Transcription factor ZFP38 is essential for meiosis prophase I in male mice

Zechen Yan¹, Dandan Fan², Qingjun Meng¹, Jinjian Yang¹, Wei Zhao¹, Fei Guo¹, Dongjian Song¹, Ruiming Guo¹, Ke Sun¹ and Jiayang Wang¹

¹Department of Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China and ²Henan Academy of Medical and Pharmaceutical Science, Zhengzhou, Henan, China

Correspondence should be addressed to J Wang; Email: wangjx72@yahoo.com

Abstract

The production of haploid gametes by meiosis is a cornerstone of sexual reproduction and maintenance of genome integrity. *Zfp38* mRNA is expressed in spermatocytes, indicating that transcription factor ZFP38 has the potential to regulate transcription during meiosis. In this study, we generated *Zfp38* conditional knockout mice (*Zfp38*^{flox/flox}, *Stra8*-Cre, hereafter called *Zfp38* cKO) and found that spermatogenesis did not progress beyond meiosis prophase I in *Zfp38* cKO mice. Using a chromosomal spread technique, we observed that *Zfp38* cKO spermatocytes exhibited a failure in chromosomal synapsis observed by SYCP1/SYCP3 double staining. Progression of DNA double-strand breaks (DSB) repair is disrupted in *Zfp38* cKO spermatocytes, as revealed by γ -H2AX, RAD51 and MLH1 staining. Furthermore, the mRNA and protein levels of DSB repair enzymes and factors that guide their loading onto sites of DSBs, such as RAD51, DMC1, RAD51, TEX15 and PALB2, were significantly reduced in *Zfp38* cKO spermatocytes. Taken together, our data suggest that ZFP38 is critical for the chromosomal synapsis and DSB repairs partially via its regulation of DSB repair-associated protein expression during meiotic progression in mouse.

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Introduction

Spermatogenesis is a highly complex cell differentiation process necessary for continued renewal of the spermatozoa population. Central to this unique process is meiosis, which is responsible for the production of haploid spermatozoa as well as for the maintenance of genome integrity (Chen & Liu 2015). Meiosis errors and genetic disruptions can cause aneuploidy and infertility (Matzuk & Lamb 2008). Prophase I of meiosis I is initiated in the primary spermatocytes and this stage can be divided into the leptotene, zygotene, pachytene, diplotene and diakinesis substages (Petronczki *et al.* 2003). During these substages of prophase I, the chromosomes undergo a number of changes that allow for the pairing of homologs and the exchange of genetic information between nonsister chromatids. Homologous chromosomes are connected by the synaptonemal complex (SC), which consists of the central element, axial/lateral elements and transverse filaments (de Boer & Heyting 2006, Costa & Cooke 2007). Homologous recombination involves the introduction of double-strand breaks (DSBs) into the genome by type II-like topoisomerase SPO11 complex (Handel 2004, Lam & Keeney 2015, Robert *et al.* 2016, Vrielynck *et al.* 2016). DSBs are subsequently repaired by the loading of RAD51 and DMC1

recombinases (Pittman *et al.* 1998). TEX15, BRCA1, BRCA2 and PALB2 have been suggested to mediate RAD51 and DMC1 loading onto sites of DSBs (Scully *et al.* 1997, Davies *et al.* 2001, Yang *et al.* 2008, Simhadri *et al.* 2014).

A previous study suggests that the *Zfp38* transcript can be detected at a highly elevated level during spermatogenesis (Chowdhury *et al.* 1992). Its expression accompanies the progression from pachytene spermatocytes to round spermatids (Chowdhury *et al.* 1992), suggesting that ZFP38 has the potential to regulate transcription during meiosis and/or spermiogenesis. However, the role of ZFP38 *in vivo* is still undiscovered.

In this study, *Zfp38* conditional knockout mice (*Zfp38*^{flox/flox}, *Stra8*-Cre; hereafter called *Zfp38* cKO) were used to study the physiological roles of ZFP38 in spermatogenesis. We found that *Zfp38* cKO male mice were completely infertile, due to meiotic arrest. Chromosomal synapsis and DSB repairs were impaired in *Zfp38* cKO spermatocytes. Furthermore, ZFP38 deletion caused significantly reduced expression of RAD51, DMC1, TEX15, BRCA1/2 and PALB2. Our study suggests that ZFP38 is a novel spermatocyte factor required for the progression of meiotic prophase I in male mice.

Materials and methods

Generation of *Zfp38* cKO mice

All procedures and care of animals were performed in accordance with the Animal Care and Use Committee (ACUC) of The First Affiliated Hospital of Zhengzhou University. This study (protocol #140312) was specifically approved by ACUC of The First Affiliated Hospital of Zhengzhou University. All mice were housed under controlled photoperiod conditions (lights on 0800–2000h) and supplied with food and ddH₂O *ad libitum*.

A targeting vector in which the *Zfp38* exon 4 was flanked by a loxP site and a loxP-Neo cassette was constructed and introduced into mouse embryonic stem cells by electroporation. Following G418 selection, surviving colonies were screened for homologous recombinants by PCR. Clones were then injected into C57BL/6 (B6) blastocysts (Kaartinen & Nagy 2001). Resultant male chimeras were identified by coat color and mated with wild-type females, resulting in successful germline transmission of the *Zfp38*^{fllox} allele. Primers for genotyping the *Zfp38* flox allele were as follows: P1, 5'-AGAGAGCAACACTGGGTCTAGTG; P2, 5'-GTGTTGTGACTGTATGAGTCAG; P3, 5'-TGTCCATG GGGAGATACAAGAC; P4, 5'-CTGTCTTTGAGCCATTTCTGCT. *Zfp38*^{+/fllox} mice were mated with mice carrying the *Stra8*-Cre transgene mice (obtained from The Jackson Laboratory, Stock No: 008208). DNA isolated from tail biopsies was used for genotyping. Genotyping for the *Stra8*-Cre transgene was performed as described previously (Sadate-Ngatchou *et al.* 2008).

Tissue collection and staining

The 4- or 8-week-old heterozygote (*Zfp38*^{fllox/+}, *Stra8*-Cre) and *Zfp38* cKO (*Zfp38*^{fllox/fllox}, *Stra8*-Cre) mice were killed, and their testes were fixed in 4% paraformaldehyde (PFA) for H&E and immunofluorescence staining. Briefly, tissue sections were deparaffinized and rehydrated followed by antigen retrieval in 10mM sodium citrate buffer. Then, sections were blocked with goat serum in 0.3% Triton X-100 and incubated with anti-ZFP38 antibody (Abcam, ab140513, 1:200) or anti-MVH antibody (Abcam, ab13840, 1:400) or anti-SYCP3 (Abcam, ab15093, 1:400) or anti-γ-H2AX (Abcam, ab2893, 1:200) at 4°C overnight followed by incubation with the FITC- or TRITC-labeled secondary antibody for 1 h. Negative control testis sections were incubated with secondary antibody in the absence of the primary antibody. The sections were counterstained with DAPI or PI (1:1000) to identify the nuclei.

Spermatocyte cell isolation for expression analysis

For isolation of spermatocytes, testes from 3-week-old mice were used. Spermatocyte cells were isolated as described previously (Baleato *et al.* 2005). Briefly, testes were isolated, decapsulated and incubated for 15 min each in 0.5 mg/mL collagenase/DMEM with agitation and then in 0.25% trypsin/EDTA in DMEM. The tubules were dissociated manually by pipetting and washed in 0.5% BSA in DMEM by

centrifugation. Cell pellets were resuspended in DMEM and filtered twice through a 70 μm membrane, then separated over a BSA gradient. The BSA gradient was prepared as described previously (Bellve *et al.* 1977).

Protein extraction and Western blot analysis

Tissue and spermatocytes were lysed in a radioimmunoprecipitation assay lysis buffer containing protease inhibitor cocktail tablets (Roche). Protein concentrations were measured using the Bradford assay. The proteins were electrophoresed under reducing conditions on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The blots were incubated with anti-ZFP38 antibody (Abcam, ab140513, 1:1000) or anti-RAD51 (Abcam, ab88573, 1:1000) or anti-DMC1 (Abcam, ab96613, 1:1000) or anti-TEX15 (Abcam, ab122364, 1:1000) or anti-PALB2 (Abcam, ab202970, 1:1000) or anti-PLZF (Santa Cruz, sc28319, 1:500) or anti-SYCP3 (Abcam, ab97672, 1:1000) or anti-WT1 (Abcam, ab89901, 1:1000) or anti-P450 Scc (Boster, Wuhan, China, BA3699, 1:1000) overnight at 4°C followed by Dye 800CW secondary antibody for 1 h at room temperature. The protein level was normalized and plotted against β-tubulin (Cell Signaling Technology, #2146, 1:2000). Signals were detected using the Western blotting Detection System (Odyssey, MA, USA).

Chromosome spreads

Chromosome spreads were prepared by the dry-down technique as described previously (Holloway *et al.* 2011, Chen *et al.* 2016). Briefly, testis tissues were dissected, and tubules were dissociated with a pipet in PBS. Cells were ruptured by adding an equal amount of hypotonic buffer (30 mM Tris-HCl pH 8.2, 50 mM sucrose, 17 mM sodium citrate, 5 mM EDTA, 0.5 mM DTT and protease inhibitors) and then mixing the solution 1:2 with 100 mM sucrose just before spreading on slides that were preincubated with 1% PFA. The slides were then dried for 1 h. Immunostaining of spermatocyte spreads was performed similar to the immunofluorescence analysis (as described above). The following antibodies were used: anti-SYCP1 (Abcam, ab15087, 1:400), anti-SYCP3 (Abcam, ab97672, 1:400), anti-γ-H2AX (Abcam, ab2893, 1:200), anti-RAD51 (Millipore, ABE257, 1:200) and anti-MLH1 (Abcam, ab92312, 1:200).

Gene expression analysis by quantitative real-time PCR

Tissue and spermatocyte RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA samples were subjected to reverse transcription using a PrimeScript RT Reagent Kit (Takara). The reactions were run in triplicate in three independent experiments. The cycle threshold (C_T) values for the samples were normalized to the corresponding *Gapdh* C_T value, and relative expression levels were calculated using the ΔΔC_T method. The primer sequences are provided in Supplementary Table 1, see section on supplementary data given at the end of this article.

Statistical analysis

Experiments were repeated at least three times. Data were evaluated for significance using Student's *t*-test. The results are presented as the mean $\pm$ S.E.M. *P* < 0.05 was deemed significant.

Results

ZFP38 expression in mouse testes

The findings of a previous study suggested that *Zfp38* mRNA is expressed from pachytene spermatocytes to round spermatids (Chowdhury *et al.* 1992). In this study, we observed that ZFP38 protein was predominantly expressed in testes (Fig. 1A). Only a small amount of ZFP38 expression was observed in ovary and kidney. Furthermore, ZFP38 expression in testis started from postnatal day 7 (PND7) and did not differ at PND 14, 21 and 28 (Fig. 1B). To evaluate the ZFP38 expression in different testicular

Figure 1 Expression of ZFP38 protein in mouse testes. (A) Expression of ZFP38 in mouse tissues, including brain, liver, spleen, ovary, kidney and testes. (B) Expression of ZFP38 in testes at different developmental stages, including E15.5, PND 3, 7, 14, 21 and 28. β -tubulin served as a protein-loading control in Western blot analysis. (C) The cellular localization of ZFP38 (red) in sections of adult testes was displayed by immunofluorescent staining. The nuclei are counterstained with DAPI (blue). Representative chromosome spreads of zygonema and pachynema are labeled with anti-ZFP38 (red) antibodies. Scale bar in a, 100 μ m; in b, 50 μ m; in c and d, 5 μ m.

cells, we performed immunofluorescence staining and observed that ZFP38 was specifically present in spermatocytes (Fig. 1C; a and b). ZFP38 was detected predominantly in chromosome axes of meiocytes and its levels increased from zygonema to pachynema (Fig. 1C; c and d).

Specific disruption of *Zfp38* in meiotic spermatocytes

To assess the *in vivo* function of ZFP38 during meiosis, we generated spermatocyte-specific *Zfp38* gene knockout (*Zfp38*^{flox/flox}, *Stra8*-Cre) mice. LoxP sites flank exon 4 of the *Zfp38* allele, and recombination of the loxP sites using *Stra8*-Cre recombinase resulted in the removal of exon 4 (Fig. 2A). The *Stra8*-Cre transgene is expressed in preleptotene-stage spermatocytes (Sadate-Ngatchou *et al.* 2008). The successful acquisition of *Zfp38* cKO mice was determined by polymerase chain reaction (PCR) amplification (Fig. 2B). We confirmed that ZFP38 was successfully depleted in *Zfp38* cKO testes using Western blot analysis (Fig. 2C). ZFP38 was absent in spermatocytes in *Zfp38* cKO mice, as evidenced by immunofluorescence staining (Fig. 2D).

Figure 2 Generation of *Zfp38* cKO mice. (A) Targeting strategy and the creation of *Zfp38* cKO mice. In the targeted allele, a loxP site and a pgk-Neo cassette flanked by two loxP sites were inserted into intron 4. (B) PCR analysis detected the presence of 5' (PCR: P1-P2) and 3' (PCR: P3-P4) loxP sites for genotyping the wild-type (+/+), heterozygous (flox/+) and homozygous (flox/flox) mice, and examined the deletion of exon 4 in the *Zfp38* Δ /+ mice (PCR: P1-P4). (C) Knockout efficiency was confirmed by Western blotting in wild-type (line 1), heterozygote (line 2) and knockout (line 3) testes. The protein level was normalized against β -tubulin. (D) Staining analysis of ZFP38 (red) in heterozygote and knockout testes. Scale bars, 25 μ m.

Figure 3 Meiotic arrest in *Zfp38* cKO male mice. (A and B) H&E staining of heterozygote and *Zfp38* cKO testis section at 4 weeks. (C and D) Expression of MVH (a germ cell marker) in heterozygote and *Zfp38* cKO testes. (E and F) Cell localization of SYCP3 in heterozygote and *Zfp38* cKO testes. (G and H) Colocalization of SYCP3 and γ -H2AX in heterozygote and *Zfp38* cKO testes. The nuclei are counterstained with PI in C–F (red). Spc, spermatocytes; R: round spermatids. Scale bars in A–B, 50 μ m; in C–H, 100 μ m.

Infertility and meiotic arrest in *Zfp38* cKO mice

Six- to eight-week-old *Zfp38* cKO males failed to produce offspring after natural mating with wild-type C57 female mice (at a ratio of 1:2) for more than 4 months. In contrast, heterozygotes (*Zfp38*^{+/-}*fllox*, *Stra8*-Cre) were fully fertile with an average litter size of 8.9 ± 1.4 (Supplementary Fig. 1A). Adult male *Zfp38* cKO mice showed no apparent morphological defects in nonreproductive organs, and there was no difference in body weight between the *Zfp38* cKO and heterozygote males (Supplementary Fig. 1B). However, testis weight of adult *Zfp38* cKO mice was less than 40% of heterozygotes (Supplementary Fig. 1C), which was likely due to the loss of postmeiotic cells. Indeed, H&E staining and MVH (a germ cell marker) immunofluorescence in 4-week-old heterozygote and *Zfp38* cKO testes showed normal postmeiotic round spermatids in heterozygote testes (Fig. 3A and C), versus few cells of pachytene-like morphology and tubules lacking haploid cells in age-matched *Zfp38* cKO testes (Fig. 3B and D). Furthermore, the meiosis in *Zfp38* cKO spermatocytes was abnormal, as revealed by immunofluorescence staining of SYCP3 (a synaptonemal complex protein) and γ -H2AX (a proxy for unrepaired breaks). Spermatocytes of heterozygotes exhibited a representative chromosomal expression pattern (wool-ball-like structure) (Fig. 3E), while no typical wool-ball-like structure of chromosomes was observed in *Zfp38* cKO spermatocytes (Fig. 3F). γ -H2AX signals in heterozygotes were restricted to one spot (asynapsed XY body) after pachytene stage (Fig. 3G), whereas the same signal was dispersed in *Zfp38* cKO spermatocytes (Fig. 3H).

Failure of chromosomal synapsis and DSB repair in *Zfp38* cKO spermatocytes

To determine the cause of meiotic arrest and to gain mechanistic insight into this defect in *Zfp38* cKO spermatocytes, we examined the assembly of the synaptonemal complex and DSB repair by surface spread analysis of spermatocytes. Using SYCP1/SYCP3 (from the central and axial/lateral elements

of synaptonemal complex) double staining of heterozygote pachytene spermatocytes, we observed perfect colocalization of SYCP1 and SYCP3 around

Figure 4 Aberrant chromosomal synapsis and failure of DSB repair in *Zfp38* cKO spermatocytes. (A) Representative chromosome spreads of spermatocytes at PND 21 labeled with anti-SYCP3 (green) and anti-SYCP1 (red) antibodies. Arrows indicated asynapsed chromosomes. The nuclei are counterstained with DAPI (blue). Scale bars, 5 μ m. (B) Chromosome spreads from PND 21 heterozygote and *Zfp38* cKO mice were stained with antibodies against γ -H2AX (a, d) or RAD51 (b, e) or MLH1 (c, f). The nuclei are counterstained with DAPI (blue). Arrows in d indicated residual expression of γ -H2AX in autosomes, and arrows in f indicated autosomes completely lacked a MLH1 focus. Scale bars, 5 μ m.

the synaptonemal complex (Fig. 4A; a–c). However, obviously, unsynapsed or partially synapsed chromosomes (arrows) were observed in *Zfp38* cKO spermatocytes (Fig. 4A; d–f). Both the number of spreads with partial synapsis (85 ± 4 for cKO vs 9 ± 2 for heterozygote; 100 cells counted from 3 males) and the average number of asynapsed chromosomes per nucleus (7 ± 2 for cKO vs ~ 0 for heterozygote; 100 cells counted from 3 males) were significantly increased in *Zfp38* cKO pachynema. γ -H2AX staining was undetectable on autosomes and restricted to the largely asynapsed XY body in heterozygote pachynema (Fig. 4B). Conversely, γ -H2AX staining of *Zfp38* cKO pachynema was sustained in asynapsed autosomal homologs (Fig. 4B; arrows). Furthermore, we detected the distribution of RAD51, which is a critical component of DNA repair complex. There was a dramatic decrease in the number of RAD51 foci (160 ± 22 in heterozygote vs 43 ± 17 in cKO; 100 cells counted from 3 males) in *Zfp38* cKO zygonema compared with the heterozygote zygonema (Fig. 4B; b and e). We also found a significant decrease in the number of MLH1 foci in *Zfp38* cKO pachynema (13 ± 3 for cKO vs 24 ± 2 for heterozygote; 100 cells counted from 3 males). In addition, 87.9% of *Zfp38* cKO pachytene cells contained at least one autosome that completely lacked a MLH1 focus compared with 3.6% of heterozygote pachynema (100 cells counted from 3 males) (Fig. 4B; c and f).

Figure 5 Attenuated expression of DSB repair-associated factors. (A) The mRNA levels of *Spo11*, *Sycp1/3*, *Rad51*, *Tex15*, *Brca1/2* and *Palb2* in heterozygote and *Zfp38* cKO spermatocytes. *Gapdh* served as the internal control gene. (B) Protein levels of RAD51, DMC1, TEX15 and PALB2 in heterozygote and *Zfp38* cKO spermatocytes. β -tubulin served as a protein-loading control. The data are expressed as the mean $\pm$ S.E.M. * $P < 0.05$.

Downregulation of DSB repair-associated factors after *ZFP38* deletion in spermatocytes

To determine the underlying reasons of aberrant chromosomal synapsis and failure of DSB repair in *Zfp38* cKO spermatocytes, we detected the expression of SPO11 (a DSB inducer), synaptonemal complex proteins (SYCP1-3) and DSB repair-associated factors (RAD51, DMC1, TEX15, BRCA1/2 and PALB2). We observed that the mRNA levels of *Spo11*, *Sycp1*, *Sycp2*, *Sycp3*, *Brca1* and *Brca2* were not significantly altered between heterozygote and *Zfp38* cKO spermatocytes. However, the mRNA levels of DSB repair-associated genes, such as *Rad51*, *Dmc1*, *Tex15* and *Palb2*, were significantly reduced in *Zfp38* cKO spermatocytes (Fig. 5A). Western blot analysis further indicated that the protein levels of RAD51, DMC1, TEX15 and PALB2 were significantly downregulated in *Zfp38* cKO spermatocytes (Fig. 5B and C).

Discussion

Expression analysis of ZFP38 strongly suggests a role in meiotic progression in male germ cells. In the male germ line, ZFP38 protein emerged from PND7. Subsequently, the expression of ZFP38 appeared relatively constant. Antibody localization of ZFP38 confirmed its presence in zygotene and pachytene spermatocytes in testis sections. Although *Zfp38* transcripts were expressed from pachytene spermatocytes to round spermatids (Chowdhury *et al.* 1992), ZFP38 protein was undetectable in round spermatids.

Conditional knockout strategy was used to investigate the role of transcription factor ZFP38 in mouse spermatogenesis. *Zfp38* cKO male mice were completely infertile and exhibited meiotic arrest. *Zfp38* cKO testes showed few spermatocytes, without round spermatids. The role for ZFP38 in meiosis was corroborated by its restricted expression in spermatocytes.

Our chromosome spread data indicated ZFP38 was required for spermatocytes to progress meiotic prophase I, as both recombination and DSB repair were affected in *Zfp38* cKO mice. Two RecA homologs, RAD51 and its counterpart DMC1, mediate homologous chromatid invasion and promote homologous chromosome pairing and synapsis in spermatocytes (Pittman *et al.* 1998, Yoshida *et al.* 1998). TEX15 (Yang *et al.* 2008), BRCA1 (Scully *et al.* 1997), BRCA2 (Davies *et al.* 2001) and PALB2 (Simhadri *et al.* 2014) mediate loading of the RAD51 and DMC1 recombinases onto sites of DSBs. Deficiency in genes encoding synaptonemal complex proteins exhibits similar phenotypes – e.g., knockouts of *Sycp1* (de Vries *et al.* 2005), *Scyp2* (Yang *et al.* 2006), *Sycp3* (Yuan *et al.* 2000), *Syce1* (Bolcun-Filas *et al.* 2009), *Syce2* (Bolcun-Filas *et al.* 2007), *Syce3* (Schramm *et al.* 2011) and *Tex12*

(Hamer *et al.* 2008) produce male infertility with an arrest during prophase. Given that the deficiency of these genes exhibits similarities to the phenotype of *Zfp38* cKO male mice, we detected the expression of genes encoding synaptonemal complex proteins and DSB repair-associated factors. The mRNA levels of *Spo11*, *Sycp1*, *Sycp2*, *Sycp3*, *Brca1* and *Brca2* were normal, while the mRNA and protein levels of RAD51, DMC1, TEX15 and PALB2 were reduced in *Zfp38* cKO spermatocytes. While the transcript abundance of *Sycp1-3* was not altered in *Zfp38* gKO spermatocytes, SYCP1 and SYCP3 proteins do not colocalize properly in these cells. Whether transcription factor ZFP38 regulates *Rad51*, *Dmc1*, *Tex15* and *Palb2* directly or indirectly needs further investigation. Genome-wide transcriptome analysis (microarray or RNA-seq) will be helpful to discern the regulatory mechanisms of transcription factor ZFP38 in meiocytes.

We found that both MLH1 foci and RAD51 foci were reduced in *Zfp38* cKO spermatocytes, which might be the consequence of the reduced expression of DSB repair-associated proteins. Although we observed that γ -H2AX foci responded equally well and appeared at the damage sites during the zygotene stage in both the control and the *Zfp38* cKO spermatocytes, with the same staining pattern and comparable intensity, we could not exclude the possibility that DSB formation was decreased in *Zfp38* cKO spermatocytes. A recent study suggests that the presence of γ -H2AX foci should not be considered an unequivocal marker of DSB (Rybak *et al.* 2016). Although the level of *Spo11* mRNA was normal in *Zfp38* cKO spermatocytes, other factors that are needed for DSB formation or forming complex with SPO11 (Robert *et al.* 2016, Vrielynck *et al.* 2016) may be limiting.

Meiotic sex chromosome inactivation (MSCI) enables the unpaired sex chromatin to bypass meiotic synapsis checkpoints and is essential for meiotic progression in males (Modzelewski *et al.* 2012). We found that ZFP38 staining was also evident in sex body at pachynema. Thus, we suggest that MSCI may be another possible reason of meiotic arrest in *Zfp38* cKO mice and whether *Zfp38* cKO spermatocytes fail to silence sex-linked transcripts and lead to apoptosis needs further investigation.

In summary, ZFP38 regulates chromosomal synapsis and DSB repair during meiotic prophase I. The meiotic arrest phenotype could be partially explained by the potential regulation of RAD51, DMC1, TEX15 and PALB2 by transcription factor ZFP38.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0225>.

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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Authors' contribution statement

J W conceived and designed the experiments. Z Y, D F, Q M, J Y and W Z performed the experiments. F G, D S, R G and K S analyzed the data. J W wrote the paper.

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