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## DNA methylation and histone post-translational modifications in the mouse germline following in-vitro maturation of fresh or cryopreserved prepubertal testicular tissue

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1 **Running title:** Epigenetic modifications in *in vitro* cultured mouse testes

2

3 **Title:** DNA methylation and histone post-translational modifications in the mouse germline

4 following *in vitro* maturation of fresh or frozen/thawed prepubertal testicular tissue

5

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16

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18 **Abstract**

19

20 **Research question:** Do freezing/thawing and *in vitro* culture procedures affect the expression  
21 of DNA methyltransferases (DNMTs) and histone modifying enzymes, as well as the  
22 establishment of DNA methylation and histone post-translational modifications (PTMs) in  
23 germ cells in prepubertal mouse testicular tissue?

24 **Design:** We investigated the expression of epigenetic modification enzymes, DNA  
25 methylation and histone PTMs, and the spermatogenic progression after *in vitro* maturation of  
26 fresh or frozen/thawed mouse prepubertal testicular tissue. Fresh or thawed testicular  
27 fragments from 6-7 days *postpartum* mice were cultured for 30 days in the presence of retinol  
28 with or without follicle stimulating hormone (FSH).

29 **Results:** The *in vitro* maturation of fresh or frozen/thawed tissue allowed the differentiation  
30 of spermatogonia into spermatozoa. Differences in the levels of transcripts encoding  
31 epigenetic modifications enzymes (*Dnmt1*, *Dnmt3a*, *Prdm9*, *Jarid1b*, *Src1*, *Sirt1*, *Hdac1*)  
32 were found between 30-day tissue cultures and age-matched *in vivo* controls.

33 DNMT1/DNMT3a expression and the presence of 5-methylcytosine (5mC) were detected in  
34 spermatogonia and leptotene/zygotene spermatocytes in cultures. The relative 5mC  
35 fluorescence intensity was similar in spermatozoa produced in cultures of frozen/thawed  
36 tissues or *in vivo*. H3K4me3, H3K9ac and H4K8ac were present in all germ cell types but  
37 differences in the proportion of germ cells containing these epigenetic marks were found after  
38 cultures.

39 **Conclusions:** Despite differences with the *in vivo* situation, DNA methylation and histone  
40 methylation and acetylation occur in the mouse germline in *in vitro* matured fresh or  
41 frozen/thawed mouse prepubertal testicular tissue, and the expression of the enzymes  
42 catalyzing these epigenetic modifications are maintained *in vitro*.

43

44 **Keywords:** mouse prepubertal testis, freezing protocols, *in vitro* spermatogenesis, epigenetic  
45 modification, DNA methylation, histone post-translational modifications.

46

47 **Key message:** In prepubertal boys with cancer, fertility preservation relies on testicular tissue  
48 freezing. *In vitro* maturation of frozen/thawed tissue is one of the approaches envisaged to  
49 restore fertility. The impact of the procedures on germline epigenetic modifications needs to  
50 be evaluated in an animal model before considering a human application.

## 51 **Introduction**

52

53 Survival rates for children with cancer have considerably increased over the past decades  
54 thanks to diagnostic and therapeutic progress. Treatments by chemotherapy and radiotherapy  
55 can however have toxic effects on gonads (Jahnukainen et al., 2015; Picton et al., 2015; Wyns  
56 et al., 2010). In prepubertal boys, the actively dividing spermatogonial stem cells (SSCs) are  
57 particularly sensitive to certain cytotoxic agents used in chemotherapy protocols (Goossens et  
58 al., 2013; Nurmio et al., 2009). At adulthood, cured patients can be diagnosed with a  
59 permanent infertility (oligozoospermia, azoospermia) with significant psychological  
60 consequences (Gies et al., 2015; Jahnukainen et al., 2015).

61 Because of the potential long-term sequelae of cancer treatment on reproductive functions,  
62 fertility preservation options have been developed and are proposed to patients prior to cancer  
63 therapy (Baert et al., 2012; Gies et al., 2015; Jahnukainen et al., 2015; Onofre et al., 2016;  
64 Picton et al., 2015; Wyns et al., 2015). For adolescents and adults, sperm can be  
65 cryopreserved before the beginning of gonadotoxic treatments and later used for assisted  
66 reproductive techniques. For prepubertal boys however, freezing the testicular tissue that  
67 contains SSCs is so far the only option. Freezing (slow freezing or vitrification) and thawing  
68 procedures have been optimized for immature testicular tissues in order to minimize ice  
69 crystal formation and cellular damage (Baert et al., 2012; Poels et al., 2013). We previously  
70 reported the development of optimal controlled slow freezing (CSF) and solid surface  
71 vitrification (SSV) protocols that preserve not only the architecture of mouse prepubertal  
72 tissue but also its exocrine and endocrine functions (Arkoun et al., 2016; Dumont et al., 2015;  
73 Milazzo et al., 2008).

74 In order to restore the fertility of cancer survivors who became sterile, thawed testicular  
75 tissues could theoretically be matured by *in vivo* or *in vitro* approaches to produce

76 spermatozoa for assisted reproduction (de Michele et al., 2017; Gies et al., 2015; Ibtisham et  
77 al., 2017; Yokonishi and Ogawa, 2016). The *in vivo* differentiation of SSCs could be achieved  
78 after autologous transplantation or autograft or eventually after xeno-transplantation or  
79 xenograft (Goossens et al., 2013; Milazzo et al., 2010; Poels et al., 2013). To avoid the  
80 potential reintroduction of malignant cells to surviving patients during auto-transplantation of  
81 cryopreserved testicular tissue or cells, especially to those treated for lymphoblastic acute  
82 leukemia, testicular tissues could be matured *in vitro* (Jahnukainen et al., 2015).

83 Tridimensional and organotypic cultures are so far the only methods that allowed the  
84 production of spermatozoa from prepubertal murine testes (Abu Elhija et al., 2012; Arkoun et  
85 al., 2016; Dumont et al., 2015; Sato et al., 2011; Yokonishi et al., 2014). Notably, a complete  
86 *in vitro* spermatogenesis could be achieved in frozen (slow freezing or vitrification)/thawed  
87 mouse prepubertal testicular tissues cultured at a gas-liquid interphase (Arkoun et al., 2016;  
88 Dumont et al., 2015; Yokonishi et al., 2014). Our data moreover showed that supplementing  
89 the culture medium with  $10^{-6}$  M retinol promoted SSCs differentiation and entry into meiosis  
90 as well as sperm production in fresh or frozen/thawed testes (Arkoun et al., 2015; Dumont et  
91 al., 2017, 2016; Rondanino et al., 2017). A recent study confirmed that retinoic acid and  
92 retinol play crucial roles in the onset of spermatogonial differentiation and meiotic initiation  
93 under organ culture conditions (Sanjo et al., 2018). The addition of follicle stimulating  
94 hormone (FSH), crucial for the initiation and progression of spermatogenesis, together with  
95 retinol in the medium had no additive effect on the spermatogenic yield (Arkoun et al., 2015).

96 We recently reported that most of *in vitro* generated spermatozoa were haploid, contained  
97 unfragmented DNA and normally condensed chromatin like their *in vivo* counterparts  
98 (Oblotte et al., 2017). Although the microinjection of *in vitro* produced spermatozoa into  
99 oocytes was capable of producing reproductively competent offspring (Sato et al., 2011;  
100 Yokonishi et al., 2014) with an almost normal state of methylation for 11 imprinted genes

101 (Yokonishi et al., 2014), the epigenetic modifications of germ cells in cultured prepubertal  
102 testes have never been studied. Epigenetic marks, including DNA methylation and histone  
103 post-translational modifications (PTMs), are however important during spermatogenesis for  
104 the proper maturation of gametes, and after fertilization the inherited marks play an essential  
105 role during embryonic development and can later influence offspring health and behavior  
106 (Champroux et al., 2018; Jenkins et al., 2017; Stewart et al., 2016; Stuppia et al., 2015; Yao et  
107 al., 2015; Boissonnas et al., 2013). DNA methylation is an epigenetic mechanism involving  
108 the covalent transfer of a methyl group onto the C5 position of cytosine to form the heritable  
109 mark 5-methylcytosine (5mC). This epigenetic modification, which is catalyzed by DNA  
110 methyltransferases (DNMT), represses the expression of numerous genes. DNMT1 is  
111 responsible for the maintenance of DNA methylation profiles during cell divisions (Uysal et  
112 al., 2016). DNMT3a and DNMT3b catalyze *de novo* DNA methylation in a heterodimer with  
113 the non-catalytic DNMT3L (Uysal et al., 2016). During mouse development, the genome of  
114 primordial germ cells undergoes a wave of demethylation between 8 and 13.5 days *post*  
115 *coitum* (Boissonnas et al., 2013). *De novo* methylation marks are then established in a sex-  
116 specific manner. In males, *de novo* DNA methylation to establish paternally imprinted loci  
117 begins at around 15.5 days *post coitum* and continues after birth in spermatogonia and  
118 spermatocytes. The process of *de novo* DNA methylation appears to be complete at the  
119 pachytene spermatocyte stage (Boissonnas et al., 2013; Yao et al., 2015). Knocking out the  
120 mouse *Dnmt1* gene was shown to induce embryonic lethality, and disruption of *Dnmt3a* and  
121 *Dnmt3l* genes led to impaired spermatogenesis (Jenkins and Carrell, 2012; Uysal et al., 2016).  
122 In men, abnormal sperm DNA methylation patterns have been associated with infertility, poor  
123 embryo quality and congenital diseases in the offspring (Cui et al., 2016; Gunes et al., 2016;  
124 Kitamura et al., 2015; McSwiggin and O'Doherty, 2018; Santi et al., 2017; Stuppia et al.,  
125 2015). Histone PTMs, such as methylation and acetylation, have been reported to be

126 associated with chromatin remodeling during spermatogenesis, including histone-to-  
127 protamine replacement occurring within germ cells during spermiogenesis. Histone  
128 methylation corresponds to the addition of one or several methyl groups on lysine (K) or  
129 arginine (R) residues by histone methyltransferases (e.g. PRDM9) and can be reversed by  
130 histone demethylases (e.g. JARID1B). PRDM9, which catalyzes the trimethylation of lysine 4  
131 of histone H3 (H3K4me3), is essential for progression through early meiotic prophase,  
132 including homologous chromosome pairing, DNA double-strand break repair and sex body  
133 formation (Hayashi et al., 2005). This mark is associated with transcriptionally active genes  
134 and is also enriched at developmental gene promoters within spermatozoa (Hammoud et al.,  
135 2011). The acetylation of histones on lysine residues, an epigenetic modification found in  
136 transcriptionally active chromatin regions, is catalyzed by histone acetyltransferases (e.g.  
137 SRC1) and reversed by histone deacetylases (e.g. SIRT1, HDAC1). An increase in the  
138 acetylation of histones H3 and H4 is observed in spermatogonia and spermatids compared to  
139 spermatocytes (Boissonnas et al., 2013; Luense et al., 2016). The H3K9ac mark is essential  
140 for the progression of spermiogenesis: alterations in H3K9ac levels induce morphological  
141 abnormalities and apoptosis of round spermatids (Dai et al., 2015). Mice with a specific  
142 deletion of *Sirt1* in male germ cells exhibit a defect in histone-to-protamine transition, altered  
143 chromatin condensation, defects in acrosome biogenesis and decreased fecundity (Bell et al.,  
144 2014; Liu et al., 2017). Moreover, a premature acetylation of histone H4 in spermatocytes or a  
145 decrease of histone acetylation in spermatids have been reported in infertile men exhibiting  
146 impaired spermatogenesis (Sonnack et al., 2002). Alterations in the genomic distribution of  
147 the heritable marks H3K4me3, H3K9ac and H4K12ac have also been described in the  
148 spermatozoa of infertile men (Hammoud et al., 2011; Steilmann et al., 2011; Vieweg et al.,  
149 2015).



150 It has been demonstrated that DNMT1 and DNMT3A expression levels, DNA methylation  
151 status of germ cells and several histone PTMs (H3K4me3, H3K9ac, H4K12ac, H4K16ac)  
152 were unchanged after *in vivo* maturation of SSCs from prepubertal mouse testes by  
153 transplantation or graft (Goossens et al., 2011). However, H4K5ac and H4K8ac marks were  
154 altered after SSCs transplantation (Goossens et al., 2011). It appears therefore imperative to  
155 ascertain that DNA methylation and histone PTMs are not altered in the germline in  
156 organotypic cultures before applying *in vitro* maturation procedures to frozen/thawed  
157 testicular tissue from prepubescent boys. So far, the maintenance of DNA methylation has  
158 only been shown in 2 to 4-day cultures of rat fetal testes (Rwigemera et al., 2017) and in 7 to  
159 21-day cultures of marmoset adult testes (Langenstroth-Röwer et al., 2017). Histone PTMs  
160 (H3K4me2, H3K4me3) were also maintained in short-term cultures (Rwigemera et al., 2017).  
161 In the present work, fresh, CSF or SSV mouse prepubertal testicular tissues were cultured for  
162 30 days at a gas-liquid interphase in the presence of retinol alone or of a retinol/FSH  
163 alternation. The progression of *in vitro* spermatogenesis was analyzed. DNA methylation,  
164 active histone marks (H3K4me3, H3K9ac, H4K8ac) and the expression of catalytic DNMTs  
165 and histone modifying enzymes were studied in germ cells prior and following *in vitro*  
166 maturation.

167

## 168 **Materials and Methods**

169

### 170 **Mice, testes collection and ethical approval**

171 Prepubertal 6-7-day-old CD-1 male mice (Charles River Laboratories, L'Arbresle, France)  
172 were euthanized by decapitation. Testes were excised, rinsed and the tunica albuginea was  
173 removed in  $\alpha$ -MEM at 4°C (Gibco by Life Technologies, Saint-Aubin, France). Testes were  
174 then divided in several batches and cultured either directly (culture of fresh tissues) or after

175 CSF or SSV and thawing (Additional File 1). In order to evaluate the impact of the freezing  
176 procedures on the testicular tissues before culture, fresh, CSF and SSV testes from mice aged  
177 6-7 *dpp* were analyzed (Additional File 1). Moreover, mice aged 36-37 *dpp* were euthanized  
178 by CO<sub>2</sub> asphyxiation and their testes were used as *in vivo* controls for testes cultured *in vitro*  
179 for 30 days (Additional File 1). In total, 42 testes (14 fresh, 14 CSF, 14 SSV) from 21 mice  
180 aged 6-7 *dpp* were used for the analyses performed before culture, 72 testes (24 fresh, 24  
181 CSF, 24 SSV) from 39 mice aged 6-7 *dpp* were used for *in vitro* maturation, and 20 testes  
182 from 13 mice aged 36-37 *dpp* were used as *in vivo* controls. A total of 134 testes from 73  
183 mice were therefore used in this study (Additional File 1).

184 This study was conducted in accordance with the 3Rs principle. All the experimental  
185 procedures were approved by the Institutional Animal Care and Use Committee of Rouen  
186 University Hospital under the licence/protocol number N/23-11-12/46/11-15.

187

#### 188 **CSF and thawing of testicular tissues**

189 **Freezing procedure.** Testes were frozen as previously described (Arkoun et al., 2016;  
190 Milazzo et al., 2008). Briefly, testes were incubated in cryotubes containing 1.3 mL of  
191 freezing medium: Leibovitz L-15 medium (Eurobio, Courtabœuf, France) supplemented with  
192 1.5 M dimethylsulfoxide (DMSO, Sigma-Aldrich, Saint-Quentin Fallavier, France), 0.05 M  
193 sucrose (Sigma-Aldrich) and 10% (v/v) fetal calf serum (FCS, Eurobio). Controlled slow  
194 freezing was performed in a programmable freezer (Nicol Freezal, Air Liquide, Marne-la-  
195 Vallée, France) with the following protocol: start at 5°C, then -2°C/minute until reaching -  
196 9°C, stabilization at -9°C for 7 minutes, then -0.3°C/minute until -40°C and -10°C/minute  
197 down to -140°C. The samples were then stored in liquid nitrogen.

198 **Thawing procedure.** Cryotubes were warmed for 1 minute at room temperature and then for 3  
199 minutes at 30°C in a water bath. Testicular tissues were then successively incubated at 4°C in

200 baths containing decreasing concentrations of cryoprotective agents during 5 minutes each  
201 (*bath 1*: 1 M DMSO, 0.05 M sucrose, 10% FCS, Leibovitz L15; *bath 2*: 0.5 M DMSO, 0.05  
202 M sucrose, 10% FCS, Leibovitz L15; *bath 3*: 0.05 M sucrose, 10% FCS, Leibovitz L15; *bath*  
203 *4*: 10% FCS, Leibovitz L15).

204

## 205 **SSV and thawing of testicular tissues**

206 ***Vitrification procedure.*** Testes were vitrified as previously described (Dumont et al., 2015).  
207 Briefly, samples were incubated for 10 minutes at 4°C in Dulbecco's modified Eagle's  
208 medium/Nutrient Mixture F12 (DMEM/F12, Sigma-Aldrich) supplemented with 1.05 M  
209 DMSO and 1.35 M ethylene glycol (Sigma-Aldrich) and then for 5 minutes at 4°C in  
210 DMEM/F12 supplemented with 2.1 M DMSO, 2.7 M ethylene glycol, 0.5 M sucrose and 20%  
211 FCS. Testis samples were then dropped onto a thin aluminium floater (VWR, Fontenay-sous-  
212 Bois, France) partially immersed in liquid nitrogen. Vitrified testes were stored in cryotubes  
213 in liquid nitrogen.

214 ***Thawing procedure.*** Cryotubes were left at room temperature for 2 seconds. Testes were  
215 incubated in DMEM/F12 supplemented with 0.5 M sucrose and 20 % FCS for 2 minutes at  
216 37°C, and then in DMEM/F12 supplemented with 20 % FCS for 2 minutes at 37°C. They  
217 were then successively transferred into four baths of DMEM/F12 supplemented with 20 %  
218 FCS for 5 minutes at 4°C.

219

## 220 **Organotypic cultures**

221 *In vitro* tissue cultures were performed at a gas-liquid interphase as previously described  
222 (Arkoun et al., 2016; Dumont et al., 2015; Sato et al., 2011; Yokonishi et al., 2014). Briefly,  
223 fresh or frozen/thawed testes were first cut into 4 fragments. They were placed on the top of  
224 two 1.5% (w/v) agarose Type I low EEO gels (Sigma-Aldrich) half-soaked in base medium

225 containing  $\alpha$ -MEM, 10% KnockOut Serum Replacement (KSR, Gibco by Life Technologies)  
226 and 5  $\mu\text{g}/\text{mL}$  gentamicin (Sigma-Aldrich). Testicular tissues were then cultured in 6-well  
227 plates (Dominique Dutscher, Brumath, France) under 5%  $\text{CO}_2$  at 34°C for 30 days (D30),  
228 which corresponds to the end of the first spermatogenic wave. In the Rol condition, testicular  
229 fragments were cultured with base medium supplemented with  $10^{-6}$  M retinol (Sigma-  
230 Aldrich). In the Rol/FSH condition, testicular samples were alternatively cultured with base  
231 medium containing  $10^{-6}$  M retinol or 500 UI/L recombinant FSH (Gonal-f, Merck Serono,  
232 Lyon, France), with retinol being added every 8 days to coincide with the entry of  
233 spermatogonia into meiosis. In total, 288 testicular fragments from 72 testes were *in vitro*  
234 cultured and analyzed. RT-qPCR analyses were repeated 3 times (from 3 different testes) for  
235 *Dnmt1* and *Dnmt3a*, and 3 times for *Prdm9*, *Jarid1b*, *Src1*, *Sirt1* and *Hdac1*.  
236 Immunohistochemical analyses were repeated 4 times (from 4 different testes) for DNMT1,  
237 DNMT3a and 5mC, and 4 times for H3K4me3, H3K9ac and H4K8ac.

238

### 239 **RNA extraction, reverse transcription and qPCR**

240 **RNA extraction.** Total RNA was extracted from testicular samples (around 2 mg) using  
241 RNeasy Micro kit (Qiagen, Courtabœuf, France) according to the manufacturer's instructions.  
242 To remove contamination with genomic DNA, RNA was incubated with 2 units of TURBO  
243 DNase (Life Technologies) for 45 minutes at 37°C.

244 **Reverse transcription.** cDNA synthesis was performed using qScript cDNA SuperMix  
245 (Quanta Biosciences by VWR) according to the manufacturer's instructions using the  
246 following program: 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C.

247 **qPCR.** cDNA amplifications were carried out in a total volume of 20  $\mu\text{L}$  containing 2  $\mu\text{L}$  of  
248 cDNA templates diluted 1:10 (10 ng) in sterile water, 10  $\mu\text{L}$  of Fast SYBR Green Master Mix  
249 (Applied Biosystems, Saint-Aubin, France) and 300 nM of each primer. Specific primers are

250 listed in Additional File 2. Reactions were performed in 96-well plates (Invitrogen by Life  
251 Technologies) in a StepOnePlus thermocycler (Applied Biosystems). Annealing temperatures  
252 were optimized for all primer sets (Additional File 2) and the efficiency of the reactions was  
253 determined using serial dilutions of cDNA samples. The amplification condition was 20  
254 seconds at 95°C followed by 40 cycles (3 seconds at 95°C, 30 seconds at the annealing  
255 temperature). Melting curves were also obtained to ensure the specificity of PCR  
256 amplifications. The size of the amplicons was verified by agarose gel electrophoresis (E-gel  
257 4%, Life Technologies). The relative expression level of each gene (*Dnmt1*, *Dnmt3a*, *Prdm9*,  
258 *Jarid1b*, *Src1*, *Sirt1* and *Hdac1*) was normalized to the housekeeping gene *Gapdh*. Data were  
259 analyzed using the  $2^{-\Delta\Delta C_t}$  method.

260

## 261 **Histological analyses**

262 ***Tissue fixation and processing.*** Testicular tissues were fixed with Bouin's solution (Sigma-  
263 Aldrich) for 2 hours at room temperature. They were then dehydrated in ethanol in the Citadel  
264 2000 tissue processor (Shandon, Cheshire, UK) and embedded in paraffin.

265 ***Tissue sectioning.*** Tissue sections (3  $\mu$ m thick) were performed with the JungRM 2035  
266 microtome (Leica) and were mounted on Polysine slides (Thermo Fisher Scientific, Waltham,  
267 MA, USA).

268 ***Immunohistochemical staining.*** Tissue sections were deparaffinized in two baths of xylene  
269 for 5 minutes and rehydrated in decreasing concentrations of ethanol. After a 5-minute wash  
270 in Tris buffer containing 10% Tween-20 (TBST, Microm Microtech, Francheville, France),  
271 sections were incubated for 40 minutes at 96°C in 10 mM citrate buffer (pH 6.0) and then  
272 cooled for 20 minutes at room temperature. Endogenous peroxidases were blocked with HP  
273 Block (Dako, Les Ulis, France) for 5 minutes at room temperature. Sections were washed for  
274 5 minutes in distilled water and blocked with Ultra-V blocking solution (Thermo Fisher

275 Scientific) for 10 minutes at room temperature. They were then incubated overnight at 4°C  
276 with primary antibodies: rabbit polyclonal anti-DNMT1 IgGs (1:100 for 36-37 *dpp in vivo*  
277 testes and 30-day cultures of testicular tissues, 1:200 for 6-7 *dpp* testes, ab19905, Abcam,  
278 Paris, France), rabbit polyclonal anti-DNMT3a IgGs (1:1250, SC-20703, SantaCruz  
279 Biotechnology, Heidelberg, Germany), mouse monoclonal anti-5mC IgGs (1:50, 16233D3,  
280 Merck Millipore, Fontenay-sous-Bois, France), rabbit polyclonal anti-H3K4me3 IgGs (1:200  
281 for 36-37 *dpp in vivo* testes and 30-day cultures of testicular tissues, 1:500 for 6-7 *dpp* testes,  
282 ab8580, Abcam), rabbit polyclonal anti-H3K9ac IgGs (1:50, ab61231, Abcam) or rabbit  
283 polyclonal anti-H4K8ac IgGs (1:1500 for 36-37 *dpp in vivo* testes and 30-day cultures of  
284 testicular tissues, 1:2000 for 6-7 *dpp* testes, ab15823, Abcam). After a 5-minute wash in  
285 TBST, biotinylated goat anti-rabbit antibodies (1:200, Santa Cruz, 25 minutes for DNMT1,  
286 DNMT3a and H3K9ac; undiluted, Thermo Fisher Scientific, 5 minutes for H3K4me3 and  
287 H4K8ac) or anti-mouse antibodies (Thermo Fisher Scientific, for 5mC) were applied at room  
288 temperature. The immunoreaction was visualized after incubation with horseradish  
289 peroxidase-conjugated streptavidin (Thermo Fisher Scientific) for 5 minutes at room  
290 temperature followed by the addition of 3,3'-diaminobenzidine (Thermo Fischer Scientific)  
291 for around 10 seconds (DNMT1 and H3K9ac), 20 seconds (DNMT3a), 30 seconds  
292 (H3K4me3) or 40 seconds (5mC and H4K8ac). Sections were finally counterstained with  
293 hematoxylin (Dako) for 15 seconds and mounted with Eukitt (CML, Nemours, France).  
294 Immunostaining with pre-immune rabbit IgGs (SC-2027, SantaCruz Biotechnology) or mouse  
295 IgGs (SC-2025, SantaCruz Biotechnology) was used as a negative control. Images were  
296 acquired at a ×500 magnification on a DM4000B light microscope (Leica, Solms, Germany)  
297 equipped with the Leica Application Suite Core v2.4 Software.  
298 In each testicular sample analyzed, the expression and localization of DNMT1 and DNMT3a,  
299 the presence of 5mC residues and modified histones (H3K4me3, H3K9ac and H4K8ac) and

300 the number of each cell type (Sertoli cells: irregular nuclei, spermatogonia: smooth spherical  
301 nuclei close to the basal membrane, leptotene/zygotene spermatocytes: irregular spherical  
302 nuclei with condensed chromatin, pachytene spermatocytes: irregular spherical nuclei with  
303 highly condensed chromatin, round spermatids: small and regular round nuclei, elongated  
304 spermatids: elongated nuclei with highly condensed chromatin) per seminiferous tubule were  
305 analyzed in 30 cross-sectioned seminiferous tubules from 3 different sections. For *in vivo*  
306 controls, the 30 seminiferous tubules were examined in multiple microscopic fields chosen at  
307 random. However, almost all seminiferous tubules present in *in vitro* matured testes were  
308 analyzed because of the small size of the testicular fragments. The percentage of each germ  
309 cell type that stained positive for DNMT1, DNMT3a, 5mC, H3K4me3, H3K9ac and H4K8ac  
310 was calculated in each seminiferous tubule.

311

### 312 **Sperm DNA methylation**

313 ***Testicular sperm extraction.*** Testicular spermatozoa were extracted from a pool of 6 testes  
314 (from 6 different mice) for each condition. Briefly, tissues were placed in  $\alpha$ -MEM pre-  
315 warmed at 37°C. After the careful removal of the central necrotic area using 27-gauge  
316 needles, tissues were mechanically dispersed. After a 10-minute centrifugation at 300  $\times$  g,  
317 pellets containing testicular cells were recovered. Cells were fixed with methanol for 30  
318 minutes at -20°C and then spread onto glass slides.

319 ***Immunofluorescent staining of 5mC.*** After 5 minutes in TBST at room temperature, cells  
320 were permeabilized in acetone for 2 minutes. Nuclei were decondensed with 1 M NaOH for 4  
321 minutes and in TBST containing 10 mM dithiothreitol and 0.1% (v/v) Triton X-100 (Sigma-  
322 Aldrich) for 20 minutes at 4°C. Slides were washed twice in TBST for 5 minutes and non-  
323 specific binding sites were blocked with 5% (w/v) horse serum albumin in TBST (Sigma-  
324 Aldrich). They were then incubated overnight at 4°C with mouse monoclonal anti-5mC IgGs

325 (1:50, 16233D3, Merck Millipore). After two washes in TBST for 5 minutes, Alexa Fluor 488  
326 conjugated-goat anti-mouse IgGs (1:200, ab150113, Abcam) were applied for 1 hour at room  
327 temperature. After a 10-minute wash, cells were counterstained with 4',6-diamidino-2-  
328 phenylindole (DAPI, Counterstain I, 30-804931, Adgenix, Voisins-Le-Bretonneux, France).  
329 A negative control was carried out by omitting the primary antibody. Images were captured at  
330 a  $\times 900$  magnification by a U-CMAD3 camera mounted on a BX61 epifluorescence  
331 microscope (Olympus), with a fixed acquisition and exposure for each condition. Image  
332 acquisitions were carried out using the Bioview 234 Duet v2.3 image analyzer (Nes Ziona,  
333 Israël).

334 ***Fluorescence intensity quantification.*** For each experimental condition, a hundred  
335 spermatozoa (from a pool of 6 different *in vitro* cultured testes or from 6 *in vivo* testes),  
336 identified by their hook-shaped heads, was examined. The border around the sperm nuclei  
337 was manually delineated according to DNA staining. After subtracting the background, 5mC  
338 and DAPI fluorescence intensities were calculated for each spermatozoon using the ImageJ  
339 v1.8 software (National Institute of Health, Bethesda, MD, USA). For each sperm nucleus,  
340 the fluorescence of 5mC was divided by the fluorescence of DAPI to obtain normalized 5mC  
341 fluorescence.

342

### 343 **Statistical analyses**

344 Statistical analyses were carried out with the statistical GraphPad Prism 6 software (GraphPad  
345 Software Inc., La Jolla, CA, USA). To evaluate the impact of the freezing procedure on  
346 testicular tissue, data obtained from fresh 6-7 *dpp* testes were compared with those obtained  
347 from CSF or SSV 6-7 *dpp* testes using the non-parametric Kruskal Wallis test followed by the  
348 Dunn *post hoc* test. Data from 36-37 *dpp* testes and from 30-day cultured tissues, as well as  
349 data from cultures of fresh, CSF and SSV testicular tissues were also compared using the non-



350 parametric Kruskal Wallis test followed by the Dunn *post hoc* test. Cultures performed under  
351 the Rol or Rol/FSH conditions were compared using the non-parametric Mann Whitney test.  
352 Finally, the normalized 5mC fluorescence intensities were compared using the non-parametric  
353 Mann Whitney test. Data are expressed as mean  $\pm$  SD. For each test, a value of  $P < 0.05$  (\*)  
354 or  $P < 0.01$  (\*\*) was considered statistically significant.

355

## 356 **Results**

357

### 358 **DNA methylation and histone PTMs in fresh or frozen/thawed prepubertal mouse** 359 **testicular tissue**

360 To determine whether the transcripts encoding epigenetic modification enzymes are present in  
361 6-7 days *postpartum* (*dpp*) testes and whether their levels are altered by a freezing/thawing  
362 cycle, qPCR analyses were performed on fresh, CSF or SSV testicular tissues. *Dnmt1* and  
363 *Dnmt3a* transcripts encoding catalytic DNMTs, *Prdm9* and *Jarid1b* transcripts encoding a  
364 histone methyltransferase and demethylase respectively, *Src1* transcripts encoding a histone  
365 acetyltransferase, and *Sirt1* and *Hdac1* transcripts encoding histone deacetylases were  
366 detected at 6-7 *dpp* (Figure 1A,B). Moreover, mRNA levels were not significantly different  
367 between fresh and frozen/thawed testes, regardless of the freezing/thawing protocol used,  
368 except for lower *Hdac1* mRNA levels in SSV *versus* fresh and CSF testes (Figure 1A,B).  
369 The expression of the catalytic DNMTs and the presence of the epigenetic marks 5mC,  
370 H3K4me3, H3K9ac and H4K8ac were then studied in prepubertal testes by  
371 immunohistochemistry (Figure 1C). At 6-7 *dpp*, seminiferous tubules only contained Sertoli  
372 cells and spermatogonia. DNMT1 and 5mC were detected in a majority of spermatogonia,  
373 and no significant difference was observed between fresh, CSF and SSV testes (Figure 1D).  
374 In contrast, spermatogonia did not express DNMT3a in fresh tissues while around 24% of

375 spermatogonia expressed this enzyme in CSF tissues ( $P < 0.05$ , Figure 1D). The three  
376 modified histones were found in a minority of spermatogonia in both fresh and frozen/thawed  
377 testes (Figure 1E).

378

### 379 **Advancement of spermatogenesis in 30-day *in vitro* cultures of 6-7 dpp testicular tissues**

380 As previously described (Arkoun et al., 2016, 2015; Dumont et al., 2017, 2016; Rondanino et  
381 al., 2017), a complete spermatogenesis can be achieved *in vitro* within fresh, CSF and SSV  
382 prepubertal testicular tissues after 30 days of culture in the presence of  $10^{-6}$  M retinol (Rol  
383 culture condition). Indeed, germ cells at all stages of differentiation were found after  
384 organotypic cultures (Table 1). In an effort to further increase the yield of *in vitro* produced  
385 spermatozoa, the supplementation of culture medium with FSH in alternation with retinol  
386 (Rol/FSH culture condition) was tested in this study. The progression of spermatogenesis in  
387 cultures of fresh, CSF and SSV tissues cultured with Rol or Rol/FSH was evaluated and  
388 compared by determining the proportion of the different germ cell types per seminiferous  
389 tubule as well as the proportion of seminiferous tubules at the most advanced stage of  
390 differentiation.

391 We first compared the progression of spermatogenesis in 30-day cultures of fresh tissues and  
392 in the 36-37 dpp age-matched *in vivo* controls. The proportion of pachytene spermatocytes  
393 and of round and elongated spermatids per tubule were lower in cultures of fresh tissues than  
394 *in vivo* (Table 1). The proportion of tubules having reached the pachytene spermatocyte and  
395 the round spermatid stages was higher in cultures of fresh tissues than in controls (Table 2). In  
396 contrast, the percentage of tubules with elongated spermatids as the most advanced stage was  
397 significantly reduced in cultures of fresh tissues compared to *in vivo* (Table 2).

398 The impact of the freezing/thawing procedures on the advancement of spermatogenesis was  
399 then investigated. The different germ cell types were also present in CSF and SSV tissues

400 following *in vitro* maturation with Rol or Rol/FSH (Table 1). The proportion of pachytene  
401 spermatocytes per tubule was similar between cultures of fresh, CSF and SSV tissues in the  
402 presence of Rol. With Rol/FSH however, the percentage of pachytene spermatocytes per  
403 tubule was higher in cultures of frozen/thawed tissues than in fresh tissues (Table 1). The  
404 proportion of round and elongated spermatids per tubule was lower in cultures of CSF tissues  
405 than in fresh tissues, whatever the medium used (Table 1). This diminution was not observed  
406 in SSV tissues. The proportion of elongated spermatids per seminiferous tubule was even  
407 more elevated in cultures of SSV tissues with Rol/FSH than in cultures of CSF tissues (Table  
408 1). The percentage of tubules with pachytene spermatocytes as the most advanced type of  
409 germ cells was more elevated in CSF tissues matured in the presence of Rol or Rol/FSH and  
410 in SSV tissues matured in the presence of Rol/FSH than in fresh tissues (Table 2). The  
411 percentage of seminiferous tubules in which round spermatids are the most differentiated  
412 germ cells were almost similar in cultures of fresh, CSF and SSV tissues. Indeed, their  
413 proportion was only lower in cultures of SSV tissues with Rol/FSH (Table 2). At last, fewer  
414 tubules reached the elongated spermatid stage in CSF tissues cultured with Rol or Rol/FSH  
415 and in SSV tissues cultured with Rol than in fresh tissues (Table 2).

416 The Rol and Rol/FSH culture conditions were finally compared. The proportion of Sertoli  
417 cells, spermatogonia, pachytene spermatocytes and round spermatids per tubule was not  
418 significantly different when FSH was added in cultures of fresh or frozen/thawed tissues  
419 (Table 1). In addition, the proportion of elongated spermatids per tubule was reduced in  
420 cultures of fresh and CSF tissues with Rol/FSH (Table 1). The percentage of tubules in which  
421 pachytene spermatocytes are the most differentiated germ cells were more elevated in fresh or  
422 SSV tissues cultured with Rol/FSH than with Rol alone (Table 2). Moreover, the percentage  
423 of tubules reaching the round spermatid stage was diminished in SSV tissues cultured with  
424 Rol/FSH (Table 2). The proportion of tubules containing elongated spermatids was higher

425 when fresh or CSF tissues were cultured with Rol than with Rol/FSH (Table 2). This  
426 parameter was however not influenced by the medium composition in cultures of SSV tissues.

427

428 **DNA methylation and histone PTMs in 30-day *in vitro* cultures of fresh 6-7 dpp**  
429 **testicular tissues**

430 Since the best spermatogenic progression was obtained in tissues cultured with Rol, this  
431 culture condition was selected for the rest of the study. The impact of organotypic culture and  
432 freezing procedures on the expression of epigenetic modification enzymes, on DNA  
433 methylation and histone PTMs was evaluated in *in vitro* matured testicular tissues.

434 Transcript levels were quantified by qPCR in fresh 6-7 dpp testicular tissues cultured *in vitro*  
435 for 30 days and compared with those measured in control 36-37 dpp testes. No significant  
436 difference in *Dnmt1* and *Dnmt3a* mRNA levels was found in tissues matured *in vitro* with Rol  
437 and *in vivo* controls (Figure 2A). While the levels of *Prdm9* and *Jarid1b* transcripts were  
438 similar in cultures of fresh tissues and in controls, *Src1*, *Sirt1* and *Hdac1* mRNA levels were  
439 lower *in vitro* (Figure 2B).

440 The expression of the DNMT1 and DNMT3a proteins, DNA methylation onto the C5 position  
441 of cytosines and the presence of the three active histone marks were detected in a majority of  
442 spermatogonia and leptotene/zygotene spermatocytes in 36-37 dpp testes (Figures 3 and 4).  
443 Moreover, more than half of round spermatids contained H3K4me3 and H3K9ac at 36-37 dpp  
444 while H4K8ac was found in a lesser proportion of these cells (Figure 4). The percentage of  
445 spermatogonia expressing DNMT1 and DNMT3a was similar in fresh tissues cultured with  
446 Rol and *in vivo* (Figure 3B). The proportion of leptotene/zygotene spermatocytes expressing  
447 DNMT1 in cultures of fresh tissues was also comparable to *in vivo* controls (Figure 3B). In  
448 contrast, a lower percentage of spermatocytes expressing DNMT3a was noticed in tissues  
449 cultured with Rol (Figure 3B). The percentage of spermatogonia with methylated DNA was

450 reduced in fresh tissues cultured with Rol compared to 36-37 *dpp* control testes (Figure 3B).  
451 However, the proportion of leptotene/zygotene spermatocytes with 5mC labelling was not  
452 significantly different in fresh tissue cultures and *in vivo* (Figure 3B). DNMT1, DNMT3a and  
453 5mC labelling in pachytene spermatocytes, round and elongated spermatids were  
454 exceptionally observed in both 36-37 *dpp* controls and 30-day cultures of 6-7 *dpp* testicular  
455 tissues. A lower proportion of spermatogonia, round and elongated spermatids containing  
456 H3K4me3 was found in cultures of fresh tissues than *in vivo* ( $P < 0.05$ , Figure 4B). The  
457 percentage of spermatogonia ( $P < 0.05$ ), round and elongated spermatids ( $P < 0.01$  and  $P <$   
458  $0.05$  respectively) containing H3K9ac was also diminished in organotypic cultures (Figure  
459 4C). Finally, H4K8ac was found in less spermatogonia and leptotene/zygotene  
460 spermatocytes in *in vitro* matured fresh tissues ( $P < 0.05$ , Figure 4D).

461

462 **Epigenetic modifications in 30-day *in vitro* cultures of CSF or SSV 6-7 *dpp* testicular**  
463 **tissues**

464 To determine whether the freezing and thawing of 6-7 *dpp* testicular tissues could influence  
465 the expression of genes encoding epigenetic modification enzymes in 30-day organotypic  
466 cultures, qPCR analyses were performed on *in vitro* matured CSF or SSV tissues and  
467 compared with the data obtained from fresh tissue cultures. Apart from 7.6-fold lower *Dnmt1*  
468 mRNA levels in SSV tissues cultured with Rol ( $P < 0.05$ , Figure 2A), *Dnmt1* transcript levels  
469 did not vary in a significant manner in cultures of CSF tissues with Rol compared to cultures  
470 of fresh tissues (Figure 2A). Furthermore, no significant difference in *Dnmt3a* transcript  
471 levels was found after culture of fresh or frozen/thawed testicular tissues (Figure 2A).  
472 Moreover, apart from 3.9-fold higher *Hdac1* mRNA levels in cultures of SSV tissues, the  
473 levels of the transcripts encoding histone modifying enzymes were not significantly different  
474 between cultures of fresh, CSF and SSV tissues (Figure 2B). However, lower *Dnmt3a* and

475 *Hdac1* mRNA levels were found in cultures of CSF tissues and lower *Dnmt1*, *Dnmt3a* and  
476 *Jarid1b* mRNA levels were found in cultures of SSV tissues compared to *in vivo* controls  
477 (Figure 2).

478 DNMT1, DNMT3a, 5mC, H3K4me3, H3K9ac and H4K8ac labelling in CSF or SSV tissue  
479 cultures were compared to those observed in fresh tissue cultures (Figures 5 and 6). DNMT1,  
480 DNMT3a and the 5mC epigenetic mark were found in a majority of spermatogonia in cultures  
481 of fresh, CSF or SSV tissues (Figure 5B). Most of the leptotene/zygotene spermatocytes  
482 found in cultures of fresh or frozen/thawed tissues expressed DNMT1 and contained 5mC  
483 residues (Figure 5B). The proportion of leptotene/zygotene spermatocytes expressing  
484 DNMT3a did not appear to be different among those under fresh, CSF, or SSV conditions  
485 (Figure 5B). As in the case of fresh tissue cultures, DNMT1, DNMT3a and 5mC were rarely  
486 detected in pachytene spermatocytes, round and elongated spermatids. The proportion of  
487 elongated spermatids containing H3K4me3 was higher in cultures of CSF and SSV tissues  
488 than in fresh tissues (Figure 6B). The proportion of spermatogonia and elongated spermatids  
489 containing H3K9ac were more elevated in cultures of CSF tissues than in fresh tissues (Figure  
490 6C). This epigenetic mark was also present in a higher proportion of spermatogonia, round  
491 and elongated spermatids in cultures of SSV tissues compared to fresh tissues (Figure 6C).  
492 Finally, a lower proportion of round spermatids contained H4K8ac in cultures of CSF tissues  
493 and the percentage of H4K8ac-positive pachytene spermatocytes was lower in cultures of CSF  
494 and SSV tissues than in fresh tissues (Figure 6D).

495

496 **DNA methylation in spermatozoa extracted from 30-day *in vitro* cultures of fresh, CSF**  
497 **or SSV 6-7 dpp testicular tissues**

498 Despite our attempts using various antigen retrieval procedures, 5mC labelling was rarely  
499 detected in germ cells beyond the leptotene/zygotene stage in both *in vivo* controls and *in*

500 *in vitro* matured testicular tissues. In order to ascertain that the genome of *in vitro* produced  
501 gametes was methylated, spermatozoa were extracted from cultures of fresh, CSF or SSV  
502 tissues, their nuclei were decondensed and the presence of the heritable 5mC epigenetic mark  
503 was investigated by immunofluorescence (Figure 7). The presence of methylated cytosines  
504 was detected in the nuclei of spermatozoa. The expression of 5mC between sperm nuclei was  
505 heterogeneous. Specifically, the normalized 5mC fluorescence intensity ranged from 0.01 to  
506 0.85 (median = 0.16) in spermatozoa extracted from 36-37 *dpp* testes (Figure 7B). Similar  
507 5mC levels were found in spermatozoa produced in cultures of CSF or SSV tissues (median =  
508 0.14 and 0.10, respectively), but lower levels were noticed in spermatozoa generated in  
509 cultures of fresh tissues (median = 0.10) (Figure 7B). Because of the low spermatogenic yield  
510 in cultures, our repeated attempts to analyze the methylation patterns of two imprinted genes  
511 (*H19* and *Igf2r*) in extracted spermatozoa by bisulfite pyrosequencing were unsuccessful.

512

## 513 **Discussion**

514

515 In the present study, no major alterations of the expression of DNMT1 and DNMT3a, two  
516 essential catalytic DNA methyltransferases, and of DNA methylation was found in germ cells  
517 after *in vitro* maturation of fresh or frozen/thawed mouse prepubertal testes, when compared  
518 to those allowed to develop *in vivo*. However, differences in the levels of transcripts encoding  
519 histone modifying enzymes and in the distribution of the H3K4me3, H3K9ac and H4K8ac  
520 marks were found between *in vitro* and *in vivo* matured testes.

521 The fertility of childhood cancer survivors could be restored by producing spermatozoa from  
522 SSCs with *in vivo* or *in vitro* approaches (de Michele et al., 2017; Gies et al., 2015; Ibtisham  
523 et al., 2017; Yokonishi and Ogawa, 2016). It has been previously demonstrated that mouse  
524 germ cells from fresh prepubertal testes go through typical DNA methylation after SSCs

525 transplantation and testicular tissue grafting, and that germ cells present alteration in H4K5ac  
526 and H4K8ac marks after SSCs transplantation but not after tissue grafting (Goossens et al.,  
527 2011). If the methylation status of 11 imprinted genes is almost normal in the offspring  
528 derived from *in vitro* produced spermatozoa, the examination of DNA methylation in germ  
529 cells following organotypic culture is warranted, as previously highlighted (Yokonishi et al.,  
530 2014). Moreover, histone PTMs have never been investigated in *in vitro* matured prepubertal  
531 testicular tissues.

532 Fresh or frozen (by CSF or SSV)/thawed 6-7 *dpp* testicular tissues were first analyzed in this  
533 study. *Dnmt1* and *Dnmt3a* gene expression was detected in these testes, which is in  
534 accordance with previous data showing that transcripts were present postnatally (La Salle et  
535 al., 2004; La Salle and Trasler, 2006; Mertineit et al., 1998). Here, no significant differences  
536 in *Dnmt1* or *Dnmt3a* mRNA levels were observed among fresh, CSF and SSV tissues. As  
537 previously reported (La Salle et al., 2004; Mertineit et al., 1998), we detected the DNMT1  
538 protein in spermatogonia in postnatal testes. In contrast, this enzyme was not found in the  
539 mouse prepubertal testis in another study (Goossens et al., 2011). Since the age of the mice  
540 was not specified, this discrepancy might come from the analysis of testes at different  
541 developmental stages. We observed that spermatogonia present in fresh 6-7 *dpp* testes  
542 contained the 5mC epigenetic mark but did not express DNMT3a. This is consistent with a  
543 previous report showing that DNMT3a was only detected in Sertoli and myoid cells while  
544 5mC was present in spermatogonia (Goossens et al., 2011). The presence of methylated  
545 cytosines in spermatogonia indicates that methylation profiles were, at least in part,  
546 established at this stage. Indeed, following a demethylation wave during embryonic  
547 development, spermatogonial precursor cells and spermatogonia then undergo *de novo* DNA  
548 methylation (Boissonnas et al., 2013; Kubo et al., 2015; Yao et al., 2015). While the presence  
549 of DNMT1 suggests that DNA methylation profiles are maintained during SSC division, the



550 lack of DNMT3a may however mean that *de novo* methylation is not occurring in  
551 spermatogonia at 6-7 dpp. In the present study, DNMT1 expression and 5mC marks were also  
552 observed in spermatogonia of thawed CSF or SSV testes. For unknown reasons and contrary  
553 to fresh tissues, DNMT3a was found in spermatogonia in frozen/thawed tissues, and may  
554 cause in these cells the appearance of abnormal DNA methylation profiles and the repression  
555 of gene expression. Moreover, we showed that the *Prdm9*, *Jarid1b*, *Src1*, *Sirt1* and *Hdac1*  
556 genes encoding histone modifying enzymes were expressed in fresh, CSF and SSV 6-7 dpp  
557 testes. In contrast to a previous study (Goossens et al., 2011), we detected the H3K9ac mark  
558 not only in Sertoli cells but also in a small fraction of spermatogonia. The H3K4me3 and  
559 H4K8ac marks were also present in these two cell types, as previously described (Goossens et  
560 al., 2011).

561 Before analyzing DNA methylation and histone PTMs in *in vitro* matured prepubertal  
562 testicular tissues, the advancement of spermatogenesis was examined. As previously shown  
563 (Arkoun et al., 2016, 2015; Dumont et al., 2016, 2015; Sato et al., 2011; Yokonishi et al.,  
564 2014), a successful differentiation of spermatogonia into spermatozoa was obtained in the  
565 present study after culture of fresh or frozen/thawed prepubertal testicular tissues at a gas-  
566 liquid interphase. The proportion of elongated spermatids per seminiferous tubule and of  
567 tubules having reached the elongated spermatid stage were lower in 30-day cultures of fresh  
568 tissues compared to *in vivo* controls. Moreover, these parameters were reduced in CSF tissues  
569 compared to fresh or SSV tissues. Our data therefore suggest that vitrification better preserves  
570 testicular tissues than slow freezing, and are in line with our previous data reporting better  
571 spermatogenic yields in cultures of SSV than CSF tissues (Dumont et al., 2015). To promote  
572 germ cell differentiation, tissues were cultured with retinol alone or with an alternation  
573 retinol/FSH. Indeed, probably because of the complex interactions between retinoids and FSH  
574 (Livera et al., 2002), no additive effect has been previously observed on *in vitro*

575 spermatogenesis when retinol and FSH were combined together (Arkoun et al., 2015). We  
576 found that the proportion of elongated spermatids per tubule and of tubules containing  
577 elongated spermatids was significantly reduced in fresh and CSF tissues cultured with  
578 retinol/FSH rather than retinol alone. In order to mimic *in vivo* conditions as closely as  
579 possible, different concentrations of FSH (corresponding to the physiological variations of  
580 this hormone) will have to be used during the course of *in vitro* cultures in future studies.  
581 Moreover, the culture medium will have to be supplemented with other factors that positively  
582 influence spermatogenesis. Indeed, recently, the use of a chemically-defined medium  
583 containing retinoic acid, retinol and a combination of four hormones (FSH, LH, testosterone,  
584 triiodothyronine) was found to promote spermatogenesis until round spermatid production  
585 (Sanjo et al., 2018).

586 In this study, *Dnmt1* and *Dnmt3a* transcripts were detected *in vivo* at 36-37 *dpp* and in fresh,  
587 CSF and SSV tissues cultured for 30 days. Differences in their levels were evidenced between  
588 *in vivo* controls and cultures of fresh or frozen/thawed tissues. DNMT1, DNMT3a and 5mC  
589 residues were mostly localized in spermatogonia and leptotene/zygotene spermatocytes *in*  
590 *vivo* and in cultures of fresh or frozen/thawed tissues. The presence of DNMT1 and DNMT3a  
591 specifically in spermatogonia and leptotene/zygotene spermatocytes during spermatogenesis  
592 is in agreement with previous studies (La Salle and Trasler, 2006; Mertineit et al., 1998;  
593 Watanabe et al., 2004). In adult testis, DNMT1 has also been detected in pachytene  
594 spermatocytes, diplotene spermatocytes, round spermatids and 5mC was also found in round  
595 spermatids (Goossens et al., 2011). The expression of DNMT3a in 36-37 *dpp* testes and 30-  
596 day cultures suggests that *de novo* methylation is occurring at this stage. Here, the epigenetic  
597 5mC mark was not found in germ cells beyond the leptotene/zygotene spermatocyte stage *in*  
598 *vivo* and in organotypic cultures. As suggested previously (Goossens et al., 2011), the lack of  
599 5mC staining in pachytene spermatocytes could be due to the fact that the methylation pattern

600 has not yet been set on daughter DNA strand. The lack of labelling in spermatids may  
601 however be explained by a poor accessibility of antibodies to the condensed DNA. To  
602 ascertain that DNA methylation marks are indeed found in most differentiated germ cells, we  
603 extracted spermatozoa from the testicular tissues after culture and performed 5mC labelling  
604 on decondensed DNA. The epigenetic mark 5mC was detected in the nuclei of spermatozoa  
605 generated in cultures of fresh or frozen/thawed testicular tissues. The relative 5mC  
606 fluorescence intensity was similar in spermatozoa produced in cultures of frozen/thawed  
607 tissues or *in vivo*, but was lower in spermatozoa extracted from cultures of fresh tissues. It has  
608 been shown that the DNA methylation patterns of a paternally methylated gene (*Igf2*) and a  
609 maternally expressed gene (*Peg1*) in epididymal spermatozoa obtained after SSC  
610 transplantation were not different from controls (Goossens et al., 2009). In our study, the  
611 analysis of the methylation status of the paternally imprinted gene *H19* and the maternally  
612 imprinted gene *Igf2r* in *in vitro* produced spermatozoa could not be achieved. Indeed, a  
613 heterogeneous cell population, among which few spermatozoa, was obtained after mechanical  
614 dissociation of *in vitro* matured testes.

615 In addition, our data showed that *Prdm9*, *Jarid1b*, *Src1*, *Sirt1* and *Hdac1* transcripts were also  
616 present in 36-37 dpp testes and in fresh or frozen/thawed tissues cultured for 30 days. The  
617 levels of *Src1*, *Sirt1* and *Hdac1* mRNA encoding histone acetyltransferase or deacetylase  
618 were lower in cultures of fresh tissues than *in vivo*. However, apart from lower mRNA levels  
619 of *Jarid1b* in cultures of SSV tissues and of *Hdac1* in cultures of CSF tissues, the levels of  
620 transcripts encoding the different histone modifying enzymes were similar between cultures  
621 of frozen/thawed tissues and *in vivo*. As previously reported (Godmann et al., 2007; Goossens  
622 et al., 2011; Hazzouri et al., 2000; Zhang et al., 2014), we found that H3K4me3, H3K9ac and  
623 H4K8ac were present in spermatogonia, leptotene/zygotene spermatocytes, round and  
624 elongated spermatids and in few pachytene spermatocytes *in vivo*. An altered distribution of

625 these three active histone marks was observed in our organotypic cultures. We observed that  
626 the H3K4me3 and H3K9ac marks, which are involved in the progression of early meiosis and  
627 spermiogenesis respectively (Hayashi et al., 2005; Bell et al., 2014), were present in a lesser  
628 proportion of spermatogonia, round and elongated spermatids and in a higher proportion of  
629 pachytene spermatocytes in cultures of fresh tissues compared to *in vivo* controls. It has been  
630 shown that a decrease in H3K4me3 or in H3K9ac could impair meiotic progression or induce  
631 morphological alterations and apoptosis in round spermatids respectively (Hayashi et al.,  
632 2005; Dai et al., 2015). This could explain, at least in part, the alteration of meiotic and  
633 postmeiotic progression and the presence of degenerating round spermatids in *in vitro*  
634 matured tissues as well as the low spermatogenic yield previously reported by us and others  
635 (Dumont et al., 2016; Rondanino et al., 2017; Yao et al., 2017). The acetylation of histones,  
636 which precedes histone-to-protamine replacement, was probably unaffected in the surviving  
637 spermatids since most of *in vitro* produced spermatozoa contain normally condensed  
638 chromatin (Oblette et al., 2017). Finally, while H4K8ac was found in less spermatogonia and  
639 leptotene/zygotene spermatocytes in our cultures of fresh tissues, the proportion of spermatids  
640 containing this mark was similar to *in vivo* controls. The freezing/thawing procedures used in  
641 this study also altered the distribution of the histone marks. A disturbed expression of histone  
642 modifying enzymes (PRDM9, JARID1B, SRC1, SIRT1, HDAC1 or others) or of their  
643 activity in organotypic cultures could explain these results.

644 Genome-wide single-cell methylome analysis and chromatin immunoprecipitation sequencing  
645 will be necessary to explore in more detail DNA methylation in *in vitro* produced  
646 spermatozoa, and especially the methylation status of imprinted genes, as well as the genomic  
647 distribution of modified histones.

648

649 In conclusion, we here report for the first time that germ cells go through DNA methylation  
650 and histone PTMs when *in vitro* matured. Importantly, in the context of fertility preservation,  
651 the present study was also performed on frozen/thawed samples. DNA methylation was found  
652 in cultures of frozen/thawed prepubertal testicular tissues and in spermatozoa generated in  
653 these tissues. Histone methylation and acetylation also occurred in germ cells in cultures of  
654 cryopreserved tissues. Before applying *in vitro* maturation to frozen/thawed prepubertal  
655 testicular tissues of pediatric cancer survivors, further studies will be necessary to investigate  
656 whether imprinted genes are properly methylated in *in vitro* produced spermatozoa, whether  
657 histone marks localize to the correct regions of the genome, and whether first and second-  
658 generation offspring are healthy, long-living and do not present behavioral disorders.

659

#### 660 **Vitae**

661 Antoine Oblette is a PhD student in the Gametogenesis and Gamete Quality team within the  
662 Institute for Research and Innovation in Biomedicine and University Hospital of Rouen in  
663 France. His area of research covers fertility preservation and restoration in young boys with  
664 cancer.

665

#### 666 **List of abbreviations**

667 CSF: controlled slow freezing; DNMT: DNA methyltransferase; *dpp*: days *postpartum*; FSH:  
668 follicle stimulating hormone; H3K4me3: trimethylated histone H3 on lysine 4; H3K9ac:  
669 acetylated histone H3 on lysine 9; H4K8ac: acetylated histone H4 on lysine 8; KSR:  
670 KnockOut serum replacement; 5mC: 5-methylcytosine; PTMs: post-translational  
671 modifications; Rol: retinol; SSC: spermatogonial stem cells; SSV: solid surface vitrification  
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## 916 **Figure legends**

917

918 **Figure 1.** Expression of epigenetic modification enzymes, DNA methylation and histone  
919 post-translational modifications in fresh and frozen/thawed prepubertal mouse testes.

920 (A) Relative mRNA levels of the *Dnmt1* and *Dnmt3a* genes normalized to the housekeeping  
921 gene *Gapdh* in fresh, CSF and SSV 6-7 *dpp* testes (n=3). Histograms are expressed as mean ±  
922 SD. \**P* < 0.05.

923 (B) Relative mRNA levels of the *Prdm9*, *Jarid1b*, *Src1*, *Sirt1* and *Hdac1* genes normalized to  
924 the housekeeping gene *Gapdh* in fresh, CSF and SSV 6-7 *dpp* testes (n=3). Histograms are  
925 expressed as mean ± SD. \**P* < 0.05.

926 (C) Localization of DNMT1, DNMT3a and the 5mC, H3K4me3, H3K9ac and H4K8ac  
927 epigenetic marks in fresh, CSF and SSV 6-7 *dpp* testes (n=4). Shown are representative  
928 microscopy images of DNMT1, DNMT3a, 5mC, H3K4me3, H3K9ac and H4K8ac labelling.  
929 Antibody binding was visualized using 3,3'-diaminobenzidine (brown staining) and tissue  
930 sections were counterstained with hematoxylin. Enlarged views of labelled and unlabeled  
931 spermatogonia are shown in the upper right corner and in the lower right corner, respectively.  
932 Bar: 50 μm.



933 **(D)** Proportion of DNMT1, DNMT3a and 5mC-positive spermatogonia in 30 cross-sectioned  
934 seminiferous tubules of fresh, CSF and SSV 6-7 *dpp* testes (n=4). Histograms are expressed  
935 as mean  $\pm$  SD. \**P* < 0.05.

936 **(E)** Proportion of H3K4me3, H3K9ac and H4K8ac-positive spermatogonia in 30 cross-  
937 sectioned seminiferous tubules of fresh, CSF and SSV 6-7 *dpp* testes (n=4). Histograms are  
938 expressed as mean  $\pm$  SD. \**P* < 0.05.

939 CSF: controlled slow freezing; *Dnmt*/DNMT: DNA methyltransferase; *dpp*: days *postpartum*;  
940 *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase; H3K4me3: trimethylated histone H3 on  
941 lysine 4; H3K9ac: acetylated histone H3 on lysine 9; H4K8ac: acetylated histone H4 on  
942 lysine 8; *Hdac1*: Histone deacetylase 1; *Jarid1b*: Jumonji AT-rich interactive domain 1b;  
943 5mC: 5-methylcytosine; *Prdm9*: PR domain-containing 9; *Sirt1*: Sirtuin 1; *Src1*: Steroid  
944 receptor coactivator 1; SSV: solid surface vitrification

945

946 **Figure 2.** Expression of genes encoding epigenetic modification enzymes in cultured  
947 testicular tissues and *in vivo* controls.

948 Relative mRNA levels of the **(A)** *Dnmt* genes (*Dnmt1*, *Dnmt3a*) and **(B)** genes encoding  
949 histone modifying enzymes (*Prdm9*, *Jarid1b*, *Src1*, *Sirt1*, *Hdac1*) normalized to the  
950 housekeeping gene *Gapdh* in 30-day cultures of fresh, CSF or SSV testicular fragments with  
951 retinol (n = 3) and in testes of mice aged 36–37 *dpp* (n = 3). Histograms are expressed as  
952 mean  $\pm$  SD. \**P* < 0.05, \*\**P* < 0.01.

953 CSF: controlled slow freezing; D30: day 30; *Dnmt*: DNA methyltransferase; *dpp*: days  
954 *postpartum*; *Gapdh*: Glyceraldehyde-3-phosphate dehydrogenase; *Hdac1*: Histone  
955 deacetylase 1; *Jarid1b*: Jumonji AT-rich interactive domain 1b; *Prdm9*: PR domain-  
956 containing 9; Rol: retinol; *Sirt1*: Sirtuin 1; *Src1*: Steroid receptor coactivator 1; SSV: solid  
957 surface vitrification

958

959 **Figure 3.** Immunolocalization of DNMT1, DNMT3a and 5mC in cultures of fresh

960 prepubertal testes and *in vivo*.

961 (A) Localization of DNMT1, DNMT3a and the 5mC epigenetic mark in fresh testicular  
962 fragments cultured for 30 days with retinol (n=4) and in 36-37 *dpp* testes (*in vivo* controls,  
963 n=4). Shown are representative microscopy images of DNMT1, DNMT3a and 5mC labelling.

964 Antibody binding was visualized using 3,3'-diaminobenzidine (brown staining) and tissue  
965 sections were counterstained with hematoxylin. Enlarged views of spermatogonia and  
966 leptotene/zygotene spermatocytes are shown in the upper right corner and in the lower right  
967 corner, respectively. Bar: 50  $\mu$ m.

968 (B) Proportion of DNMT1, DNMT3a and 5mC-positive spermatogonia and

969 leptotene/zygotene spermatocytes in 30-day cultures of fresh testicular fragments with retinol  
970 (n=4) and in control 36-37 *dpp* testes (n=4). The localization of DNMT1, DNMT3a and 5mC  
971 was analyzed in 30 cross-sectioned seminiferous tubules from three sections. Histograms are  
972 expressed as mean  $\pm$  SD. \**P* < 0.05.

973 D30: day 30; DNMT: DNA methyltransferase; *dpp*: days *postpartum*; 5mC: 5-

974 methylcytosine; L/Z: leptotene/zygotene spermatocyte; P: pachytene spermatocyte; Rol:

975 retinol; Sg: spermatogonia

976

977 **Figure 4.** Immunolocalization of H3K4me3, H3K9ac and H4K8ac in cultures of fresh

978 prepubertal testes and *in vivo*.

979 (A) Localization of the epigenetic marks H3K4me3, H3K9ac and H4K8ac in fresh testicular  
980 fragments cultured for 30 days with retinol (n=4) and in 36-37 *dpp* testes (*in vivo* controls,  
981 n=4). Shown are representative microscopy images of H3K4me3, H3K9ac and H4K8ac

982 labelling. Antibody binding was visualized using 3,3'-diaminobenzidine (brown staining) and

983 tissue sections were counterstained with hematoxylin. Enlarged views of spermatogonia,  
984 leptotene/zygotene and pachytene spermatocytes (upper right corner) and of round and  
985 elongated spermatids (lower right corner) are shown. Bar: 50  $\mu$ m.  
986 **(B-D)** Proportion of **(B)** H3K4me3, **(C)** H3K9ac and **(D)** H4K8ac-positive spermatogonia,  
987 leptotene/zygotene and pachytene spermatocytes, round and elongated spermatids in 30-day  
988 cultures of fresh testicular fragments with retinol (n=4) and in control 36-37 *dpp* testes (n=4).  
989 The localization of H3K4me3, H3K9ac and H4K8ac was analyzed in 30 cross-sectioned  
990 seminiferous tubules from three sections. Histograms are expressed as mean  $\pm$  SD. \**P* < 0.05,  
991 \*\**P* < 0.01.

992 D30: day 30; *dpp*: days *postpartum*; ES: elongated spermatid; H3K4me3: trimethylated  
993 histone H3 on lysine 4; H3K9ac: acetylated histone H3 on lysine 9; H4K8ac: acetylated  
994 histone H4 on lysine 8; L/Z: leptotene/zygotene spermatocyte; P: pachytene spermatocyte;  
995 Rol: retinol; RS: round spermatid; Sg: spermatogonia

996

997 **Figure 5.** Immunolocalization of DNMT1, DNMT3a and 5mC in 30-day cultures of  
998 frozen/thawed prepubertal testes.

999 **(A)** Localization of DNMT1, DNMT3a and 5mC in CSF or SSV testicular fragments cultured  
1000 for 30 days with retinol (n=4). Shown are representative microscopy images of DNMT1,  
1001 DNMT3a and 5mC labelling. Antibody binding was visualized using 3,3'-diaminobenzidine  
1002 (brown staining) and tissue sections were counterstained with hematoxylin. Enlarged views of  
1003 spermatogonia and leptotene/zygotene spermatocytes are shown in the upper right corner and  
1004 in the lower right corner, respectively. Bar: 50  $\mu$ m.

1005 **(B)** Proportion of DNMT1, DNMT3a and 5mC-positive spermatogonia and  
1006 leptotene/zygotene spermatocytes in CSF or SSV testicular fragments cultured for 30 days  
1007 with retinol (n=4). The localization of DNMT1, DNMT3a and 5mC was analyzed in 30 cross-

1008 sectioned seminiferous tubules from three sections. Histograms are expressed as mean  $\pm$  SD.

1009 \* $P < 0.05$ .

1010 CSF: controlled slow freezing; D30: day 30; DNMT: DNA methyltransferase; *dpp*: days

1011 *postpartum*; 5mC: 5-methylcytosine; L/Z: leptotene/zygotene spermatocyte; P: pachytene

1012 spermatocyte; Rol: retinol; Sg: spermatogonia; SSV: solid surface vitrification

1013

1014 **Figure 6.** Immunolocalization of H3K4me3, H3K9ac and H4K8ac in cultures of

1015 frozen/thawed prepubertal testes.

1016 (A) Localization of H3K4me3, H3K9ac and H4K8ac in CSF or SSV testicular fragments

1017 cultured for 30 days with retinol (n=4). Shown are representative microscopy images of

1018 H3K4me3, H3K9ac and H4K8ac labelling. Antibody binding was visualized using 3,3'-

1019 diaminobenzidine (brown staining) and tissue sections were counterstained with hematoxylin.

1020 Enlarged views of spermatogonia, leptotene/zygotene and pachytene spermatocytes (upper

1021 right corner) and of round and elongated spermatids (lower right corner) are shown. Bar: 50

1022  $\mu\text{m}$ .

1023 (B-D) Proportion of (B) H3K4me3, (C) H3K9ac and (D) H4K8ac-positive spermatogonia,

1024 leptotene/zygotene and pachytene spermatocytes, round and elongated spermatids in CSF and

1025 SSV fragments cultured for 30 days with retinol (n=4). The localization of H3K4me3,

1026 H3K9ac and H4K8ac was analyzed in 30 cross-sectioned seminiferous tubules from three

1027 sections. Histograms are expressed as mean  $\pm$  SD. \* $P < 0.05$ .

1028 D30: day 30; *dpp*: days *postpartum*; ES: elongated spermatid; H3K4me3: trimethylated

1029 histone H3 on lysine 4; H3K9ac: acetylated histone H3 on lysine 9; H4K8ac: acetylated

1030 histone H4 on lysine 8; L/Z: leptotene/zygotene; P: pachytene spermatocyte; Rol: retinol; RS:

1031 round spermatid; Sg: spermatogonia

1032

1033 **Figure 7.** DNA methylation in *in vitro* produced spermatozoa and *in vivo* controls.  
1034 (A) 5mC patterns in spermatozoa extracted from fresh, CSF or SSV testes after 30 days of  
1035 culture in the presence of retinol, and from 36-37 *dpp* testes (*in vivo* controls). A hundred  
1036 spermatozoa (from a pool of 6 *in vitro* matured testes or from 6 *in vivo* testes) were examined.  
1037 Shown are representative images of 5mC immunofluorescence staining (in green) in  
1038 spermatozoa. Cell nuclei were stained with DAPI (in blue). Representative merged  
1039 fluorescent images obtained when the primary antibody was omitted (negative controls) are  
1040 also shown (right column). Magnification:  $\times 900$ , bar: 10  $\mu\text{m}$ .  
1041 (B) Box plot distributions of normalized 5mC fluorescence intensity in spermatozoa extracted  
1042 from fresh, CSF or SSV testes after 30 days of culture in the presence of retinol, and from 36-  
1043 37 *dpp* testes (*in vivo* controls). The boxes stretch from the 25<sup>th</sup> to the 75<sup>th</sup> percentile. The  
1044 median is shown as a line across the boxes. The horizontal lines outside the boxes display  
1045 minimum and maximum. The circles represent outliers.  $**P < 0.01$ .  
1046 CSF: controlled slow freezing; D30: day 30; DAPI: 4',6-diamidino-2-phenylindole; *dpp*: day  
1047 *postpartum*; 5mC: 5-methylcytosine; Rol: retinol; SSV: solid surface vitrification  
1048

1049 **Additional File 1.** Schematic representation of the experimental design  
1050 Testes from 6-7 *dpp* prepubertal CD-1 mice were obtained and organotypic cultures were  
1051 performed either directly (from fresh tissues) or after thawing of testes frozen by CSF or SSV.  
1052 Testicular fragments were cultured at a gas-liquid interphase on agarose gels (under 5% CO<sub>2</sub>  
1053 at 34°C), with retinol alone or a retinol/FSH alternation (“Rol” and “Rol/FSH” culture  
1054 conditions, respectively) for 30 days. Testes from mice aged 36-37 *dpp* represent the  
1055 corresponding *in vivo* controls. After selection of the best culture condition, *in vitro* matured  
1056 testicular tissues and *in vivo* controls were analyzed by RT-qPCR and by  
1057 immunohistochemistry. Moreover, a hundred spermatozoa extracted from a pool of 6 *in vitro*

1058 cultured testes or from 6 *in vivo* testes were analyzed by immunofluorescence. In order to  
1059 evaluate the impact of the freezing procedures on the testicular tissues before culture, fresh,  
1060 CSF and SSV testes from mice aged 6-7 *dpp* were also analyzed by RT-qPCR and  
1061 immunohistochemistry. A total of 134 testes from 73 mice were used in this study. RT-qPCR  
1062 analyses were repeated 3 times (from 3 different testes) and immunohistochemical analyses  
1063 were repeated 4 times (from 4 different testes). For the analysis of sperm DNA methylation, a  
1064 pool of 6 testes (from 6 different mice) was used to extract spermatozoa.

1065 (\*) The same testicular samples were used for the evaluation of spermatogenic progression  
1066 and for DNMT1, DNMT3a, 5mC immunostaining

1067  $\alpha$ -MEM: minimum essential medium  $\alpha$ ; CSF: controlled slow freezing; *dpp*: days *postpartum*;  
1068 *Dnmt*/DNMT: DNA methyltransferase; FSH: follicle stimulating hormone; H3K4me3:  
1069 trimethylated histone H3 on lysine 4; H3K9ac: acetylated histone H3 on lysine 9; H4K8ac:  
1070 acetylated histone H4 on lysine 8; *Hdac1*: Histone deacetylase 1; *Jarid1b*: Jumonji AT-rich  
1071 interactive domain 1b; KSR: *knockout* replacement serum; 5mC: 5-methylcytosine; *Prdm9*:  
1072 PR domain-containing 9; Rol: retinol; RT-qPCR: quantitative PCR after reverse transcription;  
1073 *Sirt1*: Sirtuin 1; *Src1*: Steroid receptor coactivator 1; SSV: solid surface vitrification

Figure 1

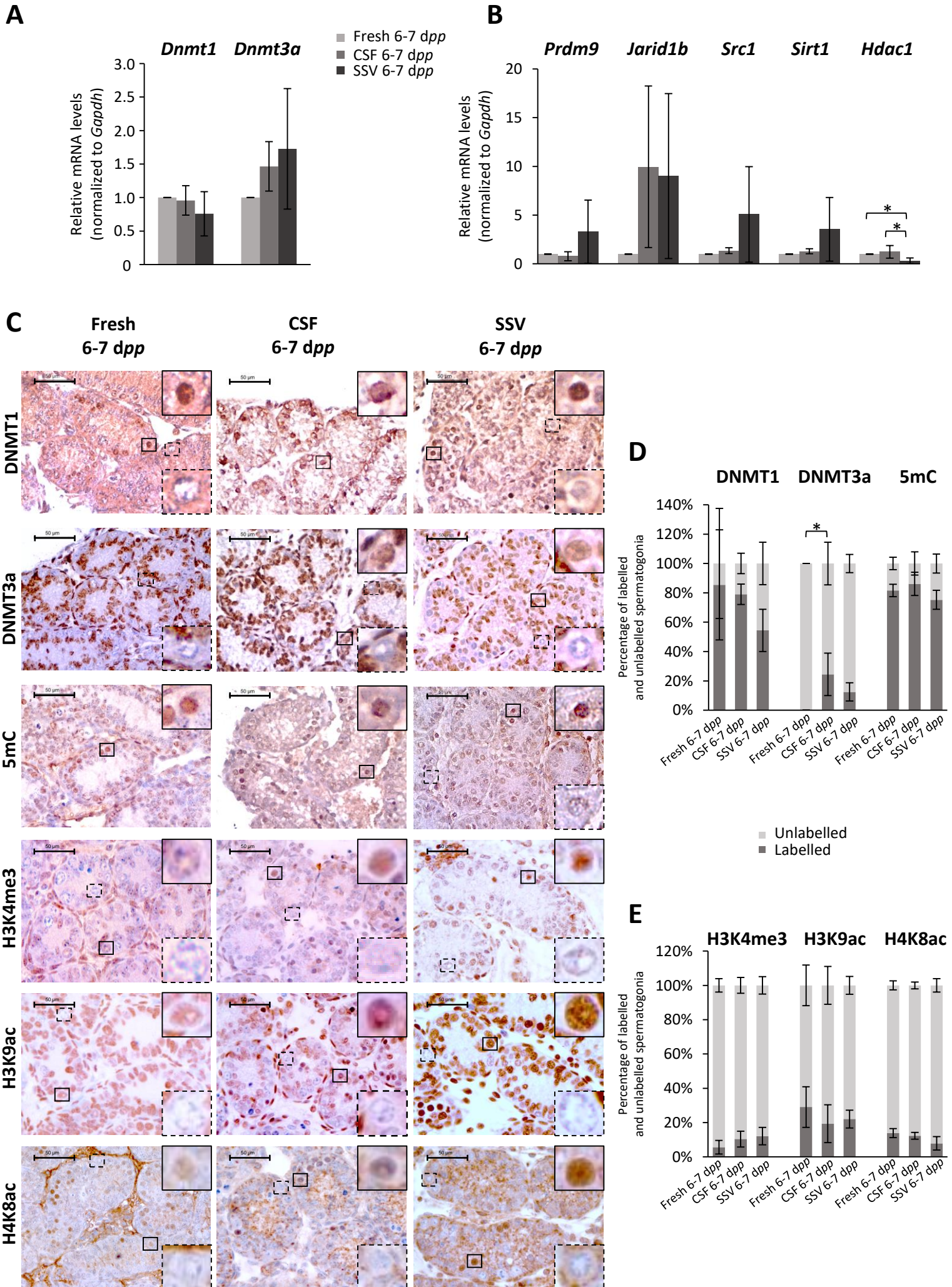


Figure 2

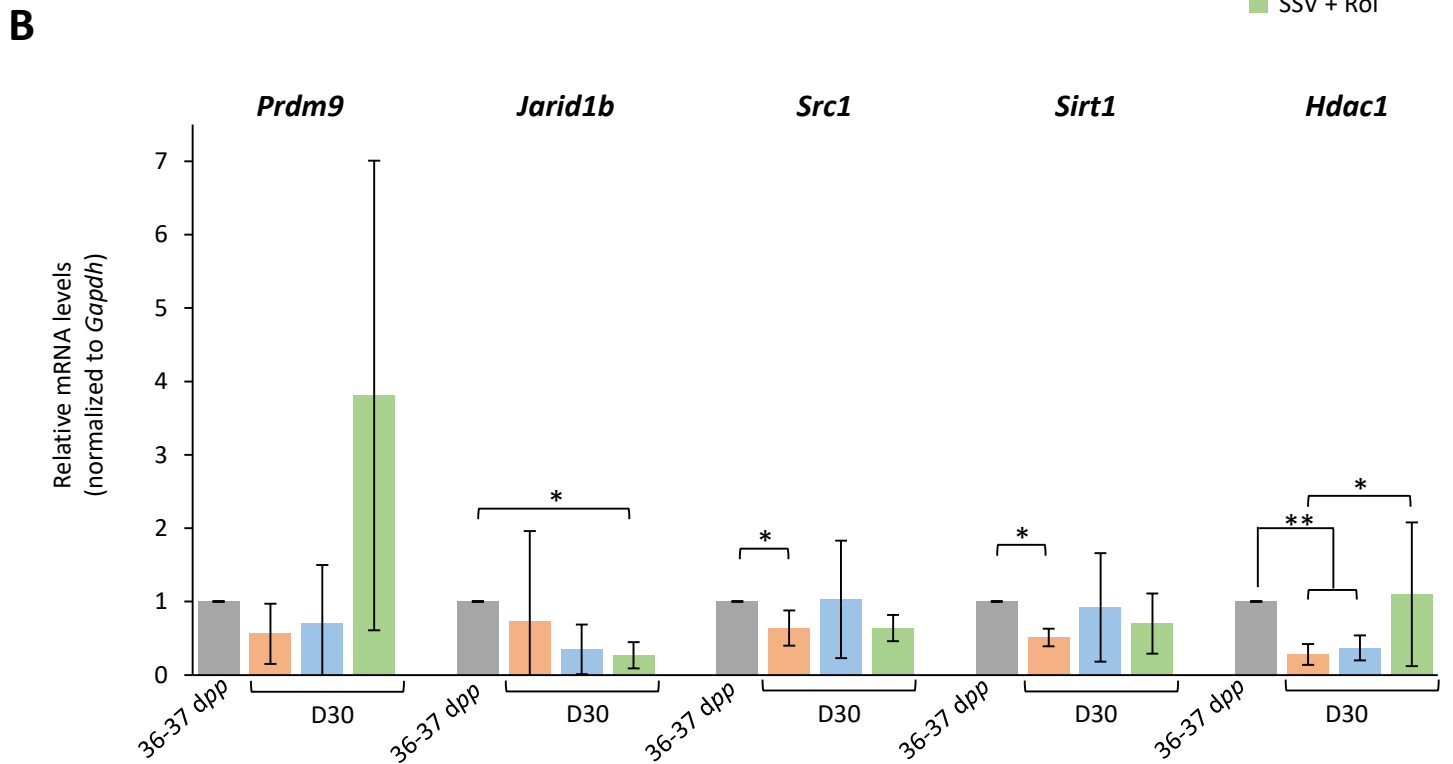
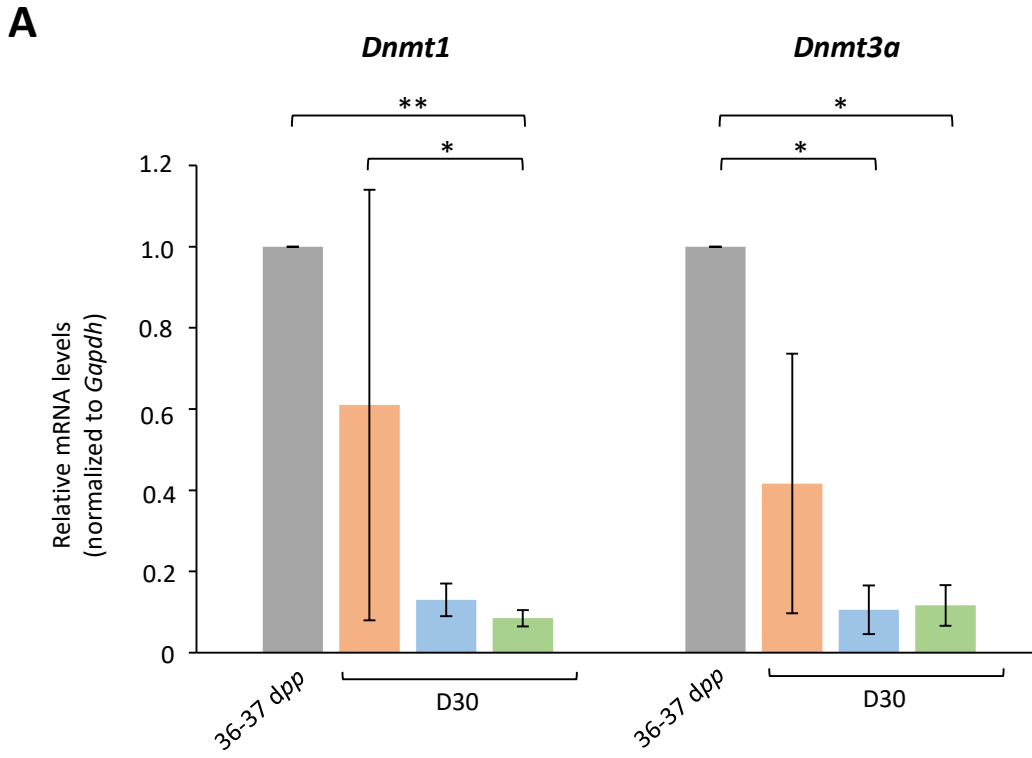
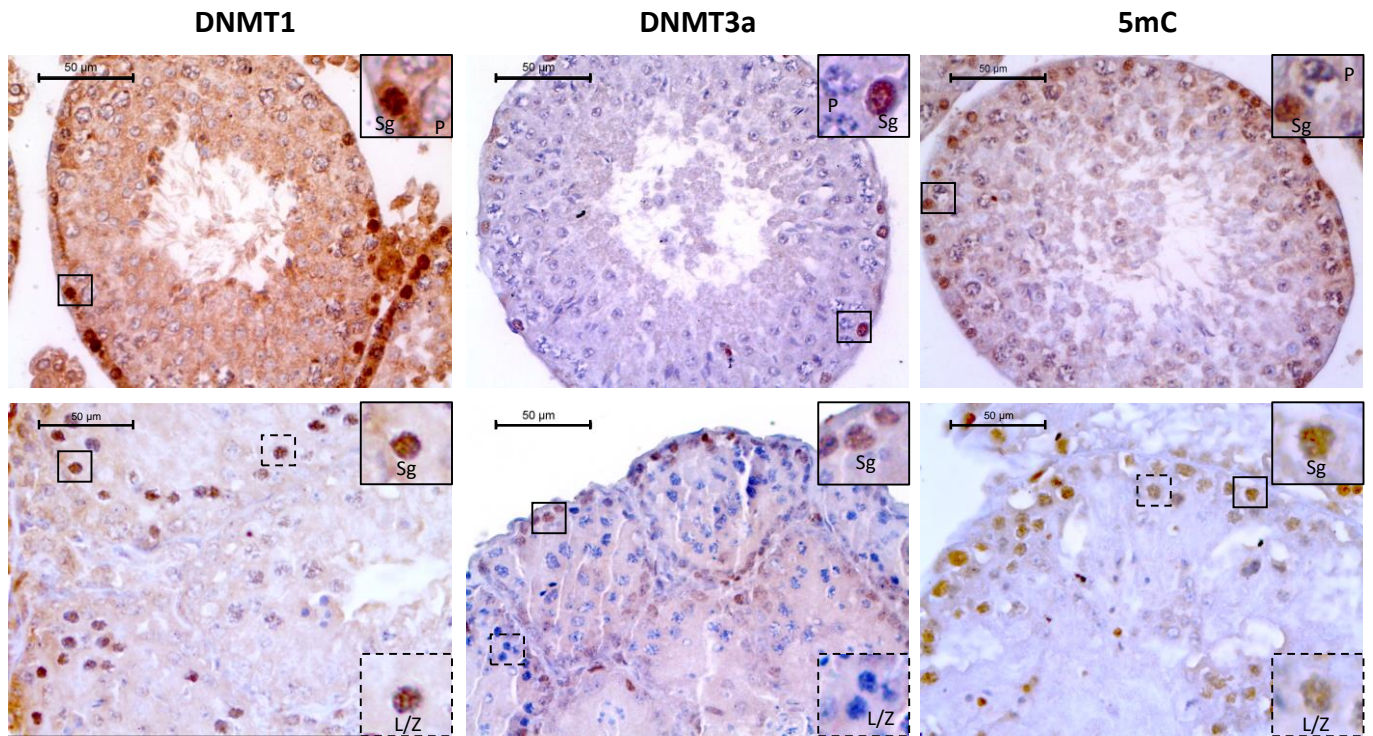


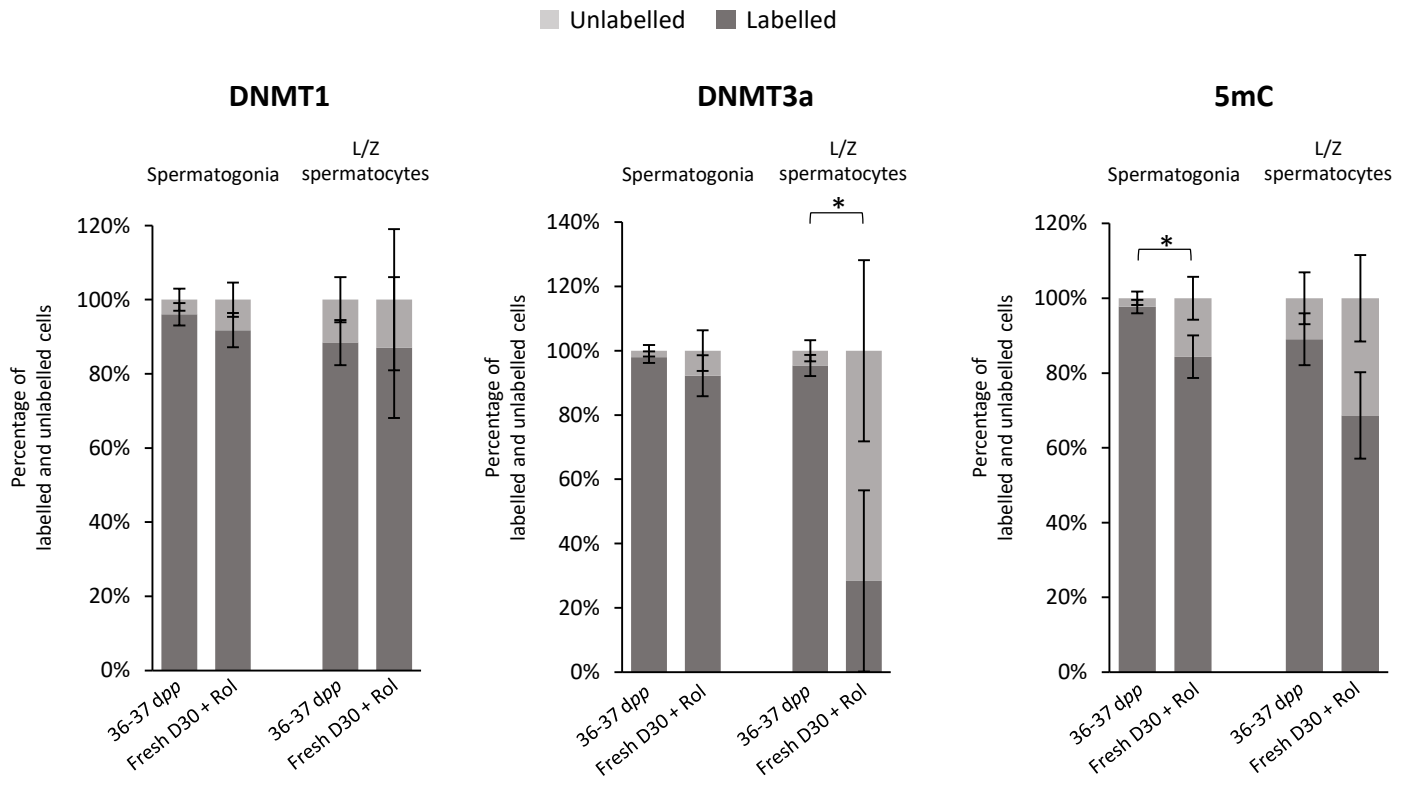


Figure 3

A



B



# Figure 4

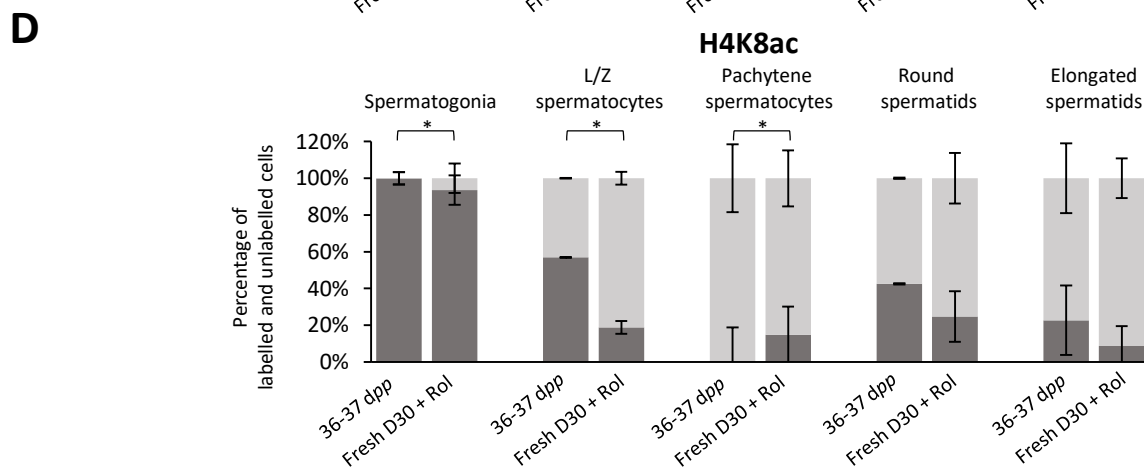
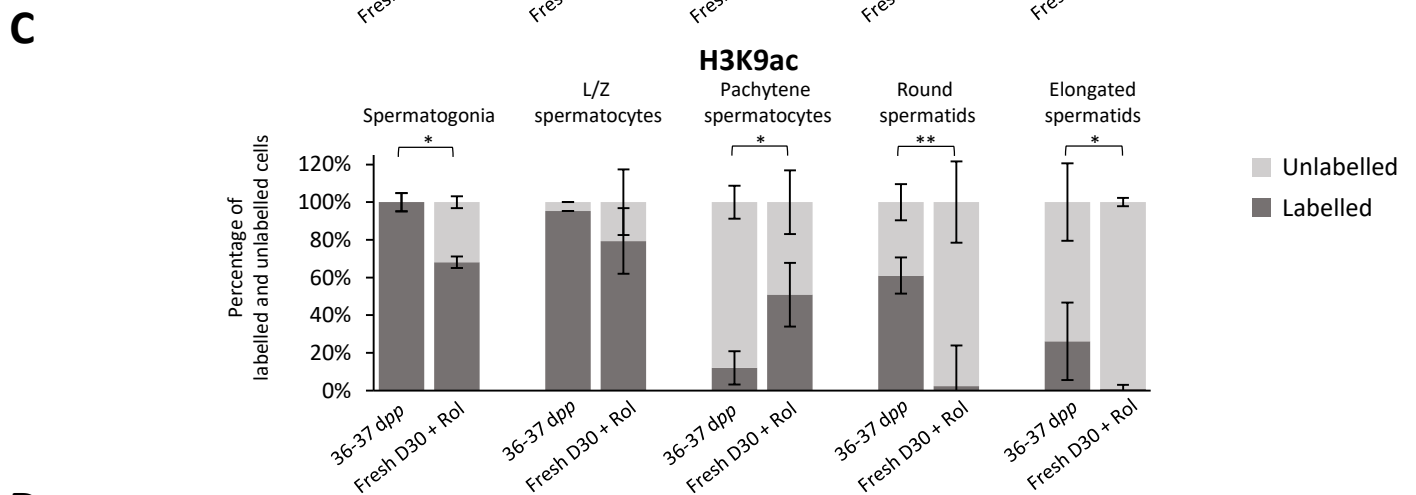
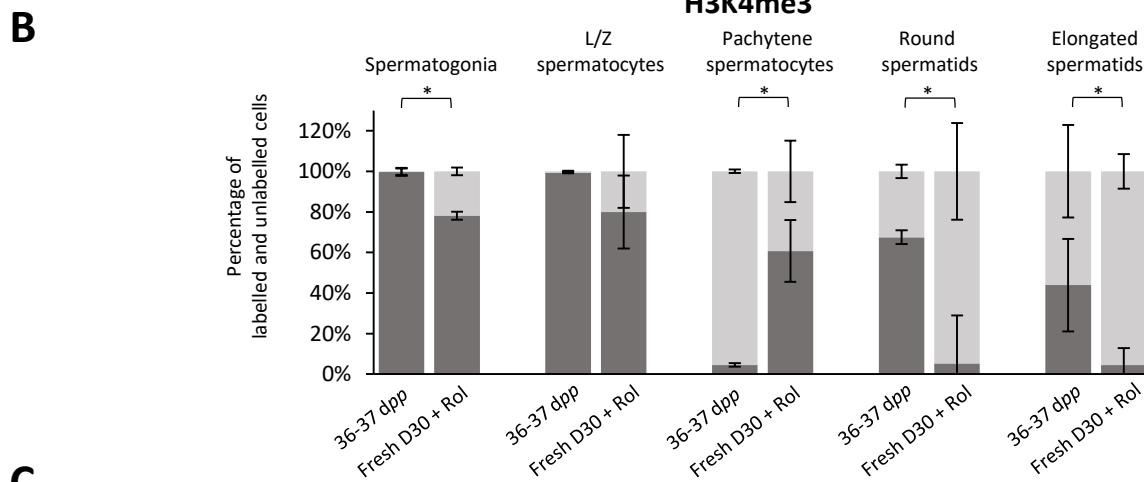
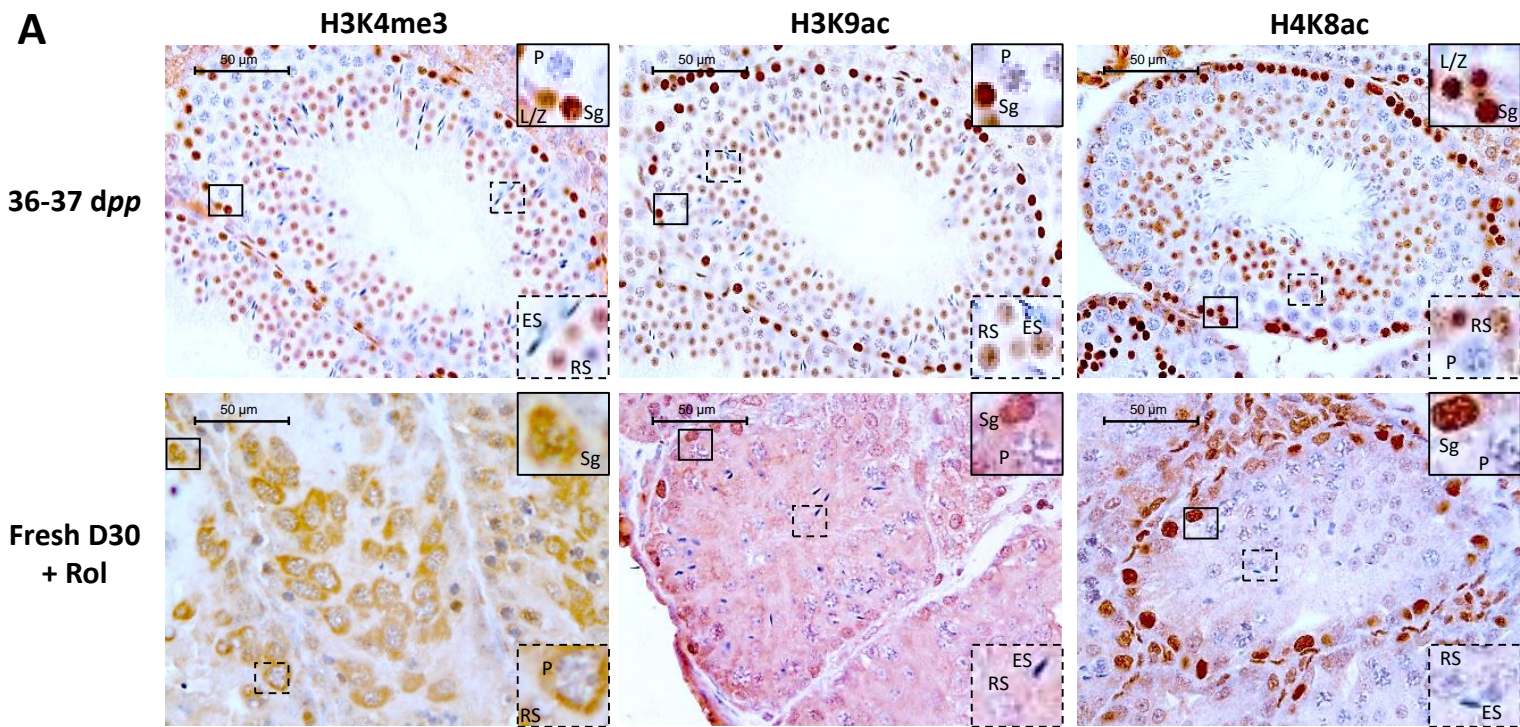
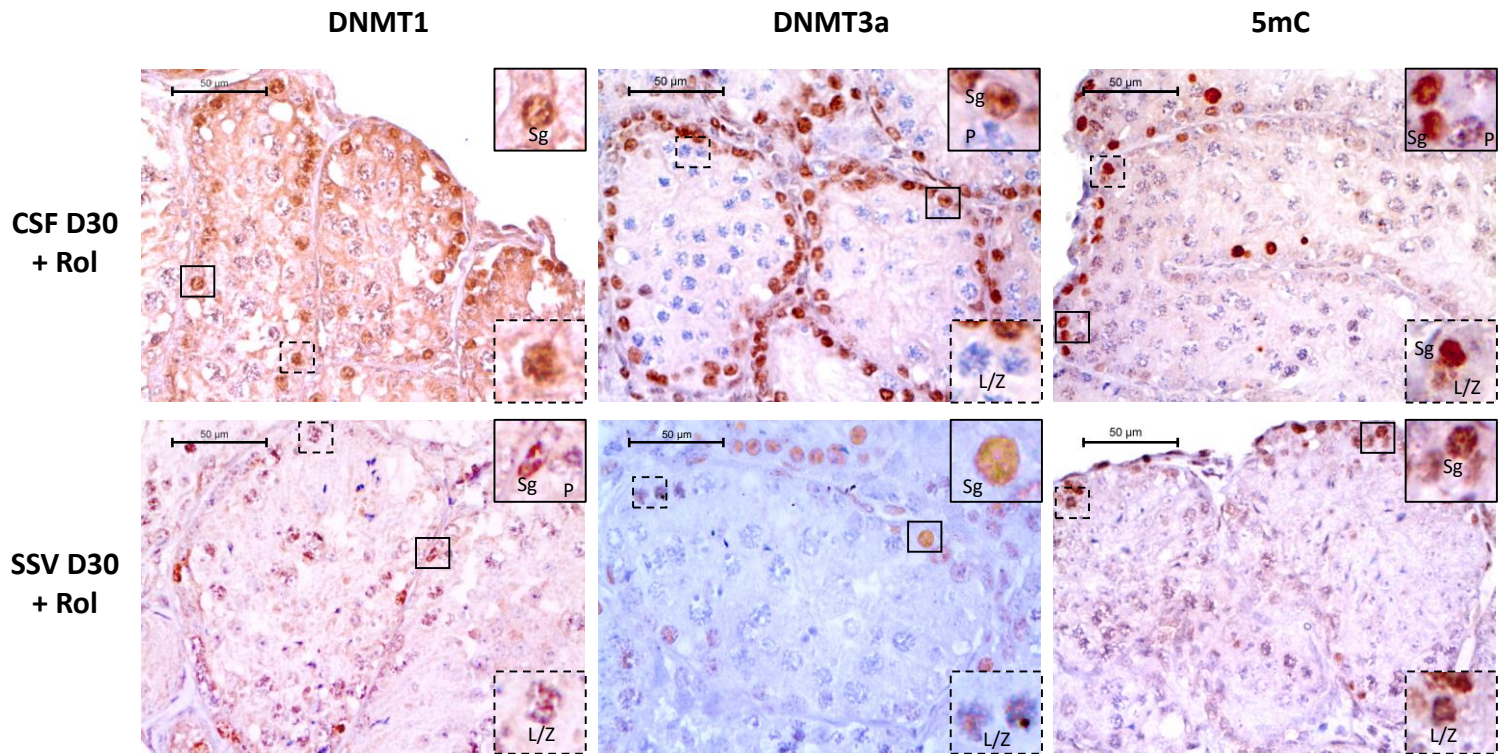




Figure 5

**A**



**B**

■ Unlabelled ■ Labelled

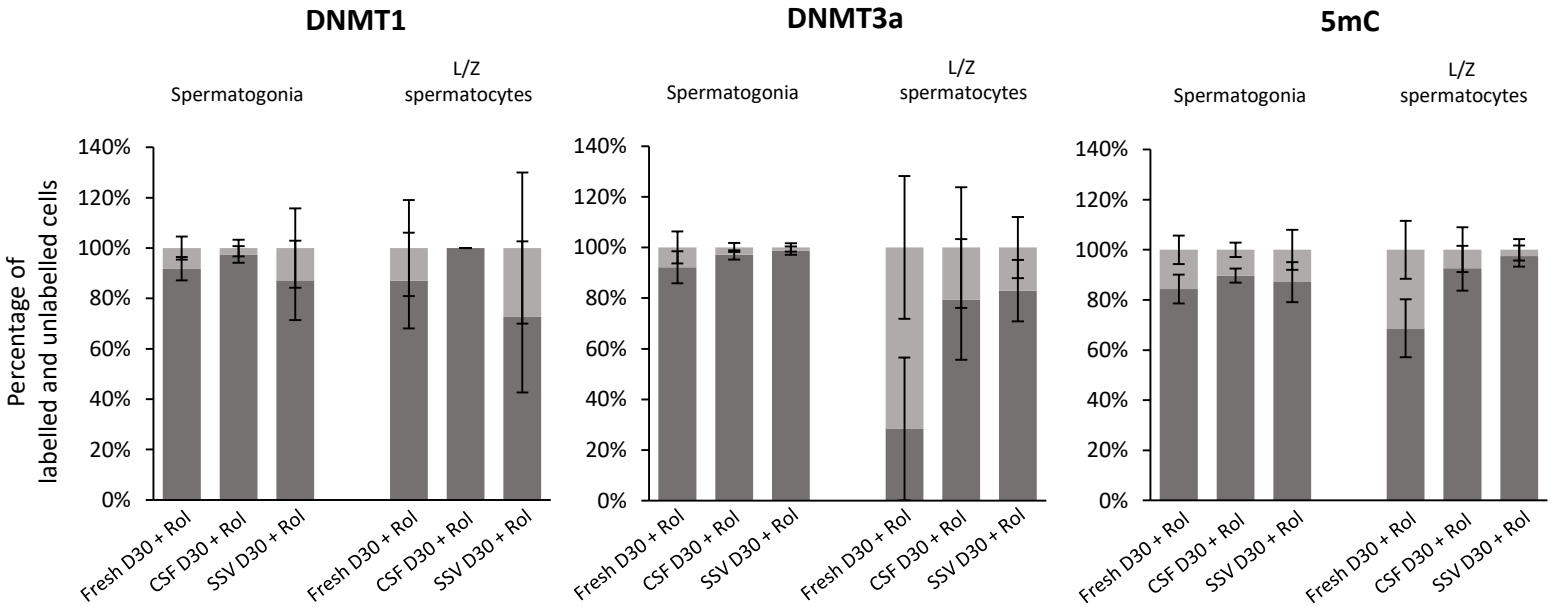


Figure 6

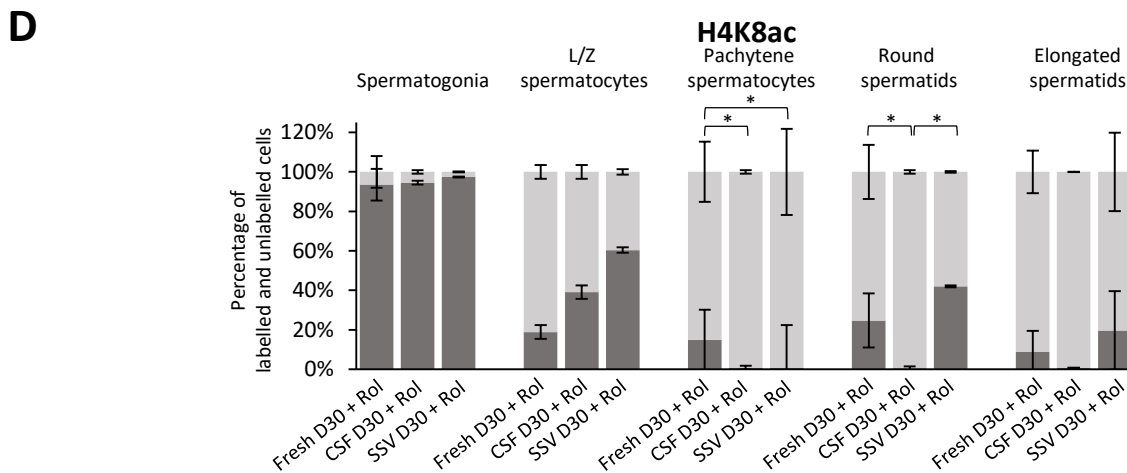
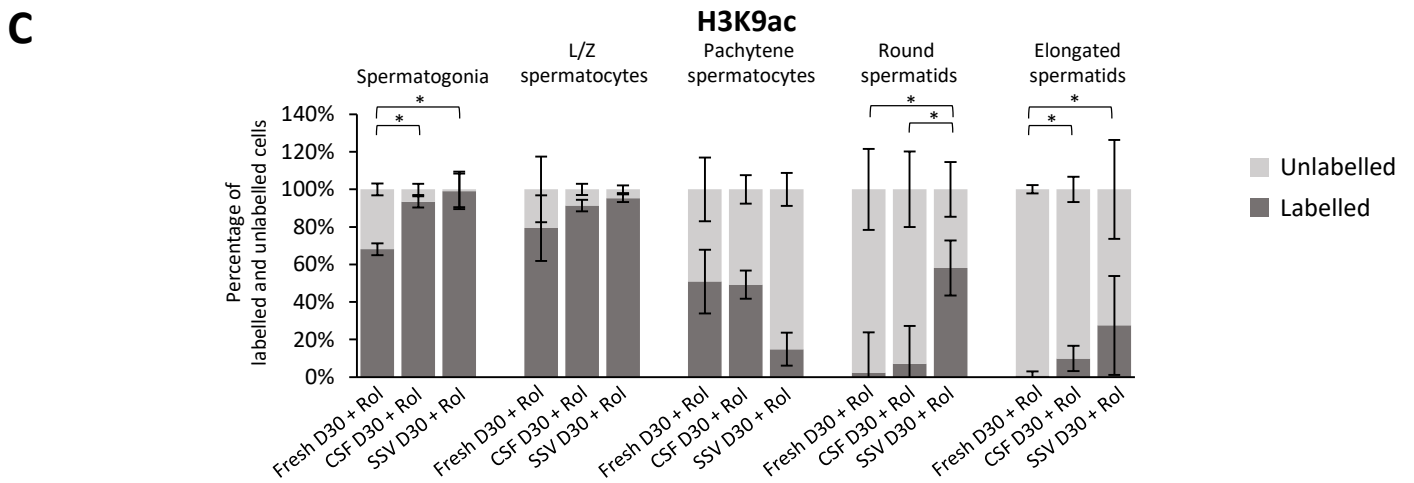
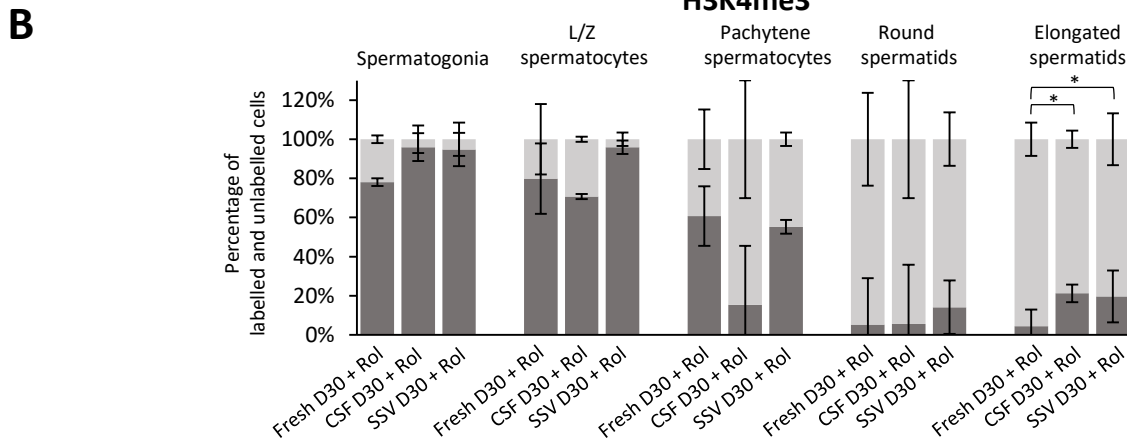
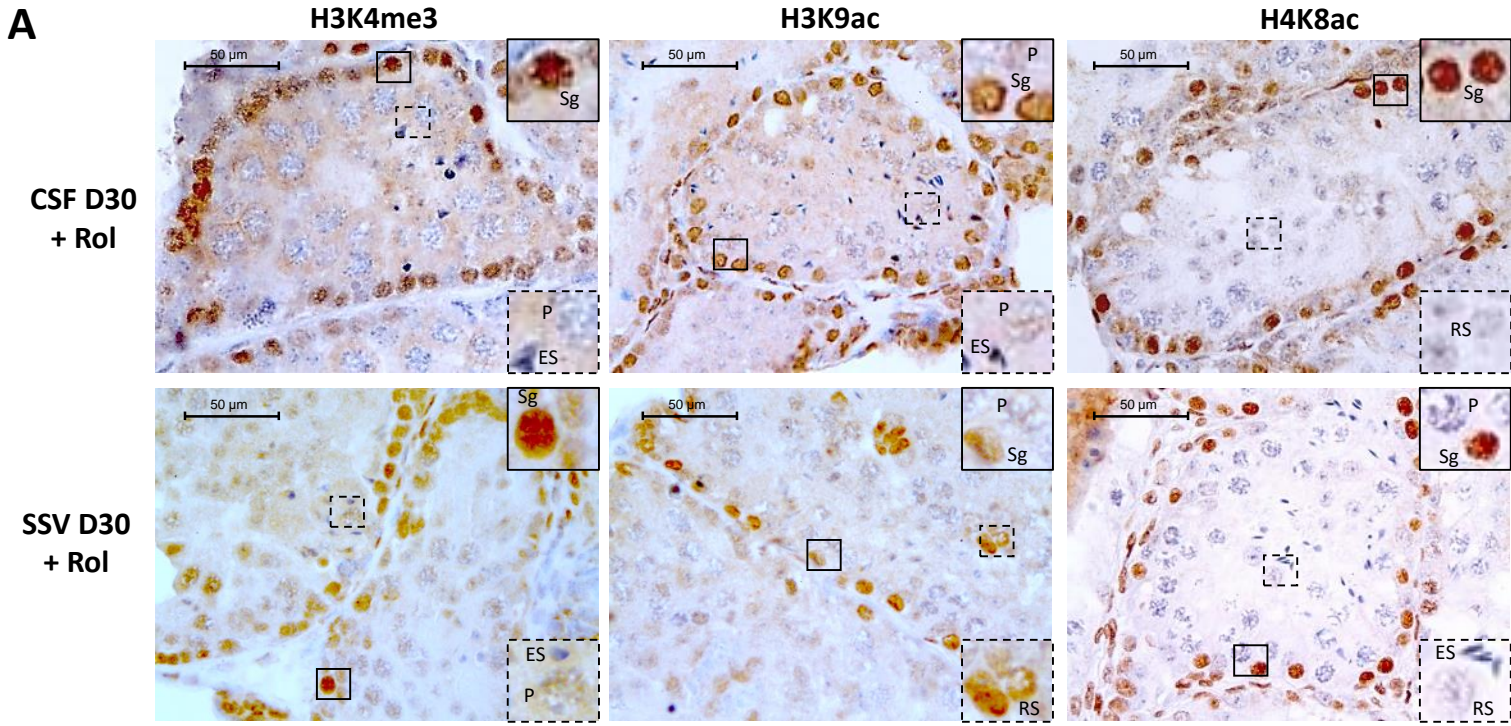
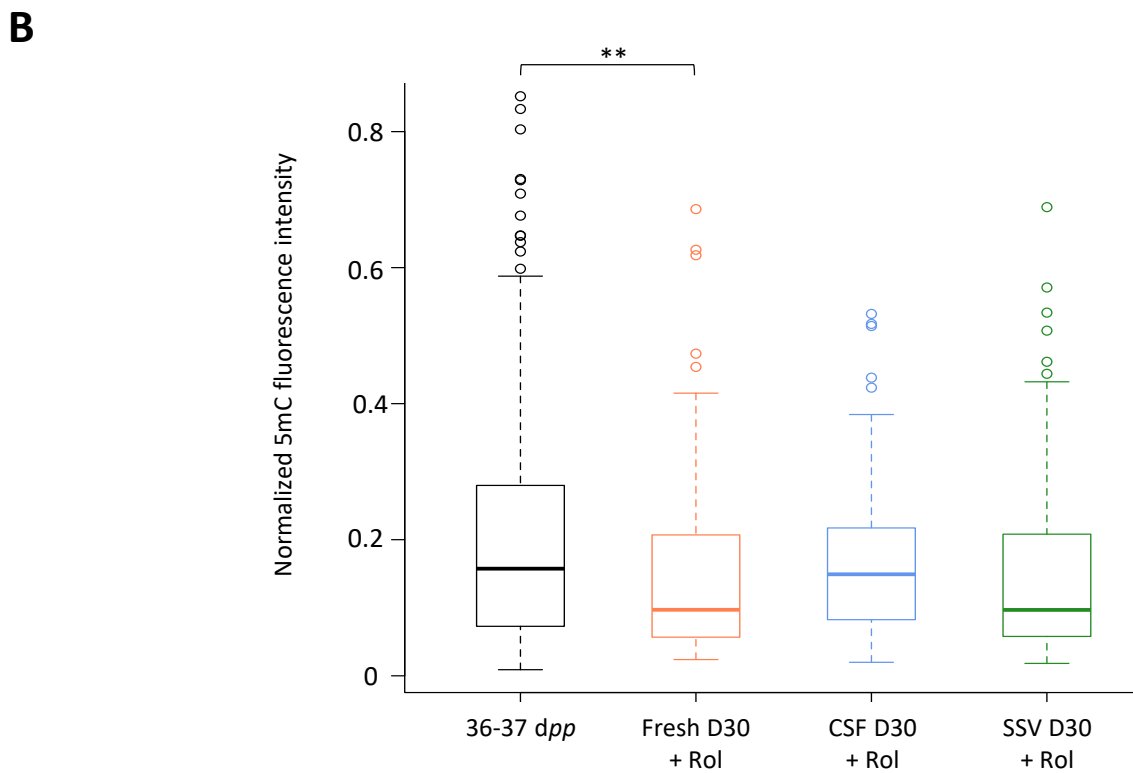
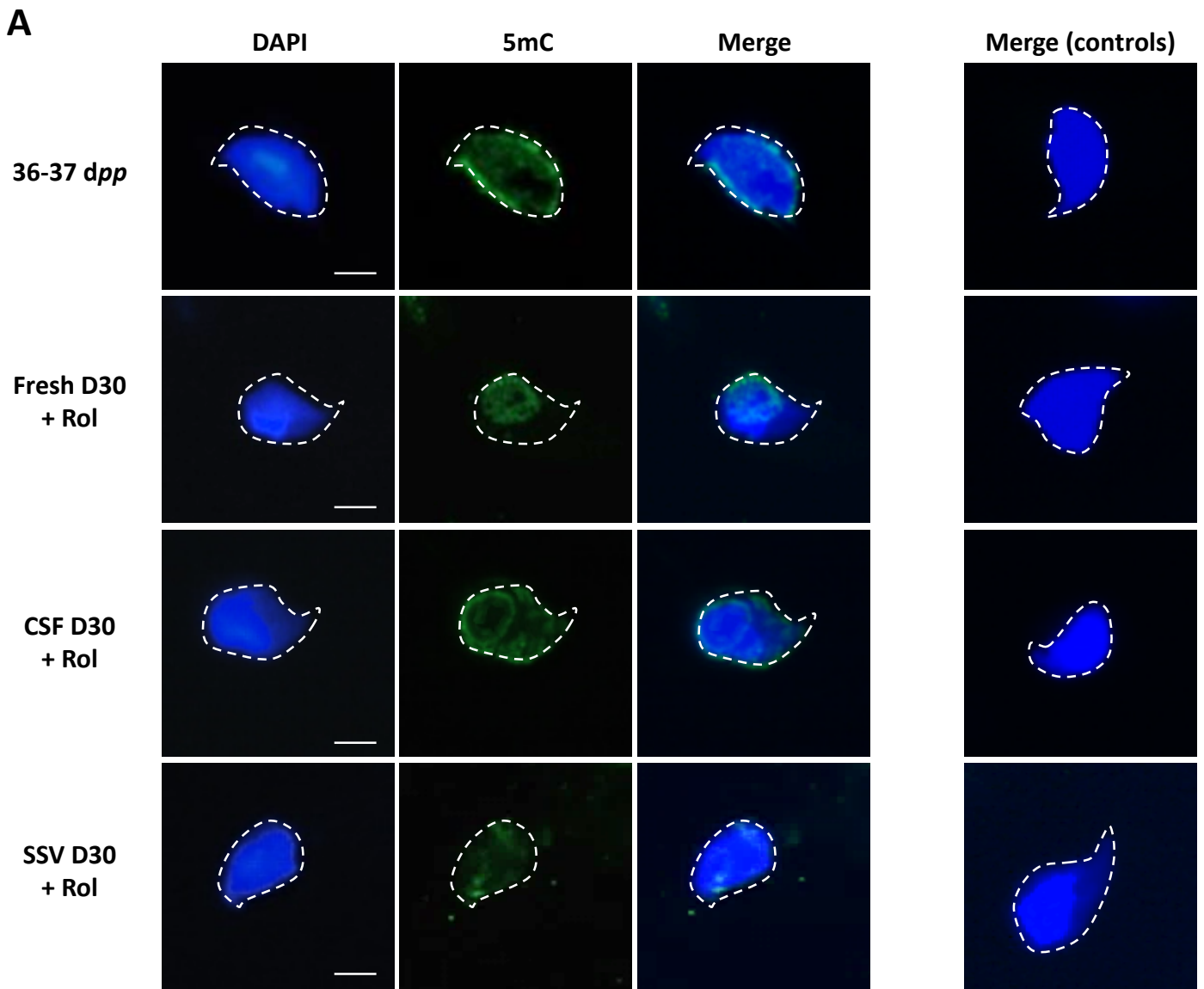


Figure 7



**Table 1.** Assessment of germ cell differentiation in seminiferous tubules

	% of cell types present in seminiferous tubules sections						
	36-37 <i>dpp</i>	Fresh + Rol D30	Fresh + Rol/FSH D30	CSF + Rol D30	CSF + Rol/FSH D30	SSV + Rol D30	SSV + Rol/FSH D30
Sertoli cells	9.11 (2.43)	33.13 (2.50) <sup>a</sup>	29.64 (5.96) <sup>a</sup>	39.17 (4.44) <sup>b</sup>	37.33 (4.78) <sup>c</sup>	33.59 (6.14) <sup>d</sup>	29.38 (5.90) <sup>e</sup>
Spermatogonia	7.89 (1.53)	14.78 (4.61) <sup>a</sup>	14.45 (3.31) <sup>a</sup>	20.12 (4.49) <sup>b</sup>	19.18 (1.92) <sup>c</sup>	15.94 (4.04)	15.65 (4.49) <sup>e</sup>
Leptotene/zygotene spermatocytes	3.24 (1.64)	4.91 (5.39)	11.91 (5.63) <sup>a,b</sup>	3.20 (1.53)	9.28 (4.28) <sup>d</sup>	3.34 (1.85)	5.69 (4.50) <sup>f</sup>
Pachytene spermatocytes	24.40 (2.97)	22.50 (6.12) <sup>a</sup>	21.63 (3.86) <sup>a</sup>	26.85 (5.60)	27.06 (6.25) <sup>c</sup>	27.42 (4.64)	32.90 (4.27) <sup>c</sup>
Round spermatids	37.70 (2.55)	15.15 (1.95) <sup>a</sup>	15.13 (5.34) <sup>a</sup>	7.31 (3.75) <sup>b</sup>	5.18 (2.60) <sup>c</sup>	13.83 (4.75) <sup>d</sup>	11.83 (3.70) <sup>e</sup>
Elongated spermatids	17.66 (4.32)	9.53 (3.06) <sup>a</sup>	7.24 (3.27) <sup>a,b</sup>	3.35 (1.05) <sup>b</sup>	1.97 (1.50) <sup>c,d</sup>	5.88 (2.20)	4.55 (2.53) <sup>e</sup>

Data are presented as the mean (SD) with n = 4 for 36-37 *dpp*, Fresh + Rol D30, Fresh + Rol/FSH D30, CSF + Rol D30, CSF + Rol/FSH D30, SSV + Rol D30, SSV + Rol/FSH D30. CSF, controlled slow freezing; D30, 30-day tissue culture; *dpp*, days *postpartum*; Rol, retinol; Rol/FSH, retinol/follicle stimulating hormone; SSV, solid surface vitrification.

<sup>a</sup>Values significantly different with 36-37 *dpp* ( $P < 0.05$ )

<sup>b</sup>Values significantly different with Fresh + Rol D30 ( $P < 0.05$ )

<sup>c</sup>Values significantly different with Fresh + Rol/FSH D30 ( $P < 0.05$ )

<sup>d</sup>Values significantly different with CSF + Rol D30 ( $P < 0.05$ )

<sup>e</sup>Values significantly different with CSF + Rol/FSH D30 ( $P < 0.05$ )

<sup>f</sup>Values significantly different with SSV + Rol D30 ( $P < 0.05$ )



**Table 2.** Percentage of seminiferous tubules containing differentiated germ cells at the most advanced stage

		% of seminiferous tubules at the most advanced stage					
	36-37 <i>dpp</i>	Fresh + Rol D30	Fresh + Rol/FSH D30	CSF + Rol D30	CSF + Rol/FSH D30	SSV + Rol D30	SSV + Rol/FSH D30
Spermatogonia	0	0.28 (0.96)	2.05 (2.72) <sup>a</sup>	3.15 (5.90)	6.17 (8.03)	3.33 (5.50)	6.13 (7.85)
Leptotene/zygotene spermatocytes	0	0.28 (0.96)	5.13 (5.89) <sup>a,b</sup>	0	6.94 (6.12) <sup>d</sup>	1.94 (4.13)	2.73 (2.29)
Pachytene spermatocytes	0	16.75 (5.08) <sup>a</sup>	27.64 (8.59) <sup>a,b</sup>	50.88 (10.82) <sup>b</sup>	55.37 (11.87) <sup>c</sup>	30.56 (15.10) <sup>d</sup>	47.11 (15.46) <sup>c,e</sup>
Round spermatids	4.72 (3.76)	33.45 (11.76) <sup>a</sup>	29.07 (9.46) <sup>a</sup>	23.33 (9.64)	18.12 (10.61)	31.11 (9.57)	17.67 (3.43) <sup>c,e</sup>
Elongated spermatids	95.28 (3.61)	49.24 (10.86) <sup>a</sup>	36.11 (10.76) <sup>a,b</sup>	22.64 (5.92) <sup>b</sup>	13.40 (7.60) <sup>c,d</sup>	33.06 (11.41) <sup>b</sup>	26.36 (14.89)

Data are presented as the mean (SD) with  $n = 4$  for 36-37 *dpp*, Fresh + Rol D30, Fresh + Rol/FSH D30, CSF + Rol D30, CSF + Rol/FSH D30, SSV + Rol D30, SSV + Rol/FSH D30. CSF, controlled slow freezing; D30, 30-day tissue culture; *dpp*, days *postpartum*; Rol, retinol; Rol/FSH, retinol/follicle stimulating hormone; SSV, solid surface vitrification.

<sup>a</sup>Values significantly different with 36-37 *dpp* ( $P < 0.05$ )

<sup>b</sup>Values significantly different with Fresh + Rol D30 ( $P < 0.05$ )

<sup>c</sup>Values significantly different with Fresh + Rol/FSH D30 ( $P < 0.05$ )

<sup>d</sup>Values significantly different with CSF + Rol D30 ( $P < 0.05$ )

<sup>e</sup>Values significantly different with SSV + Rol D30 ( $P < 0.05$ )