FANCI plays an essential role in spermatogenesis and regulates meiotic histone methylation

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INTRODUCTION

Fanconi anemia (FA) is a rare genome instability syndrome characterized by early-onset bone marrow failure, developmental defects, high predisposition to cancers, and reduced fertility [1]. It is characterized by early-onset bone marrow failure, developmental defects, high predisposition to cancers, and reduced fertility [1]. It has been revealed that mutations in FA genes, which are involved in DNA interstrand cross-links (ICLs) repair, usually lead to FA [2–4]. The FA pathway consists of 22 identified FA proteins (FANCA–FANCW) and several FA-associated proteins such as FAAP24 and FAAP100 [1, 5–8]. When DNA ICLs occur, FANC M recognizes the damage sites and recruits FA core complex proteins, which then monoubiquitinates FANCI-FANCD2 heterodimer [9, 10]. The monoubiquitination of the heterodimer is the keynote of FA pathway activation. Monoubiquitinated FANCI and FANCD2 then recruit downstream repair factors such as SLX4 (FANCP), XPF (FANCO), BRCA1 (FANCs), BRCA2 (FANCs), and RAD51 (FANCs) to complete the ICLs repair [11–15]. Currently, there are growing evidences for FA gene-associated infertility. It has been reported that mutations in FA genes including FANCA [16], FANCM [17], and FANCU [18] cause isolated non-obstructive azoospermia (NOA) in male patients. Consistently, many FA gene knockout mice showed sterility and hypogonadism in both males and females, and there were severe loss of germ cells and defects in spermatogenesis in male mice [19–23]. Besides, recent studies have reported that a network of FA proteins and DNA damage response (DDR) factors, MDC1 and RNF8, regulates the epigenetic modifications during meiosis, and FANCD2 is reported to be the central component of this network [24, 25]. Among FA proteins, FANCI is an evolutionarily related partner of FANCD2 and functions together with FANCD2 downstream of FA core complex to repair ICLs [26]. However, the exact role and mechanisms of FANCI, which is critical for FA pathway activation, in male meiosis remain unclear.

In the present study, we generated Fanci−/− mice and found that the male Fanci−/− mice were sterile and exhibited aberrant spermatogenesis. Further studies indicated that FANCI was essential for FANCD2 foci formation and regulated histone methylation during meiosis. Altogether, these findings show that FANCI, an important component of FA pathway, plays an essential role in spermatogenesis.

MATERIALS AND METHODS

Animals

Fanci-flag mice with 3xflag fusing to N-terminus of the FANCI protein were generated using the CRISPR/Cas9 technology. The detailed information of the generation of the Fanci-flag knock-in mouse was shown in Supplementary Fig. 1. Fanci−/− mice were generated by Nanjing Sync Biotech Company using the CRISPR/Cas9 technology. The deletion of 98 bp in exon 5 resulted in frameshift mutation of Fanci gene, which led to a...
truncated protein of 116 amino acids (Supplementary Fig. 2). Primer sequences were as follows: Fanci-flag, forward: TCTGTGGAATGGATG TGAAGATGT, reverse: GGTTGCTAATCAGTGTCC. Histology, immunohistochemistry, and TUNEL staining
Testes from control and Fanci−/− mice were fixed in 4% PFA overnight, and then dehydrated and embedded in paraffin. For morphological analysis, paraffin sections at 5 μm thick were stained with H&E. For immunohistochemistry, sections were incubated at 4 °C overnight with primary antibodies, after that the sections were incubated with specific secondary antibodies (Zhongshan Biotech) and then stained with DAB Substrate Kit (Vectorlabs). For the TUNEL assay, sections were treated with 15 μg/ml protease K for 10 min at 37 °C and then stained with the in situ Cell Death Detection kit (Roche). Images of histological analysis were captured on Olympus BX53 fluorescence microscope.

Meiotic chromosome spread and immunofluorescence microscopy
Meiotic chromosome spread of spermatocytes was performed as previously described [27]. Briefly, seminiferous tubules were incubated in hypotonic buffer (30 mM Tris (pH 7.5), 50 mM sucrose, 17 mM trisodium citrate, 5 mM EDTA, 1 mM PMSF, and 2.5 mM dithiothreitol) for 30 min and then minced in 100 mM sucrose to release spermatocytes. After that, the suspension was spread on slides with 1% PFA and 0.1% Triton X-100. Slides were then incubated in a humid chamber at 4 °C overnight. The following antibodies were used: SOX9 (Sigma, 1:500), DDX4 (Abcam, 1:500), PLZF (Santa Cruz, 1:100), FLAG (Sigma 1:100, FANCD2 (Novus, 1:200), SYCP1 (Abcam, 1:500), SYCP3 (Abcam, 1:500), H2AX (Cell Signaling Technology, 1:200), RNA Pol II (Sigma, 1:200), RADS1 (Invitrogen, 1:200), MLH1 (BD Biosciences, 1:100), H3K9me2 (Millipore, 1:500), H3K9me3 (Millipore, 1:500), H3K4me2 (Millipore, 1:200) and cleaved PARP1 (Cell Signaling Technology, 1:400). FITC- and TRITC-conjugated secondary antibodies (Invitrogen) were used. Fluorescence images were captured with the Dragonfly spinning disc confocal microscope (ANDOR Technology).

Meiotic stages were determined on the basis of SYCP3 signal as previously described [24]. Briefly, spermatocytes in leptonet have short and unsynapsed SYCP3 signal. In zygote spermatocytes, homologous chromosomes begin to pair, and the SYCP3 signal on lateral elements becomes longer. At pachytene, homologous chromosomes fully synapse, and the SYCP3 signal is thick and condensed. In the diplotene stage, the SYCP3 signal is compact on XY chromosomes and decreases progressively on autosomes because of the desynapsis of homologous chromosomes. The relative mean fluorescence intensity (RMFI) of histone methylation was quantified using the ImageJ software. Briefly, regions of interest (ROIs) were drawn around XY chromatin, presented as XY in Fig. 8, and the other nuclear area excluding the XY body was presented as Autosome (AU) in Fig. 8. The ROIs of XY chromatin and autosome region were standardized to the background. In this study, we calculated the RMFI of spermatocytes in both pachytene and diplotene stages.

Protein extraction and western blotting
Total protein of testes was extracted with the Total Protein Extraction Kit (Invent) and the protein concentration was examined by BCA method (Thermo). Protein samples were separated by SDS-PAGE and then transferred to PVDF membrane (Millipore). After blocking in 5% milk, the membranes were incubated with indicated primary antibodies at 4 °C overnight. The following antibodies were used: FANCi−/− males and females were normal in appearance. The body weight and the testis/body ratio of adult mice were comparable between wild-type and Fanci-Flag mice. Besides, both Fanci-Flag males and females were fertile (Supplementary Fig. 2d). Besides, the results of mRNA probe did not show any signal in testicular sections from Fanci-Flag mice showed that FANCi was localized in the nuclei of spermatogonia and spermatocytes (Fig. 1c). Consistent with our results, a single-cell RNA-seq analysis also indicated that FANCi had relatively high expression levels in spermatogonia and spermatocyte (Supplementary Fig. 3) [28]. Immunofluorescence microscopy of meiotic chromosome spreads from Fanci-Flag mice was performed and each meiotic stage was determined on the appearance pattern of chromosome axes. Results showed that FANCi was not detected in the leptonete stage. Its accumulation on the chromosome axes began in the zygote stage and increased through the mid-pachytene stage. After mid-pachytene stage, FANCi signal on the autosome region gradually decreased. Meanwhile, FANCi signal on the sex chromosomes progressively increased and spread onto the entire XY domain in the early diplotene stage. After that FANCi signal decreased on the XY domain and diffused throughout the entire chromatin in the late diplotene stage (Fig. 1c). These results indicated that FANCi was expressed in a temporal and spatial manner during meiotic prophase I.

FANCi deletion results in male sterility and causes germ cell loss in mice
To explore the function of FANCi in male fertility, we generated Fanci−/− mice by targeted deletion of 98 bp in exon 5 using the CRISPR/Cas9 technology (Supplementary Fig. S2). FANCi was not detected by western blotting in Fanci−/− testes (Fig. 2a). Furthermore, the results of mRNA probe did not show any signal in the testis of Fanci−/− mice (Fig. 2b).

Genotyping of offspring from heterozygous mating showed a normal mendelian distribution (Supplementary Fig. 2d). Besides, Fanci−/− mice appeared to be normal without microphthalmia or skeletal malformations (Supplementary Fig. 2e–g). The body weight of adult mice was comparable between wild-type and Fanci−/− mice (Fig. 2d). However, the Fanci−/− testes were smaller than those of littermate controls, with the ratio of the testis to body weight decreased significantly (Fig. 2c, e). Furthermore, both Fanci−/− males (Fig. 2f) and females were infertile. To further explore the functions of FANCi, we compared testis sections from 8 weeks old wild-type and Fanci−/− mice using H&E.
Fig. 1 Temporal and spatial expression of FANCI during meiotic prophase I. a Relative mRNA level of Fanci in various tissues. Data are presented as mean ± SD. b Western blot analysis of the wild type and Fanci-flag testes (8 weeks old). α-Tubulin was used as a loading control. c Expression and localization of FANCI in adult testes. Immunofluorescence analysis of FLAG was performed on paraffin sections from Fanci-flag testes. The nuclei were stained with DAPI. Arrowhead represents spermatogonia. Arrow represents spermatocyte. Scale bars, 20 μm. d Localization of FANCI in chromosome spread of Fanci-flag spermatocytes. Meiotic chromosome spreads were immunolabeled with anti-FLAG and anti-SYCP3 antibodies. Scale bars, 5 μm.
staining. Seminiferous tubules from wild-type mice had normal architecture and contained germ cells at all stages. In contrast, seminiferous tubules from Fanci−/− testes were predominantly degenerated and exhibited a significant decrease in the number of cells. Consistent with the histologic analysis of testis sections, sperms in epididymides of Fanci−/− mice were much fewer than controls (Fig. 3a). Additionally, in Fanci−/− testes, 46.3% of seminiferous tubules only contained Sertoli cells, and 22% of seminiferous tubules had massive cell loss. Moreover, 20% of Fanci−/− tubules had round spermatids as the most advanced spermatogenic cells, and only 12.3% tubules in Fanci−/− testes had relatively normal morphology (Fig. 3b, c). Furthermore, immunofluorescence staining of testicular sections for DDX4 (a germ cells marker) and SOX9 (a Sertoli cell marker) indicated massive germ cell loss in Fanci−/− testes (Fig. 3d), suggesting that FANCJ deletion resulted in the dramatically decrease of germ cells and relative enrichment of Sertoli cells.

**Fig. 2** FANCI deletion results in atrophy testes and male sterility. a Western blot analysis of the wild type and Fanci−/− testes (8 weeks old). α-Tubulin was used as a loading control. b The mRNA probe detection of wild type and Fanci−/− testes. Scale bars, 20 μm. c Representative images of the testes of wild-type and Fanci−/− mice. d Body weights of wild-type and Fanci−/− mice at 8 weeks after birth. Six mice were analyzed for each group, and data are presented as mean ± SD. *, P < 0.05. Student’s t-test. e Mean ratio of testis/body weight from 8 weeks old wild type and Fanci−/− mice. Six mice were analyzed for each group, and data are presented as mean ± SD. *, P < 0.05. Student’s t-test. f Fertility test result of the mean ratio of pups/litter from wild type and Fanci−/− mice. Six wild-type mice: 34 litters and 12.8 pups/litter. Six Fanci−/− mice: 0 litter. Data are presented as mean ± SD. *, P < 0.05. Student’s t-test.

**Fig. 3** FANCI deletion causes germ cell loss in mice. a H&E staining of testes and epididymides from 8 weeks old wild type and Fanci−/− mice. Scale bars, 50 μm. b Representative images of H&E stained testicular sections showing various seminiferous tubules in 8 weeks old Fanci−/− mice, including Sertoli cell-only tubules, tubules with massive cell loss, tubules with round spermatids as the most advanced spermatogenic cells, and relatively normal tubules. Scale bars, 50 μm. c Quantification of different types of seminiferous tubules in 8 weeks old wild type and Fanci−/− mice. Six wild-type mice and six Fanci−/− mice were analyzed. Data are presented as mean ± SD. *, P < 0.05. Chi-square test (Fisher’s exact test). d Immunofluorescence staining for DDX4 (a germ cell marker) and SOX9 (a Sertoli cell marker) in wild type and Fanci−/− mice testes of 8-week-old mice. Scale bars, 20 μm.
addition, massive loss of germ cells was found in Fanci−/− testes at 12 months, which was more severe than that at 8 weeks (Supplementary Fig. 4a, c). Exactly, 60.7% of seminiferous tubules only contained Sertoli cells, and only 9.0% tubules had relatively normal morphology (Supplementary Fig. 4b). Collectively, our results indicate that FANCI is required for male fertility.

FANCI deletion increases apoptosis of germ cells and affects the maintenance of undifferentiated spermatogonia

Consistent with massive germ cell loss during spermatogenesis, TUNEL assay and immunofluorescence staining for cleaved PARP1 showed that apoptosis was dramatically increased in Fanci−/− testes (Fig. 4a–c and Supplementary Fig. 5a–c), suggesting that Fanci−/− germ cells were eliminated by increased apoptosis. Furthermore, we found that most cleaved PARP1 signals were localized in SYCP3-positive cells in Fanci−/− testes, indicating that most apoptotic cells were spermatocytes. Only a few apoptotic cells were undifferentiated spermatogonia or spermatids in Fanci−/− testes (Supplementary Fig. 5d, e).

As the maintenance of undifferentiated spermatogonia is required for continuous spermatogenesis, we next investigated whether the number of undifferentiated spermatogonia was altered in Fanci−/− testes. Immunohistochemistry staining utilizing PLZF (an undifferentiated spermatogonia marker) showed that percentage of PLZF-positive tubules were significantly decreased in Fanci−/− mice compared with controls at both 8 weeks and 12 months (Fig. 4d, e). Despite the number of PLZF-positive cells per PLZF-positive tubule (f), a total of 100 tubules from three independent mice were examined at 8 weeks and 12 months, both for wild type and Fanci−/− mice. In each group, six mice were analyzed. Data are presented as mean ± SD. *, P < 0.05. Student’s t-test. d Immunohistochemistry staining against PLZF (an undifferentiated spermatogonia marker) on wild type and Fanci−/− testes at 8 weeks old and at 12 months old. Arrows represent the undifferentiated spermatogonia. Scale bars, 50 μm. e Scoring of the percentage of PLZF-positive tubules per total tubule (e) and scoring of the number of PLZF-positive cells per PLZF-positive tubule (f). A total of 100 tubules from three independent mice were examined at 8 weeks and 12 months, both for wild type and Fanci−/− mice. In each group, six mice were analyzed. Data are presented as mean ± SD. *, P < 0.05. Ns no statistical significant difference. Frequencies were compared by the Chi-square test (Fisher’s exact test). Numbers were compared by Student’s t-test.

FANCI colocalizes with FANCD2 on sex chromosomes and is required for FANCD2 foci formation in spermatocytes

It is well-known that FANCI and FANCD2 form a heterodimer and play a critical role in FA pathway activation. Thus, we firstly investigated the localization of FANCD2 and FANCI in Fanci-Flag mice, and found that FANCD2 and FANCI were mainly colocalized in spermatogonia and spermatocytes (Fig. 5a). Besides, nuclear spread analysis of spermatocytes from Fanci-Flag mice indicated that FANCD2 and FANCI were colocalized on sex chromosomes (Fig. 5c).
Fig. 5  FANCI colocalizes with FANCD2 on sex chromosomes and is required for FANCD2 foci formation in spermatocytes. 

a Expression and localization of FANCI and FANCD2 in adult testes. Immunofluorescence analysis of FLAG and FANCD2 was performed on paraffin sections from wild type and Fanci−/− testes. The nuclei were stained with DAPI. Scale bars, 20 μm.

b Immunostaining of paraffin sections from wild-type testes and Fanci−/− testes with FANCD2. DNA was stained with DAPI. Scale bars, 20 μm.

c Expression and localization of FANCI and FANCD2 in spermatocytes. Immunofluorescence analysis of FLAG and FANCD2 was performed on chromosome spread from Fanci−/− testes. The nuclei were stained with DAPI. Arrows represent the sex chromosomes. Scale bars, 5 μm.

d Immunostaining of chromosome spreads from wild type and Fanci−/− spermatocytes with FANCD2 and SYCP3. SYCP3 immunolabeling was used to distinguish meiotic stages. Scale bars, 5 μm.
foci was eliminated in microscopy of meiotic chromosome spreads revealed that FANCD2 Fanci was detected in all stages of meiotic prophase I in both wild-type and between wild type and Fanci−/− mice (Fig. 5b). Furthermore, immunolabeling for RNA Polymerase II (RNA Pol II) and SYCP3 at diplotene spermatocytes. Arrows represent the sex chromosomes. Scale bars, 5 μm.

Next, we examined the expression of FANC2 in wild type and Fanci−/− testes. Results showed that FANC2 expression was dramatically decreased in Fanci−/− testes compared with wild-type testes, and the FANC2 signal was attenuated in both spermato- gonia and meiotic cells (Fig. 5b). Furthermore, immunofluorescence microscopy of meiotic chromosome spreads revealed that FANC2 foci was eliminated in Fanci−/− cells (Fig. 5d), suggesting that FANC2 is required for FANC2 foci formation in spermatocytes.

Meiotic prophase I stage progression and meiotic recombination remain unaffected in Fanci−/− spermatocytes
To investigate the roles of FANC2 in meiotic prophase I, we analyzed multiple meiosis-related markers in wild type and Fanci−/− spermatocytes by immunofluorescence staining on meiotic chromo- some spreads. As shown in Fig. 6a, b, normal chromosome synopsis and meiotic progression were observed in Fanci−/− spermatocytes. Besides, a normal phosphorylated H2AX (γH2AX) signal was detected in all stages of meiotic prophase I in both wild-type and Fanci−/− mice (Fig. 6c). To determine whether meiotic recombination was disturbed by FANC2 deletion, we examined the accumulation of RAD51 on chromosome axes in Fanci−/− spermatocytes. Results showed that the number of RAD51 foci were comparable between wild type and Fanci−/− spermatocytes at both early stages (leptotene and zygotene) and later stage (pachytene) (Fig. 7a, b). Furthermore, we examined the accumulation of MLH1, which is the marker for meiotic crossover. We found that the number of MLH1 foci on chromosome axes were also comparable in both groups in pachytene spermatocytes (Fig. 7c, d), suggesting that crossover formation was unaffected in Fanci−/− spermatocytes. Collectively, these results reflect that FANC2 is dispensable for meiotic progres- sion and meiotic recombination.

FANC2 regulates H3K4 and H3K9 methylation on meiotic sex chromosones
It has been reported that FA core proteins and FANC2 regulate H3K4 and H3K9 methylation on meiotic sex chromosomes [24, 25]. To determine whether FANC2 is involved in such histone methylation, we performed immunostaining for H3K4me2, H3K9me2, and H3K9me3 on chromosome spreads from wild type and Fanci−/− spermatocytes and then quantified the RMFI both on the autosomes regions and XY body. Consistent with previous studies, the accumulation of H3K9me3 on sex chromosomes was increased in Fanci−/− spermatocytes in the diplotene stage (Fig. 8a), indicating that FANC2 was also involved in the regulation of H3K9 methylation. Besides, in Fanci−/− spermatocytes, H3K4me2 intensity was decreased significantly on diplotene sex chromosomes (Fig. 8c). Furthermore, H3K9me2 on XY chromatin was unaffected during the pachytene-to-diplotene transition in Fanci−/− spermatocytes (Fig. 8e).

These results suggest that FANC2 negatively regulates H3K9me3 and positively regulates H3K4me2 in the diplotene stage. To determine whether the changes in histone modifications in Fanci−/− sex chromosomes are associated with transcriptional changes, we examined the distribution of RNA polymerase II (RNA Pol II) in diplotene spermatocytes. Surprisingly, RNA Pol II signal was excluded from the sex body in diplotene spermatocytes in both wild type and Fanci−/− mice (Fig. 6d), suggesting a normal sex chromosome transcriptional inactivation in Fanci−/− spermatocytes.
compromised fertility in apoptosis in seminiferous tubules. The germline phenotypes and germline defects including massive germ cell loss and increased difference. Student consistent with many previous FA mouse models [22, 23, 25], spermatocytes and spermatids [29]. However, the exact expression and mechanism of FANCI during spermatogenesis.

These findings indicated the temporal and spatial manner of FANCI expression and may provide information for future functional studies. Importantly, we demonstrated that Fanci−/− mice exhibited germline defects including massive germ cell loss and increased apoptosis in seminiferous tubules. The germline phenotypes and compromised fertility in Fanci−/− mice in our study are consistent with many previous FA mouse models [22, 23, 25], indicating that FANCI, as an important component of FA pathway, also plays essential roles in the male germline. Previous studies have revealed that FA proteins were involved in the maintenance of hematopoietic stem cells and human pluripotent stem cells [30, 31], and it is reasonable that FA proteins are associated with undifferentiated spermatogonia. Kato et al. [25] indicated that FANCB regulated maintenance of undifferentiated spermatogonia. Consistent with this study, our findings also suggest an important role for FANCI in the maintenance of undifferentiated spermatogonia, but the underlying mechanism is uncertain and requires further exploration.

It is well-known that FA pathway is a DDR pathway that repairs DNA ICLs in the genome [1]. During meiosis, one important DDR event is meiotic recombination [32]. However, our results demonstrated that FANCI deletion did not affect the number of RAD51 foci on chromosome axes from leptotene to pachytene, suggesting an unessential role of FANCI in RAD51 recruitment and exclusion during meiosis. Similar results were also observed in Fancb mutant mice [25]. Despite the unessential role of FANCI in RAD51 accumulation during meiosis, many studies have revealed that FANCI is involved in the regulation of RAD51 functions during DDR. Studies indicated that FANCI-FANCD2 complex colocalized with RAD51 at the stalled replication forks and stabilizes the RAD51-DNA complex [33–35]. Besides, Sato et al. indicated that FANCI, but not FANCD2, was essential for the FANCI-FANCD2 complex-mediated RAD51-DNA stabilization [36]. Furthermore, a recent study also claimed that FANCI interacted directly with RAD51 and stimulated RAD51-mediated D-loop formation [29]. The underlying mechanism behind this difference is unclear and requires further exploration. Additionally, we also found the normal γ-H2AX and MLH1 signal in Fanci−/− spermatocytes from leptotene to pachytene, which is consistent with the observation in Fancb mutant mice [25]. Collectively, our results revealed the normal programmed DSB repair and meiotic recombination in Fanci−/− mice, suggesting a dispensable role of FANCI in these events.

Among the FA proteins, FANCI and FANCD2 form a protein complex (ID2 complex), and the monoubiquitination of ID2 complex is a critical step in FA pathway activation during ICLs repair [37]. When it comes to spermatogenesis, our results indicated that the global FANCD2 level was decreased in Fanci−/− testes. One possible reason...
H3K4me2 in we detected the epigenetic markers H3K9me2, H3K9me3, and reported to regulate H3K9me2 and H3K9me3 on the sex independently of FA core complex [24]. Besides, FANCB was H3K9me2 and H3K9me3, while FANCD2 regulated H3K4me2 proteins (FANCA and FANCC) and FANCD2 regulated both programming during meiosis. Alavattam et al. found that FA core complex in FA pathway activation is indispensable during spermatogenesis. 

Fig. 8 FANCI regulates epigenetic modification on sex chromosomes during meiosis. a, c, e Immunofluorescence analysis of meiotic chromosome spreads from wild type and Fanci−/− mice with indicated antibodies. Areas surrounding sex chromosomes are magnified. Scale bars, 5 μm. Quantification of H3K9me3 (b), H3K4me2 (d), and H3K9me2 (f) relative mean fluorescence intensity (RMFI) on autosomal regions (Au) and sex chromosomes (XY) in pachytene (P) and diplotene (D) spermatocytes. For each panel, 30 cells were analyzed for each group. Data are presented as mean ± SD. *, P < 0.05. Student’s t-test. 

for the global decreased FANCD2 is that FANCI and FANCD2 are partially interdependent for their protein stability [38]. In line with our findings, a previous study has also reported that meiotic FANCD2 chromosomal localization was dependent on FANCI in C. elegans, suggesting the important role of FANCI during meiosis in different species [39]. When it comes to spermatogenesis, recent studies reported that FANCD2 foci on chromosome axes were abolished in mutant spermatocytes deficient for FA core complex [24, 25], indicating the similar function of FA core complex in regulating FANCD2 foci in both somatic DNA damage response DDR and meiosis. In line with these studies, our results showed that FANCD2 foci were eliminated in Fanci−/− spermatocytes, indicating that FA pathway activation is indispensable during spermatogenesis. 

It has been reported that FA proteins regulate epigenetic programming during meiosis. Alavattam et al. found that FA core proteins (FANCA and FANCC) and FANCD2 regulated both H3K9me2 and H3K9me3, while FANCD2 regulated H3K4me2 independently of FA core complex [24]. Besides, FANCB was reported to regulate H3K9me2 and H3K9me3 on the sex chromosomes during meiosis [25]. Therefore, in the present study, we detected the epigenetic markers H3K9me2, H3K9me3, and H3K4me2 in Fanci−/− spermatocytes. Despite the unaffected H3K9me2 in Fanci−/− spermatocytes, our results clearly indicated that FANCI negatively regulated H3K9me3 and positively regulated H3K4me2 on XY chromatin in diplotene stage during meiosis. Notably, recent studies have indicated that the BRCA1-MDC1-RNF8 axis is upstream of the FA pathway in histone modification [40, 41]. Consistent with our results, FANCD2 was also reported to regulate RNF8-dependent histone modification H3K4me2, suggesting that RNF8 integrates the FA pathway and the BRCA1-MDC1-RNF8 axis [24]. Thus, our findings reinforce the intermediate role of RNF8 in this FA-DDR network, and further explorations are required to clarify the whole FA-DDR system during meiosis. 

Despite the altered modification of H3K9me3 and H3K4me2 in diplotene, the meiotic progression was not arrested in Fanci−/− mice. Besides, we observed the normal sex chromosome transcriptional inactivation in Fanci−/− spermatocytes, indicating that the altered epigenetic modification did not result in transcriptional changes. It is possible that the varied epigenetic state in Fanci−/− spermatocytes is associated with increased germ cell apoptosis. 

Recently, it was reported that a Fanci knockout model in C57BL/6J mouse strain exhibited severe hypogonadism without any spermatocytes [29]. Our Fanci−/− mice showed milder phenotypes by crossing C57BL/6J Fanci−/− mice to ICR strains. Apart from germline defects, the mixed background Fanci−/− mice in our study appeared to be healthy without any other known FA phenotypes. Notably, the difference between our study and the previous Fanci knockout model indicates the strain-dependent phenotypes. Previous studies have also revealed that FA gene knockout mice in mixed backgrounds exhibit much milder phenotypes than those in pure C57BL/6J strain [20, 21]. Our study also supports the point that strain background affects the phenotypic profiles in FA mouse models. 

In summary, we have elucidated the important role and mechanism of FANCI in spermatogenesis. Our study provides new insights into the interaction between the FA pathway and meiotic epigenetic modification underlying spermatogenesis, which will reinforce the notion that DNA repair pathways play important roles in spermatogenesis and suggest the FANCI gene may be a candidate gene for human NOA. 

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