1 **Functional importance of JMY expression by Sertoli cells in** 2 **mediating mouse spermatogenesis**

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22 Running title: Spermatid development depends on JMY expression

23 **Abstract**

24 Sertoli cells are crucial for spermatogenesis in the seminiferous epithelium because
 25 their actin cytoskeleton supports vesicle transport, cell junction, protein anchoring and
 26 spermiation. Here, we show that junction-mediating and regulatory protein (JMY), an
 27 actin regulating protein, also affects endocytic vesicle trafficking and Sertoli cell
 28 junction remodeling since disruption of these functions induced male subfertility in
 29 Sertoli cell-specific *Jmy* knockout mice. Specifically, these mice have: a) impaired
 30 BTB integrity and spermatid adhesion in the seminiferous tubules; b) high incidence
 31 of sperm structural deformity; c) reduced sperm count and poor sperm motility.
 32 Moreover, the cytoskeletal integrity in Sertoli cell-specific *Jmy* knockout mice was
 33 compromised along with endocytic vesicular trafficking. These effects impaired
 34 junctional protein recycling and reduced Sertoli cell junctions. In addition, JMY
 35 interaction with α -actinin1 and Sorbs2 was related to JMY activity and in turn actin
 36 cytoskeletal organization. In summary, JMY affects control of spermatogenesis
 37 through regulating actin filament organization and endocytic vesicle trafficking in
 38 Sertoli cells.

39

40 **Key words:** Sertoli cells, endocytic vesicle trafficking, junction-mediating and
 41 regulatory protein (JMY), junction remodeling, blood-testis barrier (BTB), spermatid
 42 development

43

44

45 **Introduction**

46 The testicular sustentacular cells, also known as Sertoli cells, are the only somatic
 47 cells in the seminiferous epithelium spanning the entire epithelium from the basement
 48 membrane into the lumen. They sustain a crucial nursing role through providing
 49 physical support, mediating nutrient transport, cleavage and eliciting paracrine signals
 50 that affect nascent sperm development (Griswold, 1998; França et al., 2016; Griswold,
 51 2018). These functional roles help account for why sperm production critically
 52 depends on functional Sertoli cells interspersed within the seminiferous epithelial
 53 layer.

54 Cell to cell junctions between juxtaposed basal Sertoli cells surround the
 55 spermatogonia and delineate the boundary between the apical and basal seminiferous
 56 epithelium. These points of contact constituting the blood–testis barrier (BTB) include
 57 tight junctions, ectoplasmic specialization (ES), gap junctions and desmosome-like
 58 junctions (Mruk and Cheng, 2015). This physical barrier isolates the developing germ
 59 cells from immune responses to immune cell infiltration and exogenous toxins
 60 trapped in the basal compartment. Therefore, this barrier provides a molecule
 61 selectivity filter which creates a unique microenvironment supporting germ cell
 62 development (Mital et al., 2011; França et al., 2016; Stanton, 2016). Meanwhile,
 63 multiple germ cell–Sertoli cell junctions including desmosome-like junction and the
 64 ES, keep germ cells adhering to Sertoli cells. Moreover, the ES localized in the apical
 65 region of the Sertoli cells are critical for orienting the elongating spermatid and
 66 releasing them once they have developed into mature spermatozoa during spermiation

67 (França et al., 2016; Li et al., 2017). Therefore, the cell junctions elaborated by Sertoli
68 cells, namely both Sertoli-Sertoli cell and Sertoli-germ cell junctions, contribute to
69 sperm production in the seminiferous epithelium.

70 It is noteworthy that multiple junctions, particularly the tight junctions in Sertoli
71 cells undergo dynamic restructuring during germ cell development. At stage VIII of
72 the seminiferous epithelial cycle during spermatogenesis, preleptotene spermatocytes
73 transit across the BTB near the basement membrane to promote the release of the
74 adherent mature spermatid cells in the apical compartment wherein multiple junctions
75 between adjacent Sertoli cells and Sertoli-germ cells instantaneously break down and
76 then rapidly recombine. It is becoming clear that some tight-, gap-, adherens-, and
77 desmosome-like junctional proteins undergo continuous endocytosis and recycling
78 back to the plasma membrane, thereby resulting in scavenging of the old junctions
79 and replacing them with new junctions (Mruk and Cheng, 2004; Du et al., 2013; Vogl
80 et al., 2014). During this process, regulation of endocytic trafficking of junctional
81 proteins provides a way of rapidly restructuring cell–cell junctions. Failure in any of
82 these steps during endocytosis by Sertoli cells can lead to structural cell junctional
83 defects in the seminiferous epithelium and subsequently an unstable environment for
84 spermatogenesis (Cheng et al., 2011; Jia et al., 2017).

85 Endocytosis by Sertoli cells is regulated by androgens and cytokines (such as
86 $\text{TNF-}\alpha$, $\text{TGF-}\beta 2$, $\text{TGF-}\beta 3$, IL-6). Androgens can promote endosomal recycling and
87 enhance formation of new cell junctions. In contrast, $\text{TGF-}\beta$, $\text{TNF-}\alpha$ and IL-6 linked
88 signaling promote the fusion of endosomes to lysosomes, which enhances the

89 degradation of cell junctions (Yan et al., 2008; Su et al., 2010). Moreover, the actin
90 cytoskeleton is essential for endocytosis and also for endosomal movement.
91 Numerous studies demonstrated the important roles of both the actin cytoskeleton and
92 actin regulating proteins in trafficking of junctional proteins and maintaining cell
93 junction integrity in Sertoli cells (Li et al., 2016). For instance, recent studies showed
94 that a class of actin-related proteins and their regulators, such as actin-related protein
95 3 (Arp3) and Wiskott-Aldrich syndrome protein (WASP), were involved in
96 maintaining Sertoli cells tight junctions and BTB function (Lie et al., 2010; Xiao et al.,
97 2014; Mok et al., 2015). More importantly, there are reports indicating that JMY, a
98 junction-mediating and regulatory protein, is a member of the WASP family and it has
99 a similar function to WASP and WHAMM (WASP homolog associated with actin,
100 membranes, and microtubules). In addition, JMY is a multifunctional protein with
101 roles in transcriptional co-activation of p53 and regulation of actin nucleation, which
102 accompanies activation of the Arp2/3 protein complex (Roadcap and Bear, 2009).
103 Analysis of the JMY protein sequence shows that its N-side contains a coiled-coil (CC)
104 domain that can combine with the p300 protein. On the other hand, the C-side has
105 three WASp Homology 2 (WH2) domains. They can combine with actin and a
106 connector and an acidic (CA) domain and in turn with the Arp2/3 complex. When in
107 the cytosol, JMY can activate Arp2/3 and produce branched F-actin filaments along
108 with unbranched filaments via its tandem WH2 domains (Coutts et al., 2009; Zuchero
109 et al., 2009; Rottner et al., 2010).

110 It is uncertain whether JMY has a functional role in mediating spermatogenesis

111 because its expression pattern has not been described in Sertoli cells. In addition,
112 the effects are unknown of JMY protein downregulation or loss of function in Sertoli
113 cells. Herein, we report that the JMY protein is highly expressed in Sertoli cells and is
114 an actin regulator. This function is crucial in the maintenance of cell junction integrity
115 which is vital for preserving spermatids adhesion and BTB integrity in Sertoli cells.

116

117 **Results**

118 **Expression and localization of JMY in Sertoli cells**

119 Immunofluorescent staining and immunoblotting detected JMY expression in the
120 seminiferous epithelium of mouse testis and primary cultured Sertoli cells (Fig. 1 A,
121 Fig. 2A, Fig. S1). JMY expression was prominent in Sertoli cells whereas it was much
122 less evident in spermatids throughout the seminiferous epithelium (Fig. 1 A, Fig. S1).
123 These results are consistent with Western blot analysis which also identified JMY
124 expression in the testis and Sertoli cells (Fig. 2 A). Additionally, JMY was localized in
125 the cytosol of cultured Sertoli cells and it was somewhat more evident at the
126 F-actin-rich domain (Fig. 1 B), which is consistent with its actin-regulatory
127 involvement.

128

129 **Conditional knock out (CKO) of *Jmy* in the Sertoli cells**

130 To identify the function of JMY in Sertoli cells, *Jmy* loxP mice were constructed.
131 Accordingly, two loxP sites were inserted into the flanking regions of the third exon in
132 the *Jmy* gene using homologous recombination (Fig. S2). Then, the *Jmy* flox (*Jmy*

133 $^{loxP/loxP}$) mice were mated with *Amh* cre mice in order to generate the expected Sertoli
134 cell specific *Jmy* CKO mice (*Jmy* $^{loxP/loxP}$; *Amh* cre⁺). They carried a Sertoli cell
135 specific *Jmy* deletion which was obtained by splicing out exon 3 using the Sertoli cell
136 specific expression of Cre recombinase. In these *Jmy* CKO mice, Western blot and
137 immunofluorescent analysis documented that JMY testicular expression was absent in
138 Sertoli cells (Fig. 2 A and B), which confirms *Jmy* gene knockout in these mice.

139

140 **Reduced male fertility and poor sperm quality in *Jmy* CKO mice**

141 To assess the fertility of the *Jmy* CKO mice, each male was mated with two mature
142 females for one week and then litter sizes were compared with their wild type (WT)
143 counterpart. As expected, the litter size of male *Jmy* CKO mice (4.65 ± 1.31) was
144 significantly less than that of both the WT and the heterozygote (Het, *Jmy* lox/-) males
145 (WT: 7.40 ± 2.01 , Het: 7.29 ± 0.95 ; Fig. 2 C). Moreover, the *Jmy* CKO sperm motility
146 and concentration also decreased in comparison with those of the WT and the Het
147 males (Sperm motility in WT: 65.60 ± 8.76 , Het: 66.20 ± 6.58 , CKO: 42.50 ± 5.74 .
148 Sperm progressive motility in WT: 26.20 ± 5.89 , Het: 23.80 ± 6.54 , CKO: $16.50 \pm$
149 6.14 . Sperm concentration, $\times 10^6$ per ml, in WT: 27.62 ± 7.82 , Het: 23.54 ± 2.62 ,
150 CKO: 15.28 ± 4.41 . Fig. 2 D and E), while the sperm head deformed ratios were
151 greatly enlarged (WT: 10.23 ± 3.38 , Het: 14.60 ± 5.33 , CKO: 43.65 ± 3.55 . Fig. 2 F
152 and G). Thus, the reduced litter size and poor sperm quality clearly indicate that male
153 subfertility was established in the *Jmy* CKO mice.

154

155 **Morphological alteration in *Jmy* CKO testes**

156 The testicular morphology of *Jmy* CKO mice was abnormal compared to that in the
 157 WT and the Het counterparts. The *Jmy* CKO testes had asyntactic Sertoli cells at the
 158 basal region of the seminiferous epithelium and poor adhesion between spermatids
 159 and Sertoli cells, leading to the abscission of round spermatids into the lumen of
 160 seminiferous tubules (Fig. 3 A), whereas in the sections of *Jmy* CKO caput
 161 epididymis, masses of round spermatids were evident in the epididymal duct lumen
 162 (Fig. 3 B).

163 To characterize the cell type alterations in the seminiferous epithelium, cell cycle
 164 analysis was performed in *Jmy* CKO and WT mice. The cell types were classified
 165 according to karyotype analysis based on comparing spermatogonia, secondary
 166 spermatocyte and Sertoli cells of diploid cells with those of: a) primary tetraploid
 167 spermatocytes in the tetraploid cells; b) haploid spermatids and spermatozoa. The
 168 results revealed a significantly higher percentage of haploid cells in *Jmy* CKO
 169 seminiferous epithelium compared to that in the WT seminiferous epithelium (Fig. 3
 170 C). Meanwhile, the number of Sertoli cells was evaluated based on the
 171 immunostaining expression pattern of vimentin, which is a Sertoli cell marker, in 8
 172 weeks old *Jmy* CKO and WT mice. The number was essentially the same between
 173 these two groups (Fig. 3 D).

174 Additionally, electron micrographs showed that cell adhesions were
 175 unconsolidated within the seminiferous epithelia of the *Jmy* CKO testes (Fig. 3
 176 E), and junctional structures between the Sertoli cells, especially the actin bundles of

177 the ES were greatly disrupted (Fig. 3 F).

178 The in vivo classical biotin permeation assay compared the BTB integrity in the
179 *Jmy* CKO with that in the WT and Het testes. As expected, the staining patterns
180 clearly defined basal spermatogonia but none were present in the upper apical cell
181 layers in the WT and Het testes. In contrast, biotin readily permeated throughout the
182 entire germinal epithelium in the *Jmy* CKO testes (Fig. 3 G).

183 In summary, loss of *Jmy* in Sertoli cells impaired Sertoli cell junctional barrier
184 formation and loosened spermatid attachment.

185

186 Disrupted cell junctions and endocytosis in *Jmy* CKO testes

187 To confirm the alteration of cell junctions in *Jmy* CKO testes, immunoblot analyses
188 evaluated junctional signature protein expression. ZO-1, claudin11 (tight junction
189 proteins), as well as β -catenin (adherens junction protein) expression levels decreased
190 in the *Jmy* CKO testes in comparison to those in WT testes (Fig. 4 A). Meanwhile,
191 immunohistochemistry analyses showed that expression of N-cadherin, specifically
192 localized in the basolateral region of adjacent Sertoli cells in WT testes decreased and
193 was more diffuse in the junctional compartment of Sertoli cells in *Jmy* CKO testes
194 (Fig. 4 B). Thus, these results show that the declines in expression of cell junctional
195 proteins impaired tight junctioncontacts and BTB integrity between neighboring
196 Sertoli cells in the *Jmy* CKO testes.

197 Notably, dynamic trafficking of tight junctionproteins is crucial for maintaining
198 barrier function and it is greatly dependent on endocytosis. Endocytic trafficking of

199 junctional proteins, including continuous endocytosis and recycling back to the
200 plasma membrane enables rapid junctional remodeling. Immunoblot analysis of
201 clathrin (an endocytic vesicle marker) and EEA-1 patterns (an early endosomal
202 marker) showed that they increased in *Jmy* CKO testes (Fig. 4 A). These changes
203 indicate that decreased levels of junctional protein expression may result from
204 endocytosis up-regulation in *Jmy* CKO testes.

205

206 Disrupted cell junctions and endocytosis in *Jmy* CKO Sertoli cells

207 Primary cultured Sertoli cells were used to confirm that alterations in cell junctional
208 integrity and endocytosis occur in *Jmy* CKO mice. As stated above, immunoblot
209 analyses showed that the tight junction protein (occludin) and the adherens junction
210 proteins (β -catenin and N-cadherin) were downregulated in *Jmy* CKO Sertoli cells. In
211 contrast, the endocytic vesicle markers (clathrin and caveolin 1), the early endosomal
212 marker (EEA-1) and the recycling endosome marker (Rab 11) were upregulated in
213 *Jmy* CKO Sertoli cells, whereas the later endosome marker (Rab 7) was unchanged in
214 *Jmy* CKO Sertoli cells (Fig. 4 C). Immunofluorescent staining analyses of cultured
215 Sertoli cells also confirmed declines in N-cadherin and ZO-1 localization at the
216 cell–cell interface of *Jmy* CKO Sertoli cells, as well as the increases in contents of
217 caveolin 1 and clathrin in *Jmy* CKO Sertoli cells (Fig. 4 D). These findings
218 unequivocally confirm losses in cell junctional formation whereas endocytosis
219 increased in *Jmy* CKO Sertoli cells. These changes suggest that JMY expression
220 contributes to controlling endocytic trafficking of junctional proteins.

Another indication of JMY involvement is that treatment of normal WT Sertoli cells with dihydrotestosterone (DHT), markedly upregulated clathrin, an activator of endocytosis and endosome recycling, and EEA-1 and Rab11 endocytosis related proteins. These changes are consistent aforementioned indications of JMY involvement in controlling BTB integrity and endocytic trafficking. Likewise, upon treatment of WT Sertoli cells with dynasore, an inhibitor of dynamin and vesicle trafficking, the contents of junctional proteins, i.e. occludin, β -catenin and N-cadherin significantly decreased, which is also consistent with their alterations in *Jmy* CKO Sertoli cells (Fig. 4 C). Therefore, the effects of loss of JMY function on Sertoli cells are similar to those induced by DHT and dynasore treatments. This correspondence strengthens the notion that JMY is involved in endocytic vesicle trafficking and endosomal recycling.

Disrupted endosomal cycling in *Jmy* CKO Sertoli cells

Endocytosis occurs through multiple steps which initially include internalization of proteins spanning the entire width of the plasma membrane followed by vesicular trafficking into early endosomes. Subsequently, the endosomes are inserted into the lysosomal degradative pathway or recycled back to the plasma membrane. To identify the trafficking fate of the endocytosed junctional proteins, we used a biotinylation assay that labels extracellular amino residues of membrane proteins and allows their trafficking to be monitored. In WT Sertoli cells, the biotin labeled proteins were initially detected at the surface of cells and endocytosis gradually moved them into the

cytoplasm. Because DHT induces endosomal recycling, the biotin labeled proteins recycled back to the plasma membrane of Sertoli cells at the cell-cell interface after either 1 hour or 3 hours culture in vitro (Fig. 5 A). Even though biotin-labeled membranous proteins were transported into the cytoplasm by endocytosis in *Jmy* CKO Sertoli cells, endocytic trafficking was not completed since the proteins accumulated in the cytoplasm (Fig. 5 A). Alternatively, treating with dynasore blocked endocytosis at a step prior to biotin labeling in *Jmy* CKO Sertoli cells, thus the biotin labeled membranous proteins remained on the cell surface (Fig. 5 B).

Moreover, immunofluorescent staining also demonstrated that a large amount of biotin labeled proteins accumulated in the cytoplasm of *Jmy* CKO Sertoli cells and colocalized with endocytic vesicle markers, clathrin and caveolin 1, rather than the endosomal markers, EEA-1 and Rab 11 (Fig. 5 C). These results suggest that the loss of *Jmy* function blocks vesicle trafficking into the early endosomes.

Identification of proteins interacting with JMY by immunoprecipitation (IP) and liquid chromatography tandem mass spectrometry (LC-MS) analysis

Due to the role of JMY as an actin regulator, we hypothesized that cooperative interaction between JMY and some other proteins contribute to endocytic vesicle trafficking. To gain insight into the molecular mechanism underlying JMY effects on vesicle trafficking, the proteins interacting with JMY in Sertoli cells were separated by IP and then identified by LC-MS analysis (Table S1). Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (<http://www.genome.jp/kegg/>) was used to

265 analyze the MS data in order to search for functional annotation terms and pathways.
 266 The analysis showed that the proteins interacting with JMY are mainly constituents of
 267 a group classified as “Regulation of actin cytoskeleton”, “Tight junction”, “Focal
 268 adhesion” and “Endocytosis” in Sertoli cells (Table S2). Similarly,
 269 Clusters of Orthologous Groups of proteins (COG) analysis revealed that 15 proteins
 270 identified by MS were classified as “Cytoskeleton” (Fig. S3). Considering the known
 271 actin-regulating activity of JMY, such we were prompted to determine if it interacts
 272 with these other actin cytoskeleton related proteins. Within this group, drebrin,
 273 actin-related protein 2/3 complex (Arp2/3), Anxa2, Cofilin, Gelsolin, spectrin have
 274 been reported to participate in regulating cell junction formation and function in
 275 Sertoli cells (Guttman et al., 2002; Lui et al., 2003; Lie et al., 2010; Li et al., 2011;
 276 Aristaeus de Asis et al., 2013; Chen et al., 2017; Chojnacka et al., 2017).

277 Previous studies demonstrated that the WH and CA domains of JMY bound to the
 278 actin and Arp2/3 complex to promote actin nucleation (Coutts et al., 2009; Zuchero et
 279 al., 2009; Rottner et al., 2010). Herein, our analysis predicted that JMY interacts with
 280 the Arp2/3 complex as well as other actin binding protein candidates that include
 281 α -actinin1, Sorbin and SH3 domain containing protein 2 (Sorbs2) in Sertoli cells. If
 282 this prediction proves to be valid, the structure of the actin and Arp2/3 complex may
 283 in fact also contain other constituents.

284

285 **Verification of interactions between JMY and two actin binding proteins,**
 286 **α -actinin1 and Sorbs2**

287 Since α -Actinin1 and Sorbs2 very prominently interact with JMY based on their
288 presence in immunoprecipitates of JMY, we presumed that they support JMY function
289 by serving as its co-factors. To confirm such an interaction, we interrogated
290 immunoblots obtained with JMY antibodies for both α -actinin1 and Sorbs2. Their
291 presence in the JMY immunoprecipitates shown in Figure 6A documents that JMY
292 interacts with α -actinin1 and Sorbs2.

293 Furthermore, immunofluorescent staining substantiates α -actinin1 and Sorbs2
294 colocalization in cultured Sertoli cells. The images clearly show that they are present
295 in close proximity to the actin cytoskeleton. More importantly, JMY colocalizes at the
296 cell edges with this complex formed between α -actinin1 and Sorbs2 and the actin
297 cytoskeleton network (Fig. 6 B). Colocalization of JMY with these proteins
298 strengthens the notion that these proteins bind to JMY. Accordingly, it is possible that
299 they may interact with one another and mediate specific biological functions such as
300 endocytosis.

301

302 **Differential expression of α -actinin 1 and Sorbs2 in *Jmy* CKO Sertoli cells**

303 JMY involvement in fostering a structured actin network was confirmed by the fact
304 that rhodamine-phalloidin stained actin filaments were disordered with highly
305 branched and extensive foci in *Jmy* CKO Sertoli cells (Fig. 7 A). Nevertheless, the
306 expression and localization of Arp3 in *Jmy* CKO Sertoli cells were clearly unchanged
307 from those in WT Sertoli cells (Fig. 4 C, Fig. 7 B and C). This invariance suggests
308 that the disorganized and unstructured actin cytoskeleton induced by loss of *JMY*

309 function was not dependent on an interaction between JMY and Arp2/3.

310 Although both α -actinin1 and Sorbs2 strongly interact with the actin cytoskeleton,
 311 their relationship to JMY function required clarification. Notably, in *Jmy* CKO Sertoli
 312 cells, both the α -actinin and Sorbs2 expression levels detected by immunoblotting
 313 were slightly reduced (Fig. 7 B). A more striking change was that loss of JMY
 314 function caused α -actinin 1 and Sorbs2 to lose their close proximity to one another.
 315 This disruption is evident based on the altered immunofluorescent staining pattern
 316 showing disorganization of the actin cytoskeletal filament network (Fig. 7 A). What is
 317 even more informative is that changes in their immunofluorescent staining pattern
 318 was delocalized and accompanied disordering of the actin filament organization (Fig.
 319 7 C). Taken together, it is apparent that JMY expression is essential for positioning
 320 α -actinin1 and Sorbs2 in locations that are commensurate for mediating development
 321 of an organized actin cytoskeleton network.

322

323 Discussion

324 There is suggestive evidence that the actin cytoskeleton and its binding proteins play
 325 an important role in maintaining Sertoli cell function and supporting BTB integrity
 326 and spermiogenesis. Here we identify a novel role for JMY, which is an actin
 327 regulating protein. It is an essential factor for enabling dynamic remodeling of the
 328 junctional proteins mediated by endocytic trafficking in Sertoli cells. JMY
 329 involvement in this process is evident because loss of *Jmy* function impaired BTB
 330 structural and integrity formation in Sertoli cells, which is obligatory for preventing

331 premature sloughing of developing germ cells from the seminiferous epithelium.
332 Such protection is necessary because it prevents dysfunctional sperm development
333 from reducing sperm fertility as a consequence of declines in sperm count and
334 motility that is associated with a high incidence of sperm head structural deformity.

335 Sertoli cells are rich in actin filaments, which play a crucial role in certain
336 biological activities, e.g., vesicle transport, ES formation and spermatid head shaping
337 (Du et al., 2013; Vogl et al., 2014; Li et al., 2016; Li et al., 2017). Our results indicate
338 that the JMY distribution pattern is reflective of a possible association between
339 changes in JMY expression and dynamic modulation of the actin cytoskeleton.
340 Specifically, JMY expression was evident in the Sertoli cells and later-stage
341 spermatogenic cells in the seminiferous epithelium. JMY expression exhibited a
342 filament-like expression localized parallel to the actin filaments in Sertoli cells,
343 whereas in the later-stage spermatogenic cells, it was specifically localized in the
344 perinuclear ring and manchette structure which was also rich in actin filaments. Such
345 observations suggested that JMY is likely involved in mediating actin-regulating
346 activity during spermatogenesis. Accordingly, we undertook a characterization of the
347 underlying molecular mechanisms mediating JMY function in spermatogenesis.

348 To determine the contribution by JMY expression in either Sertoli cells or
349 spermatogenic cells to spermatogenesis, we engineered mice both with Sertoli
350 cell-specific deletion of *Jmy* (*Jmy* flox; *Amh* cre) and germ cell-specific deletion of
351 *Jmy* (*Jmy* flox; *Mvh* cre). Even though the mice with germ cell-specific deletion of
352 *Jmy* were healthy and fertile (Fig. S4), male mice with Sertoli cell-specific deletion of

353 *Jmy* were sub-fertile. This condition was attributable to low sperm count and poor
 354 sperm motility as well as appreciable morphological deformity. Disruption of BTB
 355 integrity accompanying loss of *Jmy* function could account for declines in sperm
 356 fertility since reduced barrier function led to premature sloughing of developing
 357 spermatids.

358 In Sertoli cells, junctional structures undergo extensive organization and transition
 359 between “close” and “open” configurations to facilitate transport of germ cells across
 360 the BTB as well as control spermatid adhesion or sperm release. This dynamic
 361 remodeling of cell junctional barrier function greatly depends on endocytosis and
 362 linked endocytic trafficking pathways, which select the endocytosed proteins to be
 363 sorted for degradation or recycling (Cheng et al., 2011; Vogl et al., 2014). Moreover,
 364 ES is a unique actin-based cell-cell anchoring junction constituted by bundles of actin
 365 filaments that are situated between the cisternae of the endoplasmic reticulum and the
 366 apposing plasma membrane. In general, these structures are restricted to the interface
 367 between Sertoli cells and spermatids and are designated as the apical ES. On the other
 368 hand, those located at the Sertoli-Sertoli cell interface are referred to as the basal ES,
 369 which usually coexists with tight junctions and gap junctions to constitute the BTB
 370 structure. Interestingly, germ cell transport as well as endocytic vesicle trafficking in
 371 Sertoli cells requires rapid reorganization of these microfilament bundles, so that they
 372 are efficiently converted from a “bundled” to “unbundled /branched” configuration
 373 (Li et al., 2015). Briefly, there is no doubt that irrespective of the type of actin bundle
 374 configuration, in all cases they support and maintain cell junction integrity. On the

other hand, actin branching is a critical step weakening cell junction cohesiveness resulting in junctional reorganization in the seminiferous epithelium. Our electron microscopy analysis confirmed that these alterations occurred in the *Jmy* CKO seminiferous epithelium. Notably, the junctional BTB structures appeared disfigured and actin filament bundle density declined at the basal ES. Such changes cause the BTB to become more permeant resembling those in more leaky epithelia and endothelia. Furthermore, the phalloidin stained actin filaments of *Jmy* CKO Sertoli cells were in disarray having highly branched and enlarged as well as more numerous foci, suggesting that JMY might be essential as well for the maintenance of the effective bundled actin filaments and the ES junctions in Sertoli cells. These changes may also explain why loss of JMY function disrupts cell junction formation; namely, it occurs as a consequence of actin branching.

Additionally, we found that loss of *Jmy* function in Sertoli cells reduced expression of junctional proteins (i.e. ZO-1, claudin11, N-cadherin, occludin and β -catenin) and increased expression of endocytosis related proteins (i.e. clathrin, caveolin 1, EEA-1 and Rab 11) both in the testis and in the Sertoli cells. These effects are tentatively supportive of a mechanism, which links impaired cell junctions and BTB formation with abnormal endocytosis in Sertoli cells. To evaluate this possibility, the effects of testosterone (DHT) treatment or dynasore treatment were determined in an in vitro Sertoli cell culture system since these agents can increase or decrease BTB resistance, respectively and thereby mimic their dynamic behavior in vivo. Our usage of these modulators is based on reports in which testosterone promoted cell junction

397 integrity, apparently via enhancing protein endocytosis and recycling, while dynasore,
398 a well-characterized dynamin inhibitor effectively blocks endocytosis and vesicle
399 trafficking. In our case, testosterone treatment of WT Sertoli cells induced both
400 endocytosis and endosome recycling, whereas dynasore treatment impeded
401 endocytosis, which subsequently impaired cell junction formation. These opposing
402 effects were similar to those occurring in *Jmy* CKO Sertoli cells. Therefore, the loss
403 of JMY function disorders endocytosis which in turn impairs BTB cell junction
404 formation in Sertoli cells.

405 The biotinylation assay was performed to confirm that loss of JMY function does
406 indeed disorganize endocytosis. With this procedure, membrane protein endocytosis
407 was visualized in Sertoli cells. The results provide the first evidence that membrane
408 proteins are continuously endocytosed and recycled back to the cell surface, resulting
409 in an enhanced biotinylated barrier at the cell–cell interface. JMY involvement in this
410 process was clearly documented by showing that the loss of its function interrupted
411 endocytosis by causing the internalized protein to accumulate in the cytoplasm rather
412 than translocate into early endosomes. Interestingly, our results indicate that JMY
413 expression is not required for plasma membrane internalization but it is essential for
414 endocytosed vesicle trafficking into early endosomes. Notably, the actin cytoskeleton
415 is reported to play an important and variable role in vesicular transport (Liu, 2016;
416 Papadopoulos, 2017). This realization supports the notion that JMY induced vesicle
417 trafficking is mediated through JMY interacting with the actin cytoskeleton.

418 Our protein interaction analyses provide other indications that JMY along with

other actin binding proteins (i.e. Arp3, Sorbs2, α -actinin1) cooperate with each other to perform specific functions in Sertoli cells. One of them includes the Arp2/3 complex which JMY activates and produces branched F-actin filaments. Moreover, the Arp2/3 complex modulates invagination and pinching off of endosomes. They are then delivered to sorting endosomes as well as actin branches to promote BTB structural reorganization in a number of different vesicle-trafficking pathways (Lie et al., 2010). Remarkably, JMY was capable of nucleating actin filaments in either the presence or absence of the Arp2/3 complex (Zuchero et al., 2009). Herein, our results indicate that the effect of JMY on the actin cytoskeleton seems to be independent of the Arp2/3 complex in Sertoli cells. Its protein interaction might instead depend on its three WH2-domains that interact with other proteins.

To determine if this speculation is credible, IP and MS analysis were performed and revealed that JMY could interact with Sorbs2 and α -actinin1 in Sertoli cells. Sorbs2 (also known as Arg Kinase-binding Protein 2, ArgBP 2), contains an N-terminal SoHo (Sorbin homology) domain and three conserved SH3 domains in the C-terminal region. It has been reported to associate with actin fibers, adherens junction and tight junctions, and is involved in actin cytoskeletal organization, cell adhesion and migration (Roignot and Soubeyran, 2009). On the other hand, α -actinin1 (ACTN 1) is one of the major actin cross-linking proteins and anchors actin filaments to junctional structures and has an important role in some cellular biological processes such as cytokinesis, cell adhesion and cell migration (Murphy and Young, 2015). Recent evidence implicates that Sorbs2 can interact with α -actinin, subsequently

441 binding to actin filaments (Rönty et al., 2005; Anekal et al., 2015). The current study
 442 is the first one describing interactions between the α -actinin1, Sorbs2 and JMY triad
 443 in Sertoli cells. Such interactions are consistent with our identification of
 444 colocalization of JMY with Sorbs2 and α -actinin1 along the peripheral edges of
 445 Sertoli cells. This delimited expression was particularly evident proximal to the nuclei.
 446 These regions demarcate the basal region which delineates both Sertoli cell polarity
 447 and the boundary between the uppermost apical and basal regions constitutes the BTB.
 448 Additionally, loss of *Jmy* function could lead to disordered distribution of Sorbs2 and
 449 α -actinin1, suggesting a critical role played by JMY in cross-linking the actin-related
 450 proteins to actin filaments.

451 In conclusion, JMY, an actin regulating protein, is essential for mediating
 452 dynamic remodeling of the BTB junctional proteins in Sertoli cells. Its involvement in
 453 this process is requisite for the recycling phase of endocytic trafficking. Accordingly,
 454 JMY is crucial for sustaining the BTB structural organization commensurate with
 455 sperm health and male fertility.

456

457 **Materials and methods**

458 **Animals**

459 C57BL/6 mice were purchased from Shanghai Laboratory Animal Center.
 460 Anti-Mullerian hormone (*Amh*)-Cre mice (Jax number 007915) and *Jmy*-loxP mice
 461 (the *Jmy* exon 3 flanked by 2 loxP sites) were from Shanghai Research Center For
 462 Model Organisms (Shanghai, China). All of the mice were acclimated in the Animal

Center of Shanghai Jiao Tong University School of Medicine. Animal experiments were conducted according to the International Guiding Principles for Biomedical Research Involving Animal, as promulgated by the Society for the Study of Reproduction. This research program was approved by the ethics committee of Shanghai Jiao Tong University School of Medicine (NO. A2015-034).

Fertility evaluation

Individually housed, sexually mature male mice (8 weeks old) cohabitated with two virgin female mice (10 weeks old) for 7 days, and they were then separated from one another. Every day during cohabitation, females were examined for vaginal plugs as evidence of mating. Approximately, 20 days after the last day of cohabitation, the number of pups delivered by each mated female mouse was counted and the litter sizes were analyzed.

Sperm parameters analyses

The cauda epididymides were dissected and then placed in pre-warmed (37°C) Tyrode's Buffer (Sigma-Aldrich) to allow dispersion of spermatozoa. After 15 minutes, sperm motility, progressive motility and concentration were analyzed by computer-assisted sperm analysis (CASA) (Hamilton Thorne).

For teratozoospermia analysis, a sperm pellet was initially smeared on a glass slide. After reaching dryness at room temperature, the slide was fixed and stained as described in the Diff-Quick method (BRED Life Science Technology Inc., China).

485 The slide was viewed under a microscope (Olympus BX53) equipped with an
486 UPlanFN N 40×/0.75 objective (Olympus).

487

488 **Tissue preparation**

489 Mice were euthanized with CO₂ and then their testis and epididymis were removed.
490 For RNA analysis, tissue was immediately snap frozen in liquid N₂. For histological
491 analysis, tissues were fixed overnight either in Bouin's solution or in 2.5%
492 glutaraldehyde buffer for electron microscopy analysis.

493

494 **Histological Analysis**

495 Testes and epididymis fixed in Bouin's solution were embedded in paraffin, and
496 specimens were sliced into 5 mm thick sections and mounted on glass slides, followed
497 by deparaffinization and rehydration. The sectioned testicular and epididymal tissues
498 were then stained with hematoxylin and eosin (H&E) and observed under a
499 microscope (Olympus BX53) equipped with an UPlanFN N 20×/0.5 objective and an
500 UPlanFN N 40×/0.75 objective (Olympus).

501

502 **Transmission electron microscopy analysis**

503 Small pieces of testicular tissue were immersed in 2.5% glutaraldehyde solubilized in
504 0.1M phosphate buffer (pH 7.4) for 1 day. The tissues were then fixed in 1% osmium
505 tetroxide and dehydrated through a graded ethanol series, and embedded in Epon 618
506 (TAAB Laboratories Equipment). Ultra-thin sections (70-90 nm) in the seminiferous

507 epithelium region were stained with lead citrate and uranyl acetate, and then
508 examined at 100 kV with a Philips CM-120 (Philips).

509

510 Western blot analysis

511 Mice testes or cultured cells were homogenized in RIPA lysis buffer (Thermo Fisher
512 Scientific) containing protease inhibitor cocktail (Roche) on ice for 30 min followed
513 by centrifugation at 12,000 xg, for 10 min, at 4°C. The proteins in the supernatant
514 were collected and the protein concentrations were determined by the BCA Protein
515 Assay Kit (Thermo Fisher Scientific).

516 Protein samples (20 µg) were separated using 8% – 16% denaturing
517 polyacrylamide gels, then transferred to polyvinylidene difluoride (PVDF)
518 membranes (Millipore) using a semi-dry transfer apparatus (Bio-Rad). Membranes
519 were blocked with 5% bovine serum albumin (BSA) for 1h at room temperature and
520 immunoblotting was performed overnight at 4°C with the primary antibodies (Table
521 S3), followed by incubation with secondary antibody conjugated to HRP (Jackson
522 ImmunoResearch). Signals were generated by enhanced chemiluminescence
523 (Millipore) and detected by luminescent image analyzer (GE imagination LAS 4000).

524

525 Immunohistochemistry (IHC) and Immunofluorescence (IF) analyses

526 IHC and IF stainings were performed using standard protocols. For IHC staining,
527 paraffin sections were dewaxed and rehydrated, followed by antigen retrieval through
528 boiling the tissue for 15 min in 10 mM citrate buffer, pH 6.0. Then, the Histostain

LAB-SA Detection kits (Invitrogen) were applied according to the manufacturer's instructions, and the sections were stained using DAB and nuclei were counterstained with hematoxylin. Digital images were captured under a microscope (Olympus BX53) equipped with an UPlanFN N 20×/0.5 objective (Olympus).

For IF staining, frozen 8-μm thick sections were prepared and then fixed with 4% paraformaldehyde for 20 min at 4°C. The unspecific binding sites were blocked with 10% BSA/PBS for 60 min at room temperature, and sections were incubated with the primary antibodies (Table S3) overnight at 4°C. Then, fluorescent-labeled secondary antibodies (1:500, donkey anti-rabbit Alexa Fluor 488, donkey anti-mouse Alexa Fluor 488, or donkey anti-rabbit Alexa Fluor 555, donkey anti-mouse Alexa Fluor 555, Jackson ImmunoResearch) were used. Nuclei were counterstained with DAPI (Sigma-Aldrich). The fluorescence signals were detected under a laser scanning confocal microscope (Carl Zeiss LSM-510, Germany) equipped with an argon laser (488 nm), a He/Ne laser (543 nm), an EC Plan-NEOFLUAR 63×/1.25 objective and a LD LCI Plan-APOCHROMAT 25×/0.8 objective (Zeiss). Digital images were captured and processed using Aim software (Zeiss Systems).

Cell cycle analysis

Rate of germ cell development during spermatogenesis was studied in testis using propidium iodide (PI) staining combined with flow cytometer analysis, which resolved cell types based on differences in their DNA content. Briefly, the testes were incubated in 1 ml PBS containing 1 mg/ml collagenase (Type IV; Sigma) at 37 °C for

30 min with gentle agitation. After sedimentation for 5 min, the supernatant was removed and the seminiferous tubules were collected. Then, the seminiferous tubules were digested in accutase cell dissociation reagent (Innovative Cell Technologies) for 15 min and the samples were passed through a nylon filter followed by centrifugation at 400 xg for 5 min at room temperature. Thereafter, the pellet was re-suspended with 0.5 ml PBS-E (1 mM EDTA in PBS) and fixed with ice-cold 70% ethanol overnight at 4°C. The cells were permeabilized followed by staining with 0.5 mL PI/ribonuclease staining buffer (BD Biosciences) and incubated at room temperature for 15 min in the dark. Finally, DNA content of PI stained cells was analyzed in a flow cytometer (Beckman Coulter CytoFlex S) equipped with a 561nm laser. In each sample, 50,000 events were recorded and further analyzed by Cytexpert software (Beckman Coulter). Three different cell populations were resolved in seminiferous tubules: 1N (spermatids), 2N (spermatogonia, secondary spermatocytes and Sertoli cells) and 4N (primary spermatocytes), and their ratios were calculated.

565

566 **BTB assay**

In vivo BTB assay was performed as described (Holembowski et al., 2014). Biotin labeled reagent (10 mg/ml Sulfo-NHS-SS-Biotin, Thermo Scientific) was freshly prepared in PBS containing 1 mM CaCl₂, and then 25 µl of this solution were carefully injected into the center of the testis at low pressure. The contralateral testis was injected with the same volume of calcium chloride solution alone and served as a negative control. After 30 min exposure to biotin, the animals were euthanized, and

the testes were harvested and fixed in 10% neutral buffered formalin. Testis sections were probed sequentially with Alexa Fluor 488 conjugated Streptavidin (Jackson ImmunoResearch) to evaluate the extracellular distribution of biotin within the seminiferous epithelium. Finally, the fluorescence signal was captured under a laser scanning confocal microscope (Carl Zeiss LSM-510) equipped with an argon laser (488 nm) and a LD LCI Plan-APOCHROMAT 25×/0.8 objective (Zeiss).

Primary Sertoli cell culture

Primary Sertoli cell culture was carried out as described (Sato et al., 2013; Holembowski et al., 2014) with some modifications. Briefly, to obtain Sertoli cells for primary culture, testes of adult mice were decapsulated in HBSS and seminiferous tubules were dispersed in a HBSS solution containing collagenase (0.1%)/hyaluronidase (0.1%)/DNase (0.04%) for 20 min at 34°C. After washing with PBS, an additional digestion step was performed with accutase cell dissociation reagent (Innovative Cell Technologies) for 15 min at 34°C. The tubular pellet was washed with PBS and Sertoli cells were freed from the seminiferous epithelium by resuspending the pellet in DMEM/F12 medium. Cells in the supernatant were collected and cultured in DMEM/F12 medium containing 5% FBS overnight at 34°C. Following attachment of the Sertoli cells to the bottom of the tissue culture plate, they acquired an irregular shape, whereas the germ cells remained suspended and were easily removed by repeated washing. The purity of primary Sertoli cells was routinely analyzed by immunofluorescent staining with SOX9.

595

596 **Endocytosis assay**

597 Endocytosis was evaluated as described previously (Le et al., 1999). Briefly, cell
 598 surface proteins were biotinylated with 0.5 mg/ml Sulfo-NHS-SS-Biotin (Pierce) in
 599 PBS containing 0.9 mM CaCl₂ and 0.33 mM MgCl₂ at 4 °C for 30 min, and then
 600 quenched with 50 mM NH₄Cl in PBS at 4 °C for 15 min and subsequently, incubated
 601 at 34 °C for the indicated periods of time during which endocytosis occurred.
 602 Biotinylated cargo proteins were then stained with Streptavidin-coupled FITC
 603 (Jackson) and detected under a laser scanning confocal microscope (Carl Zeiss
 604 LSM-510) equipped with an argon laser (488 nm) and an EC Plan-NEOFLUAR
 605 63×/1.25 objective (Zeiss).

606

607 **Protein Immunoprecipitation (IP) and mass spectrometry (MS) assay**

608 Protein immunoprecipitation was characterized using the Pierce Direct IP kit (Thermo
 609 scientific) according to the manual instructions. The LC-MS analysis was performed
 610 as described (Liu et al. 2015). All MS/MS spectra were searched using Proteome
 611 Discoverer2.2 software against the mouse UniProt database. Two missing cleavage
 612 sites were allowed. The tolerances of peptides and fragment ions were set at 6 ppm
 613 and 0.5 Da, respectively.

614

615 **Statistical Analysis**

616 All data were analyzed using SAS 8.2 software, and results are presented as mean ±

SD. Group comparisons were made using Student's t-test where appropriate. One-way analysis of variance (ANOVA) test was used assuming a two-tail hypothesis with $P < 0.05$. Differences were considered statistically different when $P < 0.05$.

Supplemental material

Fig. S1 immunofluorescent staining indicates that JMY was localized in both Sertoli cells and spermatids in the seminiferous epithelium. Fig. S2 illustrates the gene engineering scheme used to obtain mice with conditional deletion of *Jmy*. Fig. S3 shows the COG analysis of proteins that immunoprecipitated with JMY. Fig. S4 shows that the testicular morphology, litter size and sperm parameters are normal in germ cell-specific deletion of *Jmy* (*Jmy* flox; *Mvh* cre) male mice. Table S1 shows results of the MS analysis of proteins immunoprecipitated with JMY. Table S2 shows the results of KEGG pathway analysis of proteins immunoprecipitated with JMY. Table S3 lists the primary antibodies used in this study.

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641

642 **Author contributions**

643 Y. Liu conducted and performed experiments, analyzed data, and prepared the
644 manuscript. J. Fan performed the animal experiments and analyzed the data. Y. Yan, X.
645 Dang, R. Zhao and Y. Xu performed part of molecular experiments. Z. Ding designed
646 and supervised the project, and provided final approval of the manuscript.

647

648 **Conflict of interest**

649 The authors declare that there is no conflict of interest that would prejudice the
650 impartiality of this work. The authors also declare no competing financial interests.

651

652 **Abbreviations**

653 ARP3, actin-related protein 3; BTB, blood–testis barrier; CKO, conditional knock out;
654 DHT, dihydrotestosterone; EEA-1, early endosomal antigen; ES, ectoplasmic
655 specialization; H&E, hematoxylin and eosin; IP, immunoprecipitation; MS, mass
656 spectrometry; JMY, junction-mediating and regulatory protein; Sorbs2, Sorbin and
657 SH3 domain containing protein 2; ZO-1, zonula occludens-1.

658

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768 **Figure Legends**

770 **Figure 1. JMY expression in testis and Sertoli cells.** (A) JMY Immunofluorescent

771 staining shows that JMY expression is localized in the Sertoli cells of mouse testis
772 (Arrows indicated). Bars: (main) 50 μm ; (enlarged) 20 μm . (B) JMY
773 immunofluorescent staining and F-actin rhodamine-phalloidin staining of primary
774 cultured Sertoli cells shows that its expression is localized in the cytosol and nucleus.
775 In the cytosol, JMY has a filamentous distribution, which in some places colocalizes
776 with F-actin. Scale Bar: 20 μm .

777

778 **Figure 2. WT and *Jmy* CKO sperm morphology analyses.** (A) Western blot
779 analysis resolves JMY expression in mouse testicular Sertoli cells. JMY expression
780 was greatly reduced in CKO testis relative to that in its WT counterpart and Het testis,
781 JMY expression was undetectable in Sertoli cells of CKO mice. GAPDH expression
782 validated loading equivalence. (B) JMY immunofluorescent staining of shows that
783 JMY expression in Sertoli cells was absent in testis from *Jmy* CKO mice. Scale Bar:
784 50 μm . (C) Litter size of mature female mice mated with *Jmy* CKO male mice was
785 greatly reduced relative to those mated with WT and Het male mice. Error bars
786 represent SD (n=10). $^{**}P < 0.01$. (D) Sperm motility analyzed by CASA shows that
787 both sperm motility and progressive motility of *Jmy* CKO mice were less than that in
788 WT and Het mice. Error bars represent SD (n=5). $^{*}P < 0.05$; $^{**}P < 0.01$. (E) Sperm
789 concentration analyzed by CASA, shows that sperm concentration of *Jmy* CKO mice
790 was lower than that in WT and Het mice. Error bars represent SD (n=5). $^{*}P < 0.05$. (F)
791 Sperm morphology of WT and *Jmy* CKO mice. Except for the prominent
792 deformations in the *Jmy* CKO sperm heads, the other regions appear unchanged from

793 the normal WT morphology (CKO, arrows indicated). Bars: (main) 50 μ m; (enlarged)
794 20 μ m. (G) Deformed sperm head ratios calculated from five independent
795 experiments of at least 200 spermatozoa in each independent experiment. Error bars
796 represent SD (n=5). $**P < 0.01$.

797

798 **Figure 3. Loss of JMY function induces testicular histological disorder.** (A-B) In
799 H&E stained testicular (A) and caput epididymal (B) sections, testicular cellular
800 disorganization is evident and spermatid epithelial adherence is less. These alterations
801 obstruct caput epididymis lumen patency in *Jmy* CKO mice. Bars: (main) 100 μ m;
802 (enlarged) 50 μ m. (C) Cell cycle analysis results resolve different cell types in the
803 seminiferous epithelium. DNA content of indicated cell types: diploid (2N), tetraploid
804 (4N) and haploid (N). Statistical results show that the proportion of haploid cells
805 increased in *Jmy* CKO mice. Error bars represent SD (n = 5; 30,000 cells per test).
806 $**P < 0.01$. (D) Vimentin immunohistochemical staining is evident in testicular
807 sections. Vimentin is one kind of intermediate filamentous protein that is an
808 established marker of Sertoli cells. Sertoli cell numbers in every seminiferous tubule
809 from WT mice and *Jmy* CKO mice are not significantly different from one another.
810 Error bars represent SD (n = 30; 5 tubules/trial). (E-F) Transmission electron
811 micrograph analysis of seminiferous epithelium shows that spermatid cell adhesions
812 are incomplete within the seminiferous epithelium of *Jmy* CKO testes (E) which are
813 accompanied by disrupted junctional structures between neighboring Sertoli cells (F).
814 Arrows in (F) indicate the actin bundles of the ES in WT mice. Bars: (E) 10 μ m; (F) 2

815 μm ; (zoom in F) 0.2 μm . (G) The biotin assay evaluated BTB integrity in vivo. Alexa
816 Fluor 488 staining visualized biotin distribution. The diffuse green fluorescent
817 staining throughout all the layers of the testicular seminiferous epithelium indicates
818 that loss of JMY function contributes to defective BTB structural formation in the he
819 *Jmy* CKO mice. Scale Bar: 100 μm .

820

821 **Figure 4. Loss of JMY function disrupts cell junctional integrity between**
822 **testicular Sertoli cells.** (A) Comparison of Western blot analyses of testicular protein
823 expression in WT and *Jmy* CKO mice. Clathrin and EEA-1, endocytosis related
824 proteins; ZO-1, β -catenin, N-cadherin and Claudin11 expressions identified junctional
825 proteins; Vimentin and GAPDH expression validated loading control equivalence. (B)
826 Immunohistochemical staining of testicular sections, junctional Claudin11 and
827 N-cadherin proteins, shows that their expression is localized to the basolateral region
828 of adjacent Sertoli cells in WT testes. In contrast, such delimited junctional expression
829 is not evident in *Jmy* CKO testicular Sertoli cells. Scale Bar: 50 μm . (C) Western blot
830 analyses of proteins expression patterns in WT Sertoli cells and *Jmy* CKO Sertoli cells,
831 as well as in those treated with either DHT or Dynasore. Vimentin and GAPDH
832 expression validated loading control equivalence. (D) Immunofluorescent staining of
833 junction proteins (N-cadherin and ZO-1) and endocytosis related proteins (Caveolin-1
834 and Clathrin), shows decreased amount of N-cadherin and ZO-1 expression whereas
835 caveolin-1 and clathrin expression increased in the *Jmy* CKO Sertoli cells. Scale Bar:
836 50 μm .

837

838 **Figure 5. Endosome cycling disrupted in *Jmy* CKO Sertoli cells.** (A) Changes in
839 cell surface biotinylation evaluated endocytosis and recycling in Sertoli cells. After
840 biotin labeling for 0h, 1h and 3h, the intracellular translocation of the biotinylated
841 membrane proteins was detected by fluorescent staining. Bars: 20 μ m. (B) Dynasore
842 treatment blocked endocytosis, which resulted in less cytoplasmic biotinylated protein
843 accumulation in the cytoplasm. Bars: 20 μ m. (C) The contribution by functional JMY
844 expression to mediating endocytosis of biotinylated membrane proteins was evaluated
845 based on its co-localization with endosomal biomarkers. In *Jmy* CKO Sertoli cells,
846 immunofluorescent staining shows that the biotinylated membrane proteins
847 accumulated in the cytoplasm and co-localized with endocytic vesicle markers
848 (clathrin and caveolin), whereas less biotinylated proteins co-localized with
849 endosomal protein markers (EEA-1 and Rab 11). Scale Bars: 20 μ m.

850

851 **Figure 6. Identification of the proteins interacting with JMY in Sertoli cells.** (A)
852 Proteins coimmunoprecipating with JMY antibodies are evident in Western blots
853 interrogated with α -actinin1 and Sorbs2 antibodies in a protein lysate of Sertoli cells
854 expressing JMY. (B) Immunofluorescent staining identifies co-localization of JMY
855 with either α -actinin1 or Sorbs2 in Sertoli cells. Scale Bars: (main) 20 μ m; (zoom) 5
856 μ m

857

858 **Figure 7. Loss of JMY function disrupts interactions between actin filament and**

859 **actin regulating proteins in Sertoli cells.** (A) Rhodamine-phalloidin staining
 860 identified numerous actin filaments foci in *Jmy* CKO Sertoli cells (Arrows highlight).
 861 Scale Bars: (main) 50 μ m; (enlarged) 20 μ m. (B) Western blot analyses of α -Actinin
 862 and Sorbs2 expression show that their expression levels are slightly less in *Jmy* CKO
 863 Sertoli cells than in the WT counterpart, but that of Arp3 is unchanged in *Jmy* CKO
 864 Sertoli cells. Vimentin and β -actin expression levels document loading control
 865 equivalence. (C) Localized Arp3, actinin1 and Sorbs2 immunofluorescent staining in
 866 WT Sertoli cells and *Jmy* CKO Sertoli cells. Arp3, actinin1 and Sorbs2 colocalize
 867 with actin filaments in WT Sertoli cells whereas their close proximity to one another
 868 is lost in *Jmy* CKO Sertoli cells. Scale Bars: 20 μ m.

869

870 **Tables and their legends**

871 **Table S1. MS identification of proteins immunoprecipitated with JMY**

872 **Table S2. KEGG pathway analysis of proteins immunoprecipitated with JMY**

873 **Table S3. Primary antibodies used in this study**

874

875 **Figure S1. JMY localization in mouse testis.** JMY immunofluorescent staining was
 876 selective and evident in the seminiferous epithelium (A). The staining was greatly
 877 localized to the Sertoli cells (B) and in the post acrosomal region of spermatids in
 878 close proximity to the manchette structure (C). Arrows indicate JMY
 879 immunofluorescent staining in Sertoli cells (B) or spermatids (C). Scale Bars: (A) 50
 880 μ m; (B and C) 20 μ m.

881

882 **Figure S2. Scheme of engineering *Jmy* Sertoli cell CKO mice.** Two loxP sites were
883 inserted into both sides of the third exon in the *Jmy* gene using homologous
884 recombination. Specific deletion of *Jmy* was carried out by a Sertoli cell specific
885 expression of Cre recombinase.

886

887 **Figure S3. Orthologous groups of proteins clusters (COG) identified based on**
888 **analysis of proteins coprecipitating with JMY in Sertoli cells.**

889

890 **Figure S4. Germ cell fertility unaltered by loss of *Jmy* function.** Male germ cell
891 fertility is unaffected in *Jmy* CKO (A-C) Litter size (A), Sperm concentration (B) and
892 Sperm motility (C). *Jmy* CKO male mice (*Jmy* flox; *Mvh* cre) germ cell fertility is
893 similar to those in WT male mice. Error bars represent SD (n=5). (D) H&E stained
894 testicular sections indicate no significant change in histological structure and integrity
895 in *Jmy* CKO testis (n = 3). Scale Bars: (main) 100 µm; (enlarged) 50 µm.

A

JMY

JMY

Control

B

JMY

F-actin

Merged

A **B** **C** **D**

A **B** **C**

A **B**

A

B

C