Ubiquitin-specific protease 26 (USP26) is not essential for mouse gametogenesis and fertility.

Natalia Felipe-Medina¹, Laura Gómez-H¹, Yazmine B. Condezo¹, Manuel Sanchez-Martín^{2,3}, José Luis Barbero⁴, Isabel Ramos¹, Elena Llano^{1,5}, Alberto M. Pendás^{1*}.

1 Molecular Mechanisms Program, Centro de Investigación del Cáncer and Instituto de Biología Molecular y Celular del Cáncer (CSIC-Universidad de Salamanca), 37007 Salamanca, Spain.

2 Departamento de Medicina, Universidad de Salamanca, Salamanca, Spain.

3 Transgenic Facility, Nucleus platform, Universidad de Salamanca, Salamanca, Spain.

4 Centro de Investigaciones Biológicas (CSIC), Madrid, Spain.

5 Departamento de Fisiología y Farmacología, Universidad de Salamanca, Salamanca, Spain.

*amp@usal.es

** To whom correspondence should be addressed*

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Abstract

USP26 is a deubiquitylating enzyme belonging to the ubiquitin-specific proteases (USPs) family with a transcription pattern restricted to the male germline. Since protein ubiquitination is an essential regulatory mechanism during meiosis, many efforts have been focused on elucidating the function of USP26 and its relationship with fertility. During the last decade, several studies have reported the presence of different polymorphisms in USP26 in patients with non-obstructive azoospermia (NOA) or severe oligozoospermia suggesting that this gene may be associated with human infertility. However, other studies have revealed the presence of these and novel polymorphisms, including nonsense mutations, in men with normal spermatogenesis as well. Thus, the results remain controversial and its function is unknown. In the present study, we describe the *in vivo* functional analysis of mice lacking USP26. The phenotypic analysis of two different *Usp26*-null mutants showed no overt-phenotype with both males and females being fertile. Cytological analysis of spermatocytes showed no defects in synapsis, chromosome dynamics, DNA repair or recombination. Histopathological analysis revealed a normal distribution and number of the different cell types in both male and female mice. Finally, normal counts were observed in fertility assessments. These results represent the first *in vivo* evidence showing that USP26 is not essential for mouse gametogenesis.

Introduction

Infertility is a major health issue with an estimated 8-12% of couples affected worldwide (Ombelet et al. 2008; Prieto et al. 2004). Men contribute to 50% and are solely responsible for 20-30% of reported cases (Sharlip et al. 2002). Many factors have been associated with male infertility such as environmental or occupational exposures and genetic abnormalities. Genetic causes are thought to underlie 15-30% of male infertilities being non-obstructive azoospermia (NOA), the complete absence of spermatozoa in the ejaculate due to a testicular failure, the most common outcome associated (Ferlin et al. 2007). Although chromosomal aberrations and microdeletions of the Y chromosome have been largely considered to be the main cause of genetic male infertility (Matzuk and Lamb 2008; Reijo et al. 1996), in the past decades a large number of fertility-related genes have been identified through the analysis of mouse models, clinical cases and genome-wide association studies (Caburet et al. 2014; Gomez et al. 2016; Hu et al. 2011). However, much work remains to be done as idiopathic azoospermia is still the most frequent diagnosis among infertile males, and it is currently believed that a high proportion are related with genetic causes (Hamada et al. 2013).

Spermatogenesis, the process through which spermatogonial stem cells generate mature spermatozoa, represents one of the most complex differentiation programs in developmental biology and requires an accurate spatio-temporal regulation of gene transcription, translation and protein turnover. In this sense, ubiquitination becomes essential for the correct completion of spermatogenesis as it targets substrates for proteasomal degradation but also can modify their biological activity, stability or subcellular localization (He et al. 2016). Moreover, the importance of ubiquitination in the regulation of protein turnover during spermatogenesis as well as in mammalian fertilization and sperm quality has been reported (Baarends et al. 2000; Muratori et al. 2005; Yi et al. 2007).

The ubiquitin-specific protease 26 (*Usp26*) is a single-exon gene firstly identified by Wang et al. (2001) from a screen for genes expressed in mouse spermatogonia. USP26 belongs to a family of cysteine-proteases that disassemble polymeric ubiquitin chains from substrates and are responsible for maintaining ubiquitin homeostasis in cells (Komander et al. 2009). *USP26* has attracted increased attention as a candidate infertility gene due to its restricted expression pattern to testis and because

1 of its genetic linkage with X-chromosome (male are hemizygous) which is enriched
2 for testis-specific genes (Nishimune and Tanaka 2006). More specifically, *USP26*
3 expression has been described in spermatogonia (types A and B), preleptotene and
4 leptotene-zygotene spermatocytes, round spermatids and at the blood-testis barrier
5 from both mouse and human testes (Lin et al. 2011; Wang et al. 2005). Additionally,
6 in humans it is also expressed in Sertoli and Leydig cells (Wosnitzer et al. 2014).
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11 Genetic polymorphisms identified in the *USP26* locus have been causally
12 associated with male infertility from Sertoli cell only-syndrome to non-obstructive
13 azoospermia or asthenozoospermia (Asadpor et al. 2013; Lee et al. 2008; Li et al.
14 2015; Ma et al. 2016; Paduch et al. 2005; Stouffs et al. 2005; Xia et al. 2014; Zhang et
15 al. 2007). By contrast, other studies have found the same frequency of *USP26*
16 polymorphisms in fertile and infertile men (Christensen et al. 2008; Luddi et al. 2016;
17 Ravel et al. 2006; Ribarski et al. 2009; Shi et al. 2011; Stouffs et al. 2006; Zhang et al.
18 2015). In addition, it has been reported that most of the described polymorphisms do
19 not abolish *USP26* enzymatic activity (Liu et al. 2018; Zhang et al. 2015).
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28 Given the absence of functional studies, we have addressed the *in vivo*
29 functional analysis of mice lacking *USP26*. The analysis of *USP26*-deficient mice
30 showed no overt-phenotype with both males and females being fertile. The
31 histopathological analysis of testis and ovaries showed normal number and distribution
32 of cell types and accordingly, the results obtained in the fertility assessment were
33 similar to the wild-type ones. Finally, spermatogenesis in absence of *USP26* proceeded
34 without any defect neither in synapsis, nor in DNA repair or meiotic recombination.
35 These results represent the first *in vivo* evidence showing that *USP26* is not essential for
36 mouse gametogenesis.
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Methods

Production of CRISPR/Cas9-Edited Mice. *Usp26*-sgRNAs were predicted at crispr.mit.edu. *Usp26*-sgRNAs were produced by cloning annealed complementary oligos (Table S1) at the BbsI site of pX330 (#42230, Addgene), generating PCR products containing a T7 promoter sequence, and then performing in vitro transcription using the MEGAshortscript™ T7 Transcription Kit (Life Technologies). The plasmid pST1374-NLS-flag-linker-Cas9 (#44758; Addgene) was used for generating CAS9 mRNA after linearization with AgeI. *In vitro* transcription and capping were performed using the mMESSAGE mMACHINE T7 Transcription Kit (AM1345; Life Technologies). Products were purified using the RNeasy® Mini Kit (Qiagen). RNA (100 ng/ul Cas9 and 50ng/ul each guide RNA) was microinjected into zygotes [F1 hybrids between strains C57BL6/J and CBA/J also known as B6CBAF1/J] as described previously (Singh et al. 2015). Edited founders were identified by PCR amplification with primers flanking the targeted regions (Table S2) and subcloned into pBlueScript (Stratagene) followed by standard Sanger sequencing. The length of the corresponding wild type and mutant alleles were 307 bp and 411 bp respectively, in the 21 mutants and 259bp vs 173bp in the catalytic mutants. The selected founder was crossed with a wild type (B6CBAF1/J) to eliminate possible unwanted off-targets. *Usp26*^(WT/edited) heterozygous mice were sequenced again by Sanger and crossed to give rise to *Usp26*^(edited/edited) homozygous. Genotyping was performed by agarose gel electrophoresis analysis of PCR products from DNA isolated from tail biopsies.

Fertility assessment. *Usp26*^{WT/Y}, *Usp26*^{21/Y} and *Usp26*^{Cat/Y} males and *Usp26*^{WT/WT}, *Usp26*^{21/21} and *Usp26*^{Cat/Cat} females (8 weeks old) were mated with wild type females and males, respectively during 3 months. 6 mice per genotype were crossed. The presence of copulatory plug was examined daily and the number of pups per litter was recorded.

Histology. For histological analysis, adult testes/ovaries were fixed in Bouin's fixative, processed into serial paraffin sections and stained with Periodic acid–Schiff (PAS) and Hematoxylin (testes) or hematoxylin-eosin (ovaries).

Immunocytology and antibodies. Testes were detunicated and processed for spreading using the "dry-down" technique and incubated with the indicated antibodies for immunofluorescence. The primary antibodies used for immunofluorescence were goat

1 α USP26 (W-12) sc-51013 (Santa Cruz, 1:5), rabbit α USP26-M (1:5, provided by Dr.
2 Yi-Wen Lin Institute of Biomedical Sciences, Taipei, Taiwan), mouse α SYCP3 IgG sc-
3 74569 (Santa Cruz, 1:60), rabbit α SYCP3 serum K921 (Prieto et al. 2004), rabbit
4 α SYCP1 IgG ab15090 (Abcam, 1:200), rabbit anti- γ H2AX (ser139) IgG #07-164
5 (Millipore, 1:150), mouse α MLH1 51-1327GR (BD Biosciences; 1:5). The secondary
6 antibodies used were TRITC α -mouse 115-095-146/ α -rabbit 111-025-144 and FITC α -
7 mouse 115-095-146/ α -rabbit 111-095-045 (Jackson ImmunoResearch, all 1:100). Slides
8 were visualized at room temperature using a microscope (Axioplan 2; Carl Zeiss, Inc.)
9 with 63 $\times$ objectives with an aperture of 1.4 (Carl Zeiss, Inc.). Images were taken with a
10 digital camera (ORCA-ER; Hamamatsu) and processed with OPENLAB 4.0.3 and
11 Photoshop (Adobe). Quantification of γ H2AX fluorescence signal was measured by
12 Image J software.

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22 **Protein extraction and western blotting.** Whole testis extracts were prepared in RIPA
23 buffer (50mM Tris Hcl (pH7,5), 150mM NaCl, 1% NP40, 0,1% SDS, 0,5% sodium
24 deoxycholate). 15 μ g of total protein were loaded onto 10% reducing polyacrylamide
25 SDS gels and transferred to 0,45 μ m Hybond ECL nitrocellulose membranes
26 (RPN303D; GE Healthcare, Life Sciences). The membranes were blocked with 5%
27 nonfat dried milk diluted in TBS-Tween. The proteins were detected by western blotting
28 with the indicated antibodies. Primary antibodies used for western blotting were rabbit
29 goat α USP26 (W-12) sc-51013 (Santa Cruz, 1:2000), rabbit α USP26-M (1:2000,
30 provided by Dr. Yi-Wen Lin, Institute of Biomedical Sciences, Taipei, Taiwan), mouse
31 anti- α -Tubulin (T9026, Sigma, 1:40000), mouse anti- β -Actin (A5441, Sigma, 1:40000).
32 Secondary horseradish peroxidase-conjugated α -mouse (715-035-150, Jackson
33 ImmunoResearch), α -rabbit (711-035-152, Jackson ImmunoResearch), or α -goat (705-
34 035-147, Jackson ImmunoResearch) antibodies were used at 1:5000 dilution.
35 Antibodies were detected by using ImmobilonTM Western Chemiluminescent HRP
36 Substrate from Millipore.

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50 **Bioinformatic analysis of RNAseq datasets.** Gene expression data were obtained from
51 each article repository and processed to extract the relevant information of the genes of
52 interest (*Usp* genes) into a dataframe using regular expressions in R language. Colors
53 have been included to represent a gradient of expression for each individual gene. Red
54 and green represent the lower and upper endpoints (stages with lower and higher
55 expression level) and between them an intermediate color gradient has been established.

Results

Usp26 CRISPR-Cas9 knockout lines

To gain further insight into USP26 function we generated a mouse model by targeting the single coding exon of *Usp26* through CRISPR-Cas9 genome editing. The selected founder carried two consecutive insertions of 85 and 19 base pairs (bp) separated by 5 bp. The first insertion changes the frame and generates a premature STOP codon at position 123 (14KDa) resulting in a predicted null-allele (herein allele²¹; Fig. 1a, left). This founder was crossed with wild-type (WT) C57BL/6J mice and the heterozygous offspring was crossed to obtain the homozygous mutant mice, which were identified by PCR (Fig. 1b).

To test whether the generated mutation was a null-allele, we analyzed the expression of USP26 in mouse testis with two different antibodies, the commercial anti-USP26 (W-12) and the anti-USP26-M kindly provided by Dr. Yi-Wen Lin (Lin et al. 2011). We first carried out an immunofluorescence (IF) analysis in spermatocyte spreads from both *Usp26*^{21/Y} and *Usp26*^{WT/Y} mice. None of the antibodies showed detectable signal in spermatocytes (leptonema, zygonema, pachynema, diplonema or diakinesis), spermatogonia or round spermatid (Fig. S1a-b and S2a-b), where the expression of USP26 has been previously reported (Lin et al. 2011; Wang et al. 2005). We also performed a Western Blot (WB) analysis of whole testis extracts from wild type and mutant mice. We did not observe any signal in the blot using the anti-USP26 (W-12) antibody (Fig. S1c) whereas the anti-USP26-M generated similar bands in *Usp26*^{21/Y} and *Usp26*^{WT/Y} extracts (Fig. S2c). No shift in the migration of any detected band was observed in the mutant extracts in comparison with the wild type (USP26 size: WT, 95 KDa vs 14 KDa, allele²¹). These results strongly suggest that the available antibodies are not able to recognize the endogenous protein and, consequently, the detected bands do not correspond to USP26.

Nonsense mutations close to the 5' of open reading frames (ORF) can lead to a translation restart from downstream AUG codons using the same original frame (Makino et al. 2016). The genomic locus of murine *Usp26* encodes an in-frame methionine at the position 147 which could be used as a downstream AUG initiation codon in our initial mutant (allele²¹) and could potentially lead to the generation of an N-terminal truncated protein (79 KDa, Fig 1a, left) with an intact catalytic domain. We

did not detect the predicted truncated proteins of 14 KDa (premature STOP codon) or 79 KDa (Restart ATG) in the *Usp26*^{21/Y} extracts by western blot (Fig. S1c and S2c). Consequently, the absence of working antibodies against USP26 that allowed us to ensure that the allele²¹ was a null mutant led us to generate a second mutant with a deletion in the proteolytic domain of USP26 (dead mutant). This domain is formed by two short motifs known as Cys and His boxes that contain the catalytic triad, one catalytic cysteine and an histidine and asparagine/aspartate that facilitate the cysteine nucleophilic attack (Davis and Simeonov 2015; Nijman et al. 2005). To abolish the protease activity of USP26 we targeted the CRISPR-Cas9 to the catalytic cysteine (Cys295) generating an 86bp deletion (including the cysteine) that produces a frameshift and consequently a premature STOP codon at position 293 (34KDa; Fig. 1a, right). Once the founder transmitted the allele, the heterozygous were crossed to obtain the homozygous mutants (herein *Usp26*^{Cat/Cat} or *Usp26*^{Cat/Y}), which were identified by PCR (Fig. 1c). Immunofluorescence and western blot analysis were performed for the new catalytic mutant and the results were identical to those obtained in the *Usp26*^{21/Y} mutant (Fig.S1a-c and S2a-c). The western blot analysis did not reveal any shift on band migration nor identify the truncated proteins of 34 and/or 56 KDa that could be expressed from the allele^{Cat} (See Fig. 1a right and S1c and S2c). Thus, although we cannot demonstrate the absence of USP26 in our mutants due to the lack of working antibodies that recognize the endogenous protein, the genetic strategy predicts that both of them are null. Especially, the resultant catalytic mutant lacks the genomic information to encode an active catalytic domain (Cys) and, consequently, represents a dead mutant without ubiquitin-specific protease activity.

USP26-deficient mice are fertile

Usp26^{21/Y} and *Usp26*^{Cat/Y} mice developed normally and showed no overt-phenotype including testis size and weight (Fig. 2a). To determine the potential effect of USP26 absence on mouse fertility we performed a fertility test. Adult males from *Usp26*^{WT/Y}, *Usp26*^{21/Y} and *Usp26*^{Cat/Y} lines were mated with *Usp26*^{WT/WT} females. The presence of copulatory plug was monitored daily to discard behavioral defects and the number of pups per litter was recorded. USP26 mutants behaved similar to their wild type counterparts and no significant differences in litter size between the three groups were observed (Table 1). The histopathological analysis of seminiferous tubules from

1 *Usp26*^{21/Y} and *Usp26*^{Cat/Y} mice showed a normal progression through the twelve stages
2 of epithelial cycle with an organization and number of germ cells similar to the wild
3 type ones (Fig. 2b). Moreover, a normal amount of spermatozoa were able to reach the
4 epididymides of USP26 mutants (Fig. 2b). Taken together, our findings demonstrate
5 that USP26 seems to have a minimal if any role in mouse male fertility.
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10 *Usp26* is not expressed in human whole ovary and mutations associated with
11 female infertility have not been reported. Accordingly, the fertility assessment and
12 histological analysis of *Usp26*^{21/21} and *Usp26*^{Cat/Cat} females did not reveal any defect
13 (Table 1 and Fig. 2c).
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20 **USP26-deficient spermatocytes showed normal synapsis, DSBs dynamics and** 21 **recombination** 22 23

24 Although *Usp26* transcription starts in spermatogonia it is transcribed at
25 maximum levels during the early stages of meiosis (leptotene and zygotene, Tables S5
26 and S6 (da Cruz et al. 2016; Margolin et al. 2014)). Moreover, the UPS has been
27 involved in the regulation of meiotic prophase in mouse (Rao et al. 2017) and USP26
28 has been associated with double-strand breaks (DSBs) repair and homologous
29 recombination in somatic cells (Typas et al. 2016). These data prompted us to analyze
30 in more detail chromosome synapsis, double-strand breaks (DSBs)
31 generation/resolution and meiotic recombination during the meiotic prophase I of
32 USP26-deficient spermatocytes.
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42 Chromosome synapsis is mediated by a proteinaceous scaffold known as
43 synaptonemal complex (SC). The SC is composed of two axial elements (AEs)
44 connected by transverse filaments (TFs) through the assembly of the central element
45 (CE), generating a tripartite zipper-like structure. In mammals the components of AEs
46 are SYCP2 and SYCP3, TFs consist of SYCP1 and five CE components have been
47 identified: SYCE1-3, TEX12 and the recently described SIX6OS1 (Bolcun-Filas and
48 Schimenti 2012; Gomez et al. 2016). To evaluate the synaptic process, we analyzed the
49 distribution of SYCP3 and SYCP1 in spermatocyte spreads from *Usp26*^{21/21},
50 *Usp26*^{Cat/Cat} and *Usp26*^{WT/WT} mice (Fig. 3). We did not observe defects on AEs
51 formation, starting as short patches in leptotene that got longer as zygotene proceeded.
52 Full synapsis was achieved at pachytene and no defects were observed as desynapsis
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1 progressed from diplotene to diakinesis. Additionally, USP26-deficient metaphase I and
2 II plates showed normal morphology (Fig. 3). Altogether, these observations suggest
3 that USP26 has no function on chromosome behavior and synapsis.
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6 Meiotic recombination establishes the physical tethering between homologous
7 chromosomes essential to ensure the correct chromosome segregation during the first
8 meiotic division. Meiotic recombination is initiated at leptotene with the formation of
9 programmed DSBs by SPO11 which are repaired during the prophase I by the
10 homologous recombination (HR) machinery (Bolcun-Filas and Schimenti 2012). Given
11 the involvement of protein ubiquitination and SUMOylation in DSBs repair (Bekker-
12 Jensen and Mailand 2011), we analyzed USP26-deficient spermatocytes for the labeling
13 of phosphorylated H2AX (γ -H2AX), the first appearing modification after DSBs
14 induction (Rogakou et al. 1998). The γ -H2AX distribution in USP26-deficient
15 spermatocytes resembled that of wild-type cells from leptotene, showing whole nucleus
16 labeling, to pachytene where the γ -H2AX signal was restricted to the sex body. These
17 data indicate that DSBs formation and repair are not compromised by the absence of
18 USP26 (Fig. 4).
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30 During early prophase I DSBs are frequently resolved as non-crossovers (NCO)
31 and only a reduced fraction of the DSBs are processed as crossovers (CO), which will
32 give rise to chiasmata. Owing to the association of the UPS to the AEs and its role in
33 regulating meiotic recombination (Rao et al. 2017), we evaluated USP26-deficient
34 spermatocytes for CO formation by MLH1 staining, a mismatch repair protein that
35 marks CO sites (Guillon et al. 2005). No differences were observed in the number of
36 MLH1 foci between *Usp26*^{21/Y}, *Usp26*^{Cat/Y} and *Usp26*^{WT/Y} spermatocytes (Fig. 5).
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Discussion

In the present study we have generated two *Usp26* knockout models, one carrying two insertions (allele²¹) and a second one lacking the catalytic Cys of the protein (dead mutant, allele^{Cat}). Although the *in silico* analysis predicts that both alleles generate truncated proteins, we have not been able to demonstrate the absence of USP26 in the mutants, likely due to the lack of sensitivity of the antibodies which failed to detect the endogenous protein. Even though we could not demonstrate the absence of protein, the generated deletion in the catalytic domain of USP26 (allele^{Cat}) ensures that the protein, if present, will not be functional as ubiquitin-specific protease leading to a reliable loss-of-function mutant.

The testis-specific USP26 belongs to the superfamily of deubiquitinating enzymes (DUBs) that remove ubiquitin (Ub) peptides from substrates, process Ub precursors and disassemble polymeric Ub chains hence being responsible for maintaining ubiquitin homeostasis in cells (Komander et al. 2009). Ubiquitination plays essential roles during spermatogenesis from the establishment and differentiation of spermatogonial stem cells to spermiogenesis (Bose et al. 2014) and to date several deubiquitinating enzymes (i.e. USP2 (Bedard et al. 2011), USP7 (Luo et al. 2015), USP9X (Kishi et al. 2017), Uchl-1(Kwon et al. 2005)) have been associated with defects at different stages of spermatogenesis and fertility impairment. However, here we show that mice lacking the deubiquitinating enzyme USP26 are fertile showing litters of the same size than those from wild type mice and a normal number and distribution of all the testis cell types.

The maximum transcription levels of *Usp26* are reached during the first stages of meiotic prophase (Tables S5 and S6 (da Cruz et al. 2016; Margolin et al. 2014)). This expression pattern along with the involvement of the UPS in the regulation of meiotic prophase in mouse (Rao et al. 2017) and the association of USP26 with DNA repair and homologous recombination in somatic cells (Typas et al. 2016), strongly suggest a potential role of USP26 on these processes. However, our cytological analysis of mutant spermatocytes did not reveal any obvious defect in synapsis, DSBs repair and meiotic recombination. These results suggest a very specific function of USP26 that seems unrelated with the regulation of the meiotic prophase I.

The association of this testis-specific protein with male infertility has focused many efforts during the last decades though with controversial conclusions. Several studies have linked *USP26* mutations with male infertility (Asadpor et al. 2013; Lee et al. 2008; Li et al. 2015; Ma et al. 2016; Paduch et al. 2005; Stouffs et al. 2005; Xia et al. 2014; Zhang et al. 2007). However, our functional studies showing that *USP26* is dispensable for mouse fertility are in agreement with previous works reporting a lack of association between *USP26* genetic polymorphisms and male infertility (Christensen et al. 2008; Luddi et al. 2016; Ravel et al. 2006; Ribarski et al. 2009; Shi et al. 2011; Stouffs et al. 2006; Zhang et al. 2015). Supporting our observations, it has been described a normozoospermic man carrying a nonsense mutation in *USP26* (c.882 C>A) that generates a premature STOP codon (Luddi et al. 2016). This mutation produces a truncated *USP26* protein at the position 293 that consequently lacks the catalytic Cys (Cys305). Our results provide new evidences that could shed light on the existing controversy about the relationship between *USP26* mutations and human male infertility. However, although the mouse is the most commonly used organism model in human disease research (Rosenthal and Brown 2007), we cannot exclude that the functional relevance of this protein could differ between both species, showing a critical role in human that does not recapitulate in the mouse where the genetic background can also drastically modulate the observed phenotypes. Nevertheless, our working models have been developed in an outbred background (B6CBAF1/J) that more closely resembles the greater genetic variability of humans.

Given the *Usp26* restricted expression pattern to testis and the importance of the maintenance of ubiquitin homeostasis in cells, the absence of phenotype in the *USP26*-deficient mice is counterintuitive. Genetic redundancy or biological robustness can be responsible for this lack of deviation in the reproductive phenotype. The *in silico* analysis of mRNA expression of different USPs during mouse spermatogenesis showed several USPs with an expression pattern highly similar to that of *USP26* (i.e *Usp9x*, *Usp37*, *Usp14*, *Usp53*, *Usp33*, *Usp28* and *Usp3*. See Tables S5 and S6 (da Cruz et al. 2016; Margolin et al. 2014)). It would be interesting to analyze which of these putative candidates (if any) are upregulated in the absence of *USP26* that would provide an indication for compensation. To proof it, a double loss of function analyses should be carried out in future studies.

1 In conclusion, this work reports for the first time a loss-of-function analysis of
2 USP26 in mice and represents *in vivo* evidence that USP26 is not essential for mouse
3 gametogenesis and fertility.
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7 **Acknowledgements**

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28 **Author contributions**

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30 NFM with the help of LGH and YBC performed the characterization of the mutant mice
31 including the cytological and histological analysis. MSM carried out the Cas9
32 injections. JLB provided the α SYCP3 serum K921 and reviewed the manuscript. IR
33 carried out infertility phenotyping of mutant mice. AMP and ELC designed the
34 experiments and helped NFM to write the manuscript with the input of the remaining
35 authors.
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Legends to figures

Figure 1. *Usp26* knockout generation by CRISPR-Cas9 genome editing. (a) Schematic representation of the wild type locus (WT) and the two genome editing strategies at the *Usp26* locus (left: knockout 21, right: catalytic knockout Cat). The sgRNAs, the single-coding exon of *Usp26* (grey) and the corresponding non-coding region (open box) are represented. Thin (non-coding) and thick (coding sequences) lines under the exon represent the expected transcripts derived from WT and *Usp26* edited alleles. ATG, start codon; TAG/TAA, stop codons; TGC, codon encoding the catalytic Cys295, Restart ATG, codons encoding the Met147(21) and Met331(Cat). Nucleotide sequence of the WT and the edited allele derived from PCR amplification of DNA from the *Usp26* edited mice is shown. In the allele^{Cat} the catalytic cysteine (Cys) is highlighted (red rectangle). The insertion (left panel) and deletion (right panel) generate a frameshift (amino acids in red) and a premature STOP codon. Double-headed arrows represent the predicted size (KDa) of the peptides that could be expressed in both alleles (from the canonical ATG to the generated STOP codon and from the first ATG after the mutation -restart ATG- to the canonical STOP codon). Arrows represent genotyping primers. (b) PCR analysis of genomic DNA from three pups from *Usp26*^{WT/21} heterozygous crosses. The PCR amplification with primers F and R (arrows) revealed a fragment of 307 bp in the case of the allele^{WT} and 411bp in the allele²¹. (WT/WT), (WT/21) and (21/21) designate wild type, heterozygous, and homozygous knockout animals, respectively. (c) PCR analysis of genomic DNA from three pups from *Usp26*^{WT/Cat} heterozygous crosses. The PCR amplification with primers F and R (arrows) revealed a fragment of 259bp in the case of the allele^{WT} and 173bp in the allele^{Cat}. (WT/WT), (WT/Cat) and (Cat/Cat) designate wild type, heterozygous, and homozygous knockout females, respectively.

Figure 2. Histopathological analysis does not show defects in USP26-deficient mice.

(a) Testis size was not affected by the absence of USP26. (b) PAS + Hematoxylin stained testis sections show a normal number and distribution of germ cells in the seminiferous tubules (**St**) and normal counts of spermatozoa in the epididymides (**Ep**) of *Usp26*^{21/Y} and *Usp26*^{Cat/Y} mice in comparison with *Usp26*^{WT/Y} mice. Bar in upper panels (St), 10 µm and in lower panels (Ep), 20 µm. (c) Comparative histopathological

analysis (hematoxylin+eosin staining) of ovaries from *Usp26*^{21/21}, *Usp26*^{Cat/Cat} and *Usp26*^{WT/WT} females at 4 months of age showing the presence of follicles, corpora lutea and a normal appearance of the stroma in absence of USP26. Bars represent 50µm.

Figure 3. Chromosome behaviour and synapsis proceed properly in spermatocytes lacking USP26. Double immunolabeling of SYCP3 (red) and SYCP1 (green) on spermatocyte spreads from *Usp26*^{21/Y}, *Usp26*^{Cat/Y} and *Usp26*^{WT/Y} mice. DNA was stained with DAPI (blue). No differences were observed on synapsis/desynapsis processes between USP26-deficient and WT spermatocytes. Bar in panels, 10 µm.

Figure 4. DSBs dynamics is not affected in absence of USP26. (a) Double immunofluorescence of γ-H2AX (green) with SYCP3 (red) in wild type and *Usp26* mutant spermatocytes showing no differences in the formation and repair of DSBs. DNA was stained with DAPI (blue). DSBs formation occurs at leptotene and they are processed during zygotene and early pachytene. Consequently, γ-H2AX labels intensely the whole nucleus at leptotene and declines until mid-pachytene where the staining is restricted to the unsynapsed sex bivalent. Plots under each panel (b) represent the quantification of fluorescence intensity at the different stages and shows no significant differences (n=60 cells from 2 different mice for each genotype). See Table S3 for numeric data; Bars represent Standard Deviation (SD). Welch's *t*-test analysis: * p<0.01; ** p<0.001; *** p<0.0001. Bar in panels, 10 µm.

Figure 5. USP26-deficient mice do not show meiotic recombination defects. (a) Immunostaining of MLH1 (green) and SYCP3 (red). DNA was stained with DAPI (blue). The number of MLH1 foci (corresponding to future chiasmata) in mutant spermatocytes resembles that observed in the wild type. Bar in panels, 10 µm. (b) Quantification of MLH1 foci in *Usp26*^{WT/Y}, *Usp26*^{21/Y} and *Usp26*^{Cat/Y} pachytene spermatocytes showing no significant differences (n= 52, 52 and 48 cells respectively from 2 different mice for each genotype). See Table S4 for numeric data; Bars represent Standard Deviation (SD). Welch's *t*-test analysis: * p<0.01; ** p<0.001; *** p<0.0001.

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Usp26^{WT/Y}
Usp26^{21/Y}
Usp26^{Cat/Y}
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Felipe-Medina Table 1

Table 1. USP26 deficiency has no impact on fertility. Numeric data (mean of pups per litter) obtained from the mating of $Usp26^{WT/Y}$, $Usp26^{21/Y}$ and $Usp26^{Cat/Y}$ males and $Usp26^{WT/W}$, $Usp26^{21/21}$ and $Usp26^{Cat/Cat}$ females with $Usp26^{WT/WT}$ females and $Usp26^{WT/Y}$ males, respectively. 8 weeks old mice were mated for 3 months (6 mice/genotype).

		Mean (offspring)	SD	n
Males	$Usp26^{WT/Y}$	7.0	1.47	15
	$Usp26^{21/Y}$	6.9	2.31	18
	$Usp26^{Cat/Y}$	6.4	2.32	16
Females	$Usp26^{WT/WT}$	7.4	2.33	14
	$Usp26^{21/21}$	7.2	1.89	16
	$Usp26^{Cat/Cat}$	7.9	2.28	16

Legends to Supplementary Figures

Figure S1. The α USP26 (W12) antibody is not able to detect the endogenous protein. (a-b) Double immunolabeling of α USP26 W12 (green) and α SYCP3 (red) in spermatocyte spreads from *Usp26*^{WT/Y}, *Usp26*^{21/Y} and *Usp26*^{Cat/Y} mice. DNA was stained with DAPI (blue). The stages where *Usp26* expression has been described are shown: (a) leptotene/zygotene and (b) spermatogonia/round spermatids (RS), Bar in panels, 10 μ m. (c) Western blot analysis of whole testis protein extracts from *Usp26*^{WT/Y} and *Usp26*^{21/Y} (left) and *Usp26*^{WT/Y} and *Usp26*^{Cat/Y} (right) animals with the α USP26 W12 antibody. Loading control was performed with anti- α Tubulin (lower panels).

Figure S2. The α USP26-M antibody is not able to identify endogenous USP26. (a-b) Double immunolabeling of α USP26-M (green) and α SYCP3 (red) in spermatocyte spreads from *Usp26*^{WT/Y}, *Usp26*^{21/Y} and *Usp26*^{Cat/Y} mice. DNA was stained with DAPI (blue). The stages where *Usp26* expression has been described are shown: (a) leptotene/zygotene and (b) spermatogonia/round spermatids (RS), Bar in panels, 10 μ m. (c) Western blot analysis of whole testis protein extracts from *Usp26*^{WT/Y} and *Usp26*^{21/Y} (left) and *Usp26*^{WT/Y} and *Usp26*^{Cat/Y} (right) animals with the α USP26-M antibody. An unspecific pattern was detected in both the wild type and the USP26-deficient extracts. Loading control was performed with anti- β Actin (lower panel).

Table S1. Sequences of the complementary oligonucleotides annealed to obtain the sgRNAs employed in the mutant mice generation by CRISPR-Cas9.

Mutant	Guide	Sequence
21	1	5'-CACCGTGGCCGAATACTACCTTGA-3' 5'-AAACTCAAGGTAGTATTCGG CCAC-3'
21	2	5'-CACCGTGAGCCTAGCACAAGTGCAC-3' 5'-AAACGTGCACTTGTGCTAGGCTCAC-3'
Cat	3	5'-CACCGAGGTGTTTCCCACATTAGGC-3' 5'-AAACGCCTAATGTGGGAAACACCTC-3'
Cat	4	5'-CACCGATTAATGATTTATTCAACCA-3' 5'-AAACTGGTT GAATAAATCATTAATC-3'

Table S2. Sequences of the primers used for PCR amplification of the CRISPR-Cas9 targeted region and mutant mice genotyping.

Mutant	Primer	Sequence
21	Forward	5'-TCCTTCAAACCTATGGCGAAGA-3'
21	Reverse	5'-AAGGTTGATGAGGATGGAAGCA-3'
Cat	Forward	5'-TTCTCCAGTTCTGTTGGAAACACAC-3'
Cat	Reverse	5'-TTTCAAACAAGCAGTTGCATCA-3'

Table S3. Quantification of γ -H2AX intensity. Measurement of the γ -H2AX intensity in *Usp26*^{WT/Y}, *Usp26*^{21/Y} and *Usp26*^{Cat/Y} spermatocytes at different stages (leptotene, zygotene, pachytene). Mean (intensity in arbitrary units, a.u.) and standard deviation (SD) are indicated, n= number of analysed nuclei (from 2 different mice for each genotype). No significant differences were obtained (Welch's *t*-test analysis: * *p*<0.01; ** *p*<0.001; *** *p*<0.0001). The measured fluorescence intensity was normalized versus the highest fluorescence level found in each replicate (encompassing one WT mouse, one 21 and one Cat) to obtain comparable values between replicates.

γ -H2AX Leptotene		Mean (intensity, a.u.)	SD	n
Spermatocytes	WT	0,475	0,208	60
	21	0,481	0,168	60
	Cat	0,489	0,201	60

γ -H2AX Zygotene		Mean (intensity, a.u.)	SD	n
Spermatocytes	WT	0,375	0,192	60
	21	0,378	0,163	60
	Cat	0,382	0,160	60

γ -H2AX Pachytene		Mean (intensity, a.u.)	SD	n
Spermatocytes	WT	0,014	0,008	60
	21	0,011	0,007	60
	Cat	0,012	0,007	60

Table S4. Quantification of MLH1 foci. Measurement of the number of MLH1 foci in *Usp26*^{WT/Y}, *Usp26*^{21/Y} and *Usp26*^{Cat/Y} spermatocytes. Mean and standard deviation (SD) are represented, n= number of analysed nuclei (from 2 different mice for each genotype). No significant differences were obtained (Welch's *t*-test analysis: * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$).

MLH1 foci		Mean	SD	n
Spermatocytes	WT	22,96	2,45	52
	21	22,76	2,14	52
	Cat	22,33	1,83	48

Table S5. mRNA expression of different USPs identified by da Cruz et al. (2016).

Min Max

Units:RPKM	2C fraction (Spermatogonia and somatic cells)	LZ fraction (Lepto-Zygotene)	PS fraction (Pachytene)	RS fraction (Round Spermatids)
Usp54	10,54	10,66	9,72	10,23
Usp33	15,75	18,81	8,31	1,42
Usp10	4,38	4,83	3,54	0,13
Usp14	10,71	14,47	4,85	3,5
Usp9x	30,43	56,25	5,35	10,5
Usp5	8,78	13,9	4,69	1,68
Usp24	20,14	26,96	15,11	7,67
Usp40	10,48	18,98	6,08	1,36
Usp28	6,97	12,99	3,5	1,98
Usp3	16,2	37,89	6,6	4,3
Usp53	7,44	12,29	5,23	3,27
Usp26	5,88	62,51	2,32	3,36
Usp34	41,24	77,15	37,99	16,42
Usp19	10,36	16,62	10,4	6,36
Usp7	33,47	41,83	29,12	32,91
Usp31	3,37	9,08	2,74	1,03
Usp38	4,61	8,48	2,87	5,07
Usp11	9,22	15,43	11,57	9,11
Usp32	13,01	73,83	33,44	14,41
Usp47	20,02	59,6	41,22	28
Usp37	7,38	19,56	15,5	16,56
Usp39	4,39	10,05	5,83	8,19
Usp36	4,73	3,94	1,16	1,11
Usp27x	3,48	2,69	0,29	0
Usp6nl	3,7	4,8	5,47	2,06
Usp22	28,1	15,28	1,43	0,52
Usp48	32,7	14,43	8,06	8,43
Usp12	6,67	6,62	4,94	15,29
Usp11	41	8,72	1,79	6,97
Usp35	8,55	1,39	0,46	0,06
Usp49	4	7,07	7,42	7,52
Usp16	12,92	25,75	32,11	36,11
Usp51	0,14	0,48	0,82	5,24
Usp1	10,56	21,4	100,04	47,7
Usp42	7,47	15,55	42,71	40,61
Usp8	28,16	33,25	103,33	48,87
Usp4	9,74	11,25	13,63	13,89
Usp15	17,33	24,08	50,52	63,35
Usp29	0,17	0,41	1,71	2,63
Usp44	0,58	1,46	8,45	24,44
Usp20	3,11	3,31	5,57	11,15
Usp45	7,58	4,18	5,42	7,41
Usp21	10,02	2,76	7,9	3,27
Usp46	4,66	3,45	3,55	6,59
Usp30	3,6	3,08	6,06	5,93
Usp25	6	1,73	14,9	41,3
Usp2	1,6	2,09	58,64	40,43
Usp50	0,49	0,58	19,72	31,74

Table S6. mRNA expression of different USPs identified by Margolin et al. (2014).

6dpp, spermatogonia; 10dpp leptotene; 12dpp zygotene; 14dpp pachytene; 16 dpp pachytene-diplo-
tene; 18dpp secondary spermatocytes; 20dpp round spermatids; 38 dpp adult mice.
Units: RPKM

Min								Max
	6dpp	10dpp	12dpp	14dpp	16dpp	18dpp	20dpp	38dpp
Usp37	19,28	31,96	29,67	26,33	24,01	20,88	19,55	24,25
Usp26	22,46	35,77	28,20	26,08	20,93	11,53	9,28	1,91
Usp9x	23,50	38,57	31,72	26,88	21,51	13,56	11,88	6,89
Usp53	13,20	21,40	16,60	14,08	11,04	8,89	10,38	5,52
Usp33	17,68	22,67	18,06	16,28	16,79	11,64	13,32	3,16
Usp14	18,03	25,38	19,78	20,24	18,29	15,30	14,22	7,89
Usp46	12,56	19,07	15,33	13,55	12,89	13,55	12,57	11,01
Usp45	3,86	6,51	4,02	4,28	4,82	4,87	5,82	3,85
Usp28	30,73	31,11	31,38	26,69	25,23	17,54	16,53	6,60
Usp3	23,55	30,36	32,21	29,69	22,35	15,74	14,58	7,27
Usp9y	1,73	3,77	3,79	4,10	2,35	1,57	1,96	0,91
Usp11	16,88	13,72	15,98	11,25	8,87	5,77	4,77	2,44
Usp35	3,09	2,29	1,84	1,63	0,94	0,67	1,13	0,97
Usp38	7,71	9,78	9,85	10,90	8,90	8,75	7,48	7,82
Usp22	59,23	44,31	43,64	33,56	29,21	19,81	16,64	7,98
Usp48	25,64	21,63	21,75	18,94	18,87	13,17	12,79	8,56
Usp13	0,75	0,47	0,53	0,34	0,69	0,27	0,23	0,02
Usp25	7,04	6,00	4,81	5,96	3,97	4,32	4,03	32,76
Usp12	8,51	10,19	12,36	11,18	11,29	6,93	7,29	14,96
Usp40	7,66	9,09	9,14	13,84	12,92	11,34	9,79	4,50
Usp7	40,10	44,71	42,68	49,40	47,25	45,11	41,41	51,80
Usp24	4,23	5,07	5,19	5,89	6,26	5,60	6,49	3,30
Usp18	1,03	0,62	0,86	0,42	0,92	0,98	0,62	0,38
Usp10	20,76	13,51	18,49	16,50	19,11	14,80	12,16	9,48
Usp32	19,63	40,05	41,44	47,38	50,53	62,04	65,97	37,34
Usp43	1,41	0,44	0,53	0,83	0,30	0,35	0,52	0,98
Usp54	8,30	10,37	14,54	16,67	17,18	14,16	11,53	6,65
Usp52	20,35	15,74	19,49	20,86	17,87	18,17	19,62	11,66
Usp11	13,07	16,49	18,72	17,57	20,34	28,36	27,89	19,44
Usp39	23,63	20,49	17,76	23,51	26,71	31,68	26,25	21,59
Usp47	25,41	36,61	38,63	48,64	48,42	62,73	68,58	57,81
Usp15	13,11	20,38	20,98	30,34	32,87	46,44	46,80	78,05
Usp16	9,11	12,95	16,74	19,82	18,62	21,18	22,95	26,52
Usp1	9,96	12,11	10,39	12,56	19,31	43,84	57,99	50,03
Usp8	24,17	31,50	38,14	46,93	45,84	81,37	96,31	104,00
Usp42	14,50	17,39	20,79	23,98	25,90	32,47	37,00	37,01
Usp5	33,10	22,64	31,03	35,51	38,75	31,92	30,11	20,99
Usp4	27,31	28,39	32,02	40,50	42,21	50,68	48,29	59,88
Usp21	18,16	13,33	15,05	16,37	15,34	19,10	20,36	17,18
Usp44	1,47	0,80	0,98	0,96	2,03	3,98	4,32	38,32
Usp49	7,89	7,29	9,02	8,73	8,38	8,74	8,52	13,29
Usp6nl	8,29	6,51	7,65	7,34	6,90	7,29	8,30	7,49
Usp20	6,34	4,10	6,59	7,28	8,79	15,15	16,00	29,45
Usp50	0,04	0,00	0,33	0,00	0,20	0,06	0,60	62,96
Usp30	11,30	7,66	8,69	10,57	9,44	12,89	12,94	12,83
Usp29	0,17	0,11	0,19	0,44	0,75	0,74	0,51	0,53
Usp2	5,04	4,48	5,75	15,15	25,94	56,47	66,82	141,24
Usp19	58,70	40,41	60,20	64,58	67,27	54,04	51,44	43,37

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