EZH2 deletion promotes spermatogonial differentiation and apoptosis

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Abstract

Spermatogenesis is crucial for male fertility and is therefore tightly controlled by a variety of epigenetic regulators. However, the function of enhacer of zeste homolog 2 (EZH2) in spermatogenesis and the molecular mechanisms underlying its activity remain poorly defined. Here, we demonstrate that deleting EZH2 promoted spermatogonial differentiation and apoptosis. EZH2 is expressed in spermatogonia, spermatocytes and round and elongated spermatids from stage 9 to 11 but not in leptotene and zygotene spermatocytes. Knocking down Ezh2 in vitro using a lentivirus impaired self-renewal in spermatogonial stem cells (SSC), and the conditional knockout of Ezh2 in spermatogonial progenitors promoted precocious spermatogonial differentiation. EZH2 functions to balance self-renewal and differentiation in spermatogonia by suppressing NEUROG3 and KIT via a direct interaction that is independent of its histone methyltransferase activity. Moreover, deleting Ezh2 enhanced the activation of CASP3 in spermatids, resulting in reduced spermatozoa production. Collectively, these data demonstrate that EZH2 plays a nonclassical role in the regulation of spermatogonial differentiation and apoptosis in murine spermatogenesis.

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Introduction

Spermatogenesis is a highly organized and consistent process that requires a balance to be established between the self-renewal and differentiation of spermatogonial stem cells (SSC). It is widely believed that SSCs, which are also known as A<sub>single</sub> (A<sub>s</sub>) spermatogonia, are the most primitive spermatogonia and that they do not form intercellular bridges (Huckins 1971, Oakberg 1971, De Rooij & Russell 2000). Spermatogenesis starts with the decision to differentiate when a committed A<sub>s</sub> spermatogonia undergoes mitosis to produce an A<sub>paired</sub> (A<sub>p</sub>) spermatogonia instead of self-renewing (De Rooij & Russell 2000). SSC<sub>s</sub> can also give rise to undifferentiated spermatogonial progenitors with different cellular states (Song & Wilkinson 2014). These spermatogonial progenitors include committed A<sub>v</sub>, A<sub>pr</sub> and A<sub>al</sub> (A<sub>a</sub>) spermatogonia, which induce the full spermatogenesis process under normal conditions. However, under specific conditions, some spermatogonial progenitors (potential stem cells) can change modes from transient amplification to self-renewal and act as SSC<sub>s</sub> (Yoshida et al. 2007).

Undifferentiated spermatogonial (A<sub>un</sub>), including A<sub>v</sub>, A<sub>pr</sub> and A<sub>al</sub> are recognized by several protein markers, such as ZBTB16 (Costoya et al. 2004), LIN28A (Zheng et al. 2009), GFRA1 and NEUROG3 (NGN3). Two previous publications described the expression of NGN3 in germ cells (Yoshida et al. 2004) and provided convincing evidence that NGN3 is expressed in A<sub>v</sub>, A<sub>pr</sub> and A<sub>al</sub> spermatogonia, all of which eventually contribute to spermatogenesis in mature LacZ-labelled testis. Furthermore, the authors noted that NGN3 was not expressed in all A<sub>v</sub>, A<sub>pr</sub> and A<sub>al</sub> spermatogonia and was expressed at low or negative levels in actual stem cells and high levels in potential stem cells (Yoshida et al. 2007). NGN3<sup>+</sup> germ cells have also been defined as spermatogonial progenitors that are committed to differentiation (Nakagawa et al. 2007, Song & Wilkinson 2014). Before meiosis is initiated, differentiating A<sub>i</sub> spermatogonia go through a series of mitoses (into A<sub>2</sub>, A<sub>j</sub>, A<sub>i</sub>, Int and B types), and these differentiating spermatogonia are recognized by KIT (Yoshinaga et al. 1991). After spermatogonial amplification, meiosis is initiated under the control of retinoic acid (RA) (Hogarth & Griswold 2010), which regulates both
STRAX (Zhou et al. 2008) and KIT (Busada et al. 2015) during spermatogenesis.

In a recent study, germline cells obtained from neonatal testes were cultured in vitro, and the SSC, proliferated for a long period of time under specific culture conditions (Kanatsu-Shinohara et al. 2003, Zhang et al. 2012b). In this culture system, the SSC colony consisted of a mixture of SSC and spermatogonial progenitors, and the SSC could not be recognized by their morphology or directly counted (Kanatsu-Shinohara et al. 2003, Zhang et al. 2012b). A widely accepted method for analysing real SSC numbers in culture is to count the number of colonies that formed after a transplantation assay was performed (Brinster & Avarbock 1994, Brinster & Zimmermann 1994). In addition, there was no difference in the number of colonized sites after 1 and 4 months, but the lengths of the colonies increased from 0.73 to 5.78 mm (Nagano et al. 1999).

Previous studies have shown that EZH2 is a key factor during cancer progression, including ovarian cancer (Lin et al. 2016), prostate cancer (Yang & Yu 2013) and testicular germ-cell tumours (Hinz et al. 2010), indicating that EZH2 plays a specific role in germ-cell progression. Furthermore, an increasing amount of evidence suggests that EZH2 is a key regulator of stem cell maintenance and differentiation during ontogenesis in processes including natural killer cell differentiation (Yin et al. 2011) and adult hippocampal neurogenesis (Zhang et al. 2014). In addition to its classical function of trimethylating histone H3K27, EZH2 also methylates non-histone targets, such as GATA4 at Lys 299 (He et al. 2012) and regulates oocyte maturation by directly binding to BUBR1 independent of its histone methyltransferase activity (Qu et al. 2016). EZH2 is expressed at high levels in adult testis (Lambrot et al. 2012), suggesting that it plays a regulatory role in spermatogenesis. In this study, we explored the localization of EZH2 in male germ cells and investigated its function in the regulation of spermatogenesis.

Materials and methods

Mouse lines and crosses

The animal experiments were approved by the Committee on Animal Care of the Institute of Zoology, Chinese Academy of Sciences. The Ezh2<sup>flox/flox</sup> mice were generated by Prof. Yang Xiao (Institute of Biotechnology, Academy of Military Medical Sciences, Beijing, China), and the Neurog3-cre mice (006333 – B6.FVB(Cg)-Tg(Neurog3-cre);C1Able/J) were obtained from the Jackson Laboratory (Schonhoff et al. 2004). All of the mice were bred in a C57BL/6 background. Ezh2<sup>flox/flox</sup> mice were mated with transgenic mice carrying Neurog3-cre to generate Ezh2<sup>flox</sup>;Neurog3-cre mice (KO mice). DNA was isolated from tails and genotyped using PCR.

Fertility valuation

The Ezh2<sup>flox/flox</sup> and Ezh2 KO mice were mated with C57BL/6 female mice, and vaginal plugs were checked every morning. Female mice with a vaginal plug were removed and replaced with new C57BL/6 female mice. The average litter size of each group was calculated from ten nests.

Sperm counts and motility analysis

Total sperm counts were obtained according to Roy et al. (2007). Briefly, epididymal caput and cauda were minced and incubated in prewarmed M<sub>16</sub> medium (Sigma-Aldrich) at 37°C in air containing 5% CO<sub>2</sub> for 30 min to allow the sperm to swim out. Then, the sperm were diluted in water and counted using a haemocytometer. To assess sperm motility, only the caudal spermatozoa were diluted in M<sub>16</sub> medium to a density of 10<sup>6</sup>/mL. They were then transferred to a MicroCell fixed-depth chamber (Conception Technologies, India) on a 37°C stage, and one hundred sperm were scored as motile or immotile. For each sample, sperm counts and motility values were obtained twice and averaged.

Immunohistochemistry and immunofluorescence

Mouse testes fixed in 4% paraformaldehyde at 4°C overnight were dehydrated, embedded in paraffin and cut into 5 μm thick sections. Rehydrated sections were subjected to antigen retrieval, blocked in 5% BSA (0.1% Triton X-100 in 5% BSA) and incubated with primary antibody at 4°C overnight. After incubation in 3% H<sub>2</sub>O<sub>2</sub> for 15 min, the section was incubated with secondary and tertiary antibodies at 37°C for 1 h each, followed by DAB staining (Zhong Shan Technology, Beijing, China), hematoxylin staining, dehydration and mounting with neutral gum. For immunofluorescence, after incubation with primary antibody, sections were incubated with secondary antibody at 37°C for 1 h and then stained with DAPI (Sigma) for 15 min. After the sections were mounted with anti-quencher fluorescence decay, images were captured using a Zeiss 780 laser scanning confocal microscope.

Real-time RT-PCR

Testicular total RNA was extracted using TRIzol reagent (Invitrogen). RNA integrity measurement and cDNA synthesis were performed as described (Barrionuevo et al. 2009). Data were normalized according to Gapdh, and the relative gene expression level was calculated according to the ΔΔCt method (Livak & Schmittgen 2001). The primer sequences are listed in Supplementary Table 1 (see section on supplementary data given at the end of this article).

Western blotting

Testes were lysed in RIPA lysis buffer (Biomed, Shanghai) containing protease inhibitor cocktail tablets (Roche) and PMSF. After electrophoresis on 10% SDS-PAGE gels, proteins were transferred to nitrocellulose membranes and blocked in 5% skimmed milk. The membranes were incubated with
primary antibody at 4°C overnight and horseradish peroxidase-labelled secondary antibody for 1 h at 37°C subsequently. Images were captured using ECL Western Blotting Substrate (Thermo Scientific Pierce).

**BrdU incorporation**

The control and KO mice were intraperitoneally injected with 50 mg/kg BrdU (Sigma) and were killed two hours later to identify BrdU-positive adult-born cells.

**Co-immunoprecipitation**

Testes were collected to perform co-immunoprecipitation experiments according to the instructions of the Pierce co-immunoprecipitation Kit (Thermo Scientific). The proteins obtained in co-immunoprecipitation assays were subjected to Western blotting and analysed using specific antibodies.

**Cell culture**

Spermatogonia were isolated, enriched and cultured according to previously described procedures (Kanatsu-Shinohara et al. 2003, Zhang et al. 2012b). Briefly, a testicular cell suspension of 5–6-dpp mouse pups (progenies of C57BL6 and DBA/2) was obtained using a two-step enzymatic digestion protocol with 1 mg/mL of type-IV collagenase (Sigma) and 0.25% trypsin (Inovitrogen). Both digestions required 500 μg/mL DNase I (Sigma) to protect against DNA degradation. The dissociated cells were centrifuged, resuspended in DPBS containing 1% FBS (HyClone, USA), and then centrifuged again in a 30% Percoll solution. Then, the spermatogonia were enriched using MACS to isolate Thy-1+ cells. Spermatogonia were cultured on MEF treated with Mitomycin C in serum-free MEM supplemented with other components, as previously described (Kanatsu-Shinohara et al. 2003).

**RNA interference of Ezh2 by shRNA**

The target sequences are listed in Supplementary Table 2. The lentiviruses were packaged according to the instructions provided in the Lenti-Easy Packaging System (Genechem). The titers of the viral particle-containing supernatants were measured using the fluorescent counting method according to the instructions mentioned above and ranged from 2 × 10⁵ to 5 × 10⁶ IU/mL. The multiplicity of infection (MOI) is the ratio of the number of viral particles to the number of target cells when approximately 80% of target cells are transfected. The MOI in our experiment was 13. The viral particles were then introduced to the cultured spermatogonia.

**Flow cytometric analysis**

The cell suspensions were fixed in 1% paraformaldehyde at RT for 15 min and then permeabilized (0.1% Triton X-100 in PBS) for 20 min, washed with DPBS twice, blocked in 0.5% BSA for 30 min at RT and incubated with primary antibodies at 4°C overnight. After the cells were washed in DPBS, they were incubated in secondary antibodies for 1 h at RT and then washed and filtered using a cell strainer with a 40 μm pore size (Becton Dickinson, USA). They were then resuspended in DPBS containing 1% FBS (HyClone) and subjected to flow cytometry. The results were analysed using a FACS-Calibur system (BD Biosciences, USA). To analyse DNA ploidy, the cells were fixed in 70% ethanol at 4°C overnight and then washed in DPBS. Then, the cells were incubated with the staining solution (0.02 mg/mL propidium iodide and 0.2 mg/mL RNase A) at 37°C for 20 min and filtered before being subjected to flow cytometry. The results were analysed using a FACS-Calibur system (BD Biosciences).

**Antibodies**

The antibodies used in this study are listed in the Supplementary Table 3.

**Statistical analysis**

All statistical analyses were performed using SPSS, version 16.0. All experiments were repeated at least three times, and data for evaluated parameters are reported as means ± S.E.M.

**Results**

**Expression pattern of EZH2 in male germ cells**

To analyse the expression pattern of EZH2 in spermatogonia, testes were obtained from 10-day-old mice, in which the most advanced germ cells are type B spermatogonial. The tissues were co-stained for EZH2, DDX4 and LIN28A. The results showed that EZH2 was localized on the nucleus of spermatogonial (DDX4+) and that immunolabelling was stronger in undifferentiated (DDX4+, LIN28A+) than in differentiating (DDX4+, LIN28A−) spermatogonia (Fig. 1A). These results suggest that EZH2 plays a potential role in maintaining an undifferentiated spermatogonial state. The expression pattern of EZH2 was also analysed in spermatocytes obtained from adult testis. Different stages of primary spermatocytes were distinguished by the expression of SYCP3 (Yuan et al. 2000, de la Fuente et al. 2007, Abby et al. 2016). Immunostaining for EZH2 was weak in preleptotene, rarely detected in leptotene and zygotene, apparent in early pachytene and strong in late pachytene and diplotene spermatocytes (Fig. 1B). Labelling persisted in round spermatids but became weaker as the spermatids elongated during progression from stage 9 to 11 and then disappeared by stage 12 (Fig. 1C), indicating that EZH2 performs a specific function during the later phases of meiosis. An overview of the expression of EZH2 showed that the strongest signals were observed in late pachytene and diplotene spermatocytes, stronger signals were observed in round spermatids and undifferentiated spermatogonial, and weak signals were observed in other germ cells (Fig. 1D).
Ezh2 conditional knockout mice have fewer sperm

To determine whether Ezh2 regulates spermatogenesis, we generated Ezh2 conditional knockout mice. In Ezh2\textsuperscript{flx/flx} mice (Fig. 2A), the fourth exon is floxed, and in Neurog3-cre mice, cre activity is initiated in Neurog3-positive spermatogonial progenitors. Ezh2\textsuperscript{flx/−};Neurog3-cre mice (KO mice) were obtained by crossing Ezh2\textsuperscript{flx/flx} mice with Neurog3-cre mice. The knockout efficiency of EZH2 in the KO mice testes was confirmed using immunostaining, Western blotting and real-time RT-PCR, which revealed that both the mRNA and the protein levels of EZH2 were significantly reduced (Fig. 2B, C and D). Moreover, EZH2 deletion in the KO mice also significantly reduced H3K27me3 (Fig. 2D and E). A histological analysis showed that the 6-month-old KO mice had normal testes (Fig. 3A and B) and released spermatozoa into the lumen during stages VII–VIII of the seminiferous epithelial cycle (Fig. 3D). However, the average litter size was significantly lower after 4 months in the KO than in the WT litters (Fig. 3C). Furthermore, the results of H&E staining of the cauda epididymidis and sperm cell counting in the epididymis at different ages showed that sperm numbers were lower in the KO mice and that the difference became more and more significant with the age (Fig. 3E and F). In addition, sperm motility was normal in the epididymis in 6-month-old KO mice (Fig. 3G), suggesting that the reduction in average litter size was caused by a lower sperm number and not reduced sperm motility.

EZH2 deficiency elevates apoptosis

To determine whether the reduction in spermatids was caused by aberrant apoptosis, we performed immunofluorescence staining of cleaved CASP3 (CAS3), which is the large fragment of activated CASP3. Compared to the cytoplasmic expression of CAS3 in the mature spermatids of control mice, CAS3 was aberrantly expressed in the nucleus of elongated spermatids in the
KO mice (Fig. 4A), and the protein level of CAS3 was increased in KO testes (Fig. 4C). These data suggest that EZH2 suppresses apoptosis by inhibiting CASP3 activation in spermatids. Moreover, the mRNA and protein levels of Ddx4 and Acr in the testis was significantly lower in the KO mice (Fig. 4B and C), indicating a reduction in germ-cell and spermatid numbers. However, the protein level of SYCP3 (Fig. 4B and C) was higher in the KO mice, indicating the observed reduction in germ cells was induced by apoptosis during a later stage of meiosis. In addition, the mRNA expression levels of Id4, which is specifically expressed in SSCs (Chan et al. 2014, Sun et al. 2015), and Ret, which is important for SSC self-renewal (Naughton et al. 2006), were significantly lower in the KO mice (Fig. 4B), suggesting a defect in SSC self-renewal. Moreover, the protein levels of ZBTB16 and STRA8 were higher in the KO mice (Fig. 4C), indicating an increase in spermatogonial progenitors and the initiation of meiosis in KO mouse testes. Finally, the protein levels of NGN3 and KIT were higher (Fig. 4C) in the KO mouse testes, suggesting that the proportion of differentiating spermatogonial was also increased.

**EZH2 deficiency impairs SSCs self-renewal and promotes spermatogonial differentiation**

To determine whether Ezh2 deletion promoted spermatogonial differentiation, we performed immunofluorescence staining for KIT, LIN28A and ZBTB16. Consistent with the observed increase in the protein level of KIT, the KO mice expressed a high level of KIT, which was aberrantly expressed in some of the undifferentiated spermatogonial (PLZF+, LIN28A+) in the KO mice (Fig. 5A). The aberrant presence of KIT indicates the precocious transition of spermatogonia from an undifferentiated to a differentiated state. Moreover, following the intraperitoneal injection of BrdU, more BrdU-positive signals were observed in stage VII–VIII seminiferous epithelial cells in the KO mice than in the controls (Fig. 5B and C). In addition, fluorescent flow cytometry analysis of total testicular cell suspensions also showed that the number of BrdU-positive cells was higher in the KO mouse (Fig. 5D), indicating enhanced DNA synthesis and proliferation in spermatogonia prior to the initiation of meiosis. Moreover, flow cytometric analysis of total testicular cell suspensions showed that the proportions of 2 N and 4 N ploidy were higher, while the proportion of 1 N ploidy was lower in the KO mice (Fig. 5E). These data also suggest an elevated rate of proliferation in spermatogonia in addition to impaired spermatogenesis. The proliferation of spermatogonia may have been increased in the KO mice as a result of the promotion of self-renewal of SSCs, enhanced differentiation in undifferentiated spermatogonia or both.

To determine the function of EZH2 in SSC self-renewal, an in vitro assay was performed using a lentivirus RNAi against Ezh2 in SSCs. Immunostaining...
performed in cultured cells (Fig. 6A) showed that EZH2 was expressed in GFRA1+ SSCs but not MEF cells (white arrow). The GFRA1+ SSCs that were cultured in the maintenance system were transfected with sh- Ezh2 viral particles (RNAi group) or sh-scramble viral particles (control group). Then, we used flow cytometry sorting to determine which cells were transfected with different lentiviral particles, and the same number of SSCs were screened in each group. The SSCs were cultured for one week, and in the RNAi group, the knockdown efficiency of Ezh2 was approximately 50% at the mRNA (Fig. 6C) and protein (Fig. 6D) levels. Moreover, the colonies that formed in the culture system were smaller in the RNAi group (Fig. 6B). The same numbers of cultured spermatogonia were selected from the RNAi and control groups and transplanted into the testes of busulfan-treated mice. The results showed that there were fewer GFP+ seminiferous epithelia and significantly fewer colonies in the RNAi than in the control tested (Fig. 6E and F). These data indicate that the self-renewal is defective in the Ezh2 knockdown SSCs.

In addition, consistent with the in vivo results, the mRNA expression level of Id4 was also significantly reduced (Fig. 6C) in the RNAi group, while the expression of NGN3, STRA8 and KIT were increased at both the mRNA and protein levels (Fig. 6C and D), confirming that spermatogonial differentiation was promoted when Ezh2 was knocked down in SSCs.

Together, these data indicate that EZH2 deficiency impairs SSCs self-renewal and promotes spermatogonial differentiation.

**EZH2 functions by directly binding to NGN3 and KIT**

In both the knockout and knockdown models of Ezh2, two protein levels of two key regulators of spermatogonial differentiation, NGN3 and KIT, were significantly higher. To determine whether EZH2 inhibits spermatogonial differentiation by directly regulating these two proteins, a co-immunoprecipitation assay was performed, and the results showed that EZH2 pulled-down NGN3 and KIT and vice versa (Fig. 7A, B, C and D), indicating that EZH2 can inhibit NGN3 and KIT via a direct interaction. Based on these data, we propose a model in which EZH2 regulates spermatogonial differentiation by maintaining spermatogonia in an undifferentiated state by inhibiting NGN3 and KIT expression. When EZH2 is deficient, NGN3 and KIT accumulate in spermatogonia, and this in turn promotes spermatogonial differentiation (Fig. 7F).

**Discussion**

EZH2 immunofluorescence was previously detected only in the nuclear apical region in round spermatids of murine testis (Lambrot et al. 2012). This is different from our observations. However, the author also mentioned
that EZH2 was detected from PND6 to PND14 using Western blot analysis, indicating that EZH2 is present from spermatogonia to spermatocytes. The author explained that this result may be because immunofluorescence is less sensitive than Western blotting (Lambrot et al. 2012). However, we bought the polyclonal EZH2 antibody with the same item number from Abcam, and the instructions included state that this antibody should not be used for immunofluorescence in paraffin sections but can be used for immunohistochemical staining. This is potentially why the results of immunofluorescence staining for EZH2 that were previously reported are not consistent with the results of Western blotting but similar to staining for IgG (Supplementary Fig. 3). These results indicate that the signal is nonspecific.

The expression pattern of EZH2 in male germ cells was validated using three different antibodies containing synthetic peptide derived from residues 50 to 150 (Abcam), 156 to 256 (BD) or 304 to 404 (CST) of human EZH2. Immunostaining for EZH2 and IgG (negative control) showed that the three different peptides for EZH2 detected similar signals in germ cells; however, no specific signals were detected in somatic cells (Supplementary Figs 1 and 2), which was similar to the results of immunostaining for IgG (Supplementary Figs 4 and 5). Hence, the weak signals that the CST antibody detected in somatic cells are likely nonspecific background signal.

The role of EZH2 in suppressing adult pluripotent stem cell differentiation has also been reported in retinal progenitors, in which EZH2 maintains the proliferation of retinal progenitors by inhibiting the expression of developmental regulators and thereby regulating the timing of late differentiation (Zhang et al. 2015). The reduction of SSCs self-renewal was confirmed using in vitro knockdown assays, and the increase in the proportion of differentiating spermatogonia and primary spermatocytes in the KO mouse testes indicated that EZH2 plays a role in regulating the balance between SSCs self-renewal and differentiation. Moreover, a previous study reported that inducing the deletion of Ezh2 in neural stem cells/progenitor cells resulted in fewer neurons (Zhang et al. 2014), similar to the effect on spermatids in our KO mice model. The induction of apoptosis by EZH2 deficiency has also been reported in tumours (Zhang et al. 2012a, Liu et al. 2015, Yu et al. 2016), and the activation of CASP3 by EZH2 may be mediated by a specific microRNA (Wang et al. 2014, Figure 4 EZH2 deficiency elevates apoptosis.

(A) Immunofluorescence staining of the cleaved CASP3 (CAS3) in the KO and control mice. (B) Real-time RT-PCR and (C) Western blotting of key marker genes in the testes of KO and control mice. Scale bars in (A): 100 µm.
Figure 5 EZH2 deficiency promotes spermatogonial differentiation. (A) Immunofluorescence staining of KIT with LIN28A and ZBTB16 in the KO and control mice testes and the white arrow indicates ZBTB16+ and LIN28A+ undifferentiated spermatogonia. (B) Immunofluorescence staining of BrdU in the KO and control mice testes two hours after the BrdU incorporation assay. (C) Quantitative analysis of the BrdU-positive cell number in the KO and control mice testes. (D) Fluorescence flow cytometry analysis of the BrdU-positive cell numbers in total testicular cell suspension in the KO and control mice. (E) DNA ploidy analysis of the total testicular cell suspension in the KO and control mice. Scale bars in (A): 50 µm, (B): 100 µm.

Figure 6 EZH2 deficiency impairs SSCs self-renewal. (A) Immunofluorescence staining of EZH2 with GFRA1 in the cultured cells. The white arrow indicates the MEF cells. (B) The cultured colony formed in the RNAi and control groups before transplantation. (C) Real-time RT-PCR and (D) Western blotting of EZH2 and several key marker genes in the RNAi and control groups before transplantation. (E) Recipient testes of the RNAi and control groups one month after the transplantation assay. (F) Quantity valuation of the colony number in the seminiferous tubules from RNAi and control groups. Scale bars in (A): 20 µm, (B): 50 µm, (E): 1 mm.
Liu et al. 2015, Yu et al. 2016). Whether the enhanced CASP3 activation observed in KO mouse testes was induced by aberrant microRNA expression needs to be further explored.

Histone modification is vital for normal spermatogenesis (Okada et al. 2007, Tachibana et al. 2007, Kolthur-Seetharam et al. 2009), and during this process, genes are regulated by the deposition of H3K27me3. In this study, we found that Neurog3 and Zbtb16 were significantly upregulated at the mRNA level when EZH2 was deficient, suggesting that these two genes might be regulated by EZH2 methyltransferase activity. However, we found that H3K27me3 was reduced but not absent in germ cells, indicating that other histone methyltransferases, such as EZH1, are compensating for EZH2. Previous reports have shown that EZH1 can compensate for the loss of EZH2 in H3K27me3 deposition in a variety of tissues (Shen et al. 2008, Bardot et al. 2013, Lui et al. 2016, Zhu et al. 2016, Mu et al. 2017). It would therefore be useful to evaluate the role of H3K27me3 deposition in the regulation of spermatogenesis using EZH1/2 single- and double-knockout mice models and to explore this regulatory network using Chip-seq analysis. The data presented in this manuscript are focused on non-canonical function of EZH2 that have been previously reported in oocyte meiotic maturation (Qu et al. 2016), in which key regulatory protein was found to directly combine with EZH2.

In conclusion, our findings indicate that EZH2 is a key regulator of spermatogonial differentiation and apoptosis, and these data provide new insights into the molecular mechanisms underlying the conservation of male fertility.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-17-0302.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Figure 7** EZH2 functions by directly binding to NGN3 and KIT. (A) The co-immunoprecipitation of EZH2 with NGN3. (B) The co-immunoprecipitation of NGN3 with EZH2. (C) The co-immunoprecipitation of EZH2 with KIT. (D) The co-immunoprecipitation of KIT with EZH2. (E) The A-single model and several genes expressed during spermatogenesis summarized from introductions. (F) Model of the nonclassical function of EZH2 in the regulation of spermatogonial differentiation.
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Role of EZH2 in spermatogenesis


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