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Mutant ZP1 in Familial Infertility

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Summary

The human zona pellucida is composed of four glycoproteins (ZP1, ZP2, ZP3, and ZP4) and has an important role in reproduction. Here we describe a form of infertility with an autosomal recessive mode of inheritance, characterized by abnormal eggs that lack a zona pellucida. We identified a homozygous frameshift mutation in *ZP1* in six family members. In vitro studies showed that defective ZP1 proteins and normal ZP3 proteins colocalized throughout the cells and were not expressed at the cell surface, suggesting that the aberrant ZP1 results in the sequestration of ZP3 in the cytoplasm, thereby preventing the formation of the zona pellucida around the oocyte.

In mammals, the zona pellucida is a glycoprotein matrix that surrounds oocytes and has an average thickness of 17 μm (Fig. 1A).[1] It is vital for the production of oocytes in early development,[2] for fertilization[3] (e.g., in gamete recognition at the zona pellucida[4] and in the prevention of polyspermia), and for the protection of early embryos before implantation.[5] In mice, the targeted knockout of either the gene encoding ZP2 or the gene encoding ZP3 precludes the development of a zona pellucida, leading to sterility.[6,7] However, to our knowledge, previous studies of defects in the zona pellucida in humans have not identified mutations in any of the four genes (*ZP1*, *ZP2*, *ZP3*, and *ZP4*) encoding the zona pellucida glycoproteins (Fig. 1B),[8,9] which are secreted by growing oocytes.[10]

Current models of the development of the zona pellucida involve the independent synthesis and transport of each zona pellucida protein through the endomembrane system toward the cell surface.[11] After the cleavage of C-terminal domains near the plasma membrane, the zona pellucida proteins are released into the extracellular space.[12] Repeating zona pellucida

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proteins form long filaments, which are cross-linked (probably by ZP1) to form the zona pellucida (Fig. 2A).[13,14]

Here we describe four sisters who received a diagnosis of primary infertility, which is defined as never having given birth to a child and not having conceived after 1 year of unprotected sexual intercourse.[15] The eggs of two of the four sisters had a similar abnormal phenotype: they were not surrounded by a zona pellucida. (We did not observe any eggs from the other two sisters.) We detected a homozygous frameshift mutation in *ZP1* in six members of the family (five sisters and one brother), resulting in the truncation of ZP1. We hypothesize that this mutation prevents the formation of the zona pellucida matrix and results in sterility.

Case Reports

The proband (family member IV-3) was 32 years old and the third sister in a family of Han Chinese origin. The determination of the family's ancestral origin was based on the proband's report that each of her four grandparents were from a rural area with little migration that had been inhabited exclusively by Han Chinese for hundreds of years. She received a diagnosis of primary infertility at 28 years of age, after 2 years of cohabitation with her partner (for 1 year before marriage and 1 year after marriage). During the 6 years preceding presentation (from 26 to 32 years of age), she had not conceived, despite unprotected sexual intercourse, with ejaculation, approximately 3 times per week. Her age at menarche was 13 years, her menstrual cycle was normal (lasting 3 to 7 days and occurring every 29 days), and she had no dysmenorrhea. Infertility-related examinations did not reveal abnormalities (see Table 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). Assisted reproductive treatments, which entailed three attempts at artificial insemination and one attempt at in vitro fertilization, were unsuccessful (for details, see the Methods section in the Supplementary Appendix).

The clinical characteristics of the fifth-born sister (family member IV-5) were similar to those of the index patient with regard to primary infertility, the basic medical history, and a full set of infertility-related examinations (Table 2 in the Supplementary Appendix). Treatment with intracytoplasmic sperm injection failed to produce a viable embryo (for details, see the Methods section in the Supplementary Appendix). Two other sisters in the family also had unexplained infertility. Thus, four of a total of six sisters in this family received a diagnosis of primary infertility.

The study was approved by two institutional ethics committees (one at the Reproductive and Genetic Hospital of CITIC–Xiangya and the other at the Institute of Reproduction and Stem Cell Engineering, Central South University) and was performed between November 2010 and August 2013. All patients provided written informed consent.

Methods

Morphologic Studies

Oocyte cumulus complexes were collected from family members IV-3 and IV-5 by means of follicular aspiration and were cultured for several hours before the removal of granular cells for in vitro fertilization or intracytoplasmic sperm injection (as detailed in the Methods section in the Supplementary Appendix). The oocytes were examined with the use of a micromanipulator and a light microscope.

Genetic Studies

We selected four candidate genes (*ZP1, ZP2, ZP3,* and *ZP4*) to screen for potential mutations[8,15,16] and used polyacrylamide-gel electrophoresis (PAGE) and polymerase-chain-reaction (PCR) sequencing to analyze the coding region and exon–intron boundaries of *ZP1*, which is located on chromosome 11 (for details, see the Methods section in the Supplementary Appendix).[1]

In Vitro Studies of Oocytes

We analyzed ZP1 and ZP3 in vitro in oocytes from family member IV-5 and controls. Immunofluorescence staining was used to tag antibodies,[7] and the stained specimens were examined with the use of a confocal laser-scanning microscope (see the Methods section in the Supplementary Appendix).

Results

Characteristics of the Eggs

Six eggs were obtained from the index patient, and four eggs from the fifth sister. The zona pellucida was not observed in any of the eggs from the index patient (Fig. 1D). The first egg from the fifth sister was examined immediately after follicular aspiration, and two other eggs were examined after culture for 3 hours and digestion with hyaluronidase to remove granular cells. None of these three eggs possessed a zona pellucida. We were unable to separate the fourth egg from granular cells with hyaluronidase (Fig. 1E). Vitrification was used to freeze all four eggs for further studies.

Genealogic Characterization

Family members III-1 and III-2, the parents of the affected women, were first cousins. Primary infertility was the only abnormal clinical feature observed among their offspring (in family members IV-1, IV-3 [the proband], IV-4, and IV-5). The zona pellucida was completely absent around the eggs that we examined from family members IV-3 and IV-5. Family members IV-6 and IV-7 were not married and had not cohabitated with a sex partner for more than 1 year. Consequently, we could not make a diagnosis with respect to fertility.[15,17] Family member IV-2 was the only person in generation IV with a biologic descendant (Fig. 1C). The pattern of inheritance of infertility in the family and the presence of consanguinity are consistent with a homozygous mutation for an autosomal recessive trait in the affected sisters.

Identification of *ZP1* Mutation

PCR primers were designed to amplify specific regions of *ZP1*, and the products were visualized by means of silver staining after PAGE. Sequencing of candidate genes *ZP1*, *ZP2*, *ZP3*, and *ZP4* revealed that six members of generation IV (the four patients and two unmarried family members) carried a homozygous frameshift deletion of 8 bp encompassing nucleotides 1169 through 1176 in *ZP1* (GenBank accession number, KJ489454). Four family members who had given birth (II-1, II-2, III-2, and IV-2) had a heterozygous deletion at the same site. (Fig. 3A and 3B, and Fig. S1 and S2 in the Supplementary Appendix). Family member III-1 was deceased, and we were unable to obtain a DNA sample for analysis. We speculate that he had either a homozygous or a heterozygous mutation. We did not detect the frameshift mutation in a sample of 210 Chinese Han controls, nor did we detect the mutation in two public databases: the 1000 Genomes Browser (www.ncbi.nlm.nih.gov/variation/tools/1000genomes), which contains genetic data for 394 people of Chinese Han origin, and the Human Gene Mutation Database (www.hgmd.org).

Characterization of Mutant ZP1 Protein

The frameshift mutation results in a premature stop codon (I390fs404X) that predicts a truncated protein of 404 amino acids, with a terminal sequence of 15 amino acids bearing no homology to the corresponding amino acid sequence of ZP1 (Fig. 3B, and Fig. S3 in the Supplementary Appendix). (The nonmutant protein consists of 638 amino acids.) Previous studies have shown that ZP1 shares features of the other three zona pellucida proteins,[5,18] with 34% to ZP2, 23% to ZP3, and 46% to ZP4. The truncated ZP1 protein is predicted to comprise the N-terminal signal sequence, the trefoil domain, and the first half of the zona pellucida domain. Other domains, such as the external hydrophobic patch, the consensus furin cleavage site, the transmembrane domain, the cytoplasmic tail, and the second half of the zona pellucida domain, are probably absent (Fig. 3C).[1,19, 20]

Absence of Zona Pellucida in Mutant Oocytes

After thawing the four frozen eggs from the fifth sister, we recovered two. In contrast to the findings in eggs obtained from controls, no evidence of extracellular ZP1 or ZP3 was detected in the two thawed eggs on immunofluorescence analysis or confocal microscopy. ZP3 and mutant ZP1 showed diffuse staining in the eggs from the fifth sister, in contrast with the concentrated staining of ZP3 and nonmutant ZP1 near the periphery of unaffected ova. Merged images showed colocalization of ZP1 and ZP3 signals throughout the cytoplasm in eggs from the patient, whereas colocalization of these signals was observed only near the plasma membrane and within the zona matrix of normal eggs (Fig. 2B, and Fig. S4 in the Supplementary Appendix).

Discussion

We detected an autosomal recessive pattern of inheritance for a homozygous deletion in *ZP1* in certain members of a family affected by infertility. Our data suggest that the mutation prevents the formation of the zona pellucida around ova: all observed oocytes from affected family members lacked a zona pellucida when examined with light microscopy (Fig. 1D and 1E, and Fig. 2B). In oocytes from family member IV-5, we did not observe the expression of

ZP1 and ZP3 in the region normally occupied by the zona matrix (Fig. 2B). We therefore conclude that oocytes from affected family members lacked the zona matrix, which accounted for the infertility.

The cosegregation between the I390fs404X mutation in *ZP1* and infertility supports but does not prove causality. Nonmutant ZP1 contains 638 amino acids, of which 270 make up the zona pellucida domain, a protein-aggregation module spanning amino acids 279 to 549[18,21]; the mutation is predicted to disrupt this domain and yet permit interaction between the mutant ZP1 protein and the other zona pellucida proteins (ZP2, ZP3, and ZP4).[22] The loss of C-terminal domains in the mutant ZP1, however, would be expected to abrogate essential normal functions, such as C-terminal cleavage,[19] transmembrane localization,[23] assembly,[20] prevention of intracellular polymerization,[12] and trafficking of the protein both intracellularly and to the exterior of the ovum.[11]

In mouse models, oocytes have been shown to secrete only three zona pellucida proteins (ZP1, ZP2, and ZP3),[1] and it has been proposed that the zonae pellucidae of mice and humans are similar in structure.[13] In mice, long filaments of repeating ZP2–ZP3 units are cross-linked by ZP1 (which has 70% homology with human ZP1) to form a meshlike matrix.[13] Complete knockout of either *Zp2* or *Zp3* in female mice results in infertility, with eggs devoid of a zona pellucida,[6,7] which is similar to the phenotype described here. However, female mice that lack *Zp1* produce two types of eggs: one with an apparently normal zona pellucida and the other with a loosely organized and swollen zona pellucida.[24]

We propose that the difference between the oocytes of mice lacking *Zp1* and those of the affected women homozygous for the truncating *ZP1* mutation described here is explained by the retention of the partial zona pellucida domain and the absence of C-terminal domains (cytoplasmictail and transmembrane domains) in the mutant protein that promotes interaction with and sequestration of the other zona pellucida proteins.[22] The intracellular sequestration of these proteins could impede their trafficking to the extracellular milieu, thus precluding the formation of a zona pellucida (Fig. 2A) and leading to infertility. The amount of truncated ZP1 protein in heterozygous subjects, in contrast, may be insufficient to sequester other zona pellucida proteins to the extent that the zona pellucida is functionally compromised.[25] The observation in an affected sister (IV-5) of the apparent absence of ZP1 and ZP3 surrounding eggs and the colocalization of ZP1 and ZP3 inside the eggs (Fig. 2B) supports our hypothesis.

Our data are consistent with family members IV-1 and IV-4 being infertile and support the prediction that family member IV-7 is infertile. The single male family member (IV-6) is homozygous for the mutation and is unmarried. Although *ZP1* is not among the 164 genes that have been found to affect male fertility,[17] whether he is fertile is not known.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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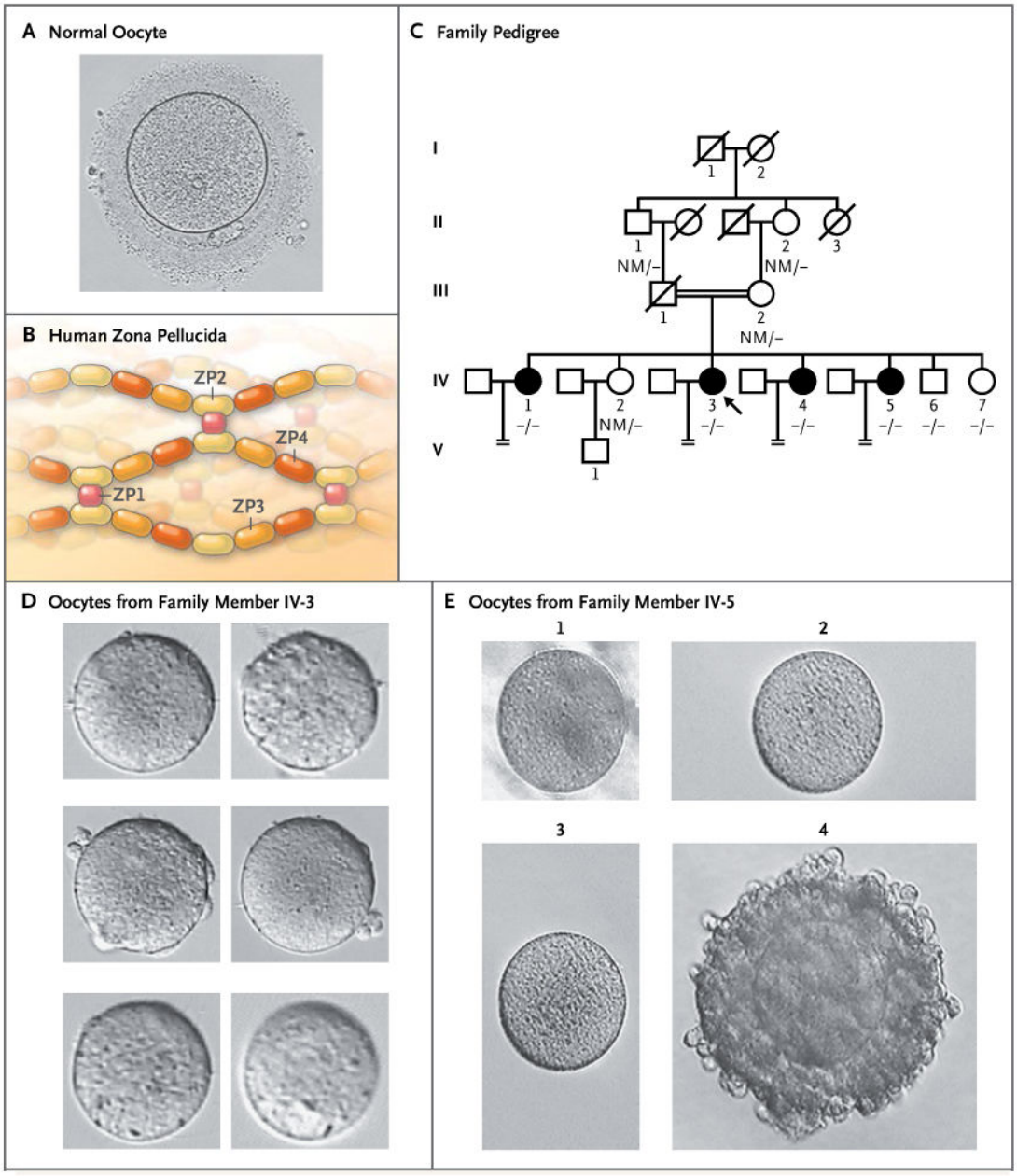

Figure 1. Phenotypic Features of the Patients

Panel A shows a normal oocyte with surrounding insoluble zona pellucida, and Panel B shows a structure diagram of the human zona pellucida. Panel C shows the pedigree of the family. Squares denote male family members, circles female family members, and solid symbols affected members; slashes denote deceased family members, equal signs infertility, and the double line a consanguineous marriage. The arrow indicates the index patient (family member IV-3). The zona pellucida 1 (*ZP1*) genotype for each person is indicated, with NM indicating no mutation in the normal allele and a dash (–) indicating the homozygous deletion of the eight nucleotides in *ZP1* that are responsible for infertility. Panel D shows six oocytes from the index patient, which were cultured for 6 hours after

follicular aspiration, separated from granulosa cells, and then imaged immediately. Panel E shows four oocytes from the fifth sister (IV-5). Oocytes 1, 2, 3, and were successfully isolated; oocyte 4 was closely surrounded by granular cells.

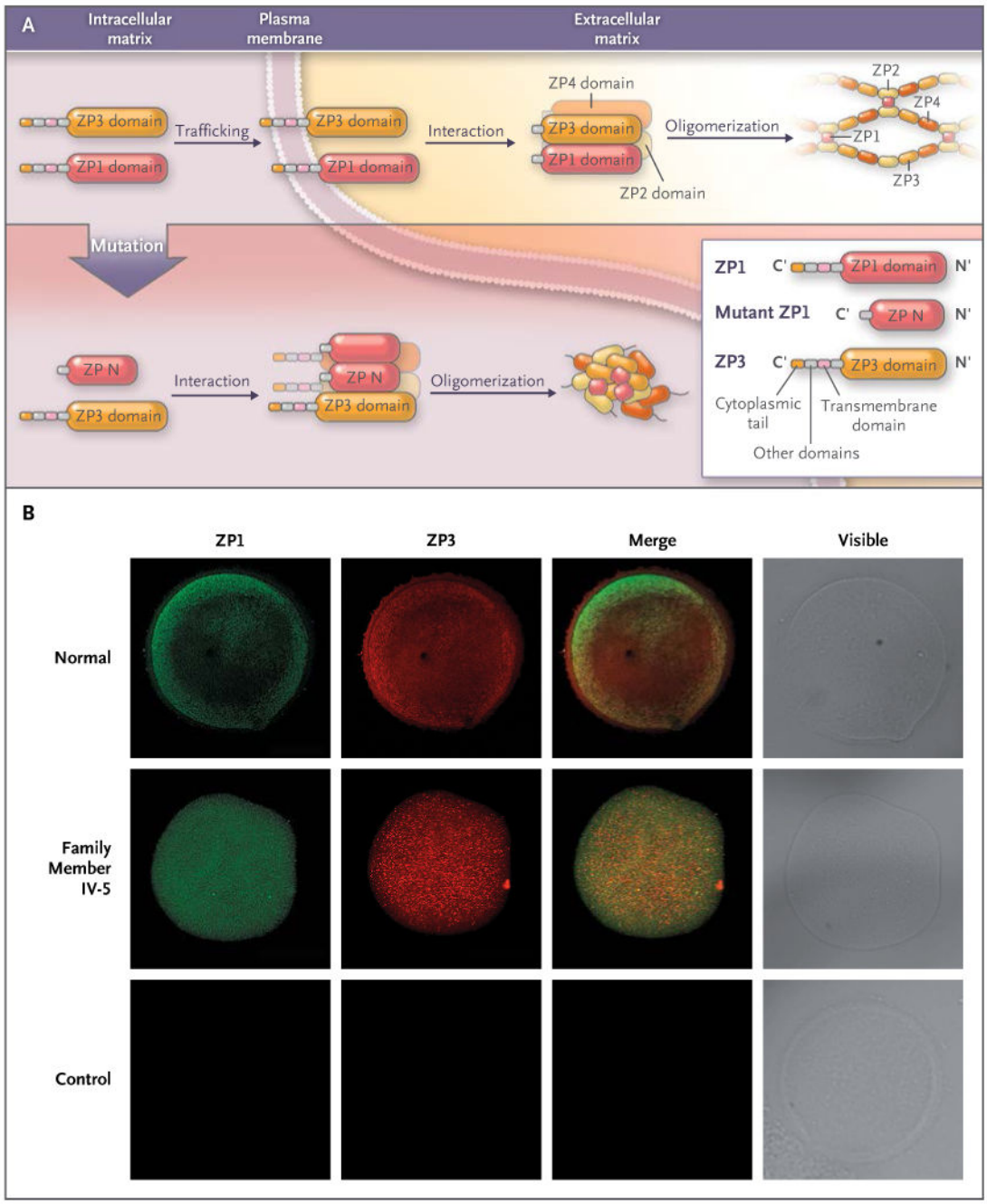

Figure 2. (facing page). Immunofluorescence in Eggs and Pathogenic Model

Panel A depicts the process through which the zona pellucida (ZP) proteins form a normal zona pellucida (upper diagram) and the hypothetical pathogenic mechanism that prevents the formation of the zona pellucida (lower diagram); ZP1, ZP2, and ZP3 are vital normal ZP proteins. Panel B shows oocytes imaged with the use of confocal laser scanning microscopy and visible inverted microscopy. The fluorescent signals of ZP proteins 1 (green) and 3 (red) were imaged individually and merged (orange). The signal pattern of normal oocytes is different from that of the oocytes from the family member IV-5.

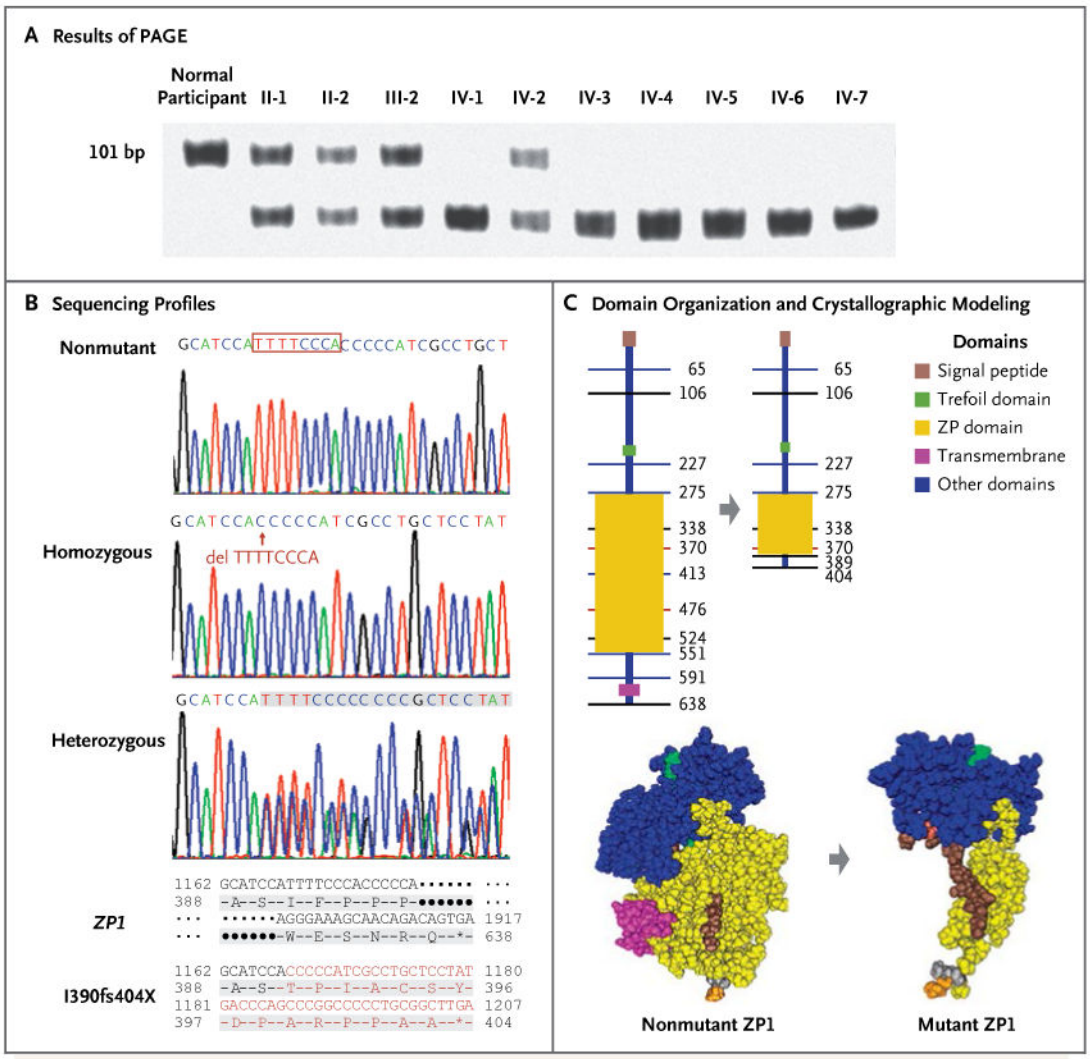

Figure 3. Genetic and Bioinformatic Analysis of *ZP1*

Panel A shows the results of polyacrylamide-gel electrophoresis (PAGE) of the products of polymerase-chain-reaction amplification of a region of exon 7. The results are consistent with six members of the family (IV-1, IV-3, IV-4, IV-5, IV-6, and IV-7) having a homozygous deletion and other family members (II-1, II-2, III-2, and IV-2) having a heterozygous mutation. Panel B shows a normal sequencing profile and profiles for the homozygous and heterozygous deletion of TTTTCCCA between codons 1169 and 1176 in *ZP1*. Six family members had a homozygous deletion, and four carried a heterozygous deletion, which is consistent with the results of PAGE. The mutation leads to a frameshift and the formation of a premature stop codon, I390fs404X. Panel C shows the domain organization (top) and the crystallographic models (bottom) of nonmutant (left) and mutant ZP1 (right). The schematic diagram shows the deleted portions of ZP1 (e.g., the transmembrane domain, part of the zona pellucida domain, and other C-terminal domains).