-fold dilution of cDNA was used. Transcript (Table S1) amounts were evaluated as described above. Cumulus cells from groups of 30 oocytes were processed as described above, except that the PCR products were evaluated by electrophoresis on 2% agarose gel. DNA was then stained with SYBR Safe (ThermoFisher Scientific) and the signal detected with the Chemidoc XRS (BioRad).

Immunoblot analysis

Groups of 100 denuded oocytes were placed into 0.2 ml tubes in 1 µl PBS, snap frozen in liquid nitrogen and kept at -80°C, until use. To compensate for the smaller diameter and, in turn, lower protein content, 200 oocytes were used in the case of *Mfn1* cKO. Samples were evaluated by western blot as previously (Wakai et al., 2014). Briefly, samples were lised, proteins size separated using 12% Bis-Tris Mini Gel and blotted onto PVDF membranes. Membranes were probed overnight with either rabbit anti-GDF9 (1000-fold dilution; Abcam) or mouse anti-TUBA1A (2000-fold dilution; ThermoFisher Scientific) primary antibodies, thoroughly washed and incubated for 1 h with HRP-conjugated secondary antibodies (10000-fold dilution; KPL). Then, membranes were treated with the Amersham ECL Select kit (GE) and the chemiluminescence

signal detected with the Chemidoc XRS. For controlling sample input, protein levels were corrected by TUBA1A. Relative protein levels were determined using ImageJ.

Statistical analysis

Statistical analysis was performed using the SAS University Edition v. 9.4 (SAS Institute Inc.). Data were tested for assumption of normal distribution and homogeneity of variance, and transformed when necessary. Experimental groups were then compared using unpaired t test (2 groups) or one-way ANOVA (4 groups), followed by Tukey post-hoc tests. Alternatively, groups were compared using a non-parametric test such as Wilcoxon Rank-Sum Test (2 groups) or Kruskal-Wallis Test (4 groups), followed by pairwise multiple comparison analysis. Binary variables were evaluated by binary logistic regression, followed by Tukey-Kramer post-hoc test. Correlation analysis was performed using Pearson's Test. Differences with probabilities (P) < 0.05 were considered significant.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Related to Figure 1. Mitofusins are not dysregulated in cumulus cells from Mfn-cKO oocytes.

(A) Cumulus cells from WT, *Mfn2*-cKO and *Mfn1&2*-cKO oocytes had *Mfn1* and *Mfn2* amplified by PCR and run onto a 2%-agarose gel. *Ppia* was used as sample input. Because *Mfn1*-cKO oocytes mostly lacked cumulus cells (Figure 1I), these could not be used in this experiment. Five replicates were considered per experimental group.

(B) Representative transmission electron microscopy micrographs of oocytes and surrounding somatic cells. Arrows indicate oocyte mitochondria whereas arrowheads denote elongated mitochondria in somatic cells. Note that mitochondrial architecture in somatic cells is not different across groups.

Figure S2. Related to Figure 2. *Mfn1* deletion arrests folliculogenesis at the preantral-to-antral follicle transition.

(A) Representative histological micrographs denoting classification of early antral follicles, corresponding to type 6 (Pedersen and Peters, 1968), into four subcategories. Type 6a: ~128 cells and ~128 μm in diameter. Type 6b: ~186 cells and ~146 μm. Type 6c: ~338 cells and ~218 μm in diameter. Type 6d: ~483 cells and ~290 μm in diameter.

(B) Representative histological micrographs of Mfn-cKO ovaries from 21- to 23-day-old mice. Arrows and arrowheads indicate normal and abnormal type 6 follicles, respectively.

(C) Percentage of follicles classified as types 6a, 6b, 6c and 6d.

In panel C, median (line inside the box), upper and lower quartiles (delimited by the box) and 1.5 times the interquartile range (whiskers).

*P < 0.05.

Figure S3. Related to Figure 3. *Mfn1* ablation results in oocytes with disrupted mitochondria.

(A) Representative transmission electron microscopy micrographs oocytes. Note that mitochondrial morphology is abnormal in *Mfn1*-cKO oocytes with evidence of organelle swelling, vesiculation of the inner membrane and lighter matrix. Arrows indicate mitochondria whereas arrowheads denote inner mitochondrial membranes and internal vesicles.

(B) Relationship between mtDNA copy number and diameter in oocytes from 12- and 32-day-old mice. Both variables correlated (R) significantly in WT (R = 0.75; P < 0.0001), but not in *Mfn1*-cKO group (R = 0.21; P = 0.09).

(C) Representative confocal microscopy micrographs of immature germinal-vesicle (GV) oocytes stained with MitoTracker CMXRos (mitochondrial – Mito) and ERTracker (endoplasmic reticulum – ER). ER staining is presented as a cytoplasmic reference. DIC = differential interference contrast. Note the intense mitochondrial aggregation in *Mfn1&2*-cKO oocytes (arrows).
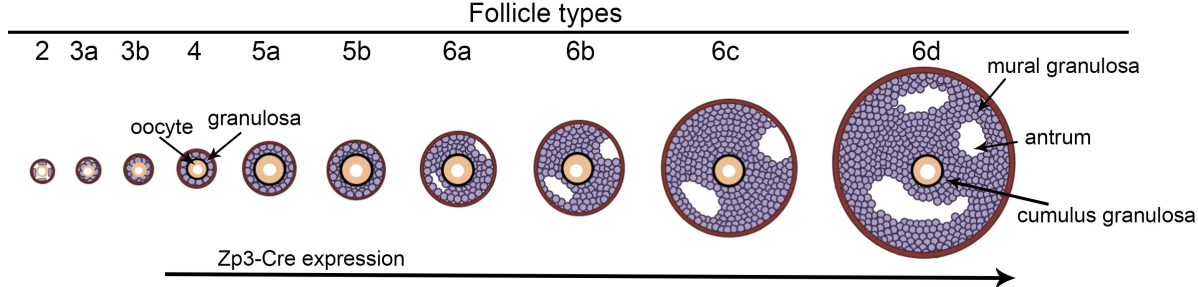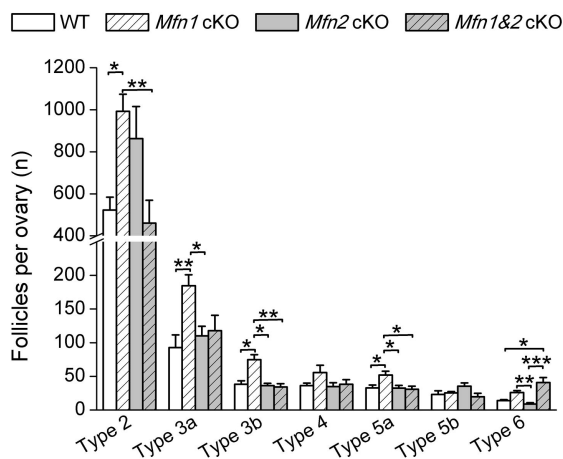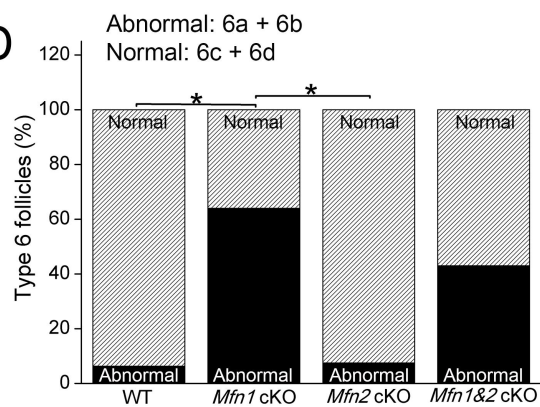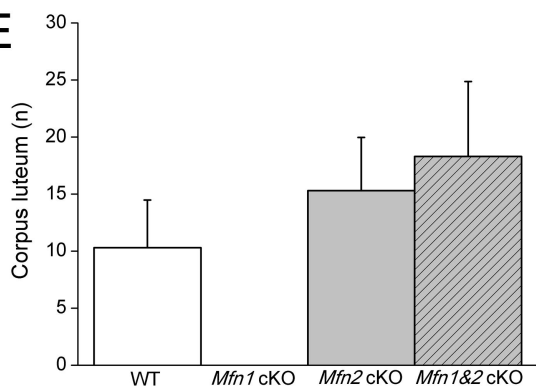

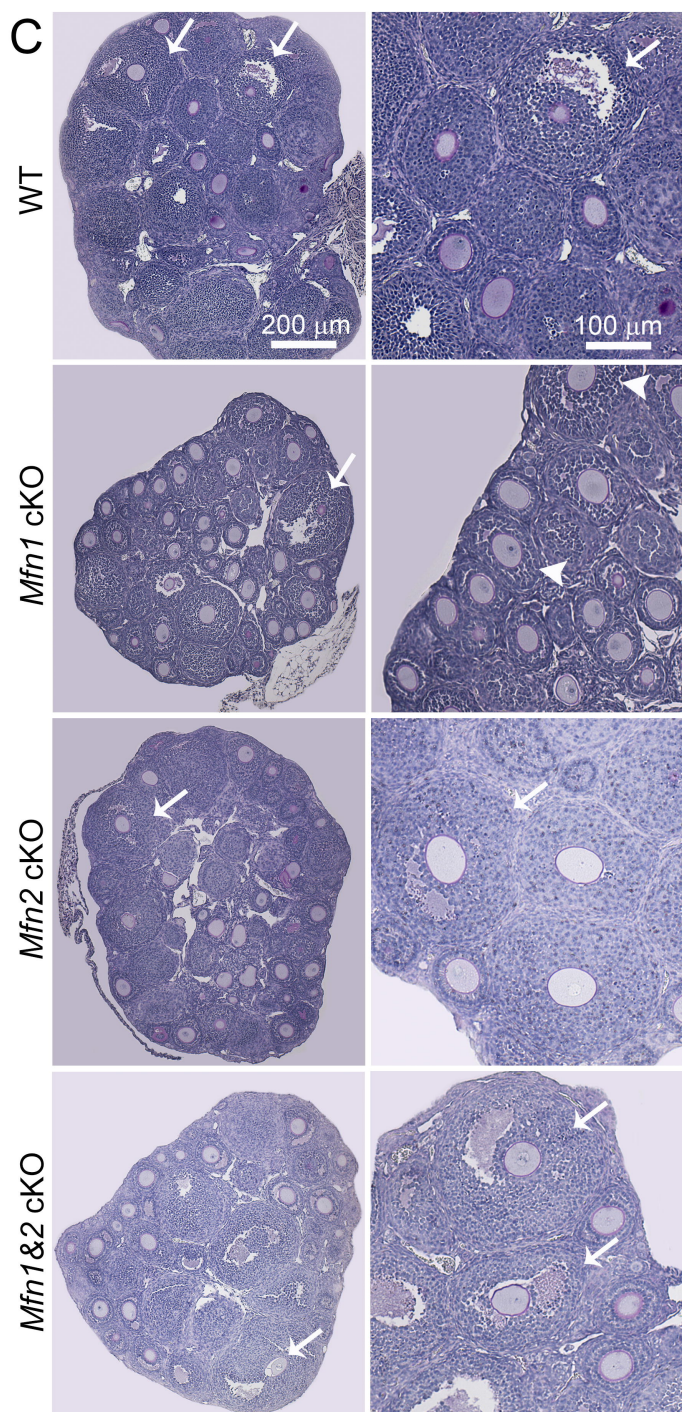**Figure S4. Related to Figure 4. Oocyte-specific deletion of *Mfn1* impairs oocyte-somatic cell communication.**

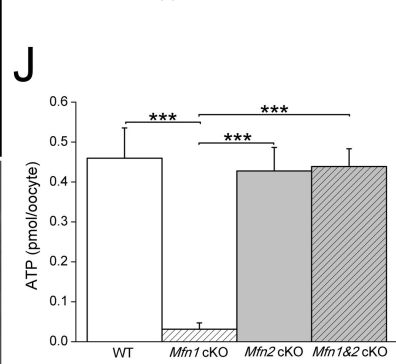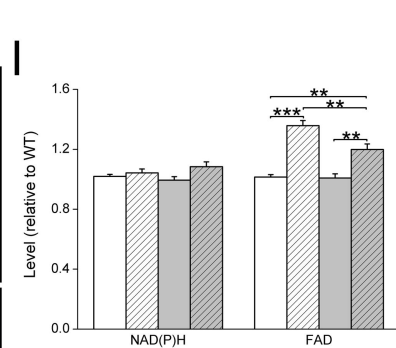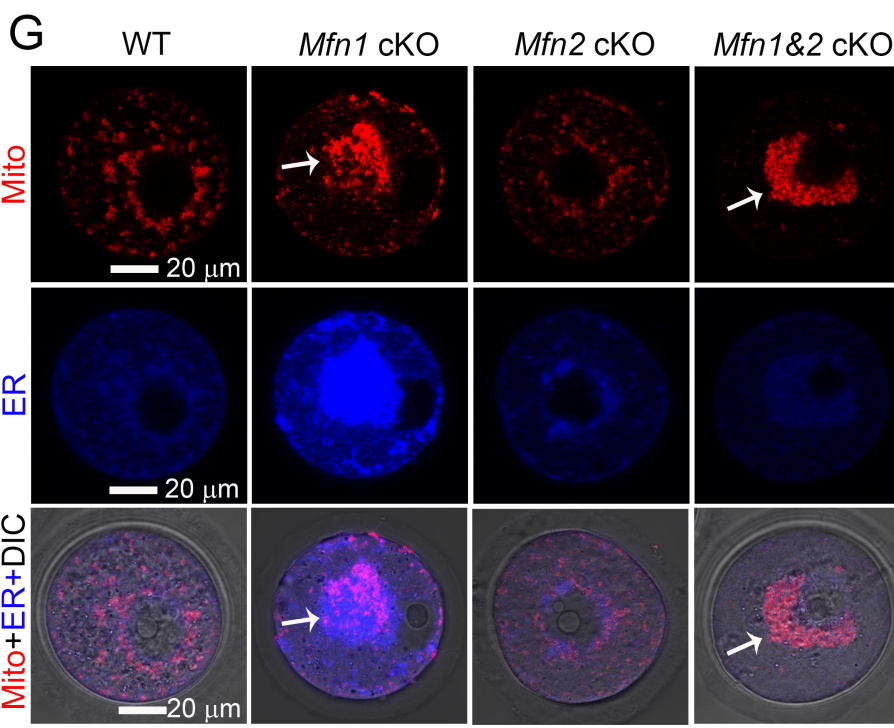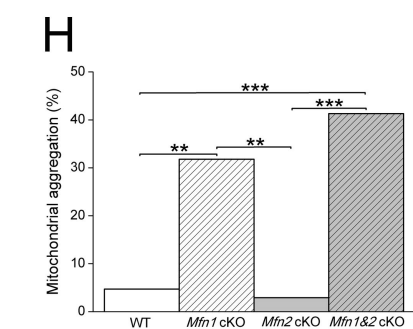(A) Representative fluorescence microscopy micrographs of BrdU-positive cells (arrowheads) in ovaries from 8-week-old mice. Ovary slides were

counterstained with DAPI to determine cell nuclei. Dotted lines denote follicle area. Note the decreased number of BrdU-positive cells in *Mfn1*-cKO ovaries. (B) Full-size images corresponding to blots presented on Figure 4D. Two different molecular weight markers were run on columns 1 and 2, whereas oocyte samples were run on columns 3-6. A total of 100 oocytes were used per group in each replicate, except for *Mfn1* cKO that 200 oocytes were used. Two biological replicates are show. Values above bands in column 1 and 2 represent molecular weights (kDa).

A Follicle types

2 3a 3b 4 5a 5b 6a 6b 6c 6d

oocyte granulosa

mural granulosa

antrum

cumulus granulosa

Zp3-Cre expression

B

WT *Mfn1* cKO *Mfn2* cKO *Mfn1&2* cKO

Follicles per ovary (n)

Type 2 Type 3a Type 3b Type 4 Type 5a Type 5b Type 6

C

WT

200 μm

100 μm

Mfn1 cKO

Mfn2 cKO

Mfn1&2 cKO

D

Abnormal: 6a + 6b
Normal: 6c + 6d

Type 6 follicles (%)

Normal Normal Normal Normal

Abnormal Abnormal Abnormal Abnormal

WT *Mfn1* cKO *Mfn2* cKO *Mfn1&2* cKO

E

Corpus luteum (n)

WT *Mfn1* cKO *Mfn2* cKO *Mfn1&2* cKO

A

| WT | *Mfn1* cKO | *Mfn2* cKO | *Mfn1&2* cKO |

2 μm

1 μm

B

Mitochondrial architecture (%)

Normal / Abnormal

WT *Mfn1* cKO *Mfn2* cKO *Mfn1&2* cKO

*** * *** *** ***

C

Mitochondrial diameter (μm)

☐ WT ▨ *Mfn1* cKO ▦ *Mfn2* cKO ▧ *Mfn1&2* cKO

Major axis   Minor axis

*** *** *** *** *** *** *** ***

D

mtDNA (copies/oocyte)

WT *Mfn1* cKO *Mfn2* cKO *Mfn1&2* cKO

*** ** *** *** ** **

E

mtDNA copies x 10³

▨ Preantral ▥ Antral

WT   *Mfn1* cKO

*** **

F

Δψₘ (relative to WT)

WT *Mfn1* cKO *Mfn2* cKO *Mfn1&2* cKO

*** *** *** ***

H

Mitochondrial aggregation (%)

WT *Mfn1* cKO *Mfn2* cKO *Mfn1&2* cKO

** ** *** ***

G

| WT | *Mfn1* cKO | *Mfn2* cKO | *Mfn1&2* cKO |

Mito

ER

Mito+ER+DIC

20 μm

I

Level (relative to WT)

NAD(P)H   FAD

*** ** ** **

J

ATP (pmol/oocyte)

WT *Mfn1* cKO *Mfn2* cKO *Mfn1&2* cKO

*** *** ***