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Detailed profiles of histone modification in male germ line cells

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of the young and aged mice

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Keywords: spermatogenesis, aging, histone modifications

Abstract

Human epidemiological studies have shown paternal aging as one of the risks for neurodevelopmental disorders such as autism in offspring. A recent study has suggested that factors other than *de novo* mutations due to aging can influence biology of offspring. Here we are focusing on epigenetic alterations in sperm that can influence offspring developmental programs. In this study, we qualitatively and semi-quantitatively evaluated histone modification patterns in male germ line cells throughout spermatogenesis based on immunostaining of testes taken from young (3 months) and aged (12 months) old mice. Although localization patterns were not obviously changed between young and aged testes, some histone modification showed differences in their intensity. Among histone modifications that repress gene expression, H3K9me3 was decreased in the male germ line cells in the aged testis, while H3K27me2/3 was increased. The intensity of H3K27ac, an active mark, was relatively low in the aged testis. Interestingly, H3K27ac was detected in putative sex chromosomes of round spermatids, while other chromosomes were occupied by a repressive mark H3K27me3. Among other histone modifications that activate gene expression, H3K4me2 was drastically decreased in the male germ line cells in the aged testis. H3K79me3 was contrastingly increased and accumulated on the sex chromosomes at M-phase spermatocytes. Therefore, aging induced alterations in the

33 amount of histone modifications, of which patterns were different in individual histone
34 modifications. Moreover, histone modification seems to be differentially regulated by
35 aging on the sex chromosomes and on others. These findings would help elucidate
36 epigenetic mechanisms underlying influence of paternal aging on offspring's
37 development.

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Introduction

Autism spectrum disorder (ASD) is one of the neurodevelopmental disorders and has been reported to show a pandemic rise in these decades. The most recent survey in the United States shows that one in 59 children aged 8 years have diagnosed as ASD (1). The reason for the rise might include changes in diagnosis, although paternal aging has been suggested for one of the biological factors. For example, meta-analyses with 5,766,794 children born during 1985–2004 in five countries have revealed significant association of advanced paternal age with a risk of ASD in children (2).

Aging induces mutations in the genome of sperm and most of the *de novo* mutations in autistic children are reported to be inherited from fathers (3). However, a study using mathematical model has demonstrated that the risk of psychiatric illness from *de novo* mutations derived from advanced paternal age has been estimated as 10-20% (4). Therefore, some factors other than genetical mutations can influence health and disease of offspring.

One possible mechanism for paternal aging effects on offspring is epigenetic information encoded in sperm. Previously, we have reported that sperm derived from aged mice have

more hypomethylated regions in their genome and it possibly affects offspring's behavior (5). On the other hand, the age-related alteration of histone modification, another major epigenetic factor, remains unclear. Sperm is produced through dynamic and complicated processes, termed spermatogenesis, including meiosis of spermatocytes and morphological changes into mature sperm cells. Considering a frequent chromatin remodeling during spermatogenesis, alteration of histone modification would have a high impact on the spermatogenic processes. Besides, most of the histone protein in the sperm cell nucleus is replaced with protamine, although some remain on genome regions that are functioning in early developmental stages (6). Therefore, features of histone moiety in spermatogenesis would provide essential clues to understand gene regulation after fertilization and subsequent development of offspring. Nevertheless, little known about localization patterns of histone modification in the male germ line cells and their alteration by aging.

Here we first demonstrated comprehensive profiles of major histone modification during murine spermatogenesis, in which individual epigenetic modifications showed dynamic and distinct localization changes. We further examined influence of aging on the histone modifications in each step of spermatogenesis, and found that levels of histone

modification were differentially affected by aging during spermatogenesis in the testis.

We also noticed accumulation of H3K79me3 on sex chromosomes. Further elucidation

of how epigenetic changes due to aging contribute to altered gene regulation in the

offspring would provide an insight to understand influence of paternal aging on health

and disease of the next generation.

Materials and methods

Animals

C57BL/6J mice at 3-months old were purchased from a breeder (Charles River Laboratories, Japan). Some mice were raised up to 5- or 12-months old at Institute for Animal Experimentation Tohoku University Graduate School of Medicine and used for histological and semi-quantitative analyses. All animals were housed in standard cages in a temperature- and humidity-controlled room with a 12-hour light/dark cycle (light on at 8 am), and had free access to standard food and water. All experimental procedures were approved by Ethic Committee for Animal Experiments of Tohoku University Graduate School of Medicine (#2017- MED210), and the animals were treated according to the National Institutes of Health guidance for the care and use of laboratory animals.

Histological analyses of histone modifications

Histological analysis was performed based on a previous our report (7). Mice were anesthetized with isoflurane and perfused with PBS (pH 7.4) to remove blood. After perfusion, testes were dissected, immersed in 4% PFA in PBS, and cut into half to be post-fixed in 4% PFA overnight at 4°C. After washing in PBS, the testes were cryoprotected by being immersed in 5 % and 10% sucrose in PBS solution for 1 hour each, 20% sucrose

102 in PBS solution overnight, and embedded in OCT compound (Sakura Finetek, Japan).

103 Frozen sections of the testes were cut at 10 μ m on a cryostat (Leica, CM3050).

104 Next, 0.01M citric acid (pH 6.0) solution was heated up using microwave and kept

105 between 90°C and 95°C on a heat plate with gentle stirring. The sections were emerged

106 in the solution for 10 minutes for antigen retrieval. The solution and sections were left to

107 cool down to 40-50°C, and washed in 1×Tris-based saline containing 0.1% Tween20

108 (TBST) (pH 7.4), and treated with 3% bovine serum albumin and 0.3% Triton X-100 in

109 PBS solution for blocking. The sections were incubated at 4°C overnight with the

110 following primary antibodies at 1/500: i.e., rabbit anti-H3K9me3 (ab8898, abcam), rabbit

111 anti-H3K27me2 (ab24684, abcam), rabbit anti-H3K27me3 (07-449, Millipore), rabbit

112 anti-H3K27ac (ab4729, abcam), rabbit anti-H3K79me2 (ab3594, abcam), rabbit anti-

113 H3K79me3 (ab2621, abcam), mouse anti-SYCP3 (ab97672, abcam). Subsequently, either

114 Cy3- or Alexa488-conjugated secondary antibody (1:500) was applied together with 4'-6-

115 diamino-2-phenylindole (DAPI), and the sections were incubated at RT for one hour in a

116 humid chamber. After washing in TBST at room temperature, specimens were mounted

117 in Vectashield (Vector Laboratories, H-1200) or ProLong™ Diamond Antifade Mountant

118 (Invitrogen, P36965). Images were captured using a confocal laser-scanning microscope

119 (LSM780, Carl Zeiss, Jena, Germany).

Semi-quantitative analyses of histone modifications

Confocal images of the testis sections were semi-quantitatively analyzed by using ImageJ. SYCP3 signals were used to identify stages of spermatogenesis. A mean intensity of DAPI and each histone modification were measured to normalize histone modification signals by DAPI signal.

DNA Fluorescence in situ hybridization (DNA-FISH) followed by immunofluorescence staining

Antigen retrieval and immunohistochemical staining were performed as described above. Rabbit anti-H3K79me3 (1:500, ab2621, abcam) and Alexa Fluor Plus 647 (1:500, A32728, Invitrogen) were used as primary and secondary antibodies, respectively, and counterstained with DAPI. The sections were mounted in Vectashield. After capturing images using a confocal laser-scanning microscope, cover glasses were removed and the sections were washed in PBS., and immersed in Histo VT One (1:10, 06380-76, Nacalai Tesque) at 90°C for 25 minutes. After washing in PBS, the sections were immersed in 70% and 100% ethanol, dried in the air, and applied with 10 µl probes for X and Y chromosomes (MXY-10, Chromosome Science Lab). The sections were covered with cover slips and heated at 80°C on a heat plate (HDB-1N, AS ONE) for 10 minutes. After 15-hour incubation in a humid chamber at 37°C, the sections were washed in 2×Standard

138 Saline Citrate (SSC) (pH 4.0), and cover slips were removed. The sections were next
 139 incubated in 50% formamid-2×SSC at 37°C for 20 minutes and washed in 1×SSC for 15
 140 minutes at room temperature. The sections were mounted in Vectashield. Images were
 141 captured using a confocal laser-scanning microscope.

142 **Statistical analysis**

143 Student's *t*-test was used to determine statistical significance. Values of **p* < 0.05 and ***p*
 144 < 0.01 were considered statistically significant.

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Results

1. Morphological profiles showing histone modification patterns during spermatogenesis

Male germ line cells drastically change their morphology during spermatogenesis. Although previous literatures show localization of some histone modifications in these cells (8-10), comprehensive localization patterns are not fully examined. Here, we first investigated localization of major histone modifications during spermatogenesis according to 12 stages based on a previous report (11). This staging helped us to morphologically follow each step of spermatogenesis without any marker staining.

(1) Histone modifications that activate gene expression

First, we examined active marks for gene expression, i.e., H3K4me2, H3K4me3, H3K27ac, H3K79me2 and H3K79me3.

(i) H3K4me2

H3K4me2 signal was observed in all stages of seminiferous tubules. At stage VIII in which the earliest meiotic spermatocytes (i.e., preleptotene cells) were located near the periphery of the tubule, a signal of H3K4me2 was detected in the whole nucleus of preleptotene cells, though constitutive heterochromatin did not show the signal (Fig. 1a,

arrowhead 1). The signal continued throughout meiosis (Fig. 1a, arrowheads 2-6). In the pachytene cells of stage X, a strong signal was detected on the XY body (Fig. 1a, arrowhead 7). The accumulation of H3K4me2 on the XY body disappeared in the M-phase spermatocytes at stage XII, and only weak signal was observed in the chromosomes (Fig. 1a, arrowhead 8). In round spermatids from stage I to VIII and elongated spermatids at stage X, the signal was widely detected in the nucleus, but not in the chromocenter (constitutive heterochromatin) (Fig. 1a, arrowheads 9-12). Thus, localization of H3K4me2 modification seems to start from preleptotene cells, and to accumulate on the XY body in late pachytene cells. Through spermatogenesis, H3K4me2 was not detected in the chromocenter (Fig. 1b).

Fig. 1 Localization of H3K4me2 in male germ line cells in the young (3 M) testis. (a)

Representative confocal images of H3K4me2 (magenta) and SCP3 (green) in the seminiferous epithelium in stages I, V, VIII, X and XII. Nuclei were counterstained with DAPI (blue). The number with arrowhead means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7; Pachytene spermatocyte at stage I, V, VIII and X

respectively, M; M phase cells at stage XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid at stage X. Scale bar: 20 μ m. (b) Magnified images of the cells indicated by arrowheads in (a), and subcellular localization of H3K4me2 in the young testis is shown in magenta in a graphical summary. pL: Preleptotene spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each parenthesis represents the stage of spermatogenesis.

(ii) H3K4me3

While H3K4me2 was observed from preleptotene cells (Fig. 1b), H3K4me3 signal was not detected from preleptotene cells to pachytene cells (Fig. 2a, arrowheads 1-7). H3K4me3 was firstly detected in M-phase spermatocytes in stage XII seminiferous tubules (Fig. 2a, arrowhead 8). The signal was broadly distributed on condensed chromosomes (Fig. 2b, arrowhead 8). After meiotic division II, the signal was detected in round spermatids at stage I to VIII, though there was no signal in the chromocenter (Fig. 2a, arrowheads 9-11). In elongated spermatids, the signal was weak and widely detected in whole nucleus (Fig. 2a, arrowhead 12). Thus, localization of H3K4me3 modification

appeared later stage of meiosis, and the signal was weak compare to H3K4me2 modification (Fig. 2b).

Fig. 2 Localization of H3K4me3 in male germ line cells in the young (3 M) testis. (a)

Representative confocal images of H3K4me3 (magenta) and SCP3 (green) in the seminiferous epithelium in stages I, V, VIII, X and XII. Nuclei were counterstained with DAPI (blue). The number with arrowhead means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7; Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid at stage X. Scale bar: 20 μ m. (b) Magnified images of the cells indicated by arrowheads in (a), and subcellular localization of H3K4me3 in the young testis is shown in magenta in a graphical summary. pL: Preleptotene spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each parenthesis represents the stage of spermatogenesis.

215 (iii) H3K27ac

216 H3K27ac signal was observed in all stages of seminiferous tubules. At stage VIII in which
 217 the earliest meiotic cells (i.e., preleptotene cells) were located near the periphery of the
 218 tubule, strong signals of H3K27ac were detected in whole nuclei of preleptotene cells
 219 (Fig. 3a, arrowhead 1). While the signal continued throughout meiosis, the intensity
 220 dramatically decreased when preleptotene cells differentiated into leptotene cells (Fig. 3a,
 221 arrowhead 2-5). In the pachytene cells of stage VIII, a strong signal was detected on the
 222 XY body (Fig. 3a, arrowhead 6). The accumulation of H3K27ac on the XY body
 223 disappeared in the pachytene cells of stage X and M-phase spermatocytes at stage XII,
 224 and only weak signal was observed in the whole nucleus or chromosomes (Fig. 3a,
 225 arrowheads 7-8). In round spermatids from stage I to V, the signal was widely detected in
 226 the nucleus, especially in the peri-chromocenter (putative sex chromosome) (Fig. 3a,
 227 arrowheads 9-10). The strong signal on the peri-chromocenter disappeared in round
 228 spermatids of stage VIII (Fig. 3a, arrowhead 11). H3K27ac signal increased again in
 229 elongated spermatids, suggesting requirement of H3K27ac for spermiogenesis (Fig. 3a,
 230 arrowhead 12). Thus, localization of H3K27ac modification seems to start from
 231 preleptotene cells, and to accumulate on the XY body in late pachytene cells and on the
 232 putative sex chromosome during early to middle stages of round spermatids (Fig. 3b).

233

234 **Fig. 3 Localization of H3K27ac in male germ line cells in the young (3 M) testis. (a)**

235 Representative confocal images of H3K27ac (magenta) and SCP3 (green) in the

236 seminiferous epithelium in stages I, V, VIII, X and XII. Nuclei were counterstained with

237 DAPI (blue). The number with arrowhead means as the followings: 1; Preleptotene

238 spermatocyte at stage VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene

239 spermatocyte at stage XII, 4-7; Pachytene spermatocyte at stage I, V, VIII and X

240 respectively, M; M phase cells at stage XII, 9-11; Round spermatid at stage I, V and VIII

241 respectively, 12; Elongated spermatid at stage X. Scale bar: 20 μ m. (b) Magnified images

242 of the cells indicated by arrowheads in (a), and subcellular localization of H3K27ac in

243 the young testis is shown in magenta in a graphical summary. pL: Preleptotene

244 spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene

245 spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each

246 parenthesis represents the stage of spermatogenesis.

247

248 (iv) H3K79me2

249 In pachytene cells from stage VIII to stage X, the signal was widely detected in the

nucleus (Fig. 4a, arrowheads 1 and 2). In M-phase spermatocytes at stage XII, the signal was broadly observed on the condensed chromosomes (Fig. 4a, arrowhead 3). In round spermatids at stage I, a weak signal of H3K79me2 was observed in the most of the nucleus except the chromocenter (Fig. 4a, arrowhead 4). Interestingly, a strong signal of H3K79me2 was observed in the peri-chromocenter (a putative sex chromosome) in round spermatids from stage V to VIII (Fig. 4a, arrowheads 5 and 6), while no signal was observed on the chromocenter itself. In elongated spermatids at stage X, the strong signal on the putative sex chromosome disappeared (Fig. 4a, arrowhead 7). Thus, localization of H3K79me2 modification seems to start from the late pachytene cells, and to accumulate on the putative sex chromosome during most stages of round spermatids (Fig. 4b).

Fig. 4 Localization of H3K79me2 in male germ line cells in the young (3 M) testis.

(a) Representative confocal images of H3K79me2 (magenta) and SCP3 (green) in the seminiferous epithelium in stages I, V, VIII, X and XII. Nuclei were counterstained with DAPI (blue). The number with arrowhead means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene

267 spermatocyte at stage XII, 4-7; Pachytene spermatocyte at stage I, V, VIII and X
 268 respectively, M; M phase cells at stage XII, 9-11; Round spermatid at stage I, V and VIII
 269 respectively, 12; Elongated spermatid at stage X. Scale bar: 20 μ m. (b) Magnified images
 270 of the cells indicated by arrowheads in (a), and subcellular localization of H3K79me2 in
 271 the young testis is shown in magenta in a graphical summary. pL: Preleptotene
 272 spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene
 273 spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each
 274 parenthesis represents the stage of spermatogenesis.

275

276 (v) H3K79me3

277 While H3K79me2 was observed from the late pachytene cells (Fig. 4b), H3K79me3
 278 signal was not detected in any pachytene cells. H3K79me3 was first detected in M-phase
 279 spermatocytes in stage XII seminiferous tubules (Fig. 5a, arrowhead 8). Unlike
 280 H3K79me2, H3K79me3 signal was not distributed on all condensed chromosomes, but
 281 on specific chromosomes (Fig. 5a, arrowhead 8). After meiotic division II, the signal was
 282 not detected in round spermatids at stage I (Fig. 5a, arrowhead 9). However, in round
 283 spermatids from stage V to VIII, H3K79me3 signal was observed again in the nucleus

broadly and strong signal was detected in the peri-chromocenter (a putative sex chromosome) (Fig. 5a, arrowheads 10-11). In elongated spermatids at stage X, the signal was widely observed in the nucleus (Fig. 5a, arrowhead 12). Thus, H3K79me3 modification appeared from the late stage of meiosis, and accumulated on the putative sex chromosome of round spermatids. A specific area of condensed chromosomes showed strong signal of H3K79me3 during M-phase (Fig. 5b).

Fig. 5 Localization of H3K79me3 in male germ line cells in the young (3 M) testis.

(a) Representative confocal images of H3K79me3 (magenta) and SCP3 (green) in the seminiferous epithelium in stages I, V, VIII, X and XII. Nuclei were counterstained with DAPI (blue). The number with arrowhead means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7; Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid at stage X. Scale bar: 20 μ m. (b) Magnified images of the cells indicated by arrowheads in (a), and subcellular localization of H3K79me3 in the young testis is shown in magenta in a graphical summary. pL: Preleptotene

spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each parenthesis represents the stage of spermatogenesis.

(2) Histone modifications that inhibit gene expression

Next, we examined repressive marks, H3K9me3, H3K27me2 and H3K27me3.

(i) H3K9me3

H3K9me3 was detected in all cell types examined in this study. Broad nuclear signals were observed from preleptotene cells at stage VIII to pachytene cells at stage V with strong dot signals in the periphery of the nucleus (Fig. 6a, arrowheads 1-5). In pachytene cells at stage VIII and stage X, only dot-like signals were detected (Fig. 6a, arrowheads 6-7). Those dotted signals matched with intense DAPI signals, indicating heterochromatin areas. Consistent with a previous report (12), H3K9me3 exceptionally localized on the sex chromosome of M-phase spermatocytes at stage XII (Fig. 6a, arrowhead 8). In round spermatids at stage I to elongated spermatids at stage X, the signal was detected on the chromocenter and peri-chromocenter (putative sex chromosome) (Fig. 6a, arrowheads 9-

12). Thus, localization of H3K9me3 modification seems to start from preleptotene cells, and to accumulate on the sex chromosome in M-phase spermatocytes until late round spermatids (Fig. 6b).

Fig.6 Localization of H3K9me3 in male germ line cells in the young (3 M) testis. (a)

Representative confocal images of H3K9me3 (magenta) and SCP3 (green) in the seminiferous epithelium in stages I, V, VIII, X and XII. Nuclei were counterstained with DAPI (blue). The number with arrowhead means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7; Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid at stage X. Scale bar: 20 μ m. (b) Magnified images of the cells indicated by arrowheads in (a), and subcellular localization of H3K9me3 in the young testis is shown in magenta in a graphical summary. pL: Preleptotene spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each parenthesis represents the stage of spermatogenesis.

335

336 (ii) H3K27me2

337 H3K27me2 was detected in the nucleus of leptotene cells in stage X to pachytene cells in
 338 stage X in a salt-and-pepper manner (Fig. 7a, arrowheads 2-7). In M-phase spermatocytes
 339 at stage XII, the signal was broadly observed on all chromosomes (Fig. 7a, arrowhead 8).
 340 In round spermatids at stage I, strong dot-like signals were observed on the chromocenter
 341 in addition to weak and broad signals in the nucleus (Fig. 7a, arrowhead 9). The dot-like
 342 signals were observed until in round spermatids of stage VIII (Fig. 7a, arrowheads 10-
 343 11), and disappeared in elongated spermatids at stage X (Fig. 7a, arrowhead 12). Thus,
 344 localization of H3K27me2 modification started from leptotene cells, and accumulated on
 345 chromocenter of all stages of round spermatids, though the signal was relatively weak
 346 through spermatogenesis (Fig. 7b).

347

348 **Fig. 7 Localization of H3K27me2 in male germ line cells in the young (3 M) testis.**

349 (a) Representative confocal images of H3K27me2 (magenta) and SCP3 (green) in the
 350 seminiferous epithelium in stages I, V, VIII, X and XII. Nuclei were counterstained with
 351 DAPI (blue). The number with arrowhead means as the followings: 1; Preleptotene

spermatocyte at stage VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7; Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid at stage X. Scale bar: 20 μ m. (b) Magnified images of the cells indicated by arrowheads in (a), and subcellular localization of H3K27me2 in the young testis is shown in magenta in a graphical summary. pL: Preleptotene spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each parenthesis represents the stage of spermatogenesis.

361

362 (iii) H3K27me3

363 H3K27me3 was detected in all cell types examined in this study except preleptotene cells. 364 In leptotene cells at stage X to pachytene cells in stage X, the signal was broadly and 365 slightly detected in the nucleus (Fig. 8a, arrowheads 2-7). In M-phase spermatocytes at 366 stage XII, the signal was widely observed on all chromosomes (Fig. 8a, arrowhead 8). In 367 round spermatids at stage I to elongated spermatids at stage X, strong dot-like signals 368 were observed on the chromocenter in addition to weak and broad signals in the nucleus

(Fig. 8a, arrowhead 12), while strong dot-like signals of H3K27me2 disappeared in elongated spermatids at stage X (Fig. 7a, arrowhead 12). Thus, localization of H3K27me3 modification seems to start from leptotene cells, the same timing as H3K27me2, and to accumulate on the chromocenter in early round spermatids (Fig. 8b).

Fig. 8 Localization of H3K27me3 in male germ line cells in the young (3 M) testis.

(a) Representative confocal images of H3K27me3 (magenta) and SCP3 (green) in the seminiferous epithelium in stages I, V, VIII, X and XII. Nuclei were counterstained with DAPI (blue). The number with arrowhead means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7; Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid at stage X. Scale bar: 20 μ m. (b) Magnified images of the cells indicated by arrowheads in (a), and subcellular localization of H3K27me3 in the young testis is shown in magenta in a graphical summary. pL: Preleptotene spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each

parenthesis represents the stage of spermatogenesis.

The above localization patterns of histone modifications during spermatogenesis are shown in Table 1. In summary, H3K4me2 was detected in preleptotene cells to elongated spermatids, and H3K27ac was detected in leptotene cells to elongated spermatids. These two modifications were detected at the early stage of spermatogenesis, while other activation markers were detected at the later stage of spermatogenesis. All repressive makers examined in this study were detected at the early stage of spermatogenesis. All activation markers except H3K4me3 showed specific accumulation on XY body in pachytene cells, or a sex chromosome in round spermatids, while all repressive makers were accumulated on the chromocenter (i.e., constitutive heterochromatin). Only H3K9me3 was accumulated on both sex chromosomes and the chromocenter.

		pL(VIII)	L(X)	Z(XII)	P(I)	P(V)	P(VIII)	P(X)	M(XII)	R(I)	R(V)	R(VIII)	E(X)	Specific note
H3K4me2	Young	+++	+++	++	++	++	+	++	++	++	+++	++	++	Accumulation on XY body
	Aged	↓↓**	↓↓**	↓↓**	↓↓**	↓↓**	↓↓**	↓↓**	↓↓**	↓↓**	↓↓**	↓↓**	↓↓**	
H3K4me3	Young	-	-	-	-	-	-	-	++	++	++	+	+	
	Aged	-	-	-	-	-	-	-	↓↓**	→	↓*	→	→	
H3K27ac	Young	++++	+	+	+++	++	++	+++	+	+++	+++	+	++++	Accumulation on XY body and sex chromosomes
	Aged	→	↓*	→	→	↓*	→	↓**	→	→	→	→	↑*	
H3K79me2	Young	-	-	-	-	-	+	+	++	++	+++	++++	+++	Accumulation on sex chromosomes
	Aged	-	-	-	-	-	→	↑**	→	→	→	↑**	↑**	
H3K79me3	Young	-	-	-	-	-	-	-	+++	-	+	++	+++	Accumulation on sex chromosomes
	Aged	-	-	-	-	-	-	-	↑**	-	→	↓*	↓*	
H3K9me3	Young	++	++	++	++	++	++	++	++	++	++	+++	+++	Accumulation on sex chromosomes and heterochromatin
	Aged	↓↓**	↓↓**	↓↓**	↓↓**	↓↓**	↓↓**	↓↓**	→	↓**	↑*	↓**	→	
H3K27me2	Young	-	++	+	+	++	+++	+++	++	+	++	+	+++	Accumulation on heterochromatin
	Aged	-	↑**	↑**	↑**	↑**	↓**	↑**	↑**	↑**	↑**	↓**	↑**	
H3K27me3	Young	-	+	+	+	+	+	+	++	++	+	+	++	Accumulation on heterochromatin
	Aged	-	↓*	↑**	↑**	↑**	↑**	↓**	→	↑**	↑**	→	→	

Table 1 Summarized expression levels of histone modifications during spermatogenesis and their alteration by aging. Expression levels of histone modifications that activate (red) and inhibit (blue) gene expression are shown. Each expression level of male germ line cells derived from young mice is indicated in + (faint), ++ (fair), +++ (bright), ++++ (very strong) or – (not detected). Arrows show expression level of male germ line cells derived from aged mice as compared to that of young mice. Single up/down arrows indicate that it was increased/decreased less than 20 % compared to young. Double up/down arrows indicate that it was increased/decreased more than 20 % compared to young. Right arrows indicate that there were no significant differences between young and aged (*p < 0.05, **p < 0.01). Specific localization or accumulation is described as specific notes.

412

413 **2. Semi-quantitative analyses of histone modifications in young** 414 **and aged testes**

415 We next performed semi-quantified analyses to examine whether intensity of histone
416 modifications are changed in young and aged testes. The signal of histone modification
417 was measured by ImageJ. The intensity of histone modification was normalized by that
418 of DAPI in the nucleus or chromosomes.

419 **(1) Histone modifications that activate gene expression**

420 No difference was observed in localization patterns of H3K4me2/3, H3K27ac,
421 H3K79me2/3, H3K9me3 and H3K27me2/3 in young and aged testes (Fig. 9a, 10a, 11a,
422 12a, 13a, 14a, 15a and 16a). However, semi-quantitative analyses revealed difference in
423 intensity levels among histone modifications observed.

424

425 **Fig. 9 Comparison of localization patterns and intensity of H3K4me2 in male germ**
426 **line cells in young (3M) and aged (12M) testes.** (a) Representative confocal images of
427 H3K4me2 (magenta) in male germ line cells in young and aged testes. Nuclei were
428 counterstained with DAPI (blue). Summarized illustration showing subcellular

localization of H3K4me2 (magenta) in male germ line cells in young and aged testes.

The number means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7; Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid at stage X. (b) Semi-quantitative analysis of H3K4me2 in male germ line cells in young and aged testes. Black bars show relative intensity of aged cells against young ones (**p < 0.01, *p < 0.05, n.d., not detected). The red and blue lines mean 20% increased and decrease to the young testis, respectively. pL: Preleptotene spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each parenthesis represents the stage of spermatogenesis.

Fig. 10 Comparison of localization patterns and intensity of H3K4me3 in male germ line cells in young (3M) and aged (12M) testes. (a) Representative confocal images of H3K4me3 (magenta) in male germ line cells in young and aged testes. Nuclei were counterstained with DAPI (blue). Summarized illustration showing subcellular localization of H3K4me3 (magenta) in male germ line cells in young and aged testes.

447 The number means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2;
 448 Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7;
 449 Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage
 450 XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid
 451 at stage X. (b) Semi-quantitative analysis of H3K4me3 in male germ line cells in young
 452 and aged testes. Black bars show relative intensity of aged cells against young ones (**p
 453 < 0.01, *p < 0.05, n.d., not detected). The red and blue lines mean 20% increased and
 454 decrease to the young testis, respectively. pL: Preleptotene spermatocyte, L: Leptotene
 455 spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R:
 456 Round spermatid, E: Elongated spermatid. Each parenthesis represents the stage of
 457 spermatogenesis.

458
 459 **Fig. 11 Comparison of localization patterns and intensity of H3K27ac in male germ**
 460 **line cells in young (3M) and aged (12M) testes.** (a) Representative confocal images of
 461 H3K27ac (magenta) in male germ line cells in young and aged testes. Nuclei were
 462 counterstained with DAPI (blue). Summarized illustration showing subcellular
 463 localization of H3K27ac (magenta) in male germ line cells in young and aged testes.
 464 The number means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2;

465 Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7;
 466 Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage
 467 XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid
 468 at stage X. (b) Semi-quantitative analysis of H3K27ac in male germ line cells in young
 469 and aged testes. Black bars show relative intensity of aged cells against young ones (**p
 470 < 0.01, *p < 0.05, n.d., not detected). The red and blue lines mean 20% increased and
 471 decrease to the young testis, respectively. pL: Preleptotene spermatocyte, L: Leptotene
 472 spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R:
 473 Round spermatid, E: Elongated spermatid. Each parenthesis represents the stage of
 474 spermatogenesis.

475

476 **Fig. 12 Comparison of localization patterns and intensity of H3K79me2 in male**
 477 **germ line cells in young (3M) and aged (12M) testes.** (a) Representative confocal
 478 images of H3K79me2 (magenta) in male germ line cells in young and aged testes.
 479 Nuclei were counterstained with DAPI (blue). Summarized illustration showing
 480 subcellular localization of H3K79me2 (magenta) in male germ line cells in young and
 481 aged testes. The number means as the followings: 1; Preleptotene spermatocyte at stage
 482 VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7;

Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid at stage X. (b) Semi-quantitative analysis of H3K79me2 in male germ line cells in young and aged testes. Black bars show relative intensity of aged cells against young ones (**p < 0.01, *p < 0.05, n.d., not detected). The red and blue lines mean 20% increased and decrease to the young testis, respectively. pL: Preleptotene spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each parenthesis represents the stage of spermatogenesis.

Fig. 13 Comparison of localization patterns and intensity of H3K79me3 in male germ line cells in young (3M) and aged (12M) testes. (a) Representative confocal images of H3K79me3 (magenta) in male germ line cells in young and aged testes. Nuclei were counterstained with DAPI (blue). Summarized illustration showing subcellular localization of H3K79me3 (magenta) in male germ line cells in young and aged testes. The number means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7; Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage

501 XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid
 502 at stage X. (b) Semi-quantitative analysis of H3K79me3 in male germ line cells in
 503 young and aged testes. Black bars show relative intensity of aged cells against young
 504 ones (**p < 0.01, *p < 0.05, n.d., not detected). The red and blue lines mean 20%
 505 increased and decrease to the young testis, respectively pL: Preleptotene spermatocyte,
 506 L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M:
 507 M phase, R: Round spermatid, E: Elongated spermatid. Each parenthesis represents the
 508 stage of spermatogenesis.

509

510 **Fig. 14 Comparison of localization patterns and intensity of H3K9me3 in male**
 511 **germ line cells in young (3M) and aged (12M) testes.** (a) Representative confocal
 512 images of H3K9me3 (magenta) in male germ line cells in young and aged testes. Nuclei
 513 were counterstained with DAPI (blue). Summarized illustration showing subcellular
 514 localization of H3K9me3 (magenta) in male germ line cells in young and aged testes.
 515 The number means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2;
 516 Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7;
 517 Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage
 518 XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid

at stage X. (b) Semi-quantitative analysis of H3K9me3 in male germ line cells in young and aged testes. Black bars show relative intensity of aged cells against young ones (**p < 0.01, *p < 0.05, n.d., not detected). The red and blue lines mean 20% increased and decrease to the young testis, respectively pL: Preleptotene spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each parenthesis represents the stage of spermatogenesis.

Fig. 15 Comparison of localization patterns and intensity of H3K27me2 in male germ line cells in young (3M) and aged (12M) testes. (a) Representative confocal images of H3K27me2 (magenta) in male germ line cells in young and aged testes. Nuclei were counterstained with DAPI (blue). Summarized illustration showing subcellular localization of H3K27me2 (magenta) in male germ line cells in young and aged testes. The number means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7; Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid at stage X. (b) Semi-quantitative analysis of H3K27me2 in male germ line cells in

young and aged testes. Black bars show relative intensity of aged cells against young ones (**p < 0.01, *p < 0.05, n.d., not detected). The red and blue lines mean 20% increased and decrease to the young testis, respectively. pL: Preleptotene spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each parenthesis represents the stage of spermatogenesis.

Fig. 16 Comparison of localization patterns and intensity of H3K27me3 in male germ line cells in young (3M) and aged (12M) testes. (a) Representative confocal images of H3K27me3 (magenta) in male germ line cells in young and aged testes. Nuclei were counterstained with DAPI (blue). Summarized illustration showing subcellular localization of H3K27me3 (magenta) in male germ line cells in young and aged testes. The number means as the followings: 1; Preleptotene spermatocyte at stage VIII, 2; Leptotene spermatocyte at stage X, 3; Zygotene spermatocyte at stage XII, 4-7; Pachytene spermatocyte at stage I, V, VIII and X respectively, M; M phase cells at stage XII, 9-11; Round spermatid at stage I, V and VIII respectively, 12; Elongated spermatid at stage X. (b) Semi-quantitative analysis of H3K27me3 in male germ line cells in young and aged testes. Black bars show relative intensity of aged cells against young

ones (**p < 0.01, *p < 0.05, n.d., not detected). The red and blue lines mean 20% increased and decrease to the young testis, respectively. pL: Preleptotene spermatocyte, L: Leptotene spermatocyte, Z: Zygotene spermatocyte, P: Pachytene spermatocyte, M: M phase, R: Round spermatid, E: Elongated spermatid. Each parenthesis represents the stage of spermatogenesis.

(i) H3K4me2

Intensity levels were lower in all the cells of the aged testis than in the young testis (Fig. 9b).

(ii) H3K4me3

Intensity levels were lower in M-phase spermatocytes at stage XII and round spermatids at stage V of the aged testis than in the young testis (Fig. 10b). In other cells, there were no statistical differences.

(iii) H3K27ac

Intensity levels were lower in leptotene cells at stage X, pachytene cells at stage V and X of the aged testis than in the young testis (Fig. 11b). However, higher intensity was

detected in stage X elongated spermatids in the aged testis. In other cells, there were no statistical differences.

(iv) H3K79me2

Comparing with the young testis, higher intensity was detected in pachytene cells at stage X, round spermatids at stage VIII, and elongated spermatids at stage X (Fig. 12b) of the aged testis. However, other cells showed no difference in the intensity level.

(v) H3K79me3

Higher intensity was detected in M-phase spermatocytes at stage XII of the aged testis than in the young testis, while lower intensity was observed in round spermatids at stage VIII and elongate spermatids at stage X (Fig. 13b).

(2) Histone modifications that inhibit gene expression

(i) H3K9me3

Intensity levels were lower in almost all the cells of the aged testis than in the young testis (Fig. 14b). However, higher intensity was detected in stage V round spermatids in the aged testis. There were no statistical differences in other cell types.

(ii) H3K27me2

Intensity levels were lower in pachytene cells at stage VIII and round spermatids at stage VIII of the aged testis than in the young testis (Fig. 15b). However, other cells showed higher intensity in the aged testis.

(iii) H3K27me3

Similar to the result of H3K27me2, almost stages of cells showed higher intensity in the aged testis (Fig. 16b). In stage X leptotene cells and stage VIII pachytene cells, lower intensity was detected in the aged testis. There were no statistical differences in other cell types.

The above intensity of histone modifications in the young and aged testis is shown in Table 1. In summary, histone modifications were altered by aging and the alteration patterns were different in individual histone modifications. Among the repressive marks, H3K9me3 level was decreased in the aged testis, while H3K27me2 and H3K27me3 level was increased during spermatogenesis. On the other hand, the intensity level of an activation mark, H3K4me2, was drastically decreased in the aged testis during spermatogenesis. Other histone modifications also showed differences at some stages.

3. Accumulation of H3K79me3 on sex chromosomes

As described above, H3K79me3 was detected in M-phase spermatocytes, round spermatids and elongated spermatids. It is previously reported that H3K79me3 accumulates on XY body in late pachytene cells and on a sex chromosome in round spermatids (13). In this study, H3K79me3 signal was strongly detected on specific chromosomes in M-phase spermatocytes at stage XII (Fig. 17a). Therefore, we suspected that the chromosome on which H3K79me3 accumulated might be sex chromosomes.

To identify the sex chromosome, we established a new protocol by combining immunostaining together with fluorescence in situ hybridization (FISH) on the same slide. First, immunostaining was performed to detect H3K79me3 signal in M-phase spermatocytes (Fig. 17b). After images were captured using confocal microscopy, FISH was performed using probes that recognize X and Y chromosomes, respectively, followed by capturing FISH images of the same slides to obtain triple signals (Fig. 17c). Signals of H3K79me3 was overlapped with those of X and Y chromosomes at the same position within the nucleus of M-phase spermatocytes. Finally, we revealed that H3K79me3 was specifically accumulated on the sex chromosomes in M-phase spermatocytes. This result is consistent with another study that suggest the accumulation of H3K79me3 on the sex chromosomes in late pachytene cells and round spermatids (13).

623

624 **Fig. 17 H3K79me3 localization on sex chromosomes in the spermatocyte at M phase.**

625 (a) Summarized illustration showing subcellular localization of H3K79me3 (magenta).

626 Lower panels show representative confocal images of H3K79me3 (magenta) and α -

627 tubulin (green) in M phase cells (red square in the summarized illustration) in young and

628 aged testes. Nuclei were counterstained with DAPI (blue). Magnified images of the cells

629 indicated with numbers are shown in the box. (b) Representative confocal images of

630 H3K79me3 (magenta) in M phase cells. Nuclei were counterstained with DAPI (blue). M

631 phase cells are indicated with dotted line and arrowheads. (c) Representative images of

632 FISH showing X (red) and Y (green) chromosomes in M phase cells on the same section

633 as (b). Nuclei were counterstained with Hoechst (blue). The same M phase cells as (b)

634 are indicated with dotted lines and arrowheads.

635

636

Discussion

In this study, we have made a catalog showing transition of histone modification localization during spermatogenesis and its alteration caused by aging. Individual histone modifications showed distinct localization patterns and different timing to start their expression. Even among the histone modifications that have similar functions showed different localization patterns. Moreover, intensity of histone modifications was altered by aging. Aged testes showed differential increased/decreased signals of histone modifications at different stages. Therefore, responses to aging seem to be different among histone modifications. We also identified a chromosome in which H3K79me3 accumulated in the M-phase spermatocyte. H3K79me3 was accumulated on sex chromosomes in M-phase spermatocytes and round spermatids. Morphologically, it is revealed that not only H3K79me3, but also other histone modifications such as H3K79me2, H3K27ac and H3K9me3 accumulated on putative sex chromosomes or XY body. The intensity of these histone modification specifically accumulated on the putative sex chromosomes or XY body was also altered by aging at some stages. Therefore, epigenetic regulation of histone modifications may be different between sex chromosomes and other chromosomes, which may also be affected by aging.

654 In the present study, histone modifications that inhibit gene expression showed more
655 drastic changes in the aged testis. At the pachytene stage of meiosis I, genes on sex
656 chromosomes are transcriptionally shut down by meiotic sex chromosome inactivation
657 (MSCI), and reactivated after meiosis (14). It is reported that H3K9me3 is involved in
658 silencing of sex chromosomes in meiosis and that if there is no enrichment of H3K9me3,
659 remodeling and silencing of sex chromosomes failed and cause germ cell apoptosis (12).
660 In post-meiotic spermatids, 87% of X-linked genes remain suppressed, while autosomes
661 are largely active (15). Moreover, spermatids form a distinct post-meiotic sex chromatin
662 (PMSC) area that is enriched with H3K9me3 (15, 16). Thus, H3K9me3, a repressive mark,
663 has various roles during meiosis and in the post-meiosis phase. In our results, H3K9me3
664 appeared from preleptotene cells to elongated spermatids, and drastically decreased in the
665 aged testis during meiosis and in most of the stages of round spermatids. It is assumed in
666 the aged testis that failure of correct chromosome segregation during meiosis or leaky
667 expression of X-linked genes might occur in the post-meiotic spermatids even though the
668 male germ line cells safely pass meiosis.

669 Another repressive mark, H3K27me3, appeared from leptotene cells to elongated
670 spermatids. A study has reported that H3K27ac may act as an antagonist of Polycomb-
671 mediated silencing through inhibition of H3K27me3 (17). H3K27ac showed signals in

all stages of the male germ line cells examined, especially in preleptotene cells and elongated spermatids. It is very interesting that H3K27ac and H3K27me3 showed exclusive localization in round spermatids; the former was accumulated on the sex chromosome, while H3K27me3 on the chromocenter, a heterochromatin region. Therefore, the absence of H3K27ac (an activation mark) and presence of H3K27me3 (a repressive mark) in the chromocenter may be reasonable in regard with gene regulation. We found that H3K27me3 was dramatically increased in the aged testis, while H3K27ac showed slightly decreased at certain stages. H3K27me3 inhibits transcription by inducing a compact chromatin structure (18, 19). Thus, gene expression influenced by H3K27me3 might be more repressed in the aged testis.

It is not easy to elucidate the function of histone modification and its alteration by aging. However, progenia models can be used to know aging-induced phenotypes and their underlying mechanisms. Hutchinson-Gilford progeria syndrome (HGPS) patients show premature aging, and cultured cells derived from HGPS patients exhibit an increase of H4K20me3 and a reduction of both H3K9me3 and H3K27me3 (20), i.e., histone modifications inducing heterochromatin regions (21). Although this result seems to be rather puzzling based on our general knowledge about parallel regulation of H3K9me3 and H4K20me3, it might be considered that heterochromatin regions are decreased in

690 HGPS patients. Indeed, loss of heterochromatin is considered to be one of the mechanisms
 691 involved in aging (22, 23). However, H3K27me2/3 was increased at the most of stages in
 692 the aged testis. A similar increase of H3K27me3 due to aging is observed in quiescent
 693 muscle stem cells (24). Therefore, it may reasonable to assume that increase/decrease of
 694 H3K27me3 may depend on tissues or cell types. One possible reason of these difference
 695 can be explained by activities of histone methyltransferases or demethylases, some of
 696 which have tissue- or cell line-specific function (25, 26). Polycomb-repressive complex
 697 2 (PRC2) that catalyzes the methylation of H3K27 transcriptionally represses soma-
 698 specific genes and facilitates homeostasis and differentiation during mammalian
 699 spermatogenesis (27). This specificity can be associated with different alteration patterns
 700 of histone modification in each tissue or cell line. It might be interesting to know whether
 701 H3K27me3 is increased by PRC2 and affected to spermatogenesis.

702 The sex chromosomes seemed to be unique in localization of histone modifications. In
 703 this study, we confirmed accumulation of H3K79me3 on the sex chromosomes in
 704 spermatocytes at M phase. A similar localization of H3K9me3 has been shown in a
 705 previous study (12). Moreover, H3K27ac was accumulated on XY body and sex
 706 chromosomes in spermatocytes and in round spermatids, respectively. H3K79me2 also
 707 showed a signal on the sex chromosomes in round spermatids. We found that intensity of

708 these histone modifications specifically accumulated on the sex chromosomes and/or XY
 709 body were also affected by aging at certain stages. What do those results mean?

710 According to the database collecting risk genes for autism (SFARI at June 25, 2019), X
 711 chromosome has 81 risk genes, which is comparable to 95 and 89 risk genes on the larger
 712 chromosomes 1 and 2, respectively. It may be reasonable to assume that these three
 713 chromosomes have a large number of autism risk genes depending on their size. However,
 714 X chromosome has 16 “syndromic” genes, whereas chromosome 1 and 2 have only four
 715 and five, respectively. Enrichment of the autism risk genes, especially syndromic ones,
 716 on X chromosome might explain severer symptoms in female patients because X
 717 chromosome of father inevitably transmitted only to a daughter. Therefore, more detailed
 718 analyses would be interesting to focus on target genes of histone modifications that
 719 located on X chromosome.

720 Our results demonstrated aging-related alteration of histone modifications in
 721 spermatogenesis. In literatures, the possibility for the effect of paternal epigenetic factors
 722 on offspring’s health is suggested (28-31). Currently, however, there is no report
 723 explaining how the paternal epigenome is actually inherited to offspring. This will be a
 724 next important question in regard with the paternal impact on physical conditions of

725 offspring. Although most of histone protein is replaced with protamine during
726 spermatogenesis, some histone modifications in sperm are remained at promoter regions
727 that are known to regulate embryonic development (6). Further studies are needed to
728 reveal paternal aging impact on epigenetic mechanisms that may cause transgenerational
729 influence on health and disease of progeny.

730

731 **Acknowledgements**

732 The authors thank Prof. Yasuhisa Matsui for providing insightful comments and
733 assistance, and Ms. Sayaka Makino for animal care. We are also grateful to all members
734 of our laboratory for fruitful discussions and advice. This work was supported by
735 KAKENHI (17K19372).

736

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Fig. 1 a

b

(Modified from Hess & de Franca, 2008)

Fig. 2 a

b

(Modified from Hess & de Franca, 2008)

Fig. 3 a

b

(Modified from Hess & de Franca, 2008)

Fig. 4 a

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(Modified from Hess & de Franca, 2008)

Fig. 5 a

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(Modified from Hess & de Franca, 2008)

Fig. 6 a

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(Modified from Hess & de Franca, 2008)

Fig. 7 a

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(Modified from Hess & de Franca, 2008)

Fig. 8 a

b

(Modified from Hess & de Franca, 2008)

Fig. 9 a

Fig. 10 a

Fig. 11 a

Fig. 12 a

Fig. 13 a

Fig. 14 a

(Modified from Hess & de Franca, 2008)

Fig. 15 a

Fig. 16 a

(Modified from Hess & de Franca, 2008)

Fig. 17 a

b

c