

1 **NF-κB-repressed Sirt3 mediates testicular cholesterol metabolism and**
2 **cytoskeleton assembly via P450scc/SOD2 deacetylation during spermatogenesis**

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15 **Running head:** SIRT3 during spermatogenesis

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27

28 **Abstract**

29 Testicular homeostasis requires the balanced interplay between specific molecules in
30 Sertoli cells, Leydig cells, germ cells. Loss of this coordination can lead to the
31 disruption of spermatogenesis, even male infertility. By operating the upregulation
32 and downregulation of Sirt3 in our male subfertility rats model and two testicular cells
33 models, we indicated that Sirt3 overexpression and activator ameliorated cholesterol
34 metabolism via P450scc deacetylation in Leydig cells, and cytoskeleton assembly via
35 PDLIM1 with SOD2 deacetylation in Sertoli cells and elongating spermatids. In terms
36 of the upstream regulator of Sirt3, the phosphorylation of NF- κ B p65^{Ser536} stimulated
37 the nuclear translocation of NF- κ B subunits (p50, p65, RelB), which bound to TFBS1
38 and TFBS2 synchronously in the promoter of Sirt3, repressing Sirt3 transcription.
39 This study demonstrates that NF- κ B-repressed SIRT3 acts directly on cholesterol
40 metabolism of Leydig cells and cytoskeleton assembly of Sertoli cells via
41 P450scc/SOD2 deacetylation to regulate sperm differentiation, influencing
42 spermatogenesis, even male fertility.

43

44 **Research organism: Rat, mouse**

45

46 **Key words:** SIRT3; Spermatogenesis; Cholesterol metabolism; Cytockleton
47 assembly; NF- κ B; P450scc/SOD2 deacetylation

48

49 **Introduction**

50 Spermatogenesis is a well-defined dynamic process that consists of spermatogonial
51 mitosis, spermatocytic meiosis and spermogenesis (Griswold, 2016). During this
52 highly specialized process, Sertoli cells provide structural and energy support for
53 germ cells differentiation, growth and spermiation (Yokonishi et al., 2020); Leydig
54 cells transform cholesterol to produce testosterone (Li et al., 2016), which is required
55 for meiosis (Deng et al., 2016), releasing mature sperm (O'Donnell et al., 2009),
56 maintaining blood-testis barrier (BTB) (Mrk and Cheng, 2015). Ectoplasmic
57 specialization (ES), as an actin microfilament-rich anchoring device, comprises
58 Sertoli-elongating spermatid interface (apical ES) and Sertoli-Sertoli cell interface
59 (basal ES) (Mrk and Cheng, 2015). The apical ES interacts with the acrosome,
60 reshaping the sperm head, facilitating spermatid morphology and spermiation; the
61 basal ES with the tight/gap junctions constitutes BTB, protecting from external
62 stimulus (Cheng and Mrk, 2012). Except for ES, the microtubule is also a vital
63 cytoskeleton component. In fact, the function of Sertoli cells in nourishing the
64 developing spermatozoa depends on Leydig cells-secreted testosterone (Makela et al.,
65 2019), providing a communication network in testis. Thus, loss of this coordination
66 can lead to the disruption of spermatogenesis, even male infertility.

67 Sirtuin 3 (SIRT3) is the primary mitochondrial acetyl-lysine deacetylase that
68 modulates various proteins for mitochondrial function, ROS generation, cell death
69 (Pellegrini et al., 2012) and metabolism (Hor et al., 2020; Palomer et al., 2020).
70 SIRT3 cooperates with SIRT1, targeting PGC-1 α in antioxidant defense systems
71 (Chen et al., 2018b). Sirt1 deficiency in mice leads to spermatogenesis derangement
72 via meiotic arrest (Heo et al., 2017) and acrosome biogenesis disruption by autophagy
73 (Liu et al., 2017a). Despite the high expression of SIRT3 in mammalian testis (Yu et
74 al., 2014; Yue et al., 2014), the role of SIRT3 in testis remains ambiguous. Our
75 previous study has evidenced that testicular cell injury induced by cadmium (Cd) in
76 rats is associated with mitochondrial autophagy and oxidative stress (Wang et al.,
77 2020). Then, a question appears: whether manipulating Sirt3 can also regulate
78 testicular injury induced by Cd? Even, what's the functional role of Sirt3 in testis?

79 Herein, our male subfertility model indicated that SIRT3 activator melatonin (Mel)
80 rescued cholesterol metabolism (and testosterone biosynthesis) by Abca1, Hsd17b3,
81 Scap, Srebf2, Star and F-actin-containing cytoskeleton assembly by ectoplasmic
82 specialization and microtubule-based manchette during spermiogenesis, facilitating
83 sperm motility and count in Cd-treated rat testis. The expression of testosterone
84 biosynthesis-associated markers (NF- κ B p65 and P450scc) and cytoskeleton markers
85 (PDLIM1, SOD2), especially SIRT3 presented responsive changes. Further,
86 generating Sirt3 knockdown/overexpression models in TM3 (mouse Leydig) cells and
87 TM4 (mouse Sertoli) cells respectively, we found that like Mel, Sirt3 overexpression
88 ameliorated cholesterol metabolism (and testosterone biosynthesis) via P450scc
89 deacetylation in Cd-treated TM3 cells and cytoskeleton assembly via PDLIM1 with
90 SOD2 deacetylation in Cd-treated TM4 cells. Sirt3 knockdown-induced cell
91 disruption failed to be salvaged by Mel. In terms of the upstream regulator of SIRT3,
92 phosphorylation of NF- κ B p65^{Ser536} stimulated the nuclear translocation of NF- κ B
93 subunits (p50, p65, RelB), which bound to TFBS1 and TFBS2 in the promoter of
94 Sirt3 synchronously, repressing Sirt3 transcription. Collectively, this study
95 demonstrates the novel role of SIRT3 in testicular cells: **Sirt3 transcriptional**
96 **repression by NF- κ B orchestrates cholesterol metabolism via P450scc**
97 **deacetylation in Leydig cells; whereas, in Sertoli cells, SIRT3 regulates**
98 **cytoskeleton assembly via PDLIM1 with SOD2 deacetylation, facilitating sperm**
99 **differentiation during spermiogenesis.** Our findings shed light on the multi-faceted
100 action of SIRT3 and establish a novel signaling network in the crosstalk between
101 testicular different cells. Understanding the role of SIRT3 in cholesterol metabolism
102 and cytoskeleton assembly will contribute to the development of spermatogenesis.
103 SIRT3 may serve as a potential therapeutic target for spermatogenesis disorganization,
104 male infertility, even other metabolic diseases.

105

106 **Results**

107 **SIRT3 activator Mel rescues male reproductive function injury induced by Cd in** 108 **adult rats, including cholesterol metabolism and structure disruptions**

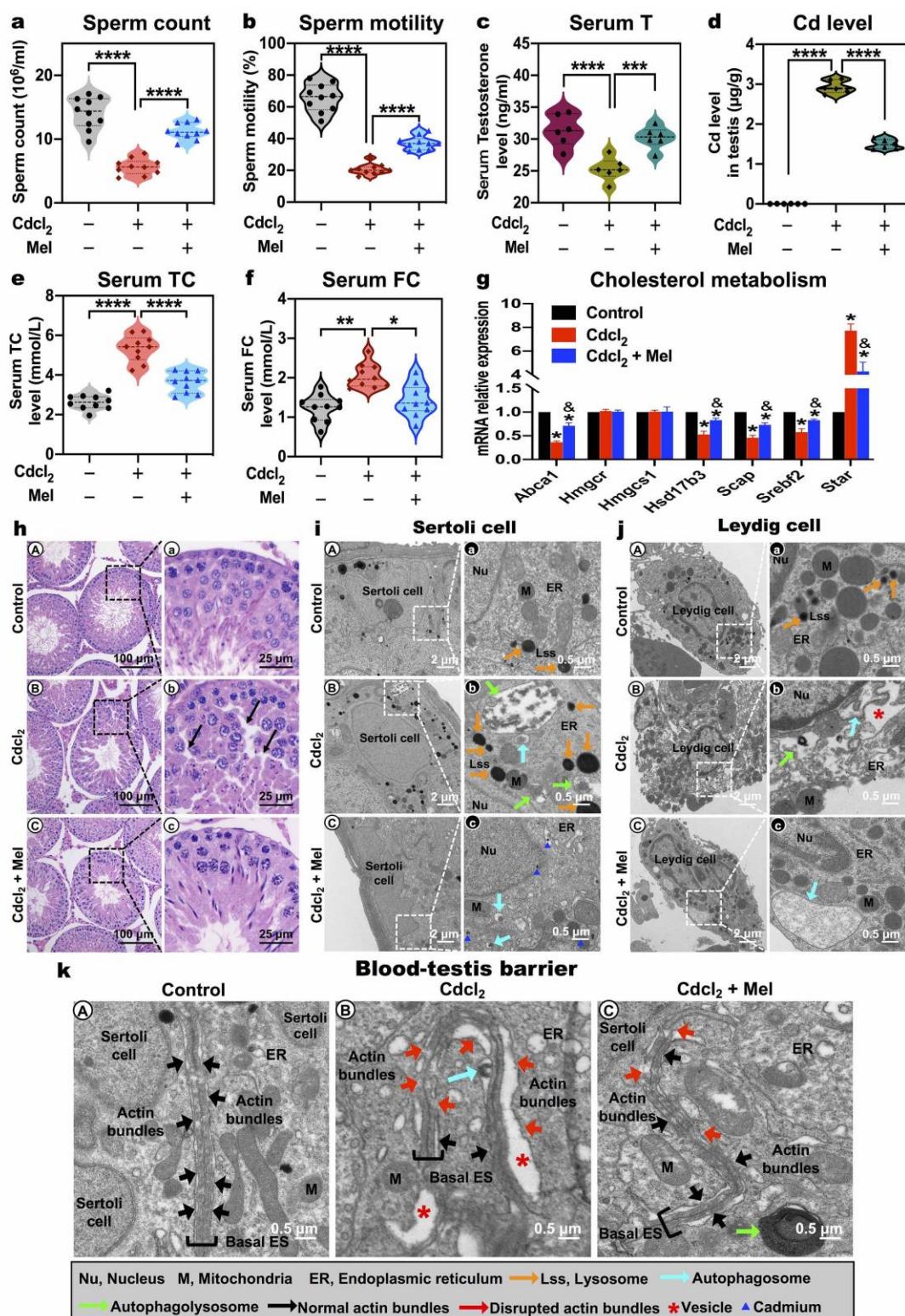
109 To investigate whether SIRT3 was involved in the testicular function, we first
110 examined the effects of SIRT3 activator Mel by a previous subfertility model in

111 Cd-treated adult male SD rats. Results showed that Mel significantly reversed sperm
112 count, sperm motility, serum testosterone reduction induced by Cd (**Fig. 1a-c**). The
113 concentration of Cd in the testis was apparently reduced by Mel (**Fig. 1d**). Like other
114 steroid hormones, testosterone was biosynthesized from cholesterol, so we explored
115 the levels of serum total cholesterol (TC) and free cholesterol (FC). Interestingly, Cd
116 provoked the augment of serum TC and FC rather than the decline of those, but Mel
117 overturned the above changes (**Fig. 1e, f**).

118 To elucidate the reason, we checked cholesterol metabolism markers, including a
119 series of steroidogenic enzymes, which are responsible for testosterone biosynthesis.
120 Cd suppressed the expression of cholesterol efflux marker Abca1, which was reversed
121 by Mel (**Fig. 1g**), suggesting that Mel could regulate cholesterol efflux blocked by Cd
122 in testicular cells. Cholesterol biosynthesis markers Hmgcr and Hmgcs1 (Luo et al.,
123 2020) didn't present any differences in Cd with or without Mel group when compared
124 to the control group (**Fig. 1g**), indicating that whether Cd or Mel was independently
125 associated with cholesterol biosynthesis in the testis. Besides, the expression of Star, a
126 key transporter of cholesterol from cytoplasm into the mitochondria for testosterone
127 biosynthesis (Rubinow, 2018), was dramatically increased in Cd-treated group, and
128 Mel reversed the augment of Star in the testis (**Fig. 1g**). As predicted, more cholesterol
129 could be delivered into the mitochondria, and then produced more testosterone in
130 Cd-treated group. Nonetheless, the fact was that Cd diminished testosterone production.
131 Given that Scap/SREBP system was sensitive to the concentration of cholesterol in the
132 plasma membrane and regulated genes transcription of cholesterol metabolism (Das et
133 al., 2014), we subsequently inspected the expression of Scap and Srebf2, which were
134 significantly dwindled in Cd-treated group and raised back in Cd with Mel group (**Fig.**
135 **1g**). Hsd17b3, as an indispensable enzyme for testosterone biosynthesis, specifically
136 catalyzed the conversion of androstenedione to testosterone (Marshall et al., 2002). Mel
137 salvaged the reduction of Hsd17b3 expression induced by Cd (**Fig. 1g**). Above results
138 revealed that SIRT3 activator Mel rescued cholesterol metabolism disruption in male
139 subfertility model induced by Cd.

140 Mel decreased the morphologic damage of the seminiferous tubules was decreased
141 by Mel. As depicted in **Fig. 1h**, testicular cells in Cd-treated group were arranged
142 loosely with a large number of voids (**Fig. 1h (Bb)**), while whether the control group or
143 Cd with Mel group exhibited an intact and dense lumen structure (**Fig. 1h (Aa, Cc)**).

144 For further exploring which of testicular cells were affected in this study, we analyzed
145 the ultrastructure of the testis by transmission electron microscopy (TEM). Both Sertoli
146 cells and Leydig cells presented complete nuclear membrane structure, and normal
147 organelles (such as mitochondria, endoplasmic reticulum) with a low number of
148 lysosomes in the control group (**Fig. 1i (Aa), j (Aa)**). By contrast, Cd apparently
149 induced lots of lysosomes, autophagosomes and enormous autophagolysosomes in
150 Sertoli cells (**Fig. 1i (Bb)**), which were consistent with autophagy observed in previous
151 study (Wang et al., 2020). However, Cd with Mel group showed nearly normal
152 organelles with sporadic autophagosomes, and tiny black unidentified objects
153 (probably Cd) (**Fig. 1i (Cc)**). In Leydig cells, Cd evoked massive cytoplasmic
154 vacuolization, while Mel obviously protected against the abnormal ultrastructure
155 induced by Cd (**Fig. 1j (Bb, Cc)**). Given that Leydig cells were the primary source of
156 testosterone production (Makela et al., 2019), the ultrastructural changes in Leydig
157 cells corresponded to above cholesterol metabolism and testosterone biosynthesis
158 regulation. Our previous study indicated that Cd disturbed BTB via oxidative stress
159 (Chen et al., 2018a), so we asked whether Mel could protect from BTB injury. The
160 basal ES and other junctions constituted the BTB between adjacent Sertoli cells. The
161 control group exhibited adjacent Sertoli cells and normal basal ES with the actin
162 bundles; Cd disrupted the basal ES and actin bundles with large vacuoles (asterisks),
163 but Mel rescued BTB disruption induced by Cd (**Fig. 1k**). Actually, the disruption of
164 BTB led to germ cell loss and male infertility (Cheng and Mruk, 2012), which might
165 account for sperm count reduction.



166

167 **Fig. 1 SIRT3 activator Mel rescues male reproductive function injury induced by**
 168 **Cd in adult rats, including cholesterol metabolism and structure disruptions. a**
 169 **Sperm count. b Sperm motility. c Serum testosterone (T) level. d Cadmium (Cd) level**
 170 **in testis. e Serum total cholesterol (TC) level. f Serum free cholesterol (FC) level. g**

171 mRNA expression levels of cholesterol metabolism markers of *Abca1*, *Hmger*,
172 *Hmgcs1*, *Hsd17b1*, *Scap*, *Srebf2*, *Star* in testis. **h** Histological results of the testis in
173 rats. **i-k** Representative transmission electron micrographs (TEM) depicting the
174 ultrastructure of Sertoli cell, Leydig cell, blood-testis barrier (BTB) in testis. Areas for
175 the cell in the left column (Scale bar, 2 μ m) have been shown for further details in the
176 right column (Scale bar, 0.5 μ m) in **i** and **j**. Nu, nucleus; M, mitochondria; ER,
177 endoplasmic reticulum; orange arrowheads indicate lysosome; turquoise arrowheads
178 indicate autophagosome; spring arrowheads indicate autophagolysosome; black
179 arrowheads indicate normal actin bundles in BTB; red arrowheads indicate disrupted
180 actin bundles in BTB; red asterisk indicates vesicle; blueberry triangles indicate
181 potential Cd in testis. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$ in **a-f**. $*p <$
182 0.05 compared with the control. $\&p < 0.05$ compared with the Cd-treated group in **g**.
183

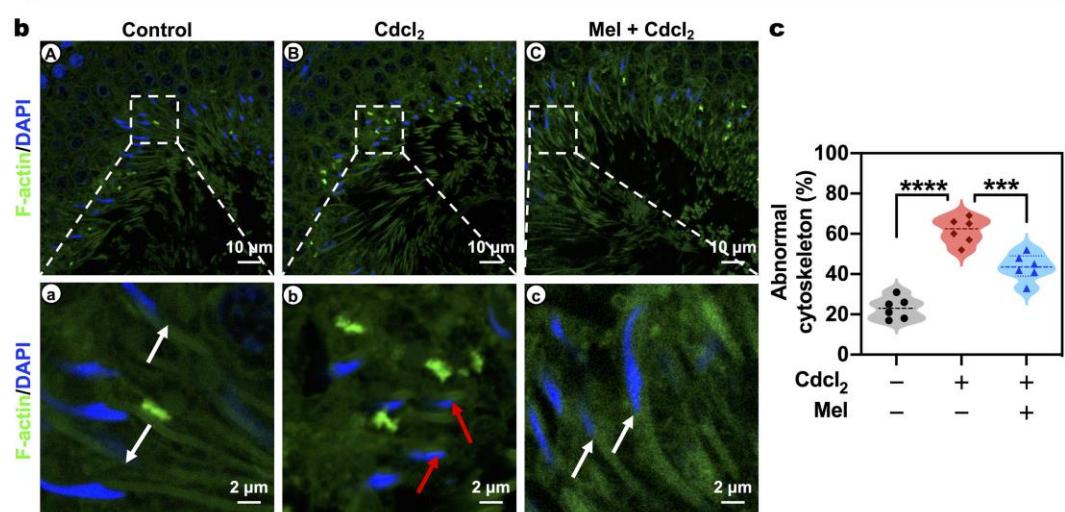
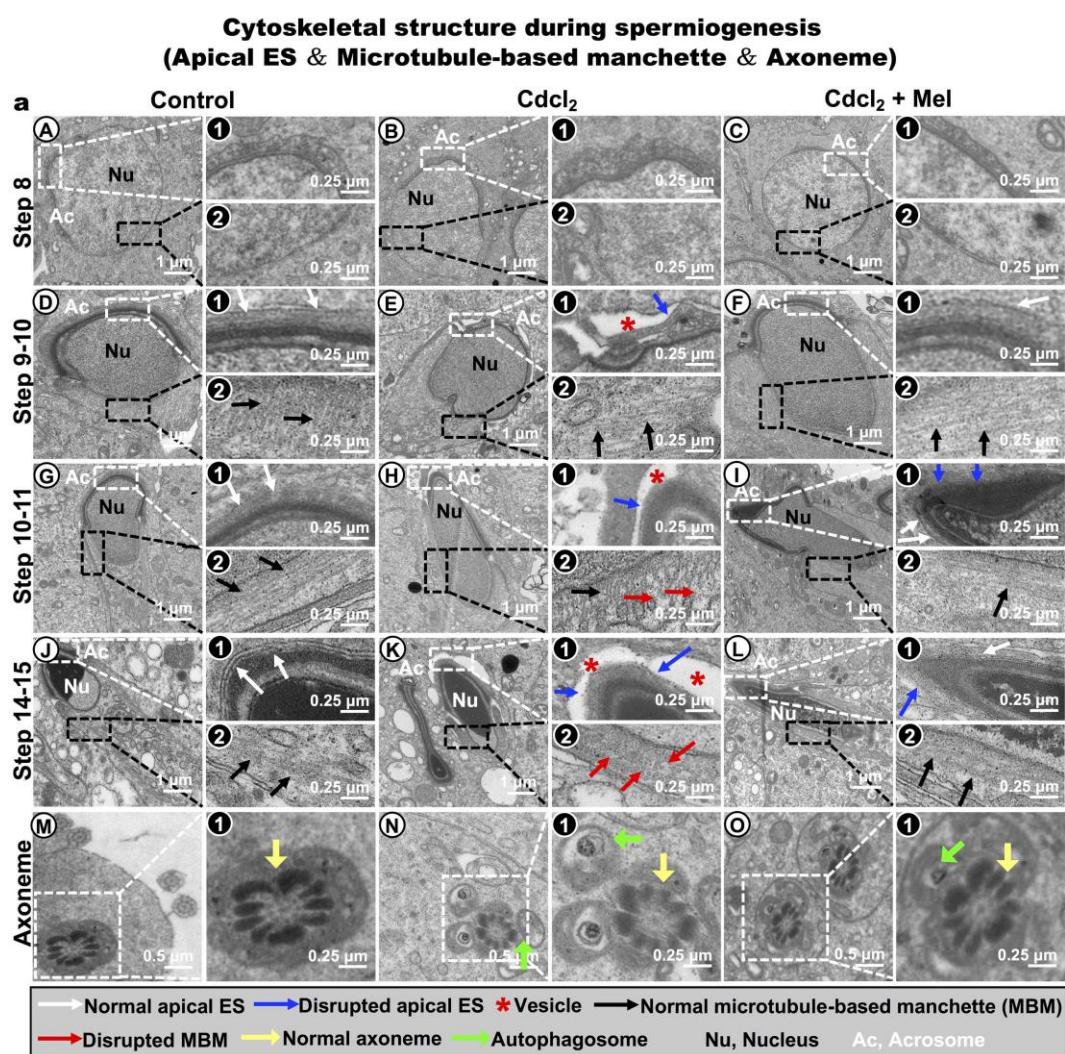
184 Above results proved that Mel regulated Sertoli cells, Leydig cells and BTB,
185 intimating that SIRT3 might engage in cholesterol metabolism and testosterone
186 synthesis by Leydig cells, and affect sperm count by BTB and Sertoli cells.
187

188 **SIRT3 activator Mel rescues cytoskeleton (apical ES and microtubule-based
189 manchette) assembly disruption induced by Cd in the elongating spermatid
190 during spermiogenesis**

191 Except for sperm count, Mel rescued sperm motility reduction induced by Cd (**Fig. 1b**).
192 The cytoskeleton assembly of apical ES and microtubule-based manchette in sperm
193 head and flagellum during spermiogenesis, is required for sperm motility acquirement
194 (Pleuger et al., 2020). To test the hypothesis if SIRT3 activator Mel protects sperm
195 motility by regulating cytoskeleton assembly, we screened the spermatid deformation
196 process step by step during spermiogenesis through TEM analysis.

197 Spermiogenesis begins with round spermatid elongation at step 8, so our
198 observation started from this stage. There were no significant differences between Cd
199 with or without Mel group and the control group at step 8 (**Fig. 2a (A-C)**). From step
200 9 to 10, bundles of microtubules were assembled to form the manchette structure
201 (black arrow), which was tightly arranged in different groups (**Fig. 2a (D2-F2)**). The
202 apical ES (white arrow), an imperative component for shaping sperm head, was
203 constituted by actin bundles surrounding the acrosome's outer layer. We found that

204 the sperm head was disturbed with large vacuoles (red asterisk) in Cd-treated group,
205 but Mel reversed the disruption of apical ES and acrosome induced by Cd (**Fig. 2a**
206 (**D1-F1**)). In the early elongating spermatids of step 10-11, a well-assembled sperm
207 head and long microtubule-based manchette could be observed in the control group
208 (**Fig. 2a (G)**); whereas, in the Cd-treated group, apical ES disappeared with large
209 vacuoles in sperm head, and the manchette was undermined with short microtubules
210 (red arrow) (**Fig. 2a (H)**); however, in Cd with Mel group, apical ES and manchette
211 were slightly impaired (**Fig. 2a (I)**). In the late elongating spermatids of steps 14 to 15,
212 well assembled apical ES and acrosome were finely arranged surrounding the
213 condensed nucleus, and the long manchette could be detected clearly (**Fig. 2a (J)**);
214 instead, in Cd-treated group, gigantic vacuoles replaced the well apical ES, only
215 round dots (red arrow) rather than long microtubules were observed (**Fig. 2a (K)**); yet
216 Cd with Mel group presented slightly apical ES and manchette injury (**Fig. 2a (L)**).
217 Besides, flagella axonemes (yellow arrow), which were associated with sperm
218 motility, were identified. We did not discover any differences in axoneme between
219 three groups, only with some autophagosomes around the axoneme in Cd-treated
220 group (**Fig. 2a (M-O)**). Ultrastructural results displayed that Mel could extricate
221 apical ES and manchette injury caused by Cd.



222

223 **Fig. 2 SIRT3 activator Mel rescues spermatid cytoskeleton (apical ES and**
 224 **microtubule-based manchette) assembly disruption induced by Cd during**
 225 **spermiogenesis. a** Ultrastructural analysis of the spermiogenesis in rat testis during

226 different developmental stages. Areas for the cell (A-O) in the left column (Scale bar,

227 1 or 0.5 μ m) have been shown for further details (1, 2) in the right column (Scale bar,
228 0.25 μ m). (A-C) the ultrastructures of step 8 round spermatids before manchette
229 assembly; (D-F) the ultrastructures of step 9-10 spermatids; (G-I) the ultrastructures
230 of step 10-11 early elongating spermatids spermatids; (J-L) the ultrastructures of step
231 14-15 late elongating spermatids; (M-O) the ultrastructures of developing axoneme. 1
232 indicates acrosome, and 2 indicates nuclear membrane in A-C; 1 indicates apical ES,
233 and 2 indicates microtubule-based manchette in D-L. 1 indicates zoom-in axoneme in
234 M-O. Nu, nucleus; Ac, acrosome; white arrowheads indicate normal apical ES;
235 blueberry arrowheads indicate disrupted apical ES; red asterisk indicate vesicle; black
236 arrowheads indicate normal microtubule-based manchette; red arrowheads indicate
237 disrupted microtubule-based manchette; yellow arrowheads indicate normal axoneme;
238 spring green arrowheads indicate autophagosome. **b** Representative confocal
239 microscope imaging of F-actin (a marker of cytoskeleton) in spermatids by the
240 immunofluorescent analysis of phalloidin. Areas (A-C) in the upper panels (Scale bar,
241 10 μ m) have been shown for further details (a-c) in the low panels (Scale bar, 2 μ m).
242 White arrowheads indicate normal F-actin structure; red arrowheads indicate
243 disrupted F-actin structure. **c** The percentages of spermatids with abnormal
244 F-actin-containing cytoskeleton. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$
245 in **c**.

246

247 F-actin is a marker of cytoskeleton, especially in apical ES and microtubule of the
248 testis. To further confirm the role of SIRT3 activator Mel in the cytoskeleton, we
249 scrutinized the distribution of F-actin in spermatids by the immunofluorescent
250 analysis of phalloidin, which labeled F-actin. Confocal microscopy imaging showed
251 that the F-actin structure was well oriented in linear arrays parallel to the elongating
252 spermatid nucleus's long axis in the control group (**Fig. 2b (Aa)**). Similar to the TEM
253 experiments, Mel rescued the F-actin structure disruption induced by Cd (**Fig. 2b (Bb,**
254 **Cc)**), and evidently lowered the percentages of spermatid with abnormal
255 F-actin-containing cytoskeleton (**Fig. 2c**).

256 Taken together, these results manifested that SIRT3 activator Mel regulated
257 cytoskeleton assembly, including apical ES and microtubule-based manchette in the
258 elongating spermatid during spermiogenesis. Therefore, SIRT3 might participate in

259 cytoskeleton assembly of elongating spermatids during spermiogenesis, influencing
260 the sperm motility.

261 **Mel regulates SOD2 deacetylation and PDLIM1 by SIRT3 in Cd-induced
262 testicular cytoskeleton assembly disruption**

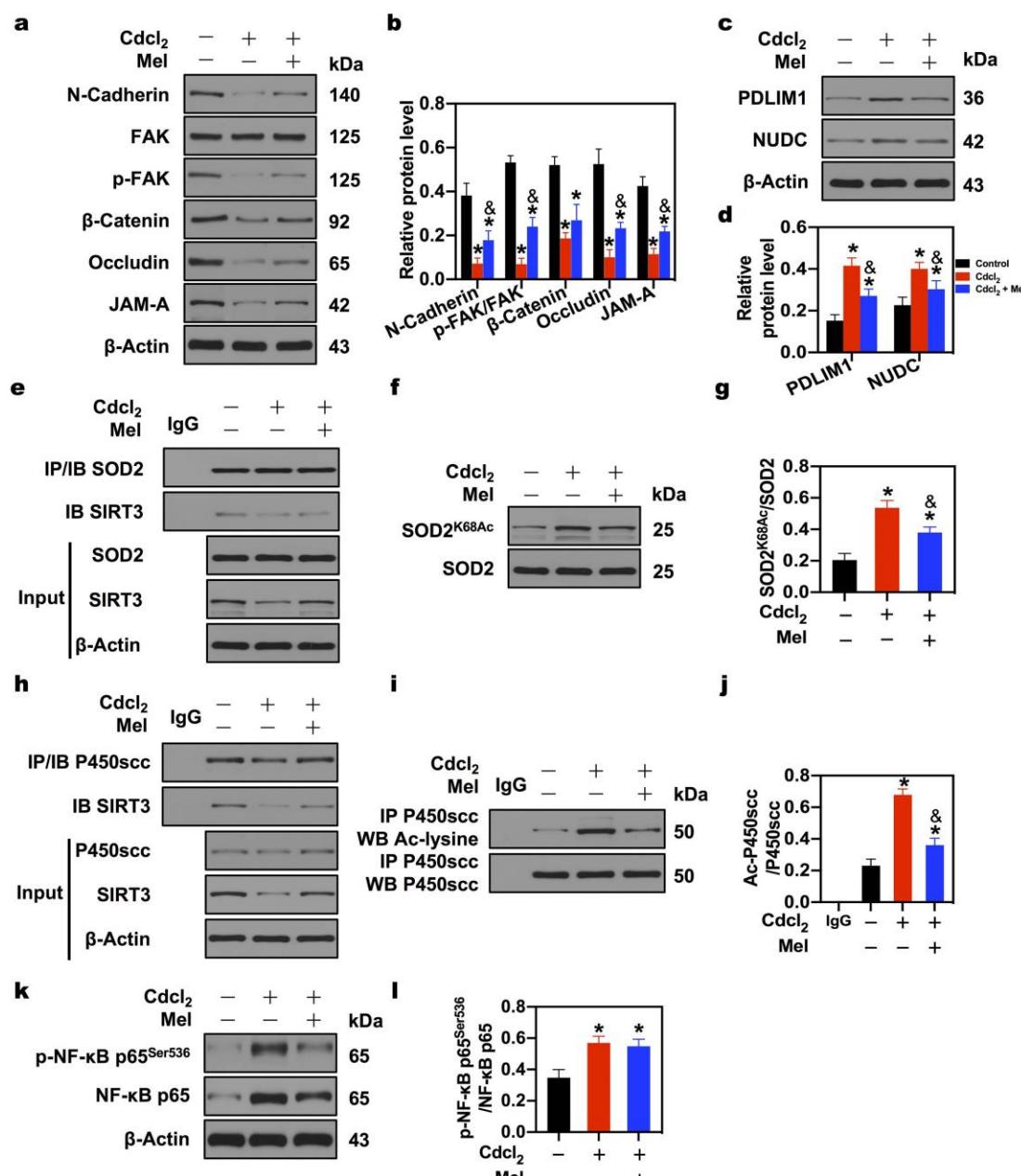
263 Above TEM and immunofluorescent analysis displayed that Mel modulated
264 cytoskeleton assembly, including BTB with basal ES, apical ES and manchette in the
265 elongating spermatids. To further elucidate the regulatory mechanisms at the
266 molecular level, we asked how Mel modulated cytoskeleton assembly in testis. The
267 protein expression of cytoskeleton assembly markers was investigated. Cd
268 significantly decreased the protein expression of BTB markers—integral membrane
269 proteins (Occludin, JAM-A, N-cadherin), adaptor protein (β -catenin) and regulatory
270 proteins (FAK, p-FAK-Tyr407) (**Fig. 3a, 3b**). In addition to β -catenin, the reduction
271 of other proteins was successfully reversed by Mel. Actually, p-FAK-Tyr407 is a
272 molecular ‘switch’ in the apical ES–BTB axis to regulate BTB restructuring and
273 apical ES adhesion. These results corresponded to the changes of TEM in BTB and
274 apical ES. PDLIM1, NUDC are the identified F-actin networks negative regulator,
275 whose overexpression results in the disassembly of the F-actin net structure in Sertoli
276 cells (Liu et al., 2016). Besides, PDLIM1 is critical for ES assembly. Our results
277 showed that SIRT3 activator Mel reversed the protein expression augment of
278 PDLIM1 and NUDC induced by Cd in testis (**Fig. 3c, 3d**). Therefore, Mel might
279 regulate cytoskeleton assembly by PDLIM1.

280 Our previous studies showed that Cd elevated testicular ROS level and reduced
281 SOD activity in testis (Chen et al., 2018a) and serum (Wang et al., 2020). SIRT3 is
282 the most robust mitochondrial deacetylase, and SOD2 (also named MnSOD) is just
283 the identified downstream of SIRT3 in the liver (Tao et al., 2010). To investigate the
284 correlation of SIRT3 and SOD2 in testis, we examined the interaction of SIRT3 and
285 SOD2 by co-immunoprecipitation (co-IP). The results indicated that Cd decreased the
286 protein expression of SIRT3 and disturbed the interaction of SIRT3 and SOD2;
287 whereas, SIRT3 activator Mel could effectively increase the protein expression of
288 SIRT3 and the affinity of SIRT3 and SOD2 (**Fig. 3e**). Notably, Cd or Mel had no
289 significant effect on the protein expression of SOD2 (**Fig. 3e**). Then, what is the direct

290 mechanism of the interaction between SIRT3 and SOD2? Whether does SIRT3 exert
 291 its role as deacetylase?

292 To test the hypothesis, SOD2 acetylation levels were determined by the anti-SOD2
 293 antibody with anti-SOD2^{K68Ac} antibody. Significantly, Cd elevated the acetylation of
 294 SOD2; Mel reduced the acetylation of SOD2 compared with the Cd-treated group
 295 (**Fig. 3f, 3g**), suggesting that SIRT3 regulated SOD2 deacetylation by directly
 296 interacting with SOD2 to mediate oxidative stress in testis.

297 These data indicated that Mel regulated SOD2 deacetylation and PDLIM1 by
 298 SIRT3 in Cd-induced testicular cytoskeleton assembly disruption.



299

300 **Fig. 3 Mel regulates SOD2/P450scc deacetylation by SIRT3, and PDLIM1,**
301 **NF-κB p65^{Ser536} phosphorylation and P450scc deacetylation by SIRT3 in**
302 **Cd-induced testicular injury. a-b** A representative immunoblot and quantification
303 analysis of BTB markers N-Cadherin, β-Catenin, Occludin, JAM-A and apical ES–
304 BTB axis switch FAK, p-FAK-Tyr407 in testis. **c-d** A representative immunoblot and
305 quantification analysis of cytoskeleton negative regulators PDLIM1, NUDC in testis.
306 **e** Co-immunoprecipitation (Co-IP) of SOD2 and SIRT3 in testis. **f-g** A representative
307 immunoblot and quantification analysis of SOD2 acetylation level. **h** Co-IP
308 of P450scc and SIRT3 in testis. **i-j** IP of P450scc and Ac-lysine, and quantification
309 analysis of P450scc acetylation level in testis. **k-l** A representative immunoblot and
310 quantification analysis of NF-κB p65^{Ser536} phosphorylation. * $p < 0.05$ compared with
311 the control. & $p < 0.05$ compared with the Cd-treated group.

312

313 **Mel regulates P450scc deacetylation by SIRT3 and NF-κB p65^{Ser536}**
314 **phosphorylation in Cd-induced testicular cholesterol metabolism disruption**
315 In cholesterol metabolism, cholesterol is delivered into the mitochondria through
316 StAR (gene name Star), and converted to pregnenolone by the catalysis of P450scc
317 (Hsu et al., 2006), which has been considered the rate-limiting step of testosterone
318 biosynthesis. Although Cd increased the expression of Star and cholesterol level, the
319 testosterone level is decreased (Fig. 1). A study proposed that the deacetylation of
320 P450scc was associated with SIRT3 and SIRT5 in resveratrol-mediated cortisol
321 biosynthesis (Li et al., 2012). Thus, we speculated whether Mel operated P450scc
322 deacetylation via SIRT3 in this process. Co-IP was performed to verify the interaction
323 of SIRT3 and P450scc. Cd decreased the protein expression of SIRT3 and disturbed
324 the interaction of SIRT3 and P450scc; whereas, Mel effectively increased the protein
325 expression of SIRT3 and the affinity of SIRT3 and P450scc (Fig. 3h). Whether Cd or
326 Mel had no significant effect on the protein expression of P450scc (Fig. 3h).
327 Synchronously, P450scc acetylation levels were determined by immunoprecipitation
328 with an anti-P450scc antibody, followed by immunoblot analysis of acetylated-lysine.
329 Cd elevated the acetylation of P450scc; Mel reduced the acetylation of P450scc
330 compared with the Cd-treated group (Fig. 3i, 3j), suggesting that SIRT3 regulated

331 P450scc deacetylation by directly interacting with P450scc to mediate cholesterol
332 metabolism in testis.

333 Then, how does Mel exert its role on SIRT3 in testis? Given that Mel could
334 remarkably alleviate inflammasome-induced pyroptosis by blocking NF- κ B p65
335 signal in mice adipose tissue (Liu et al., 2017b), and the inhibition of NF- κ B p65
336 regulated testosterone production by Nur77 and SF-1 (Hong et al., 2004), thus the
337 activity of NF- κ B p65 was observed. We noticed that Cd indeed stimulated NF- κ B
338 p65^{Ser536} phosphorylation, and Mel mitigated the phosphorylation of NF- κ B p65^{Ser536}.
339 Meanwhile, Mel reversed Cd-induced protein expression changes of NF- κ B p65 and
340 p-NF- κ B p65^{Ser536} respectively. Nonetheless, the ratio of p-NF- κ B p65^{Ser536}/NF- κ B
341 p65 did not exhibit a significant difference between the Cd-treated group and Cd with
342 Mel group (**Fig. 3k, 3l**). Above results evidenced that Mel regulates P450scc
343 deacetylation by SIRT3 and NF- κ B p65 in Cd-induced testicular cholesterol
344 metabolism disruption.

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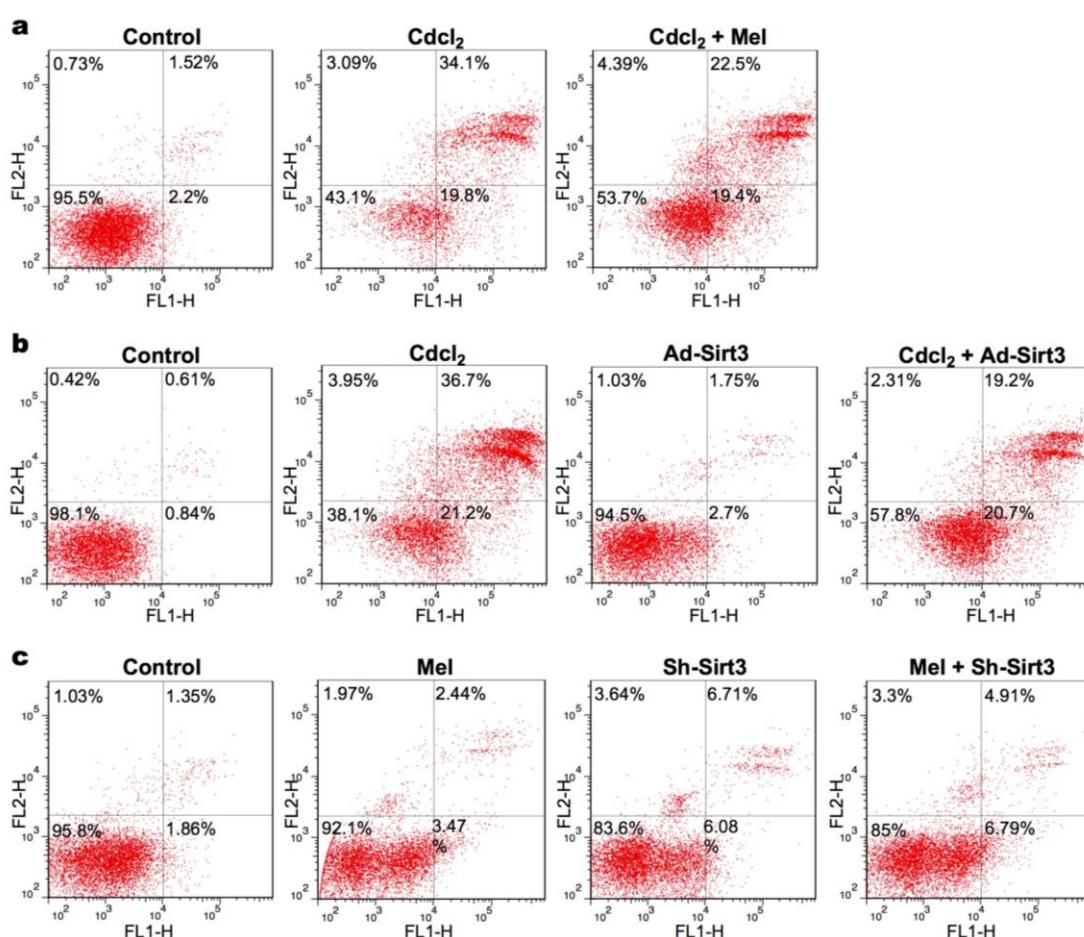
346 **Mel-mediated cholesterol metabolism protection is dependent on Sirt3, which is**
347 **the upstream regulator of P450scc deacetylation, but not of NF- κ B p65^{Ser536}**
348 **phosphorylation in TM3 mouse Leydig cells**

349 To further validate the role of SIRT3 in cholesterol metabolism in Leydig cells, we
350 generated Sirt3 overexpression and knockdown models in TM3 mouse Leydig cells
351 (TM3 cells). The efficiencies of Sirt3 overexpression and knockdown were examined
352 (**supplementary Fig. 1c**).

353 Initially, we got the half-maximal inhibitory concentration (IC50) of Cd for TM3
354 cells—8.725 μ g/ml by the concentration gradient method (Wang et al., 2020), which
355 was exploited in subsequent experiments. Cell Counting Kit-8 (CCK-8) assay
356 indicated that Mel significantly rescued cell viability reduction induced by Cd (**Fig.**
357 **4a₁**—the first panel in Fig. 4a), implying that Mel blocked the specific injury process.
358 To determine whether Mel-mediated protection depends on SIRT3 or not, Sirt3
359 overexpression adenovirus (Ad-Sirt3) and Sirt3 knockdown adenovirus with shRNA
360 (Sh-Sirt3) were utilized. Like Mel, the overexpression of Sirt3 rescued cell viability
361 reduction induced by Cd (**Fig. 4a₂**), suggesting that Sirt3 participated in the specific
362 protection process. Interestingly, the knockdown of Sirt3 decreased TM3 cell viability,
363 but Mel failed to rescue sh-Sirt3-induced cell viability reduction (**Fig. 4a₃**), revealing

364 that Sirt3 dominated the protection process, and scilicet Mel-mediated cell viability
365 protection was dependent on Sirt3 in TM3 cells.

366 Flow cytometry analysis confirmed the results of the CCK-8 assay. Cd significantly
367 decreased the percentage of viable cells and increased the percentage of necrotic,
368 early and late apoptotic cells; remarkably, Mel reversed Cd-triggered TM3 cell
369 apoptosis (**Supplementary Fig. 2a; Fig. 4b₁**). Moreover, the overexpression of Sirt3
370 rescued apoptosis induced by Cd, but Mel did not rescue sh-Sirt3-induced apoptosis
371 in TM3 cells (**Supplementary Fig. 2b, 2c; Fig. 4b_{2,3}**), demonstrating that
372 Mel-mediated cell apoptosis protection was dependent on Sirt3 in TM3 cells.



373
374 **Supplementary Fig. 2 Mel-mediated cell apoptosis protection is dependent on**
375 **Sirt3 in TM3 cells.** Representative flow cytometry analysis: **a** Mel reverses
376 Cd-triggered TM3 cell apoptosis. **b** The overexpression of Sirt3 rescues apoptosis
377 induced by Cd in TM3 cells. **c** Mel fails to rescue sh-Sirt3-induced apoptosis in TM3
378 cells.

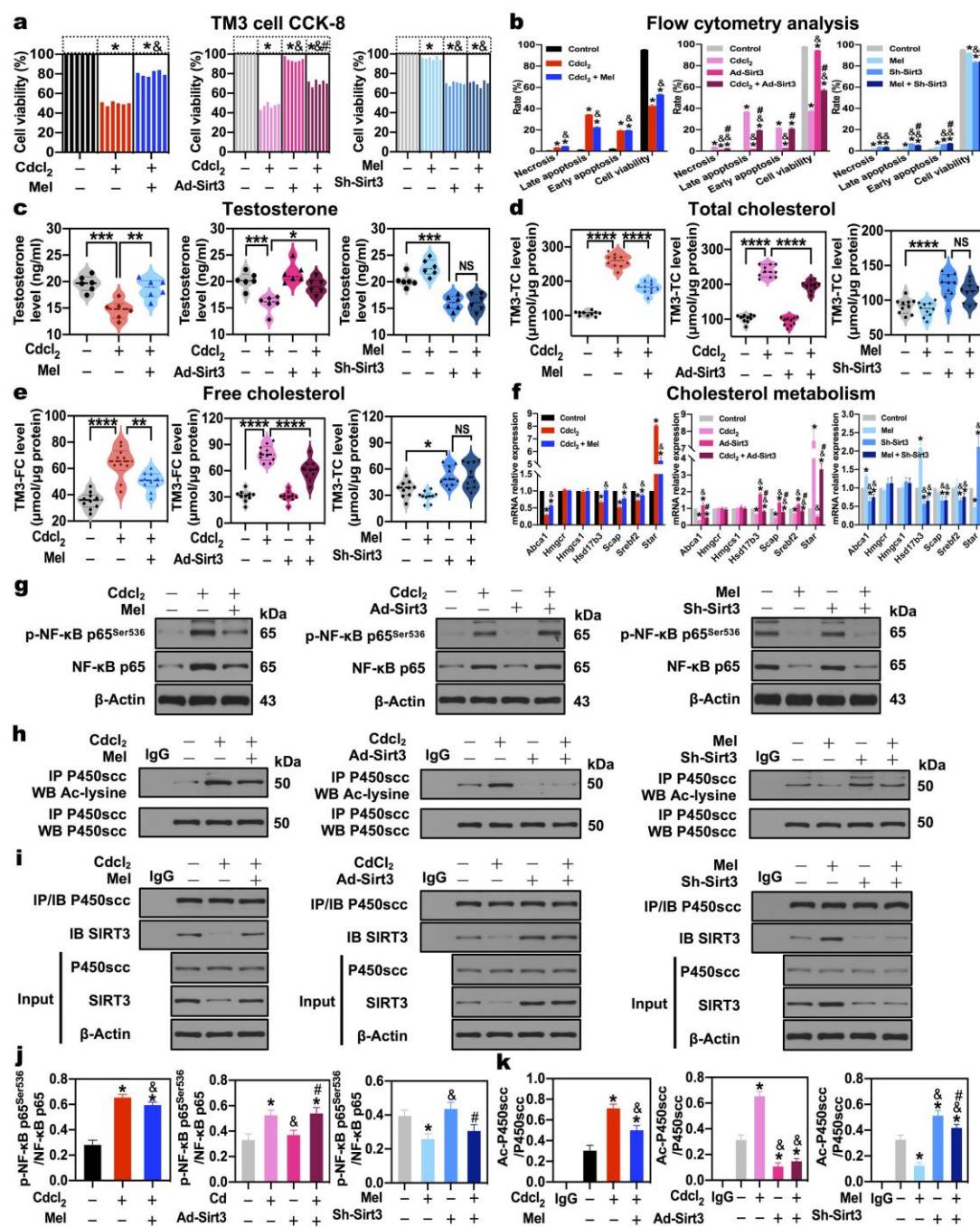
379

380 Subsequently, the level of testosterone, total cholesterol (TC), and free cholesterol
381 (FC) in TM3 cells were scrutinized. Consistent with in vivo results, Cd decreased
382 testosterone level and increased TC and FC level, which were reversed by Mel (**Fig.**
383 **4c₁, 4d₁, 4e₁**). Likewise, the overexpression of Sirt3 defended testosterone, TC, and
384 FC disruption induced by Cd in TM3 cells (**Fig. 4c₂, 4d₂, 4e₂**). The knockdown of
385 Sirt3 led to analogous testosterone, TC, and FC disruption as Cd, which did not be
386 salvaged by Mel (**Fig. 4c₃, 4d₃, 4e₃**), indicating that Mel-mediated testosterone and
387 cholesterol protection was dependent on Sirt3 in TM3 cells.

388 Testosterone synthesis is derived from the cholesterol mechanism. To further
389 clarify, in Leydig cell, the specific effect of SIRT3 on regulating testosterone and
390 cholesterol, we detected cholesterol metabolism markers, including Hmgcr and
391 Hmgcs1 (cholesterol biosynthesis markers), Abca1 (cholesterol efflux marker), Star
392 (mitochondrial cholesterol transporter), Scap/Srebf2 (cholesterol metabolism
393 regulators), Hsd17b3 (testosterone biosynthesis marker). Both Hmgcr and Hmgcs1
394 were not disturbed by Cd, Mel, Ad-Sirt3 and sh-Sirt3, suggesting that Sirt3 did not
395 correlate with cholesterol biosynthesis (**Fig. 4f**). Cd significantly diminished the
396 expression of Abca1 and elevated the expression of Star, implying that Cd enhanced
397 cholesterol influx; Cd reduced the expression of Scap, Srebf2 and Hsd17b3, implying
398 that Cd impaired the regulation of cholesterol metabolism; however, Mel rescued
399 cholesterol metabolism disruption by reversing the expression of Abca1, Star, Scap,
400 Srebf2 and Hsd17b3 (**Fig. 4f₁**). In parallel, the overexpression of Sirt3 protected from
401 cholesterol metabolism disruption induced by Cd in TM3 cells (**Fig. 4f₂**). Instead, the
402 knockdown of Sirt3 induced cholesterol metabolism disruption similar to Cd, which
403 did not be rescued by Mel (**Fig. 4f₃**), indicating that Mel-mediated cholesterol
404 metabolism was dependent on Sirt3 in TM3 cells.

405 In vivo study, we found Mel might regulate NF- κ B p65^{ser536} phosphorylation to
406 mediate SIRT3 with P450scc deacetylation in Cd-induced testicular cholesterol
407 metabolism disruption. Herein, NF- κ B p65^{ser536} phosphorylation and P450scc
408 deacetylation were investigated in TM3 mouse Leydig cells. As expected, Cd
409 significantly stimulated NF- κ B p65^{Ser536} phosphorylation, which was weakened by
410 Mel in TM3 cells (**Fig. 4g₁, 4j₁**). Nonetheless, the overexpression of Sirt3 showed no
411 difference in NF- κ B p65^{Ser536} phosphorylation compared with the control group and
412 failed to reverse Cd-stimulated NF- κ B p65^{Ser536} phosphorylation (**Fig. 4g₂, 4j₂**).

413 Despite that Mel indeed inhibited NF- κ B p65^{Ser536} phosphorylation, the knockdown of
 414 Sirt3 didn't influence NF- κ B p65^{Ser536} phosphorylation (Fig. 4g₃, 4j₃). Results
 415 suggested that, in TM3 cells, Mel regulated NF- κ B p65^{Ser536} phosphorylation, which
 416 was not dominated by Sirt3.



417
 418 **Fig. 4 Mel-mediated cholesterol metabolism protection is dependent on Sirt3,**
 419 **which is the upstream regulator of P450scc deacetylation, but not of NF- κ B**
 420 **p65^{Ser536} phosphorylation in TM3 mouse Leydig cells. a** Cell viability analysis by
 421 CCK-8 assay. **b** Necrosis, late apoptosis, early apoptosis, cell viability analysis by

422 flow cytometry. **c** Testosterone level in TM3 cells supernatants. **d** Total cholesterol
423 level in TM3 cells. **e** Free cholesterol level in TM3 cells. **f** mRNA expression levels
424 of cholesterol metabolism markers of *Abca1*, *Hmger*, *Hmgcs1*, *Hsd17b1*, *Scap*,
425 *Srebf2*, *Star* in TM3 cells. **g, j** A representative immunoblot and quantification
426 analysis of NF- κ B p65^{Ser536} phosphorylation in TM3 cells. **h, k** IP of P450scc and
427 Ac-lysine, and quantification analysis of P450scc acetylation level in TM3 cells. **i**
428 Co-IP of P450scc and SIRT3 in TM3 cells. NS, $p > 0.05$; * $p < 0.05$; ** $p < 0.01$;
429 *** $p < 0.001$; **** $p < 0.0001$ in **a-e**. * $p < 0.05$ compared with the first group
430 (control). & $p < 0.05$ compared with the second group, # $p < 0.05$ compared with the
431 third group in each panel of **f, j, k**.

432

433 Subsequently, we found that Cd promoted P450scc acetylation, which was rescued
434 by Mel in TM3 cells (**Fig. 4h₁, 4k₁**). Noticeably, the overexpression of Sirt3 lessened
435 P450scc acetylation and reversed Cd-induced P450scc acetylation (**Fig. 4h₂, 4k₂**); the
436 knockdown of Sirt3 dramatically boosted P450scc acetylation and reversed
437 Mel-induced P450scc deacetylation (**Fig. 4h₃, 4k₃**), indicating that Mel regulated
438 P450scc deacetylation, which was dominated by Sirt3.

439 To further confirm the interaction between SIRT3 and P450scc, co-IP experiment
440 was performed in TM3 cells. Consequently, Cd, Mel, Ad-Sirt3 or sh-Sirt3 had no
441 significant effect on the protein expression of P450scc; strikingly, Cd reduced the
442 interaction of SIRT3 and P450scc, which was reversed by Mel (**Fig. 4i₁**). The
443 overexpression of Sirt3 contributed to the interaction of SIRT3 and P450scc and
444 rescued Cd-induced disruption (**Fig. 4i₂**); the knockdown of Sirt3 impaired the
445 interaction of SIRT3 and P450scc, which failed to be reversed by Mel (**Fig. 4i₃**),
446 displaying that SIRT3 directly interacted with P450scc to mediate P450scc
447 deacetylation.

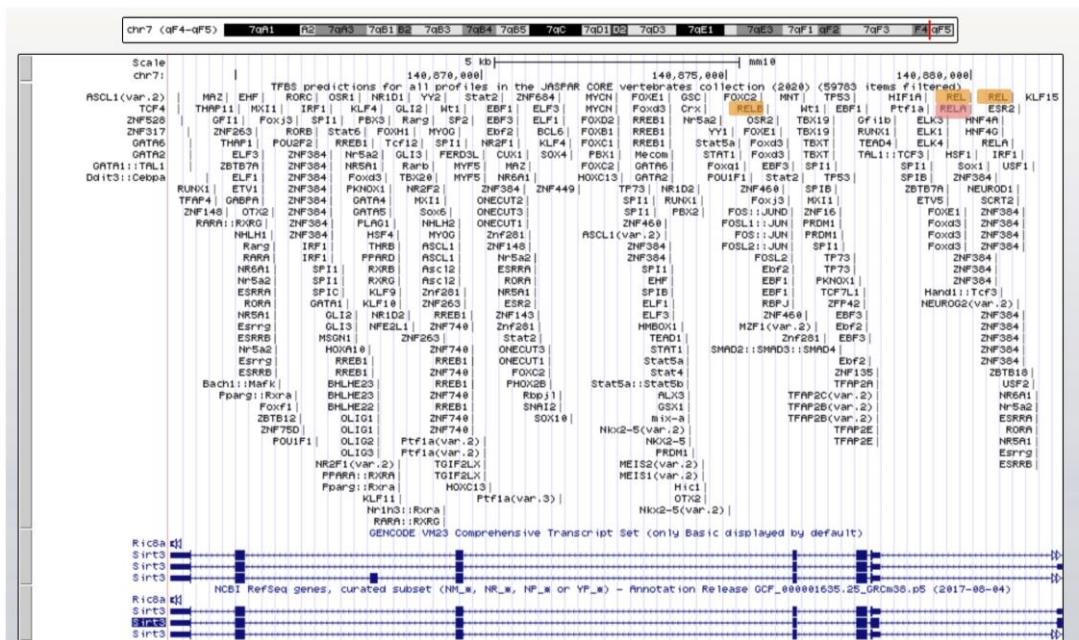
448 Above results demonstrated that Mel-mediated cholesterol metabolism protection
449 was dependent on Sirt3, which was the upstream regulator of P450scc deacetylation,
450 but not of NF- κ B p65^{Ser536} phosphorylation in TM3 mouse Leydig cells.

451

452 **NF- κ B, as the upstream transcription factor, represses Sirt3 transcription by**
453 **binding to TFBS1 and TFBS2 of Sirt3 promoter in TM3 mouse Leydig cells**

454 Due to three facts as follows: (1) Mel-mediated cholesterol metabolism was
455 dependent on Sirt3 in TM3 cells; (2) Mel regulated NF- κ B p65^{Ser536} phosphorylation
456 in TM3 cells; (3) Sirt3 was not the upstream regulator of NF- κ B p65^{Ser536}
457 phosphorylation in TM3 cells, we speculated that Sirt3 was the downstream of NF- κ B
458 p65 in Mel-mediated cholesterol metabolism. Phosphorylation of p65 at Ser536 led to
459 nuclear localization of the transcriptionally active complex, and NF- κ B mediated
460 transactivation of several downstream genes (Mai et al., 2020). To test the conjecture,
461 we examined the cytoplasmic and nuclear localization of NF- κ B molecules in Cd
462 with or without Mel-treated TM3 cells. As shown in **Fig. 5a**, Cd resulted in slightly
463 increased nuclear translocation of c-Rel and robust nuclear translocation of p50, p65
464 and RelB, which could be ameliorated by Mel, suggesting that phosphorylation
465 of p65 at Ser536 indeed stimulated nuclear translocation of NF- κ B molecules in TM3
466 cells.

467 Querying UCSC Genome browser (<https://genome.ucsc.edu>) with expanded
468 JASPAR database, NF- κ B subunits, including RELA (also named p65), RELB and
469 REL, were predicted as putative upstream transcription factors for mouse Sirt3 gene
470 (**Supplementary Fig. 3**). To confirm the interaction of NF- κ B and Sirt3 promoter
471 region in TM3 mouse Leydig cells, chromatin immunoprecipitation (ChIP) assay was
472 performed as described in **Fig. 5b**. Protein-DNA complexes were extracted from TM3
473 cells and immunoprecipitated using anti-NF- κ B (p50, p65, RelB respectively) with
474 precipitation of normal IgG as the negative control. DNA fragments were amplified
475 with primers specific for the Sirt3 promoter sequence. ChIP-qPCR assay manifested a
476 physiological binding of p65, RelB and especially p50 to the Sirt3 promoter in TM3
477 cells (**Fig. 5c**).



478

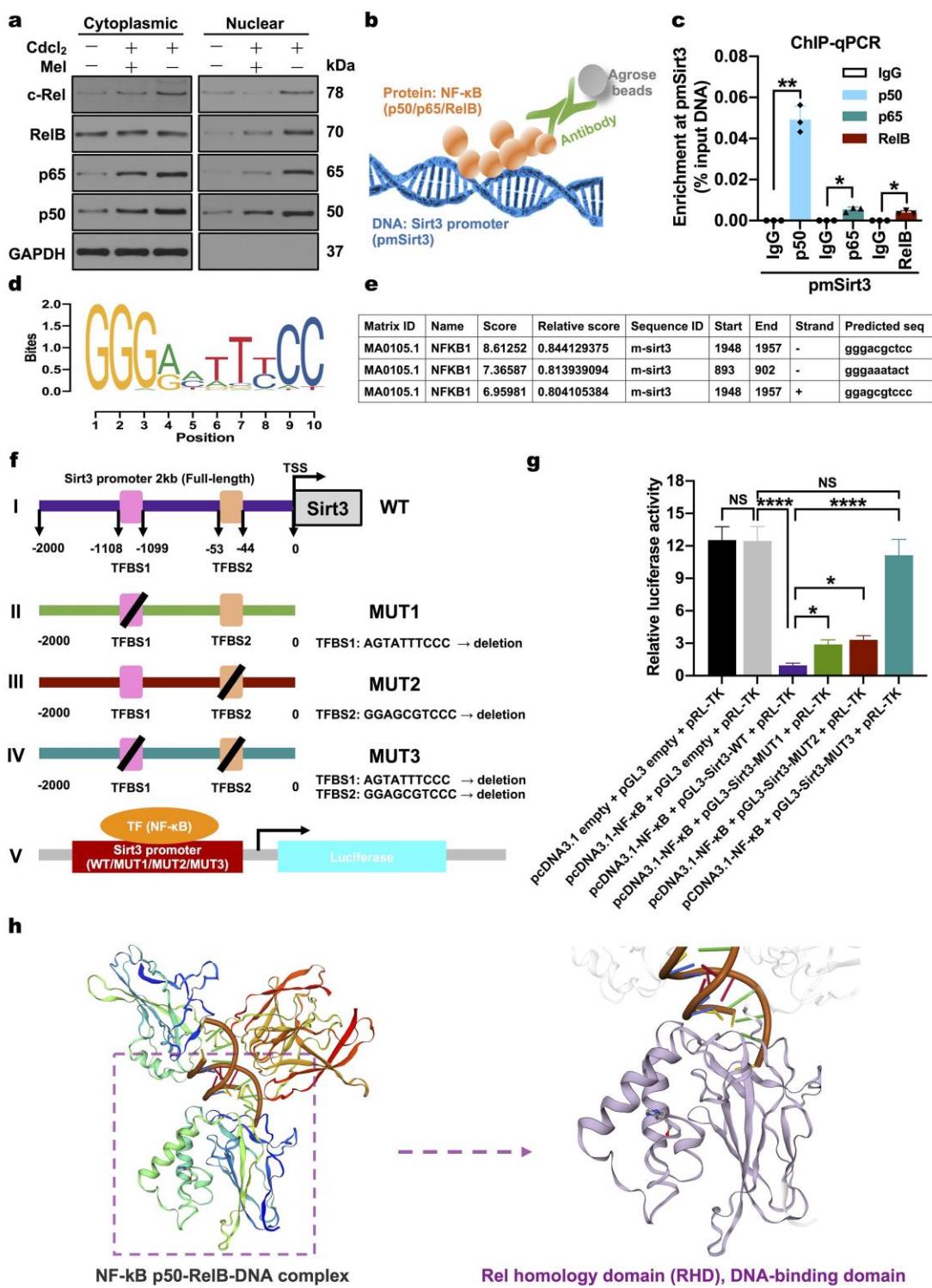
479 **Supplementary Fig. 3 Prediction of upstream transcription factors for mouse**
480 **Sirt3 gene.** By Querying UCSC Genome browser (<https://genome.ucsc.edu>) with
481 expanded JASPAR database, NF-κB subunits, including RELA (also named p65),
482 RELB and REL, were predicted as putative upstream transcription factors for mouse
483 Sirt3 gene.

484

485 To further substantiate the transcriptional regulation of Sirt3 by NF-κB and
486 recognize the specific transcription factor (TF)-DNA binding sequences, we identified
487 the consensus sequence of mouse NF-κB p50 by analyzing structural profiles of
488 transcription factor binding sites (TFBSs) from JASPAR and UniPROBE databases.
489 In fact, the consensus sequence reflected p50 TF-DNA binding preferences, which
490 were represented by a position weight matrix (PWM) and visualized as motif logo
491 (**Fig. 5d**). Based on the selected matrix models, three fragments in full-length mouse
492 Sirt3 promoter (2kb) were predicted as TFBSs with a relative profile score of greater
493 than 0.8 by the JASPAR database (**Fig. 5e**). Given that gggacgctcc on reverse (minus)
494 DNA strand (1948-1957) overlapped with ggagcgtccc on forward (plus) DNA strand
495 (1948-1957), there were actually two fragments as putative TFBS1 and TFBS2. In the
496 full-length (FL) Sirt3 promoter from -2000 bp to 0 transcription start site (TSS),
497 TFBS1 (AGTATTTCCC) locates in -1108 to -1099, and TFBS2 (GGAGCGTCCC)
498 locates in -53 to -44 (**Fig. 5f(I)**). Wild-type (WT) or TFBS1/TFBS2 mutant Sirt3
499 promoter (MUT1/MUT2/MUT3) luciferase reporter vector was transiently

500 co-transfected with NF-κB p50 overexpression plasmid (pcDNA3.1-NF- κB)
501 into HEK 293T cells (**Fig. 5f**), and relative luciferase activity was measured as a
502 function of NF-κB p50-dependent Sirt3 transcription. Conspicuously, NF-κB p50
503 with WT-Sirt3 promoter inhibited the transcription, while NF-κB p50 with
504 TFBS1/TFBS2 mutant Sirt3 promoter (MUT1/MUT2/MUT3) attenuated the
505 transcriptional suppression; especially, MUT3 (simultaneous deletion of TFBS1 and
506 TFBS2) had no difference in transcription compared with empty pGL3-promoter
507 vector (**Fig. 5g**). Results demonstrated that NF-κB p50 could suppress Sirt3
508 transcription by binding to TFBS1 and TFBS2 in Sirt3 promoter (**Fig. 5h**), which
509 responded to the decreased protein expression of SIRT3 in Cd-treated testis and TM3
510 cells.

511 Above stringing shreds of evidence supported the inference that, **in Leydig cells, phosphorylation of NF-κB p65^{Ser536} stimulated the nuclear translocation of NF-κB molecules (subunits p50, p65, RelB), which bound to the promoter of Sirt3; NF-κB (particularly p50) repressed Sirt3 transcription by binding to TFBS1 and TFBS2 in Sirt3 promoter; then, the deficiency of SIRT3 disturbed cholesterol metabolism and testosterone synthesis by impairing P450scc deacetylation in male subfertility model induced by Cd.**



518

519 **Fig. 5 NF-κB, as upstream transcription factor, represses Sirt3 transcription by**
 520 **binding to TFBS1 and TFBS2 of Sirt3 promoter in TM3 mouse Leydig cells. a**
 521 **Western blot analysis of the cytoplasmic and nuclear localization of NF-κB molecules**
 522 **p50, p65, RelB, c-Rel. b ChIP assay model for NF-κB molecules p50, p65, RelB and**
 523 **Sirt3 promoter. c ChIP assay of p50, p65 and RelB, especially p50, binding to Sirt3**
 524 **promoter in TM3 cells. d Position weight matrix (PWM) and motif logo of NF-κB1**

525 p50 TF-DNA binding preferences. **e** Predicted transcription factor binding sites
526 (TFBSs) with a relative profile score of greater than 0.8 by JASPAR database. **f** A
527 diagram showing the relative positions of full-length (FL) and predicted TFBSs
528 of Sirt3 promoter reporters and wild-type (WT) and mutant promoter constructions
529 followed by dual-luciferase reporter assay. I the relative positions of FL and predicted
530 TFBSs of Sirt3 promoter (WT); II deletion of TFBS1 (-1108 to -1099,
531 AGTATTTCCC) for constructing mutant Sirt3 promoter 1 (MUT1); III deletion of
532 TFBS2 (-53 to -44, GGAGCGTCCC) for constructing mutant Sirt3 promoter 2
533 (MUT2); IV simultaneous deletion of TFBS1 and TFBS2 for constructing mutant
534 Sirt3 promoter 3 (MUT3); V a schematic of dual-luciferase reporter assay for
535 detecting the interaction between NF- κ B1 p50 and Sirt3 promoter
536 (WT/MUT1/MUT2/MUT3). **g** Dual-luciferase reporter assay results showing NF- κ B1
537 p50-dependent suppression of Sirt3 transcription by simultaneously binding to TFBS1
538 and TFBS2 in Sirt3 promoter in HEK293T cells. **h** 3D structure of NF- κ B
539 p50-RelB-DNA complex from Swiss-Model. Left panel, NF- κ B p50-RelB-DNA
540 complex; right panel, Rel homology domain (RHD), DNA-binding domain. NS, $p >$
541 0.05; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

542

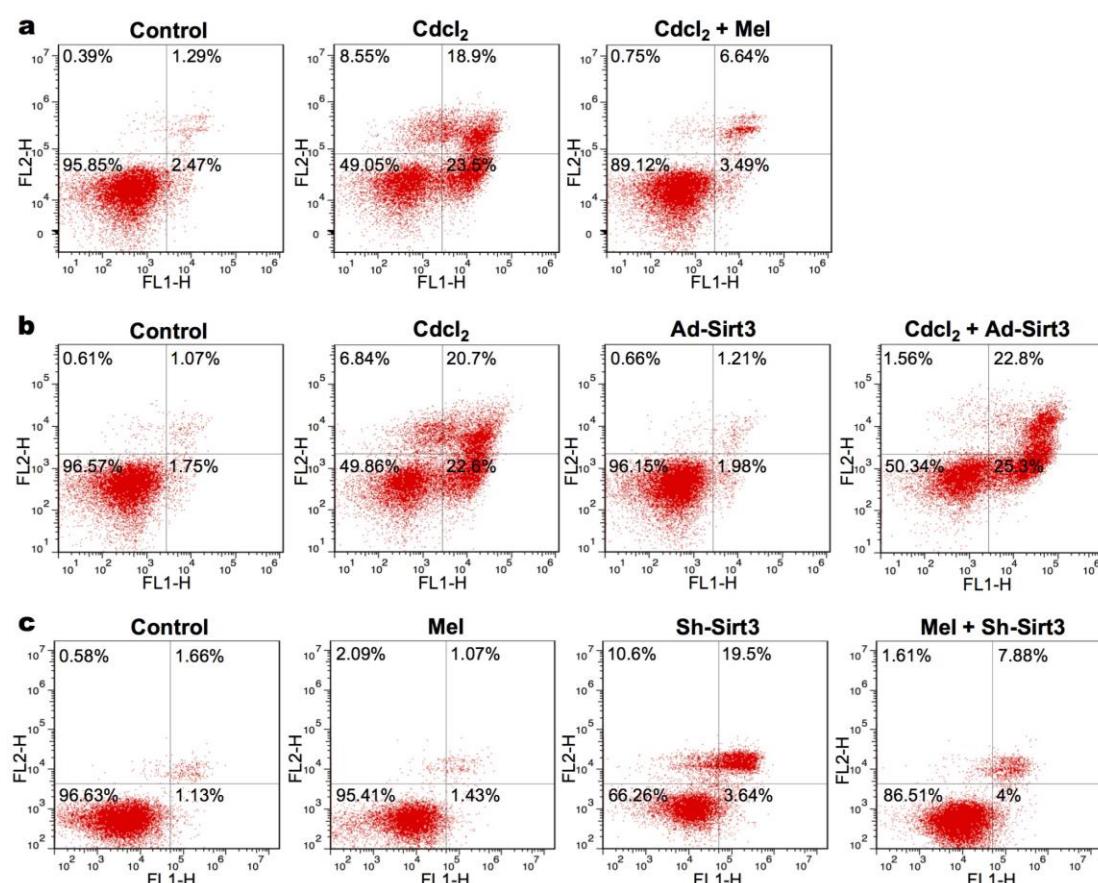
543 **Overexpression and knockdown of SIRT3 blunts and enhances PDLIM1 and**
544 **SOD2 acetylation in Mel-mediated TM4 mouse Sertoli cell protection**

545 Except for the role of Sirt3 in cholesterol metabolism of Leydig cell, our *in vivo* study
546 displayed that Mel might regulate SOD2 deacetylation and PDLIM1 by SIRT3 in
547 Cd-induced the disruption of cytoskeleton assembly, including basal ES
548 (Sertoli-Sertoli cell interface), apical ES (Sertoli-elongating spermatid interface) and
549 microtubule-based manchette. Considering that the disturbed cytoskeleton was related
550 to the Sertoli cell, and PDLIM1 in Sertoli cell could regulate ES (Liu et al., 2016), we
551 hypothesized that SIRT3 orchestrated cytoskeleton assembly by PDLIM1 and SOD2
552 deacetylation in Sertoli cell.

553 To verify the hypothesis, Sirt3 overexpression and knockdown models in TM4
554 mouse Sertoli cells (TM4 cells) were established. The efficiency of Sirt3
555 overexpression and knockdown were examined (**supplementary Fig. 1d**). IC50 of Cd
556 for TM4 cells—12 μ g/ml by the concentration gradient method (Wang et al., 2020)
557 was exploited in subsequent experiments. Similar to TM3 cells, CCK-8 assay

558 indicated that both Mel and overexpression of Sirt3 rescued TM4 cell viability
559 reduction induced by Cd (**Fig. 6a_{1,2}**); the knockdown of Sirt3 decreased cell viability,
560 but Mel failed to rescue sh-Sirt3-induced cell viability reduction (**Fig. 6a₃**), hinting
561 that Sirt3 dominated the protection process. Scilicet Mel-mediated cell viability
562 protection was dependent on Sirt3 in TM4 cells.

563 Flow cytometry analysis detected the cell viability and apoptosis. Cd significantly
564 decreased the percentage of viable cells and increased the percentage of necrotic,
565 early and late apoptotic cells; whereas, Mel reversed Cd-triggered TM4 cell apoptosis
566 (**Supplementary Fig. 4a; Fig. 6b₁**). The overexpression of Sirt3 rescued necrosis but
567 not apoptosis in Cd-treated TM4 cells; the knockdown of Sirt3 facilitated apoptosis,
568 which couldn't be attenuated by Mel in TM4 cells (**Supplementary Fig. 4b, 4c; Fig.**
569 **6b_{2,3}**), proving that Mel-mediated cell apoptosis protection was dependent on Sirt3 in
570 TM4 cells.



571
572 **Supplementary Fig. 4 Mel-mediated cell apoptosis protection is dependent on**
573 **Sirt3 in TM4 cells.** Representative flow cytometry analysis: **a** Mel reverses
574 Cd-triggered TM4 cell apoptosis. **b** The overexpression of Sirt3 rescues necrosis but

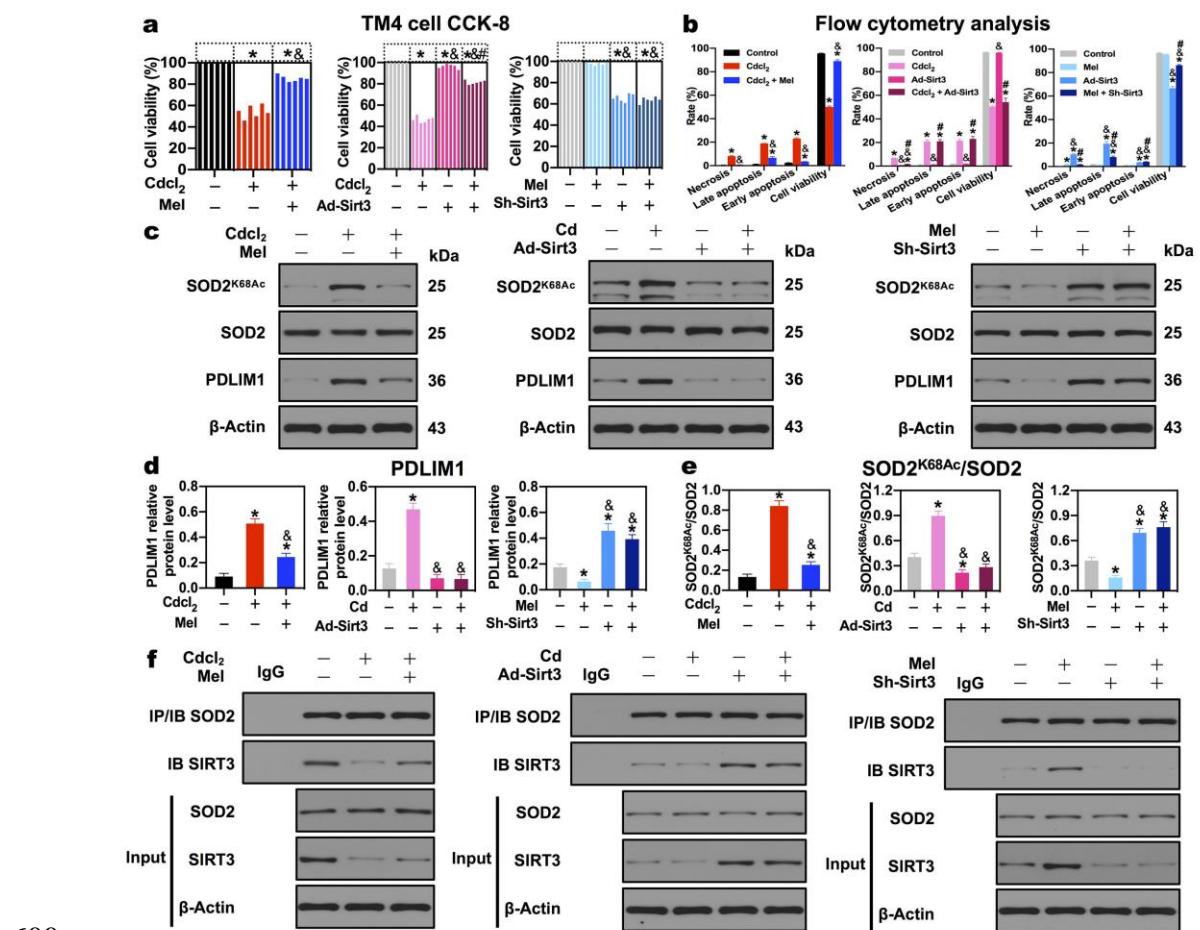
575 not apoptosis in Cd-treated TM4 cells. **c** Mel fails to rescue sh-Sirt3-induced
576 apoptosis in TM4 cells.

577

578 To address whether SIRT3 regulated SOD2 deacetylation and PDLIM1 in Sertoli
579 cells, we assessed the PDLIM1 protein levels and SOD2 acetylation when Sirt3 was
580 overexpressed or restrained. PDLIM1, as negative cytoskeleton regulator, was ignited
581 in Cd-treated TM4 cells; whereas, Mel extinguished the augment of PDLIM1 protein
582 level induced by Cd (**Fig. 6c₁, 6d₁**). Likewise, the overexpression of Sirt3 reversed the
583 augment of PDLIM1 protein level induced by Cd (**Fig. 6c₂, 6d₂**). Prominently, the
584 knockdown of Sirt3 provoked the protein expression of PDLIM1, which couldn't be
585 salvaged by Mel (**Fig. 6c₃, 6d₃**), intimating that Sirt3 was indispensable for the
586 regulation of PDLIM1 in Mel-mediated TM4 cells protection.

587 In terms of SOD2 acetylation, both Mel and the overexpression of Sirt3 parallelly
588 reversed Cd-induced SOD2 acetylation (**Fig. 6c_{1,2}, 6e_{1,2}**). Whereas, the knockdown of
589 Sirt3 impede SOD2 deacetylation and reversed Mel-mediated SOD2 deacetylation
590 (**Fig. 6c₃, 6e₃**), suggesting that Sirt3 was imperative for SOD2 deacetylation in
591 Mel-mediated TM4 cells protection.

592 To further confirm the interaction between SIRT3 and SOD2, co-IP experiment
593 was performed in TM4 cells. Consequently, Cd barricaded the interaction of SIRT3
594 and SOD2, which was reversed by Mel and the overexpression of Sirt3 (**Fig. 6f₁, 6f₂**).
595 The overexpression of Sirt3 significantly facilitated the interaction of SIRT3 and
596 SOD2 (**Fig. 6f₂**); whereas, the knockdown of Sirt3 impaired the interaction of SIRT3
597 and SOD2, which failed to be reversed by Mel (**Fig. 6f₃**). Taken together, these data
598 approved that SIRT3 mediated SOD2 deacetylation by directly interacting with SOD2
599 in TM4 cells.



601 **Fig. 6 Overexpression and knockdown of SIRT3 blunts and enhances PDLIM1**
602 **and SOD2 acetylation in Mel-mediated TM4 mouse Sertoli cell protection. a** Cell
603 viability analysis by CCK-8 assay. **b** Necrosis, late apoptosis, early apoptosis, cell
604 viability analysis by flow cytometry. **c** A representative immunoblot of cytoskeleton
605 negative regulator PDLIM1, SOD2 and SOD2^{K68Ac} in TM4 cells. **d** Quantification
606 analysis of cytoskeleton negative regulator PDLIM1 protein level in TM4 cells. **e**
607 Quantification analysis of SOD2 acetylation level in TM4 cells. **f** Co-IP of SOD2 and
608 SIRT3 in TM4 cells. *p < 0.05 compared with the first group (control). &p < 0.05
609 compared with the second group, #p < 0.05 compared with the third group in each
610 panel of **a, b, d, e**.

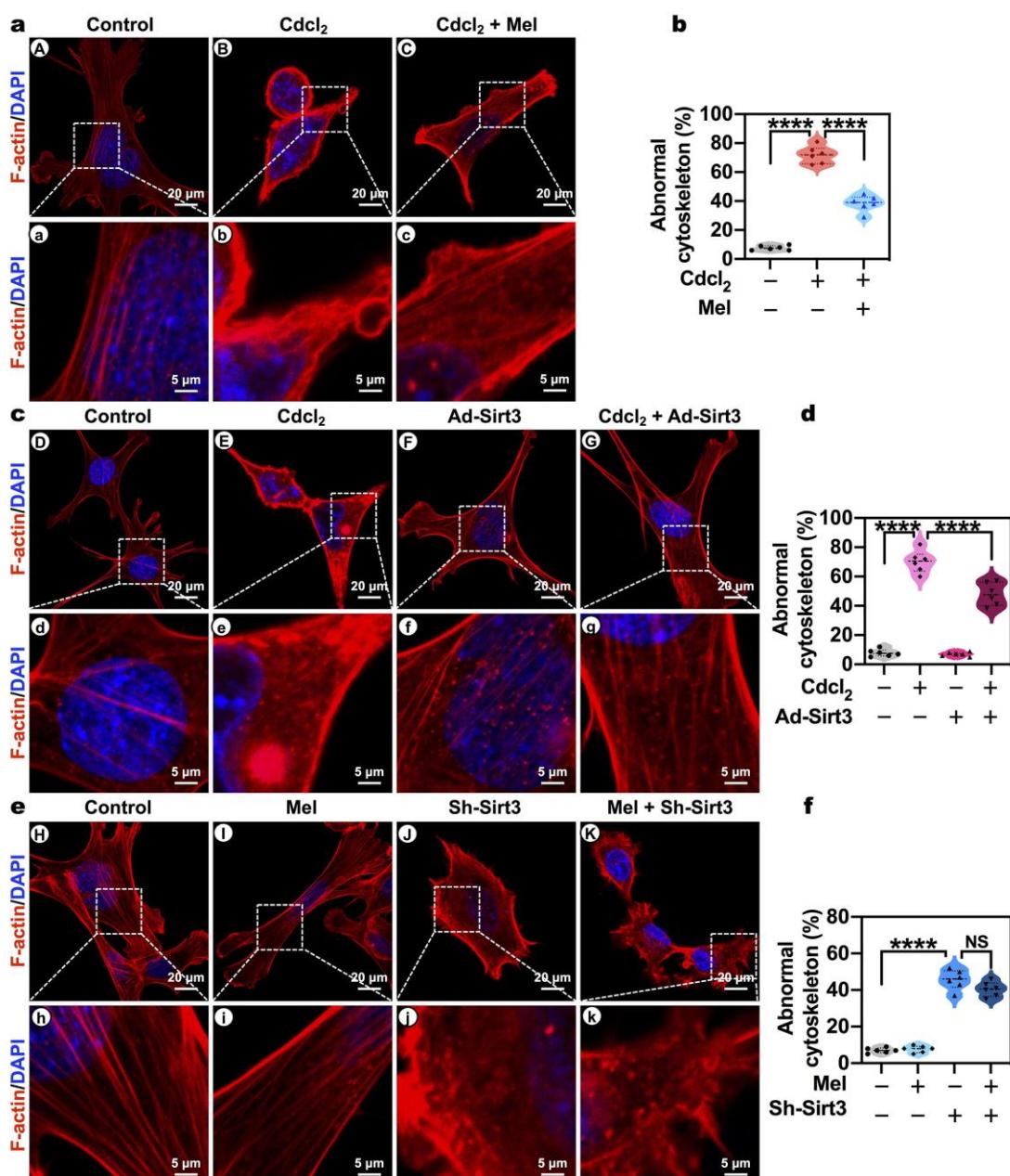
611
612 **Overexpression and knockdown of Sirt3 protects and impairs the**
613 **F-actin-containing cytoskeleton assembly in Mel-mediated TM4 mouse Sertoli**
614 **cells protection**

615 F-actin, as a pivotal component of the cytoskeleton, was proposed to be associated
616 with PDLIM1 (also named CLP36) twenty years ago (Bauer et al., 2000). A recent

617 study has demonstrated that PDLIM1 competitively binds to ACTN4, which
618 disaffiliates from F-actin, hampering F-actin organization (Huang et al., 2020).
619 Critically, PDLIM1 was responsible for ES assembly in Sertoli cell (Liu et al., 2016).
620 It's rational to deem that SIRT3 regulated PDLIM1 and SOD2 deacetylation to
621 operate cytoskeleton assembly. To authenticate the role of SIRT3 in cytoskeleton
622 assembly, we monitored the F-actin distribution by using rhodamine-phalloidin (red)
623 in Sirt3 overexpression and knockdown models of TM4 mouse Sertoli cells.

624 As depicted in **Fig. 7a**, in normal TM4 cells, a highly ordered F-actin network
625 wove cytoskeleton to formulate the intact cell; Cd collapsed the F-actin organization,
626 yet Mel ameliorated the F-actin disruption induced by Cd. The percentages of
627 abnormal cytoskeletal Sertoli cells per 200 cells were calculated in 6 independent
628 experiments for each group. Cd increased the rate of abnormal Sertoli cells with
629 severely F-actin disruption, which was rescued by Mel (**Fig. 7b**), implying that Mel
630 protected the cytoskeleton assembly in Sertoli cells. The group with Sirt3
631 overexpression presented a well-organized F-actin structure and salvaged the F-actin
632 disruption induced by Cd (**Fig. 7c**). Meanwhile, the overexpression of Sirt3 decreased
633 the rate of abnormal F-actin Sertoli cells compared with Cd (**Fig. 7d**), suggesting that
634 Sirt3 participated in the Mel-mediated cytoskeleton assembly. Furthermore, the
635 knockdown of Sirt3 apparently disturbed the F-actin network, which couldn't be
636 rescued by Mel (**Fig. 7e, 7f**), revealing that Mel-mediated cytoskeleton assembly
637 protection was dependent on Sirt3 in TM4 cells.

638 Overall, these results indicated that **SIRT3, in Sertoli cells, regulated SOD2**
639 **deacetylation and negative cytoskeleton protein PDLIM1 to orchestrate**
640 **F-actin-containing cytoskeleton assembly, which accounted for basal ES (Sertoli**
641 **cell-Sertoli cell interface) and apical ES (Sertoli cell-spermatid interface), even**
642 **microtubule-based manchette in elongating spermatids.**



643

644 **Fig. 7 Overexpression and knockdown of Sirt3 protects and impairs the**
 645 **F-actin-containing cytoskeleton assembly in Mel-mediated TM4 mouse Sertoli**
 646 **cells protection. a, c, e Representative confocal microscope imaging of F-actin (a**
 647 **marker of cytoskeleton) in TM4 cells by the immunofluorescent analysis of phalloidin.**
 648 **Areas (A-K) in the upper panels (Scale bar, 20 μm) have been shown for further**
 649 **details (a-k) in the low panels (Scale bar, 5 μm). (A-C) Mel protects the cytoskeleton**
 650 **assembly in TM4 cells; (D-G) Sirt3 overexpression presents well-organized F-actin**
 651 **structure, and salvages the F-actin disruption induced by Cd; (H-K) the knockdown of**
 652 **Sirt3 apparently disturbs the F-actin network, which fails to be rescued by Mel. b, d, f**

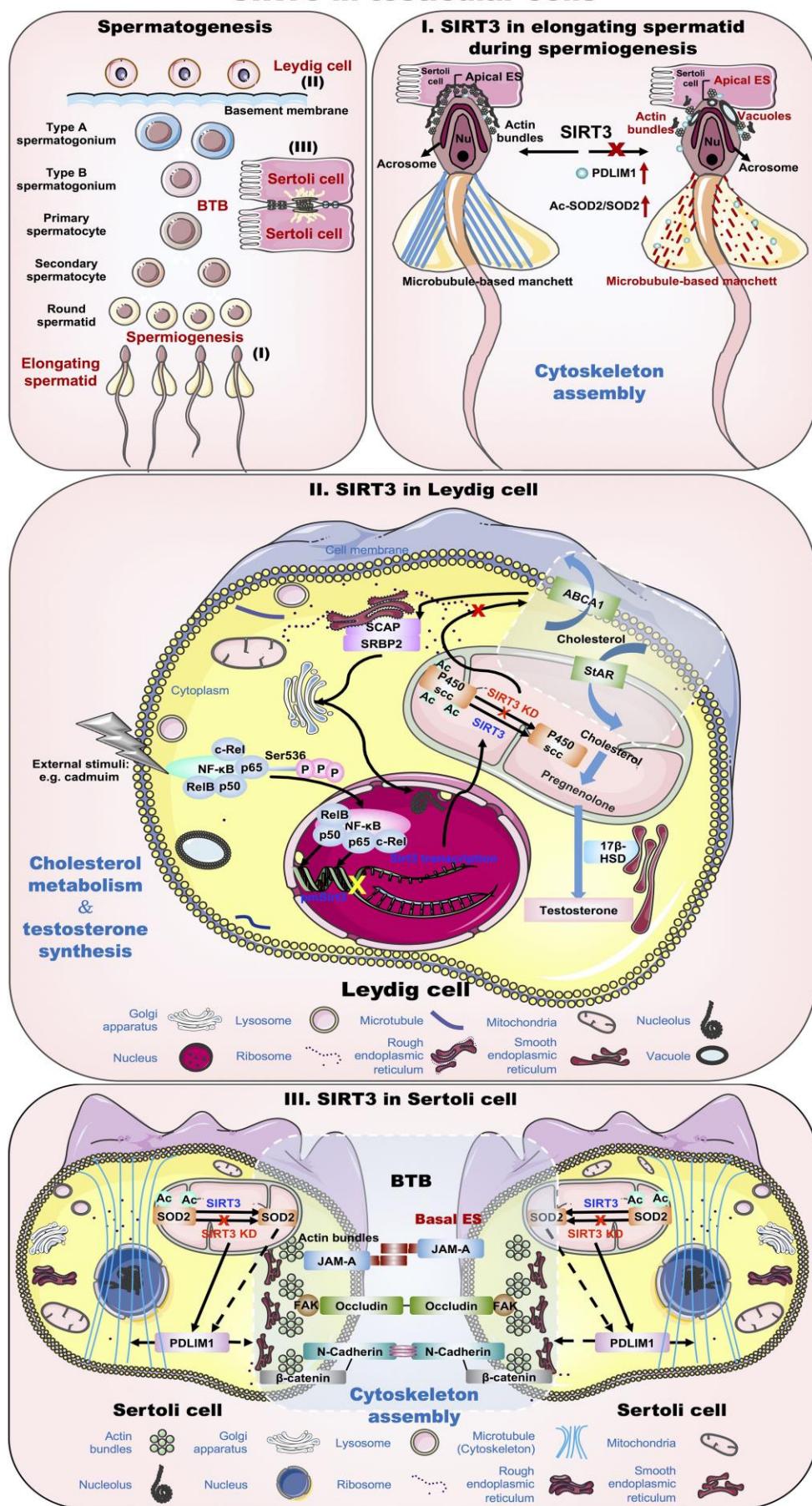
653 The percentages of TM4 cells with abnormal F-actin-containing cytoskeleton per 200
654 cells. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

655

656 Discussion

657 Mammalian spermatogenesis requires highly-coordinated Sertoli cell and Leydig cell
658 for germ cell differentiation through mitosis, meiosis, and spermiogenesis (**Fig. 8**). The
659 absence of this homeostasis impairs sperm count and motility, eliciting male infertility
660 (Garolla et al., 2013; Wang et al., 2019). Mammalian spermatogenesis requires
661 highly-coordinated Sertoli cells and Leydig cells for germ cells differentiation
662 through mitosis, meiosis, and spermiogenesis. Here, we elucidate that SIRT3 exerts a
663 dual role in testis: regulating cholesterol metabolism and cytoskeleton assembly
664 during spermatogenesis. Mechanistically, in Leydig cells, phosphorylation of NF- κ B
665 p65^{Ser536} stimulates the nuclear translocation of NF- κ B molecules (subunits p50, p65,
666 RelB), which bind to the promoter of Sirt3; particularly, p50 represses Sirt3
667 transcription by simultaneously binding to TFBS1 and TFBS2 in Sirt3 promoter; then,
668 inhibited SIRT3 disturbs cholesterol metabolism markers ABCA1, StAR and P450scc
669 deacetylation (**Fig. 8(II)**). Whereas, in Sertoli cells, SIRT3 regulates SOD2
670 deacetylation and negative cytoskeleton protein PDLM1 to orchestrate
671 F-actin-containing cytoskeleton assembly, including microtubule, basal ES (Sertoli
672 cell-Sertoli cell interface) in blood-testis barrier (BTB) via JAM-A, Occluding,
673 N-Cadherin, β -catenin (**Fig. 8(III)**). SIRT3 mediates cytoskeleton assembly of
674 elongating spermatids, including apical ES (Sertoli cell-spermatid interface), even
675 microtubule-based manchette during spermiogenesis (**Fig. 8(I)**). To sum up,
676 NF- κ B-repressed SIRT3 acts directly on cholesterol metabolism of Leydig cells and
677 cytoskeleton assembly of Sertoli cells via P450scc/SOD2 deacetylation to regulate
678 sperm differentiation, influencing spermatogenesis, even male fertility.

SIRT3 in testicular cells



680 **Fig. 8 Summary diagram for the role of SIRT3 in testicular cells.** Mammalian
681 spermatogenesis requires highly-coordinated Sertoli cells and Leydig cells for germ
682 cells differentiation through mitosis, meiosis, and spermiogenesis. Here, we elucidate
683 that SIRT3 exerts a dual role in testis: regulating cholesterol metabolism and
684 cytoskeleton assembly during spermatogenesis. Mechanistically, in Leydig cells,
685 phosphorylation of NF- κ B p65^{Ser536} stimulates the nuclear translocation of NF- κ B
686 molecules (subunits p50, p65, RelB), which bind to the promoter of Sirt3; particularly,
687 p50 represses Sirt3 transcription by simultaneously binding to TFBS1 and TFBS2 in
688 Sirt3 promoter; then, inhibited SIRT3 disturbs cholesterol metabolism markers
689 ABCA1, StAR and P450scc deacetylation (**II**). Whereas, in Sertoli cells, SIRT3
690 regulates SOD2 deacetylation and negative cytoskeleton protein PDLIM1 to
691 orchestrate F-actin-containing cytoskeleton assembly, including microtubule, basal
692 ES (Sertoli cell-Sertoli cell interface) in blood-testis barrier (BTB) via JAM-A,
693 Occluding, N-Cadherin, β -catenin (**III**). SIRT3 mediates cytoskeleton assembly of
694 elongating spermatids, including apical ES (Sertoli cell-spermatid interface), even
695 microtubule-based manchette during spermiogenesis (**I**). To sum up, NF- κ B-repressed
696 SIRT3 acts directly on cholesterol metabolism of Leydig cells and cytoskeleton
697 assembly of Sertoli cells via P450scc/SOD2 deacetylation to regulate sperm
698 differentiation, influencing spermatogenesis, even male fertility.

699

700 Cholesterol metabolism is prerequisite for testosterone biosynthesis in testicular
701 Leydig cells (Gao et al., 2018). In vivo study, Mel, as SIRT3 activator (Zhai et al.,
702 2017; Zhang et al., 2017), salvaged male reproductive function injury—sperm count
703 and motility reduction, testosterone reduction and cholesterol augment of serum in
704 Cd-induced subfertility rats model (**Fig. 1a-f**). Cholesterol and testosterone level
705 mirror a dynamic balance of cholesterol metabolism: biosynthesis, uptake, export and
706 esterification (Luo et al., 2020). Our observations about cholesterol metabolism
707 markers that Cd-induced inhibition of Abca1 (cholesterol efflux marker), Scap/Srebf2
708 (cholesterol metabolism regulators) and Hsd17b3 (testosterone biosynthesis marker),
709 and stimulation of Star (mitochondrial cholesterol influx transporter) were ameliorated
710 by Mel in testis, corresponded to the changes in the level of cholesterol and testosterone.
711 The results suggest that SIRT3 activator Mel participates in cholesterol metabolism,
712 affecting testosterone production in testis.

713 Testosterone is essential for several crucial plots during spermatogenesis: BTB
714 (basal ES/Sertoli-Sertoli adhesion), meiosis, apical ES (Sertoli-spermatid adhesion),
715 and sperm release (Smith and Walker, 2014). Our data show that SIRT3 activator Mel
716 rescued the structure disorganization of Sertoli cell, Leydig cell, and BTB induced by
717 Cd, which accounts for the above male reproductive function injury. Apart from basal
718 ES in BTB, cytoskeleton such as apical ES, microtubule and axoneme, are responsible
719 for sperm head reshaping and flagella formation during spermiogenesis, promoting
720 sperm motility acquirement (Pleuger et al., 2020). To identify whether Mel protects
721 cytoskeleton assembly, facilitating sperm motility, we detected the dynamic process of
722 spermiogenesis by TEM. Despite the normal axoneme, Cd undermined apical ES and
723 microtubule-based manchette in elongating spermatids, which were alleviated by Mel.
724 Parallelly, Mel rescued F-actin-containing cytoskeleton disruption induced by Cd.
725 Molecularly, Mel-mediated cytoskeleton assembly is concomitant with the changes in
726 BTB markers (Occludin, JAM-A, N-cadherin) (Cheng and Mruk, 2012), apical ES–
727 BTB axis regulators (FAK, p-FAK-Tyr407) (Cheng and Mruk, 2012), and
728 cytoskeleton negative regulators (PDLIM1) (Shang et al., 2016). Taken together, these
729 results echo our hypothesis that Mel engages in cytoskeleton assembly, including basal
730 ES, apical ES, and microtubule-based manchette during spermatogenesis.

731 Upregulation of SIRT3 following Mel has been demonstrated as a protective factor
732 in a diversity of tissues and cells, such as myocardium (Zhai et al., 2017), hepar (Song
733 et al., 2017), cerebrum (Liu et al., 2019), which is accordant with our data that Mel
734 indeed stimulated SIRT3, expediting SOD2 deacetylation by directly interacting with
735 SOD2 in testis. SIRT3-SOD2 axis regulates the scavenge of ROS (Kim et al., 2017),
736 while the balance of ROS is required for actin cytoskeletal dynamics (Gourlay and
737 Ayscough, 2005; Hunter et al., 2018). These data hint that Mel may regulate SOD2
738 deacetylation and cytoskeleton negative regulator PDLIM1 by SIRT3 in testicular
739 cytoskeleton assembly.

740 Except for SOD2, Mel also regulated P450scc deacetylation by the interaction of
741 SIRT3 and P450scc in testis. P450scc, as a catalyst converting cholesterol to
742 pregnenolone, is indispensable for testosterone biosynthesis (Jing et al., 2020).
743 Besides, the data showing that Mel reversed NF-κB p65^{Ser536} phosphorylation in
744 Cd-treated rat testis, is consistent with the work by Liu et al., who manifested that
745 Mel blocked NF-κB p65 signal in mice adipose tissue (Liu et al., 2017b). Actually,

746 the inhibition of NF- κ B p65 regulates testosterone production by Nur77 and SF-1
747 (Hong et al., 2004). Therefore, P450scc acetylation and NF- κ B p65^{Ser536}
748 phosphorylation are responsible for cholesterol metabolism and testosterone
749 biosynthesis. Plus, Winnik et al. delineated that Sirt3 knockout mice failed to address
750 a high-cholesterol diet, and resulted in endothelial cell dysfunction (Winnik et al.,
751 2016), displaying that SIRT3 was associated with cholesterol metabolism in
752 endothelial cells. These data and ours imply Mel may regulate NF- κ B p65^{Ser536}
753 phosphorylation and P450scc deacetylation by SIRT3 in testicular cholesterol
754 metabolism.

755 In vitro study, to confirm the role of SIRT3 in cholesterol metabolism in Leydig
756 cells, we generated Sirt3 overexpression and knockdown models in TM3 mouse
757 Leydig cells (TM3 cells). Like Mel, Sirt3 overexpression in TM3 cells rescued cell
758 apoptosis and cholesterol metabolism disruption induced by Cd; however, the
759 knockdown of Sirt3 induced cholesterol metabolism disruption similar to Cd, which
760 failed to be rescued by Mel, revealing that Mel-mediated cholesterol metabolism was
761 dependent on Sirt3 in TM3 cells.

762 SIRT3 dominated P450scc deacetylation in TM3 cells, which is consistent with in
763 vivo study. Strikingly, whether Sirt3 overexpression or Sirt3 knockdown conducted
764 no influence on NF- κ B p65^{Ser536} phosphorylation, despite Mel indeed reversed
765 Cd-stimulated NF- κ B p65^{Ser536} phosphorylation, suggesting that Mel regulated NF- κ B
766 p65^{Ser536} phosphorylation, which was not dominated by Sirt3 in TM3 cells. Since
767 Sirt3 was not the upstream regulator of NF- κ B, what's the relationship between
768 NF- κ B and Sirt3? Our data displaying that NF- κ B p65^{Ser536} phosphorylation
769 stimulated nuclear translocation of NF- κ B subunits p50, p65, and RelB, is supported
770 by three studies pinpointing that NF- κ B can translocate to the nucleus through Ser⁵³⁶
771 phosphorylation (Yu et al., 2020). Using bioinformatics analysis, we predicted that
772 NF- κ B could be the upstream transcription factor of Sirt3. Our ChIP-qPCR assay
773 demonstrated the combination between Sirt3 promoter and NF- κ B subunits (p50, p65,
774 RelB), especially p50. A dual-luciferase reporter assay identified that p50 repressed
775 Sirt3 transcription by binding to TFBS1 and TFBS2 in Sirt3 promoter (**Fig 5**). In
776 contrast, Liu et al. deemed that NF- κ B activated SIRT3 transcription in irradiated
777 tumor cells (Liu et al., 2015). This paradox may be caused by the different types of

778 cells. Taken together, Sirt3 transcriptional repression by NF- κ B operates cholesterol
779 metabolism via P450scc deacetylation in Leydig cells.

780 When it comes to cytoskeleton assembly, both basal ES (Sertoli-Sertoli cell
781 interface) and apical ES (Sertoli-elongating spermatid interface) are associated with
782 Sertoli cell. We investigate the role of SIRT3 in cytoskeleton assembly by Sirt3
783 overexpression and knockdown models in TM4 mouse Sertoli cells (TM4 cells).
784 Similar to Mel, Sirt3 overexpression rescued F-actin-containing cytoskeleton
785 assembly disruption and cell apoptosis in Cd-treated TM4 cells; however, the
786 knockdown of Sirt3 elicited cytoskeleton assembly disruption, which failed to be
787 rescued by Mel, indicating that Mel-mediated cytoskeleton assembly protection was
788 dependent on Sirt3 in TM4 cells (Fig 6, 7). Molecularly, SIRT3 dominates SOD2
789 deacetylation by directly interacting with SOD2, and Mel-mediated SOD2
790 deacetylation depends on SIRT3, in response to in vivo study. In terms of PDLIM1,
791 both Mel and the overexpression of Sirt3 extinguished the augment of PDLIM1
792 protein level induced by Cd; the knockdown of Sirt3 evoked PDLIM1 accumulation,
793 impairing cytoskeleton assembly, which didn't be reversed by Mel. Additionally, the
794 overexpression of Pdlim1 has been demonstrated to disturb cytoskeleton assembly in
795 Sertoli cells (Liu et al., 2016). SIRT3-SOD2-mediated ROS clearance is indispensable
796 for cytoskeleton assembly (Gourlay and Ayscough, 2005; Hunter et al., 2018). These
797 data and ours support the assumption that SIRT3 controls cytoskeleton assembly by
798 PDLIM1 and SOD2 deacetylation in Sertoli cells.

799 Although the knockdown of Sirt3 by shRNA exerts an effective interference in
800 TM3 (mouse Leydig) cells and TM4 (mouse Sertoli) cells, more high-efficiency
801 methods such as the conditional knockout (cKO) of Sirt3 in Leydig cells and Sertoli
802 cells should be involved in future study. Our models in TM4 cells can partially
803 respond to the ectoplasmic specialization (ES) assembly including apical/basal ES, but
804 the phenotype about ES in Sirt3 cKO mice will be required to confirm or refute this
805 hypothesis. Meanwhile, the accurate mechanism by which SIRT3 regulates
806 microtubule-based manchette remains to be elucidated in elongating spermatids. In
807 addition, the direct modes of actions between SIRT3 and cholesterol metabolism
808 markers (Abca1, Scap/Srebf2, Hsd17b3, and Star) deserve intensive investigation,
809 which may offer a novel orientation for exploring cholesterol metabolism during
810 spermatogenesis.

811 Overall (Fig. 8), the most striking finding of this study is that **SIRT3 exerts a dual**
812 **role in testis: Sirt3 transcriptional repression by NF-κB orchestrates cholesterol**
813 **metabolism via P450scc deacetylation in Leydig cells; whereas, in Sertoli cells,**
814 **SIRT3 regulates cytoskeleton (basal ES, microtubule) assembly via PDLIM1**
815 **with SOD2 deacetylation; the regulation of Leydig cells and Sertoli cells may**
816 **account for apical ES and microtubule-based manchette assembly in elongating**
817 **spermatids, influencing sperm differentiation during spermiogenesis.** Distinct
818 molecular mechanisms establish a novel signaling network, highlighting the complex
819 and multi-faceted action of SIRT3 as robust deacetylase in testicular cells.
820 Understanding the role of SIRT3 in regulating cholesterol metabolism and
821 cytoskeleton assembly during spermatogenesis may allow for the development of
822 additional rational combination therapies using SIRT3 activators such as Mel to
823 improve therapies for male subfertility, even infertility.

824

825 **Methods**

826 **Chemicals and reagents.** Cdcl₂ was from Sinopharm (CFSR-10005416, Shanghai,
827 China). Melatonin (Mel) was from MedChemExpress (Cat#HY-B0075, USA).
828 Testosterone ELISA kit was from Goybio (Cat#GOY-088B, Shanghai, China). Total
829 and free cholesterol assay kits were from Applygen Technologies (Cat#E1015-50,
830 Cat#E1016-50, Beijing, China). TRIzol reagents were from Invitrogen. PrimeScript
831 RT-PCR and SYBR Premix ExTaq Kits were from Takara (Cat#2641A, Cat#RR420,
832 Japan). BCA protein assay and ECL kits were from Beyotime Institute of
833 Biotechnology (Shanghai, China). N-Cadherin, β-Catenin, Occludin, JAM-A, β-Actin,
834 SOD2, SOD2(acetyl K68), PDLIM1, NUDC, GAPDH antibodies were from Abcam
835 (Cat#ab18203, Cat#ab68183, Cat#ab167161, Cat#ab125886, Cat#ab8226,
836 Cat#ab13534, Cat#ab137037, Cat#ab129015, Cat#ab109318, Cat#ab37168,
837 Cambridge, UK); FAK, p-FAK-Tyr407 antibodies were from Bioss (Cat#bs-1340R,
838 Cat#bs-3164R, MA, USA); SIRT3, P450scc, Acetylated-Lysine, NF-κB p50, NF-κB
839 p65, Phospho-NF-κB p65^{Ser536}, RelB, c-Rel, normal IgG antibodies from Cell
840 Signaling Technology (Cat#5490S, Cat#14217S, Cat#9441S, Cat#3035S, Cat#8242S,
841 Cat#3033S, Cat#10544S, Cat#4727T, Cat#2729S, MA, USA). Co-IP and ChIP kits
842 were from Thermo Fisher Scientific (Cat#26140, Cat#26156, MA, USA). CCK-8 Kit
843 was from Dojindo (Kumamoto, Japan). An Annexin V-FITC and PI Detection Kit

844 was from BD Biosciences (New Jersey, USA). DMEM was from HyClone (Logan,
845 Utah, USA). Collagenase and FBS were from Gibco (Australia). FITC Phalloidin
846 FITC and TRITC Phalloidin rhodamine were from Yeasen (Cat#40735ES75,
847 Cat#40734ES75, Shanghai, China).

848

849 **Experimental design**

850 **Animal model.** 2-mo-old adult male SD rats (230 ± 30 g) were purchased from
851 Tongji Medical College Animal Center. Animals were adapted for 7d to the new
852 environment, and fed ad libitum. The conditions were maintained as follows: a 12-h
853 light/dark phase, temperature (22–26°C) and humidity (50 ± 5%). Thirty-six rats were
854 randomly divided into 3 groups. Restricted randomization was not applied. According
855 to our previous subfertility/infertility model (Wang et al., 2020), all rats except
856 controls were intraperitoneally injected with CdCl₂ (0.8 mg/ kg) for consecutive 7 d.
857 Some rats were intraperitoneally injected with Mel (2 mg/kg) (SIRT3 activator (Zhai
858 et al., 2017)(Song et al., 2017)(Liu et al., 2019)) at 2 h before Cd treatment. Given
859 that both CdCl₂ and Mel were dissolved in 0.9% NaCl, the control group was treated
860 with 0.9% NaCl. All the animal experiments were permitted by the IACUC of Tongji
861 Medical College, Huazhong University of Science and Technology (**Supplementary**
862 **file**) (Permit IACUC Number: 2061), and were implemented ethically as the Guide
863 for the Care and Use of Laboratory Animal guidelines.

864 **Cell models.** TM3 mouse Leydig cells and TM4 mouse Sertoli cells were from the
865 Institute of Reproductive Health, Tongji Medical College. After tested for
866 mycoplasma contamination, TM3 and TM4 cells were respectively cultured in
867 DMEM, which was supplemented with 10% FBS, at 37°C in a humidified atmosphere
868 with 5% CO₂/95% air. According to our previous study (Wang et al., 2020), IC₅₀ of
869 Cd for TM3 cells—8.725 µg/ml and IC₅₀ of Cd for TM4 cells—12 µg/ml were
870 exploited in subsequent cell models. Considering that 10 µM CdCl₂ could be rescued
871 by 1 µM Mel in HepG2 cells, we determined 1.1 µg/ml Mel to protect against 8.725
872 µg/ml CdCl₂ in TM3 cells and 1.5 µg/ml Mel to protect against 12 µg/ml CdCl₂ in
873 TM4 cells by unit conversion (**Supplementary Table 1**).

Supplementary Table 1. Unit conversion.

	Cdcl ₂		Melatonin	
TM3 cells	8.72 µg/mL	47.57 µM	1.1 µg/mL	4.757 µM
TM4 cells	12 µg/mL	65.4588 µM	1.5 µg/mL	6.54588 µM

874 **Formula: Mass (mg) = Concentration (mM) * Volume (mL) * Molar Mass (g/mol)**

875 (<https://www.promega.com/resources/tools/biomath/molarity-calculator/>)

876

877 To explore if Mel alleviated the Cd-induced TM3 cells injury, TM3 cells were
878 randomly divided into 3 groups in model 1: control, Cdcl₂ (8.725 µg/ml), Cdcl₂ (8.725
879 µg/ml) with Mel (1.1 µg/ml). To identify if SIRT3 alleviated the Cd-induced TM3
880 cells injury, TM3 cells were randomly divided into 4 groups in model 2: control,
881 Cdcl₂ (8.725 µg/ml), Sirt3 overexpression (Ad-Sirt3), Cdcl₂ (8.725 µg/ml) with Sirt3
882 overexpression (Ad-Sirt3). To examine if Mel-mediated cell protection was
883 dependent on SIRT3, TM3 cells were randomly divided into 4 groups in model 3:
884 control, Mel (1.1 µg/ml), Sirt3 knockdown (sh-Sirt3), Mel (1.1 µg/ml) with Sirt3
885 knockdown (sh-Sirt3).

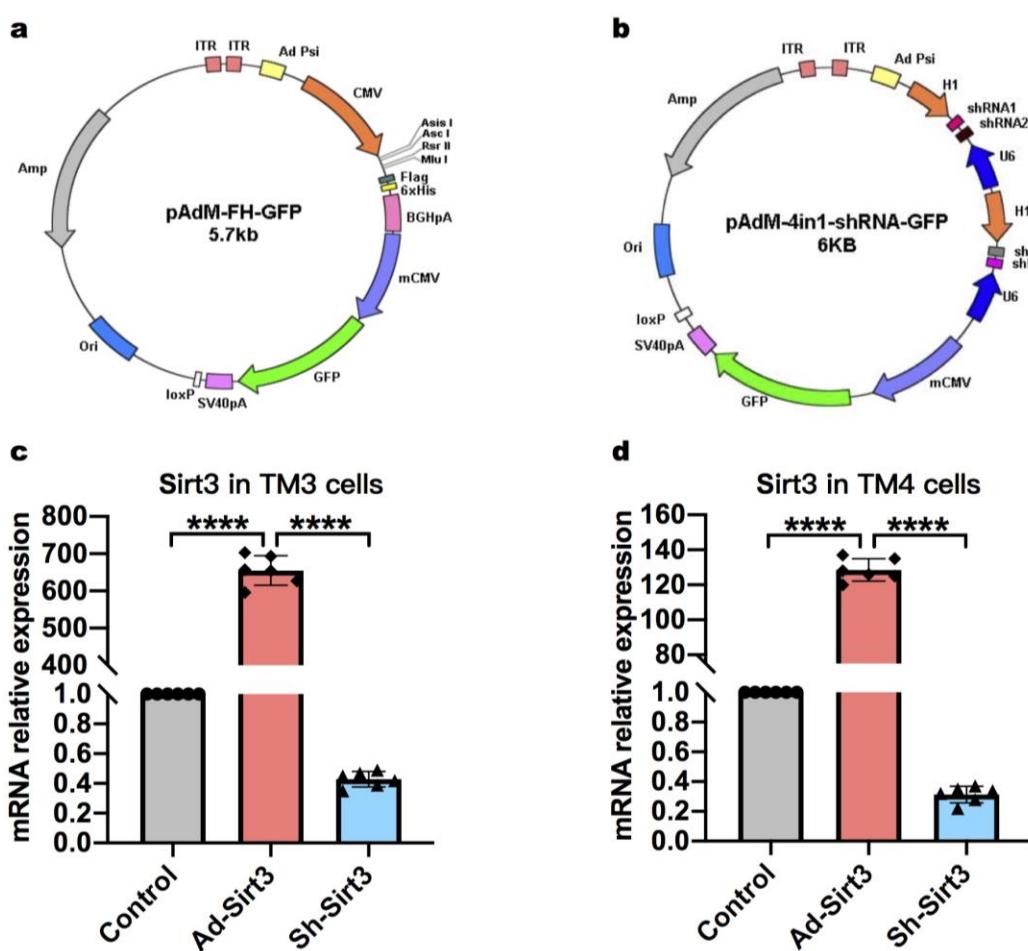
886 In parallel, to investigate the role of SIRT3 in TM4 cells, TM4 cells were randomly
887 divided into 3 models—TM4-model 1: control, Cdcl₂ (12 µg/ml), Cdcl₂ (12 µg/ml)
888 with Mel (1.5 µg/ml); TM4-model 2: control, Cdcl₂ (12 µg/ml), Sirt3 overexpression
889 (Ad-Sirt3), Cdcl₂ (12 µg/ml) with Sirt3 overexpression (Ad-Sirt3); TM4-model 3:
890 control, Mel (1.5 µg/ml), Sirt3 knockdown (sh-Sirt3), Mel (1.5 µg/ml) with Sirt3
891 knockdown (sh-Sirt3).

892 24 h post Ad-Sirt3 or sh-Sirt3 transfection, some cells were exposed to Cd for 24 h.
893 Some cells were pretreated with Mel for 2 h prior to Cd treatment. Researchers and
894 statistical analysts were blind to the allocation of groups.

895

896 **Sirt3 overexpression and knockdown by adenovirus vectors.** Mouse Sirt3
897 (NM_001177804.1) overexpression adenovirus were synthesized by a
898 pAdM-FH-GFP vector (Vigene Biosciences) (**Supplementary Fig.1a**). The construct
899 was confirmed by DNA sequencing. Sirt3 knockdown adenovirus was designed
900 according to four sequences of shRNA as follows: (5' → 3' orientation)
901 Sirt3-shRNA1: GGCTCTATA

902 CACAGAACATCGTTCAAGAGAGACGATGTTCTGTGTATAGAGCCTTTTT;
903 Sirt3-shRNA2: GGCAATCTAGCATGTTGATCGTTCAAGAGAGC
904 ATCAACATG CTAGATTGCCTTTTT; Sirt3-shRNA3:
905 AGACAGCTCCAACACAGTTACTTCA
906 AGAGAGTAAACGTGTTGGAGCTGTCTTTTT; Sirt3-shRNA4: GCGTTGTGA
907 AACCCGACATTGTTCAAGAGAGACAATGTCGGGTTCACAAACGCTTTTT,
908 which were constructed in a pAdM-4in1-shRNA-GFP vector (Vigene Biosciences)
909 (**Supplementary Fig.1b**). Sirt3 overexpression and knockdown adenoviruses were
910 respectively transfected into TM3 cells or TM4 cells with ADV-HR (FH880805)
911 (Vigene Biosciences) for the Sirt3 overexpression or knockdown assay. The
912 efficiencies of Sirt3 overexpression and knockdown were examined in TM3 cells and
913 TM4 cells (**supplementary Fig. 1c, 1d**).



914
915
916 **Supplementary Fig. 1 The efficiency of Sirt3 overexpression or knockdown in**
917 **TM3 cells and TM4 cells. a** The adenovirus vector for Sirt3 overexpression. **b** The
918 **shRNA-containing adenovirus vector for Sirt3 knockdown. c** The efficiency of Sirt3

919 overexpression and knockdown in TM3 cells by mRNA expression level analysis. **d**
920 The efficiency of Sirt3 overexpression and knockdown in TM4 cells by mRNA
921 expression level analysis. *** $p < 0.0001$.

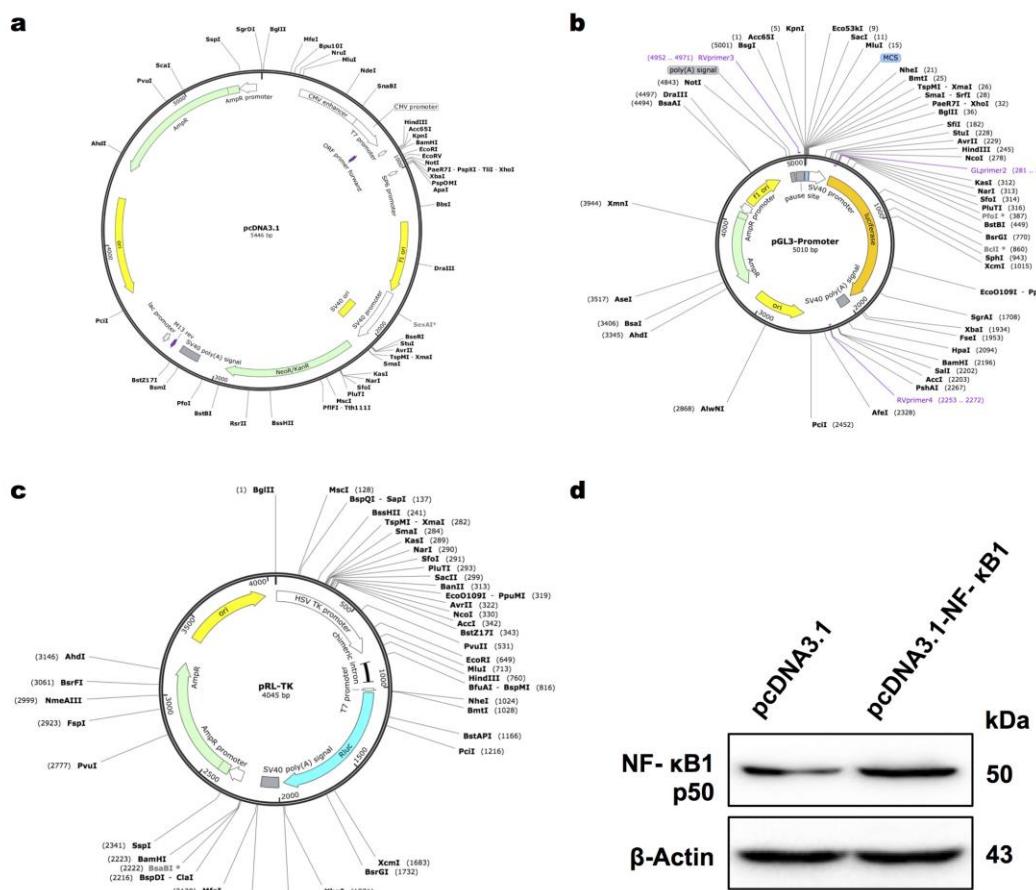
922

923 **Plasmid constructions.** Mouse Nfkb1 (NM_008689.2) coding sequence was
924 synthesized by gene synthesis (General Bio), and cloned into the pcDNA3.1
925 eukaryotic expression vector (**Supplementary Fig. 5a**). Sirt3-WT promoter and the
926 mutant promoters Sirt3-MUT1 (deletion of TFBS1), Sirt3-MUT2 (deletion of TFBS2),
927 Sirt3-MUT3 (simultaneous deletion of TFBS1 and TFBS2) (**Fig. 5f**) were constructed
928 by PCR-based amplification and cloned into pGL3-Promoter vector (**Supplementary**
929 **Fig. 5b**). Sequences of the primers for amplification of promoters are shown in
930 **Supplementary Table 3**. All constructs were confirmed by DNA sequencing
931 (**Available from authors**). NF- κ B1 protein coding capacity in HEK293T cells was
932 tested by western blot assay (**Supplementary Fig. 5d**).

Supplementary Table 3. Sequences of the primers for amplification of promoter.

Gene	Primers (5'-3')	
	Forward	Reverse
In ChIP-qRT PCR		
Sirt3	GTTCTGAGGAAGAGGTGTTTTTC	CTGACTGCTTAGTGCTGTGTTGT
In Dual luciferase reporter assay		
Sirt3 (WT)	gcgtgctagccgggctcgagCCATTGGCCCC TTTGGT	atgcagatcgagatctcgagCTCCCGGACCCC ACAGTC
Sirt3 (MUT1)	TCAAACACCAAGGTCCCCTGCAC AGCCAGGAG	TCAAACACCAAGGTCCCCTGCACA GCCAGGAG
Sirt3 (MUT2)	GGGCATGCTGAAATATTAAACTA GCCTCACGGTTGCGGTCGTC	TGAGGCTAGTTAATATTCAGCA TGCCCCGCGCGCCCCCGGGT

933



934

935 **Supplementary Fig. 5 Plasmid constructions for dual luciferase reporter assay. a**
936 pcDNA3.1 eukaryotic expression vector for overexpression of NF-κB1 in HEK293T
937 cells. **b** pGL3-Promoter vector for insertion of Sirt3 wild-type or mutant promoter in
938 HEK293T cells. **c** pRL-TK vector as an internal control for transfection. **d** NF-κB1
939 protein coding capacity in HEK293T cells by western blot assay.

940

941 **Dual-luciferase reporter assay.** By Lipofectamine® 3000 transfection reagent
942 (Invitrogen), HEK293T cells (from Tongji Medical College) were transiently
943 transfected with the NF-κB p50 expression vector (pcDNA3.1- NF-κB), or empty
944 control vector as well as WT or TFBS1/TFBS2 mutant Sirt3 promoter
945 (MUT1/MUT2/MUT3) luciferase reporter vector (pGL3-Promoter) and Renilla
946 luciferase-reporter vector (pRL-TK, as an internal control for transfection)
947 (**Supplementary Fig. 5c**). Luciferase activities were determined after 24-h incubation
948 with the Dual-luciferase reporter assay system (Promega). The firefly luciferase
949 activity was normalized to renilla luciferase activity. The relative luciferase activity
950 was analyzed as a function of NF-κB p50-dependent Sirt3 transcription.

951

952 **Chromatin immunoprecipitation (ChIP) assay.** The ChIP assay was implemented
953 by a ChIP assay kit according to the manufacturer's instructions (Thermo Fisher
954 Scientific). 1% formaldehyde was utilized to cross-link the histones and genomic
955 DNA in TM3 mouse Leydig cells. Cell lysates were prepared, and chromosomal
956 DNA was sonicated to obtain average sizes between 200 and 1000 bp. The chromatin
957 was incubated and precipitated with antibodies against p50, p65, RelB or normal
958 rabbit IgG (CST) as controls at 4 °C overnight. DNA fragment that contained the
959 NF- κ B binding site of the Sirt3 promoter was amplified. The amplified products were
960 analyzed by quantitative real-time PCR.

961

962 **Sperm parameter analysis.** According to "WHO laboratory manual for the
963 examination and processing of human semen (Fifth edition, 2010)", the sperm count
964 was calculated by multiplying the semen volume and concentration, which was
965 measured via an improved Neubauer hemocytometer. Sperm motility was analyzed
966 through the progressive sperm count per 200 sperm under a microscope.

967

968 **Cd level in the testis.** Cd level in testis was detected by graphite furnace atomic
969 absorption spectrometry (GFAAS) as the previous method (Wang et al., 2020).

970

971 **Testosterone and cholesterol levels.** Testosterone levels in rat serum and cultured
972 TM3 cells supernatants were qualified by testosterone ELISA kit following the
973 manufacturer's instructions (Goybio). Total cholesterol (TC) levels in testis and TM3
974 cells were measured by total cholesterol assay kit; free cholesterol (FC) levels were
975 determined by free cholesterol assay kit according to the manufacturer's instructions
976 (Applygen Technologies).

977

978 **Quantitative real-time PCR assay.** The mRNA expression levels of Abca1, Hmgcr,
979 Hmgcs1, Hsd17b1, Scap, Srebf2, Star in testis and TM3 cells, and Sirt3 in TM3/TM4
980 cells were analyzed by quantitative real-time PCR assay. SYBR RT-PCR kit (Takara)
981 and LightCycler (Roche) were exploited according to the standard procedures. Data
982 were normalized to β -actin expression. Sequences of the primers for quantitative
983 RT-PCR are in **supplementary Table 2**.

Supplementary Table 2. Sequences of the primers for quantitative real time PCR of mRNA levels.

Gene	Primers (5'-3')	
	Forward	Reverse
β-Actin	CTGAGAGGGAAATCGTGCCT	CCACAGGATTCCATACCCAAGA
Abca1	GAGACCAACCAGGCAATCCA	GCCCAGAACCTCCTCTCGTC
Hmger	CCATCGAGCCACGACCTAAT	AGCTGGATATGCTTGGCATT
Hmgcs1	GGGCCAAACGCTCCTCTAAT	ACAGGGTACTCGGAGAGCAT
Hsd17b1	AGTTTGCCTCGAAGGTTG	CATCTGCTCGTCCAGAGCC
Scap	AGACCCATGGCGACATTACC	CCCATAATTACGCGGGCAGA
Srebf2	CGGTGGAGTCCTGGTAAA	ACGGAAC TGCTGGAGAATGG
Star	AGTGACCAGGAGCTGTCCTA	TTAGCACTCGTCCCCGTTTC
Sirt3	CATCGACGGGCTTGAGAGAG	TTACAAAGGTCCCCTGGGC

984

985

986 **Histopathological analyses.** Testes were fixed in Bouin's fixative for 24 h, and
987 embedded in paraffin, and sectioned. Testes sections were stained with hematoxylin
988 and eosin (H&E) and observed under a light microscope for structure.

989

990 **Transmission electron microscopy (TEM).** Testes were fixed with 2.5%
991 glutaraldehyde for 2 h at 4 °C, postfixed in 1% osmium tetroxide, and embedded in
992 Epon 812 as the previous method (Wang et al., 2020); the ultrastructure of Sertoli cell,
993 Leydig cell, BTB, and cytoskeleton during spermiogenesis in testis was investigated
994 by TEM (JEM 1200-EX; Hitachi, Ltd, Tokyo, Japan) at 80 kV.

995

996 **F-actin examination by immunofluorescence and confocal microscopy.** Fixed
997 testis sections were stained with FITC Phalloidin FITC (Yeasen), which labeled
998 F-actin. TM4 cells were washed and fixed with 4% paraformaldehyde, and then
999 stained with TRITC Phalloidin rhodamine (Yeasen), which labeled F-actin. Under
1000 Nikon A1 laser scanning confocal microscope (Nikon America Inc., Melville, NY),
1001 F-actin structure was investigated. The percentages of spermatids or Sertoli cells with
1002 abnormal F-actin-containing cytoskeleton per 200 elongating spermatids or TM4 cells
1003 were calculated in 6 independent experiments for each group.

1004
1005 **Western blotting.** The protein concentration was detected by a BCA protein assay kit.
1006 Proteins were denatured, separated on SDS-PAGE gels, and transferred to a PVDF
1007 membrane as the previous method (Wang et al., 2020). The membranes were
1008 incubated with antibodies according to the manufacturer's instructions, including
1009 N-Cadherin, β -Catenin, Occludin, JAM-A, β -Actin, SOD2, SOD2(acetyl K68),
1010 PDLIM1, NUDC, GAPDH, FAK, p-FAK-Tyr407, SIRT3, P450scc,
1011 Acetylated-Lysine, NF- κ B p50, NF- κ B p65, Phospho-NF- κ B p65^{Ser536}, RelB, c-Rel.
1012 β -Actin and GAPDH proteins were used as a loading control for total and cytoplasmic
1013 proteins respectively. The membranes were incubated with corresponding secondary
1014 antibodies; the target proteins were detected with ECL; the density was analyzed by
1015 Image J.
1016
1017 **Co-immunoprecipitation (Co-IP) assay.** co-IP was performed by the Thermo
1018 Scientific Pierce co-IP kit according to the manufacturer's protocol (Thermo Fisher
1019 Scientific). For the detection of interaction between SOD2 and SIRT3, 60 μ l of
1020 anti-SOD2 antibody, 60 μ l of anti-SIRT3 antibody, and 30 μ l of IgG antibody were first
1021 immobilized for 2 h using AminoLink Plus coupling resin, which was washed and then
1022 incubated with 200 μ l (500 μ g of proteins) of lysate. Next, the resin was precleaned
1023 with control agarose resin for 1 h and incubated. Using the elution buffer, the coupling
1024 resin was again washed and protein was eluted. For the detection of interaction between
1025 P450scc and SIRT3, the method was as above. Samples were analyzed by
1026 immunoblotting.
1027
1028 **CCK-8 assay.** Cell viabilities of TM3 and TM4 cells were assessed by CCK-8 assay
1029 kit according to the manufacturer's instructions as the previous method (Wang et al.,
1030 2020).
1031
1032 **Flow cytometry analysis.** To analyze the effects of the indicated treatments on cell
1033 survival, we stained the cells with an Annexin V-FITC and PI Detection Kit and
1034 analyzed them by flow cytometry; flow cytometry data were assessed using BD
1035 FACSDiva Software v7.0 (Becton-Dickinson, USA) (Wang et al., 2020).
1036

1037 **Statistical analysis.** The data were expressed as the mean \pm S.D. Differences among
1038 two groups were analyzed by Student's *t*-test, the Mann-Whitney *U*-test, or
1039 Generalized estimating equation (if non-normal distribution). Differences among
1040 multiple groups were analyzed by one-way analysis of variance (ANOVA). Multiple
1041 comparisons for subgroups were determined by Dunnett's T3 tests. Statistical
1042 significance was considered as follows: NS, $p > 0.05$; *, &, #, $p < 0.05$; ** $p < 0.01$;
1043 *** $p < 0.001$; **** $p < 0.0001$. The showed experiments were replicated 4-10 times.

1044

1045 **Data availability**

1046 All data generated or analyzed during this study are included in the manuscript and
1047 supporting files. Source data files have been provided for Figures 1-7 and
1048 supplementary files.

1049

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1056

1057 **Author contributions**

1058 M.W., Y.Z.Z. and P.S. conceived the project and designed the experiments; M.W.,
1059 L.Z., Y.X., X.F.W., L.C. performed the experiments; M.W., F.W., analyzed the data,
1060 and wrote the paper; M.W., and Y.X. edited the manuscript; Y.Z.Z. and P.S.
1061 supervised the study; Y.Z.Z. obtained fundings for the work.

1062

1063 **Declaration of interest**

1064 The authors report no conflicts of interest.

1065

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华中科技大学同济医学院实验动物伦理委员会审批报告
The Institutional Animal Care and Use Committee at Tongji Medical College,
Huazhong University of Science and Technology

[2018] 伦审字 (S2061) 号
[2018] IACUC Number: 2061

项目名称 Title of Project	The crosstalk of autophagy and apoptosis in cadmium-induced injury/recovery of male reproduction		
申请单位 Application Institute	Family Planning Research Institute, Tongji Medical College, Huazhong University of Science and Technology		
项目负责人 Principal Investigator	Ping Su	职称 Title	Associate Professor
报 送 资 料 Material submitted	课题研究方案 Research Plan	有 <input checked="" type="checkbox"/> 无 <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	
	观察记录表 Observational Recording Table	有 <input checked="" type="checkbox"/> 无 <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	
	研究人员名单 List of Researchers	有 <input checked="" type="checkbox"/> 无 <input type="checkbox"/> Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	
审 查 Censor	研究者资格 Qualification of Researchers	符合要求 <input checked="" type="checkbox"/> 不符合要求 <input type="checkbox"/> Meet <input checked="" type="checkbox"/> Not meet <input type="checkbox"/>	
	课题研究方案 Research Plan	适当 <input checked="" type="checkbox"/> 不适当 <input type="checkbox"/> Appropriate <input checked="" type="checkbox"/> Not appropriate <input type="checkbox"/>	
有效期 Period of Validity	2018.02.15---2019.03.15		
审评意见: Remarks:	本伦理委员会审阅并讨论了上述相关资料, 该课题研究符合《湖北省实验动物管理条例》和《华中科技大学同济医学院实验动物伦理委员会章程》, 经伦理委员会审核, 同意该课题实施。 The Tongji Medical College, HUST Institutional Animal Care and Use Committee has reviewed and discussed the above mentioned materials, and accepted the proposal and approve the research plan as described		
华中科技大学同济医学院实验动物伦理委员会 Institutional Animal Care and Use Committee Tongji Medical College, Huazhong University of Science and Technology			
负责人签字:  Signature: _____			
批准日期: 年 月 日 Date: _____			