1 **Mutant *Zp1* results in Zona Pellucida Lacking** 2 **and Female Infertility in Rats and Humans**

3 **Running head: *Zp1* Mutation Causes ZP lacking and Infertility**

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5 Chao Lv^{1,*}, Hua-Lin Huang^{1,2,*}, Yan Wang¹, Tian-Liu Peng¹, Hang-Jing Tan¹,
6 Ming-Hua Zeng¹, Ru-Ping Quan¹, Hong-Wen Deng^{1,3,†}, and Hong-Mei Xiao¹,

7 †

8 ¹Center of Reproductive Health and System Biology and Data Information,
9 Institute of Reproductive & Stem Cell Engineering, School of Basic Medical
10 Science, Central South University, Changsha 410008, China; ²Guangzhou
11 Institutes of Biomedicine and Health, Chinese Academy of Sciences,
12 Guangzhou 510530, China; ³Tulane Center for Bioinformatics and
13 Genomics, Department of Global Biostatistics and Data Science, School of
14 Public Health and Tropical Medicine, Tulane University, New
15 Orleans, 70112, U.S.

16 * These authors contributed equally to this work.

17 † Authors for correspondence: Hong-Mei Xiao: hmxiao@csu.edu.cn;

18 Hong-Wen Deng: hwdeng@tulane.edu

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1 Summary statement

2 Rat model mirrored completely the phenotypes observed in humans,
3 infertility and abnormal eggs that lack a zona pellucida, through the
4 negative effects of *ZP1* mutation.

5

6 Abstract

7 Zona pellucida (ZP) plays a vital role in reproductive processes including
8 oogenesis, fertilization and preimplantation development of embryo. The
9 ZP of humans is composed of four glycoproteins (ZP1-ZP4), same as rats
10 ZP. Our previous research reported a first case of human infertility due to
11 *ZP1* mutation, but the mechanism was unclear. Here we developed a
12 genome editing *in vivo* rat model and a co-transfected *in vitro* cell model
13 to investigate the pathogenic effect. In rat homozygous for the
14 homologous mutation, ZP were absent in all of collected eggs. Further the
15 growing and fully grown oocytes in the mutant ovaries completely lack a
16 ZP but with detectable intracellular ZP1 protein. After mating with male
17 rats, none of the mutant female rats got pregnant. Moreover, the co-
18 transfected cell experiments and the ovarian experiments showed that
19 the truncated ZP1 sequestered intracellularly ZP3 and ZP4 to impede their
20 release outside, resulting in an intracellular accumulation of ZP1, ZP3 and
21 ZP4, leading to absence of ZP in mutant oocytes. Our results clearly

1 establish the causal role of *ZP1* mutation on ZP defects and female
2 infertility.

3 **Keywords:** zona pellucida; infertility; Zp1; oocytes; rat model

4

5 INTRODUCTION

6 The mammalian zona pellucida (ZP) is a glycoprotein matrix
7 surrounding oocytes, eggs and embryos up to the time of blastocyst
8 hatching (Fig. 1A) (Wassarman, 2008). It plays an important role in
9 production of oocytes, in recognition of gametes, in induction of
10 acrosome reaction, in prevention of polyspermy and in protection of the
11 embryo during its transfer through the fallopian tube (Gupta et al., 2009;
12 Matzuk et al., 2002). When oocytes enter the growth phase, the *ZP* genes
13 were activated early or late, encoding the zona glycoproteins to form the
14 ZP, which is critical for mature, ovulation and fertilization of oocytes *in vivo*
15 (Avella et al., 2014; Wassarman, 1999). During follicular development, the
16 ZP can be first observed as extracellular patches surrounding oocytes in
17 primary follicles which then gradually increase in width around growing
18 oocytes (Gook et al., 2008).

19 Our previous study reported a form of familial infertility with an
20 autosomal recessive mode of inheritance (OMIM 615774), characterized

1 by eggs lacking a ZP (Fig. S1) (Huang et al., 2014). We determined that a
2 frameshift mutation (I390fs404X) in the *ZP1* gene was co-segregated with
3 the ZP defects and infertility in the family. *ZP1* encodes one of the four
4 zona glycoproteins in humans, which is vital for the formation of zona
5 matrix (Ganguly et al., 2010; Nishimura et al., 2019). Based on both
6 humans and rats ZP were constructed of four zona glycoproteins (ZP1-ZP4)
7 (Boja et al., 2005; Lefievre et al., 2004), we developed a rat model that
8 carried a homologous deletion in the *Zp1* gene and a cell model co-
9 transfected with genes encoding the normal and mutant zona proteins
10 (Ma et al., 2017), to investigate the causal effects of this mutation on ZP
11 defects and infertility.

12 Materials and Methods

13 CRISPR/Cas9-mediated gene editing

14 By analyzing the homologous genomic sequences of humans and rats,
15 we determined the 8-bp deletion site in the rat *Zp1* genomic DNA
16 sequence (GenBank accession number MK527841), which is located on
17 chromosome 1. Using VectorBuilder software (www.vectorbuilder.com),
18 we designed a *Zp1* guide RNA (gRNA) and a donor oligo (Table S1) bearing
19 the locus-specific homologous sequence and the intended 8-bp deletion
20 according to the quality scores and off-target analysis (Fig. S2). The Cas9

1 mRNA, gRNAs generated by transcription *in vitro* and donor oligo were
2 coinjected into fertilized eggs to generate the 8-bp deletion in the rat *Zp1*
3 gene (Table S1). The injected zygotes were transplanted into the womb of
4 foster mothers (Sprague Dawley rat, SD rat) (Sander and Joung, 2014) .
5 The founder mice (F0) were obtained in approximately 3 weeks. All animal
6 experiments were approved by Department of Zoology, Central South
7 University. The positive F0 offspring were selected to mate with wild type
8 (WT) rats to generate hybrid F1 offspring, some of which could carry
9 heterozygous *Zp1* mutation with stable inheritance. The F1 heterozygous
10 rats were cross-hybridized with each other to generate additional F2
11 homozygous mutant type (MT) rats. The *Zp1*^{WT/WT} and *Zp1*^{MT/MT} female rats
12 were hybridized with *Zp1*^{WT/WT} male rats to study their fertility.

13 **DNA, RNA and protein analysis**

14 The test primers were designed to match the segment of gDNA and
15 transferred anti-cDNA containing the 8-bp deletion (Table S2). Genomic
16 DNAs were obtained from tail biopsy specimens and detected by Sanger
17 sequencing. According to the results of the off-target analysis, the
18 designed primers were located on the promoter region of the *Zp1* gene
19 and the potential off-target sites (Table S3). RNAs from ovarian tissues of
20 the rats were dealt with reverse transcription-polymerase chain reaction

(RT-PCR) to subject to polyacrylamide gel electrophoresis (PAGE) analysis and Sanger sequencing analysis to identify the targeted 8-bp deletion.

Glycoproteins from ovarian tissues were extracted from 8-week-old female rats. The ovarian tissues were homogenized with M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA) supplemented with Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific). Following centrifugation (16,000 g, 4°C), ovarian lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes, which were probed with antibodies directed against a peptide that had been mapped near the N-terminus of ZP1 (G-20, Santa Cruz, CA, USA, Fig. S4) or against GAPDH (Santa Cruz) and detected by secondary antibodies (Santa Cruz) with an ECL western blotting kit (Pierce Biotechnology).

***In Vitro* Studies of ZP**

The oocytes collected from the patient in the family were observed under a light microscope (Fig. S1) (Huang et al., 2014). All collection and experiments involving samples from patients with infertility were approved the Institute of Reproduction and Stem Cell Engineering, Central South University. All patients provided written informed consent.

1 Some 8-week-old female rats were sacrificed 21 hours after injection
2 of human chorionic gonadotropin (hCG, 45 IU), and others were sacrificed
3 69 hours after injection of pregnant mare serum gonadotropin (PMSG, 45
4 IU). Oocyte cumulus complexes (OCCs) were recovered from *Zp1*^{MT/MT}
5 female rats by ovarian puncture. The OCCs were immersed in
6 hyaluronidase droplets, and the cumulus granule cells were gently
7 removed by pipetting to isolate eggs for observation under a visible light
8 microscope.

9 To highlight the ZP, periodic acid Schiff (PAS) staining was conducted.
10 Ovaries from 8-week-old females (during the sexual maturity period)
11 expressing one of three genotypes were of similar size and morphological
12 appearance. After fixation in 4% paraformaldehyde solution (16-20 hours,
13 room temperature, RT), sections of paraffin-ovarian tissue chips were
14 sliced to a thickness of 4 µm at 100 µm intervals. The mounted sections
15 were stained with PAS reagent and hematoxylin and scanned with a slide
16 scanner (Pannoramic MIDI, 3D HISTECH).

17 **Protein-Protein Interaction Analysis**

18 The expression vectors for ZP1^{WT}, ZP1^{MT} (I390fs404X), ZP2, ZP3 and
19 ZP4 were cloned and inserted into the eukaryotic expression vector
20 plasmid pENTER. In addition, different tags were fused to the C-terminus

1 of genes into *the* vector plasmid pcDNA3.1 (+) to encode ZP1^{MT}-FLAG,
2 ZP1^{WT}-FLAG, ZP2-MYC, ZP3-V5 and ZP4-HA to avoid the interference of
3 endogenous proteins from tool cells (Fig. S3). The information for the ZP
4 cDNA sequence was acquired from NCBI (www.ncbi.nlm.nih.gov).

5 Human embryonic kidney 293T (HEK293T) cells (China Center for
6 Type Culture Collection, Wuhan, China) were grown (37°C, 5% CO₂) to 70-
7 80% confluence in dulbecco's modified eagle medium (DMEM)
8 supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher
9 Scientific, Waltham, MA, USA). Transient transfections were performed
10 with Lipofectamine 3000 (Thermo Fisher Scientific). For each transfection,
11 6 µl of the Lipofectamine 3000 transfection reagent was added to 125 µl
12 of Opti-MEM (Gibco) to which 2.5 µg of template plasmid had been added
13 (cotransfection of ZP1^{MT} or ZP1^{WT} with ZP2, ZP3 or ZP4) and incubated (5
14 minutes, RT). The mixture was added to the wells with growing cells.
15 Transiently transfected cells were harvested at 24~48 hours for analysis.

16 For coimmunoprecipitation (co-IP) analysis, the proteins from
17 cotransfected cell lysates after 24 hours were precipitated with an
18 antibody against the N-terminus of ZP1 (G-20, Santa Cruz). Then, the
19 collected precipitates were analyzed by immunoblotting (IB) with
20 antibodies raised against ZP2 (C-7, Santa Cruz), ZP3 (H-300, Santa Cruz) or
21 ZP4 (I-14, Santa Cruz) to detect coprecipitated zona glycoproteins (Fig. S4).

1 For IB analysis of ZP1, ZP2, ZP3 and ZP4, zona proteins with specific
2 tags 48 hours after transfection from the culture medium were enriched
3 by IP with a FLAG-tag antibody for labeling ZP1 (AT0022, CAMTAG, WI,
4 USA), a MYC-tag antibody for labeling ZP2 (AT0045, CAMTAG), an V5-tag
5 antibody for labeling ZP3 (AT0497, CAMTAG) and a HA-tag antibody for
6 labeling ZP4 (AT0046, CAMTAG).

7 **Fluorescent staining**

8 For immunofluorescence (IF) staining, ovarian tissues were isolated and
9 fixed in 3% paraformaldehyde (3-5 hours or overnight, RT), rinsed and
10 transferred to 70% ethanol. The tissues were dehydrated and embedded
11 in methacrylate and were cut into 4 mm sections, which were rehydrated
12 and rinsed three times with phosphate-buffered saline (PBS). The sections
13 were blocked with 5% bovine serum albumin (BSA) for 1 hour and
14 incubated (1 hour at 37°C) with antibodies directed against ZP1 (D-4,
15 Santa-Cruz), ZP2 (PA5-75949, Abcam, Cambridge, United Kingdom), ZP3
16 (G-1, Santa-Cruz Science) or ZP4 (PA5-37086, Abcam), respectively, and
17 washed three times with PBS, incubated with Alexa-Fluor 555-conjugated
18 donkey anti-rabbit secondary antibodies (Life Technologies, Ghent,
19 Belgium), washed three times in PBS, and counterstained with DAPI
20 (Technologies). Confocal images were obtained with a microscope (ZEISS

1 LSM 880 + Airyscan, Jena, Germany).

2 Following the above protocol, cells that were double-transfected with
3 plasmids carrying ZP1^{WT} or ZP1^{MT} and ZP2, ZP3 or ZP4, were examined by
4 confocal laser-scanning microscopy, using Alexa-Fluor 488- and Alexa-
5 Fluor 594-conjugated secondary antibodies (Abcam, Cambridge, United
6 Kingdom).

7 **Statistical Analysis**

8 For each group, the mean $\pm$ standard error (s.e.) was calculated.
9 Kruskal-Wallis Test and chi-square test were used for statistical analysis of
10 enumeration data; significance was assumed at $P < 0.05$.

11 **Results and Discussion**

12 **Targeted mutation in *Zp1* gene**

13 Since ZP of both humans and rats are composed of four zona
14 glycoproteins, while the mice ZP consists of three glycoproteins(ZP1-ZP3),
15 the basic structure of ZP in humans and rats may be more similar than that
16 in mice (Boja et al., 2005; Familiari et al., 2006; Lefievre et al., 2004).
17 Although most previous studies exploring the *in vivo* role of the ZP in
18 fertility have been based on mouse models, particularly those utilizing
19 knockouts of ZP genes(Liu et al., 1996; Rankin et al., 1999; Rankin et al.,

2001), we chose rat as the animal model for our study to explore the role of truncated ZP1 protein contributing to infertility.

Based on bioinformatics analysis, wild type ZP1 of rat and humans resemble one another, with 67.9% sequence identity (<https://www.uniprot.org/blast/uniprot>), and the 8-bp deletion at nucleotides 1174-1181 (TCTTCTCA) of the CDS (Coding sequence) of rat *Zp1* (NM_053509.1) resulted in a premature stop codon expected to give rise to a protein truncated at amino acid 401 (I379fs401X) (Fig. 2A). The genotypes of the mutant rats were confirmed using Sanger sequencing (Fig. 2B). The results of PAGE and sequence also showed the mRNA of *Zp1*^{MT/MT} rat carrying the 8-bp deletion (Fig. 2C).

The results of western blotting to examine the proteins from the *Zp1*^{MT/MT} female rats showed a truncated ZP1 protein (Fig.2D), suggesting the retention of the N-terminal domains (e.g., N-terminal signal sequence, the trefoil domain, and the first half of the ZP domain) and the absence of the C-terminal domains (e.g., external hydrophobic patch, consensus furin cleavage site, transmembrane domain, cytoplasmic tail, and the second half of the ZP domain) (Fig.2E) (Ganguly et al., 2010; Gupta, 2018; Monne et al., 2008; Zhao et al., 2003). Similarly, the truncated ZP1 protein of rat, generated for this study by an 8bp deletion, closely resembles the truncated mutant ZP1 that we identified in humans, with 65% sequence

1 identity.

2 **The homozygous mutant rats were infertile and produced eggs** 3 **lacking ZP**

4 The fertility of the mutant rats was assessed. Wild-type males were
5 caged with $Zp1^{WT/W}$ and $Zp1^{MT/MT}$ females over at least one pregnant cycle
6 in a breeding period of 6 months. Live births occurred 3 weeks later. While
7 $Zp1^{WT/WT}$ females repeatedly gave birth to normal litter sizes, $Zp1^{MT/MT}$
8 female rats never gave birth when caged with several fertile males
9 ($p=0.003$, Table 1). A continuous breeding period of 12 months was
10 observed for $Zp1^{MT/MT}$ females, indicating that $Zp1^{MT/MT}$ females were
11 sterile.

12 In order to get insight into the mechanisms underlying the infertility
13 elicited by ZP1 mutation, oocytes were recovered from oviducts of the two
14 genotypes of female rats after hyperstimulation. In agreement with the
15 infertility of $Zp1^{MT/MT}$ females, all of the mutant eggs completely lacked a
16 ZP (Fig. 1A). These findings suggest that the significantly infertility from
17 $Zp1^{MT/MT}$ female rats may be attributed to the eggs without a ZP lost their
18 capacity for *in vivo* fertilization or early embryonic development.

19 To elucidate the defect in oogenesis of $Zp1^{MT/MT}$ rats, we performed
20 detailed ovarian morphological analyses. The ovarian sections detected by

1 morphological analysis with PAS staining showed the absence of ZP in
2 mutant homozygous oocytes and disorganized follicular structure at all of
3 the follicular development stages, especially among the antral follicles (Fig.
4 1B).

5 The rat model mirrored completely the phenotype observed in our
6 patients (Fig. 1A) (Huang et al., 2014), which is similar to the phenotypes
7 in female mice that lack either *Zp2* or *Zp3* (Liu et al., 1996; Rankin et al.,
8 2001), and is more severe than those in mice carrying mutations in *Zp1*
9 gene (Rankin et al., 1999; Wang et al., 2019), suggesting rat may be a more
10 suitable animal model for studying human infertility due to ZP defect.

11 **The intracellular abnormality of zona proteins leads to ZP** 12 **lacking**

13 To examine the location and the trafficking of zona pellucida proteins
14 in oocytes, ovarian sections were stained with monoclonal antibodies
15 specific to ZP1, ZP2, ZP3, or ZP4, respectively, and the IF stained specimens
16 were detected with confocal laser scanning microscopy. Mutant ZP1,
17 normal ZP3 and ZP4 were not detected in the extracellular ZP area but
18 were found throughout the cytoplasm in the oocytes of mutant
19 homozygotes, whereas ZP2 was observed only on the cytoplasmic
20 membrane. In contrast, all four zona proteins were detected within the

1 zona matrix of normal growing oocytes but not inside ova (Fig. 3A). As
2 reported earlier, the absence of ZP3 appeared to preclude the formation
3 of a visible extracellular zona matrix. These results suggested that mutant
4 ZP1 cannot be transported out by itself and also affects the transport of
5 ZP3 and ZP4, so their non-release out of ova can cause absence of ZP.

6 To investigate the functional mechanisms of mutant ZP1 on the
7 intracellular trafficking of zona proteins, co-IP and IF analysis was
8 performed to explore the interactions between ZP1 and other zona
9 proteins. Truncated ZP1 interacted with ZP3 and ZP4 but not ZP2 inside
10 the co-transfected cells, where normal ZP1 did not interact with ZP2, ZP3
11 or ZP4 (Figs. 3B and S5).

12 To test the secretion of zona proteins, IB of IP-enriched secreted
13 proteins with specific tags from the medium showed that only ZP2 was
14 detected in the medium from the ZP1^{MT} cotransfected system, while all
15 four ZP proteins were detected in the medium from the ZP1^{WT}
16 cotransfected system (Fig. 3C).

17 These data indicated that the mutant ZP1 protein prevented the
18 secretion of ZP3, ZP4 and itself out of the ova to interact with ZP2 to form
19 the basal filaments of the zona matrix, resulting in lack of formation of ZP
20 of oocytes (Fig. 3D). These results could be explained by the retention of
21 the partial ZP domain and the absence of cytoplasmic-tail and

1 transmembrane domains in the mutant ZP1 protein that promotes
2 intracellular sequestration of ZP3 and ZP4 proteins, thus impeding their
3 section outside of ova to form the ZP (Jimenez-Movilla and Dean, 2011).
4 The pathogenic mechanism of the familial infertility was identified as a
5 gain of function in recessive mutations that the abnormal protein in
6 homozygotes causes disease by affecting the function of other proteins in
7 addition to the loss of its own function (Hastings et al., 2009; Wilkie, 1994).

8 *Zp1* mutation can lead to congenital deficiency that loss of ZP which
9 leads to infertility, which is verified in the rat model. The interaction
10 between truncated ZP1 and ZP3 or ZP4 is gained in the cell model, which
11 affects their normal transport and section. Our results suggest that normal
12 ZP1 is crucial for structure of ZP and fertility of oocytes.

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1 Competing interests:

2 The authors declare no competing financial interests.

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18

19

1 Tables

2 Table 1. Fertility of $Zp1^{WT/WT}$ and $Zp1^{MT/MT}$ female rats

	$Zp1^{WT/WT}$	$Zp1^{MT/MT}$
Pregnancy cycle*	2.75±0.43 (4)	0 (6)
Litter size*	11±1.87 (4)	0 (6)
*Average ± s.e. (numbers of animals).		

3

1 Figures and Legends

2 Figure 1

3

4 Fig. 1 Morphological analysis of ZP in human and rat models

5 **A**, oocytes from humans, which were cultured and separated from
6 granulosa cells (left). The morphology of the ZP in ovulated eggs from rats
7 under a light microscope: eggs of wild-type rats and mutant eggs lacking
8 a ZP (right). **B**, High magnification images of oocytes at different stages of
9 follicular development from *Zp1^{MT/MT}* and *Zp1^{WT/WT}* rats. Arrows point to
10 ova. Following the primary follicle stage, the oocytes showed a

- 1 disorganized follicular structure with the absence of the zona pellucida in
- 2 mutant oocytes; the antral oocytes were especially clean without any
- 3 nearby granulosa cells.

1 **Figure 2**

2

3 **Fig. 2 Analysis of *Zp1* mutation in rat models**

4 **A**, the schematic diagram for the generation of mutant *Zp1* rats by the

5 CRISPR/Cas9 system. The mutant *ZP1* rats showed a homozygous 8bp-

6 deletion at nucleotides 1174-1181 (TCTTCTCA) of rat *ZP1* CDS

7 (CCDS37918), leading to a frameshift and the formation of a premature

8 stop codon, I379fs401X. **B**, Genotyping of *Zp1*^{MT/MT} rats was performed by

9 PCR and Sanger sequencing of the genomic DNA. Mutant *Zp1* rats

10 exhibited a homozygous mutation [an 8 bp deletion at nucleotides 1174-

1 1181 (TCTTCTCA) of the CDS of rat *Zp1* gene], while a heterozygous
2 mutation was observed in heterozygotes. **C**, The results of PAGE and
3 sequence of *Zp1* cDNA in *Zp1*^{MT/MT} and *Zp1*^{WT/WT} rats. **D**, The ZP1 proteins
4 from the ovaries of *Zp1*^{MT/MT} and *Zp1*^{WT/WT} rats detected with a special
5 antibody raised against ZP1. GAPDH was used as an internal standard. **E**,
6 The domain organization of wild-type (upper) and mutant (lower) ZP1. The
7 predicted structure shows the deleted portions (e.g., part of the ZP
8 domain in yellow, consensus furin cleavage site in violet, the
9 transmembrane domain in blue and other C-terminal domains).

1 **Figure 3**

2

3 **Fig. 3 Functional study and Pathogenic model of *Zp1* mutation**

4 **A**, The growing oocytes imaged with the use of confocal microscopy. The

5 ovaries were isolated from *Zp1*^{MT/MT} and *Zp1*^{WT/WT} rats, fixed, sectioned

6 and stained with antibodies against ZP1 (first row), ZP2 (second row), ZP3

7 (third row) or ZP4 (fourth row). Fluorescent signals indicating mutant ZP1

1 were not detected in the extracellular ZP of *Zp1^{MT/MT}* rat ovaries but were
2 detected inside the oocytes, which was similar to ZP3 and ZP4, whereas
3 only ZP2 was detected on the cytoplasmic membrane. In normal oocytes,
4 ZP1, ZP2, ZP3 and ZP4 were present in the ZP surrounding the oocytes but
5 were not detected internally. **B**, The interactions of ZP1^{WT} and ZP1^{MT} with
6 ZP glycoproteins. Lanes 1 and 3 show ZP1^{WT} cotransfected with ZP2, ZP3
7 and ZP4; lane 3 shows IP with an anti-ZP1 antibody; and lane 1 shows the
8 input. Lanes 2 and 4 show ZP1^{MT} cotransfected with ZP2, ZP3 and ZP4; lane
9 4 shows co-IP with an anti-ZP1 antibody; and lane 2 shows the input. **C**,
10 The media assayed by IP and IB, using monoclonal tagged antibodies
11 against FLAG, MYC, V5 and HA. **D**, The model of the mechanism that
12 prevents the formation of the ZP in homozygotes. ZP1, ZP2, ZP3 and ZP4
13 are vital normal zona proteins expressed during the formation of a normal
14 ZP in humans. Blue shapes denote wild-type alleles and yellow shapes
15 refer to mutant alleles.