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Female Fertility and the Mammalian Egg's Zona Pellucida

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Short title: Zona Pellucida and Fertility

Abstract: All mammalian eggs are surrounded by a relatively thick extracellular matrix (ECM) or zona pellucida (ZP) to which free-swimming sperm bind in a species-restricted manner during fertilization. The ZP consists of either three (e.g., *Mus musculus*) or four (e.g., *Homo sapiens*) glycosylated proteins, called ZP1-4. These proteins are unlike those found in somatic cell ECM, are encoded by single-copy genes on different chromosomes, and are well conserved among different mammals. Mammalian ZP proteins are synthesized as polypeptide precursors by growing oocytes that will become ovulated, unfertilized eggs. These precursors are processed to remove a signal-sequence and carboxy-terminal propeptide and are secreted into the extracellular space. Secreted ZP proteins assemble into long, crosslinked filaments that exhibit a structural repeat due to the presence of ZP2-ZP3 dimers every 140 Å or so along filaments. Filaments are crosslinked by ZP1 and are oriented either perpendicular, parallel, or randomly to the plasma membrane of eggs depending on their position in the ZP. Free-swimming mouse sperm recognize and bind to ZP2 or ZP3 that serve as sperm receptors. Acrosome-intact sperm bind to ZP3 oligosaccharides and acrosome-reacted sperm bind to ZP2 polypeptide. ZP filaments fail to assemble in the absence of either nascent ZP2 or ZP3 and results in mouse eggs that lack a ZP and female infertility. Gene sequence variations due to point, missense, or frameshift mutations in genes encoding ZP1-4 results in human eggs that lack a ZP or have an abnormal ZP and female infertility. These and other features of the mouse and human egg's ZP are discussed here.

Keywords: Oogenesis, Extracellular Matrix, Female Fertility, Gene Targeting, Gene Sequence Variations, Sperm Binding, Zona Pellucida, ZP Domain, ZP Genes, ZP Proteins, ZP Subdomains.

Abbreviations: AI, acrosome-intact; AR, acrosome-reacted; aa, amino acid; N-terminus, amino-terminus; CG, cortical granules; CTP, carboxy-terminal propeptide; C-terminus, carboxy-terminus; CFCS, concensus furin cleavage-site; ECM, extracellular matrix; Ig, immunoglobulin; kD, kilodaltons; MW, molecular weight; SS, signal-sequence; TD, trefoil domain; TMD, transmembrane domain; ZP, zona pellucida; ZPD, zona pellucida domain; ZP-C, ZPD carboxy-terminal subdomain; ZP-N, ZPD amino-terminal subdomain.

Introduction

The extracellular matrix (ECM) that surrounds animal somatic cells can affect cellular adhesion and migration, cell-to-cell communication, as well as gene expression, differentiation, and morphogenesis (Hynes, 2009; Franz et al., 2010). Somatic cell ECM consists of proteoglycans, such as hyaluronic acid, heparin-, chondroitin-, and keratin-sulfate, and fibrous proteins, such as collagens, elastins, fibronectins, and laminins. On the other hand, ECM of mammalian oocytes and eggs, called the zona pellucida (ZP) (**Fig. 1**), is composed of a unique set of glycosylated proteins, called ZP1-4, that differ from proteins present in ECM of somatic cells (Litscher and Wassarman, 2015, 2018, 2020a,b). Genes encoding ZP proteins are expressed solely in females by growing oocytes at a time when the ZP first appears and then continues to thicken throughout oocyte growth. Eggs from fish, amphibia, reptiles, and birds are also surrounded by ECM, called the vitelline envelope or ZP, that consists of several proteins closely related to ZP1-4. Like somatic cell ECM, the mammalian ZP can affect cellular adhesion and communication during ovarian follicular development that culminates in production of a Graafian follicle. The ZP is the site of receptors for species-restricted binding of free-swimming sperm during fertilization of eggs and participates in prevention of polyspermy following fertilization.

The mouse and human egg's ZP are ≃6 μm and ≃20 μm thick, respectively, are a viscoelastic ECM that consists of an extensive network of long, crosslinked filaments, and are porous to small viruses and relatively large macromolecules, such as antibodies and enzymes. The ZP can be dissolved under conditions that do not result in breaking of covalent bonds (e.g., at relatively low pH), suggesting that its structural integrity is maintained by non-covalent interactions between ZP proteins. Following fertilization, the ZP undergoes physical changes, but remains around cleavage-stage embryos until the expanded blastocyst stage when embryos hatch from the ZP and implant in the uterus.

Results of experiments with homozygous knockout mice strongly suggest that the presence of both ZP2 and ZP3 is essential for ZP formation during oogenesis and the absence of either protein causes female infertility. Furthermore, evidence from *in vitro* fertilization (IVF) clinics strongly suggests that mutation of genes encoding human ZP1-4 can prevent normal ZP formation during oogenesis and cause female infertility. The latter findings are of considerable interest since female infertility is currently estimated to affect about 10% of married women worldwide.

Here we discuss a number of features of the mouse and human ZP, from their synthesis, structure, and assembly into filaments to deleterious effects on female fertility that result from failure to produce a normal ZP during oogenesis.

Mammalian Oogenesis

Oogenesis is the complex process by which unfertilized eggs are produced in the female's ovaries and ensures an increase in genotypic variation, a decrease in egg ploidy from diploid (2n) to haploid (n), and an accumulation of small molecules, macromolecules, and organelles used to regulate and sustain early embryogenesis (**Fig. 2**).

Oogenesis begins during fetal development with formation of primordial germ cells (PGC) in the yolk-sac endoderm and the region of the allantois arising from the primitive streak (Austin and Short, 1972; Conti and Chang, 2016; Larose et al., 2019; Telfer et al., 2023). PGC migrate through the endodermal epithelium of the hindgut into dorsal mesentery and then to the genital ridges in the roof of the coelom, the site of gonad (ovary or testis) development. Genital ridge formation occurs at day-10 in mice and week-5 in humans under the influence of several transcription factors, such as Gata4, Fog2, and WT1. PGC proliferate during migration and for a short time after arriving at the genital ridges where they become either female (oogonia) or male (spermatogonia) germ cells. In females, PGC become oogonia that proliferate mitotically following gonadal differentiation into ovaries. Oogonia are transformed into meiotic oocytes that have four-times the haploid complement of DNA, are at various stages of meiotic prophase (leptotene, zygotene, pachytene, and diplotene), and whose chromosomes exhibit chiasmata due to pairing of homologous chromosomes and crossing-over. This pool of non-growing oocytes is the sole source of unfertilized eggs in sexually mature adults and the number of oocytes entering the growth phase is a function of the size of the pool of non-growing oocytes (Krarup et al., 1969; Peters et al., 1973; Yoshihara et al., 2023). Following birth, most non-growing oocytes are arrested in late diplotene (dictyate stage) of meiosis, where they remain until stimulated to grow and resume meiosis (meiotic maturation) at the time of ovulation. Only fully-grown oocytes resume meiosis and follicular growth is controlled by pituitary gonadotropins.

Oocytes are contained within ovarian follicles that grow concomitantly with oocytes under control of the hypothalamic-pituitary-gonadal axis. Oocyte growth is regulated within the ovary and is a period of intense metabolic activity as reflected in marked changes in oocyte ultrastructure

(Wassarman and Josefowicz, 1978). Oocytes initially are surrounded by a single layer of somatic cells that becomes several layers of granulosa or cumulus cells by the time oocytes complete their growth phase. A thecal layer is first distiguishable, outside of and separated by a basal lamina from the cumulus cells, when the cumulus layer is a few layers thick. When oocyte growth is complete, granulosa cells continue to proliferate extensively and produce a Graafian follicle with a large cavity or antrum filled with follicular fluid. As the antrum expands, the oocyte takes up an acentric position surrounded by a few layers of cumulus cells, the *cumulus oophorous*, with the innermost layer constituting the *corona radiata*.

The ovarian follicle is a functional syncytium that provides routes of bidirectional communication between oocytes and follicle cells, with follicle cells regulating oocyte growth and oocytes regulating follicle development (Doherty et al., 2022; Marchais et al., 2022). Long processes from the *corona radiata* penetrate the ZP and form gap junctions with oocyte microvilli (Matzuk et al., 2002; Wassarman, 2002; Li and Albertini, 2013; Clarke, 2018). Gap junctions link cytoplasm of different cells and allow electrical and biochemical coupling of cells, as well as exchange of ions, metabolites, and second- messengers between cells. These junctions permit passage of small molecules, <1,000 MW, into oocytes from the surrounding syncytium of granulosa cells (Simon and Goodenough, 1978; Marchais et al., 2022; Crozet et al., 2023). Gap junctions are responsible for bidirectional communication between oocytes and follicle cells in the ovary and support the health of growing oocytes and developing follicles. It has been proposed that the presence of a ZP is required to stabilize contacts between oocyte microvilli and innermost follicle cell (*corona radiata*) projections that traverse the ZP and form gap junctions with oocyte plasma membrane (Wassarman and Litscher, 2022a).

In sexually mature adults, fully-grown oocytes in Graafian follicles undergo meiotic maturation in response to a surge of luteinizing hormone. Meiotic maturation involves dissolution of the nuclear (germinal vesicle, GV) membrane, condensation of chromatin into bivalents, separation of homologous chromosomes, emission of a polar body containing one set of chromosomes, and arrest of meiosis at metaphase II. Completion of meiosis, with separation of chromatids and emission of a second polar body, is triggered by fusion of a single sperm with an egg and restores the egg to a diploid (2n) state.

At birth, human females have 1-2 million oocytes, but by puberty only about 300,000-500,000 remain. The number of oocytes decreases continually with age, reaching about 5,000-10,000 at

age 40 and zero-1,000 at menopause, age 45-55. Since only one egg is ovulated during each reproductive cycle, with only about 300-400 eggs ovulated over 30 years, the substantial decrease in oocytes is attributable to massive degeneration of oocytes over time. This pattern of oocyte loss with age is characteristic of virtually all female mammals.

Appearance of the ZP during Oogenesis

Non-growing oocytes that populate mammalian ovaries following birth are in contact with a few flat mitotic cells and are not surrounded by a ZP. As soon as oocytes begin to grow, genes encoding ZP proteins are expressed, nascent ZP proteins are synthesized, and a thin ZP is laid down around oocytes that continues to thicken throughout oocyte growth (**Fig. 3**). For example, for mouse oocytes the ZP increases from zero (non-growing oocyte) to ≃6 μm (fully-grown oocyte) thick as oocytes grow from ≃12 μm to ≃80 μm in diameter over 14-21 days (Wassarman and Litscher, 2022a). At the same time, the few mitotic cells associated with non-growing oocytes differentiate and multiply profusely during and especially after oocyte growth, and give rise to a very large Graafian follicle (≃600 μm diameter in mice; ≃20 mm in humans) from which unfertilized eggs are ovulated in response to hormones. At about the time of ovulation, fully-grown oocytes resume meiosis (meiotic maturation), undergo the first meiotic reductive division (separation of homologous chromosomes), emit the first polar body, and arrest at metaphase II in the oviduct (unfertilized eggs). Unfertilized eggs remain arrested in meiosis until stimulated to complete meiosis, with emission of a second polar body (separation of chromatids), due to sperm-egg fusion (fertilization). The follicle left behind in the ovary following ovulation becomes an endocrine gland, the *corpus luteum*, that secretes progesterone and supports pregnancy.

Expression of ZP Genes during Oogenesis

Mouse and human ZP proteins are encoded by single-copy genes located on different chromosomes (Gupta, 2018; Lunsford et al., 1990). In *Mus musculus*, genes encoding ZP1-3 are located on chromosomes 19, 7, and 5, respectively, *ZP4*, a pseudogene, is located on chromosome 13 (**Table 1**). In humans, genes encoding ZP1-4 are located on chromosomes 11, 16, 7, and 1, respectively (Table 1). ZP genes are highly conserved among mammals (e.g., coding regions and 5'-flanking regions) and permits the human ZP promoter to utilize transcriptional machinery of mouse oocytes (Liang and Dean, 1993). ZP genes are expressed coordinately and exclusively by

growing oocytes and not by somatic cells. *Cis*-acting sequence elements in the 5'-flanking regions of ZP genes and *trans*-acting factors regulate oocyte-specific expression (Wassarman and Litscher, 2021).

The number of copies of ZP mRNA present per mouse oocyte or egg increases from undetectable levels (< 1,000 copies) in non-growing mouse oocytes (≃12 μm diameter) to hundreds-of-thousands of copies in mid-stage growing (≃50 μm diameter) and fully-grown (≃80 μm diameter) oocytes (Roller et al., 1989). ZP mRNA is undetectable in oocytes that have undergone meiotic maturation and become unfertilized eggs (< 1,000 copies). ZP transcripts that accumulate during oocyte growth are selectively degraded during meiotic maturation and ovulation, a period when oocyte chromosomes condense and transcription is terminated. ZP mRNA is undetectable in cleavage-stage embryos.

Characteristics of ZP proteins

The mouse (*Mus musculus*) ZP consists of three heterogeneously glycosylated proteins, called ZP1-3, that have apparent MWs of ≃200, ≃120, and ≃83 kD, respectively (Bleil and Wassarman, 1980a; Shimizu et al., 1983; Wassarman, 1988). However, it should be noted that the ZP of several other mouse species consists of four proteins, ZP1-4 (Lefievre et al., 2004; Izquierdo-Rico et al., 2021); *ZP4* is a pseudogene in *Mus musculus* (Goudet et al., 2008). The human ZP consists of four heterogeneously glycosylated proteins, called ZP1-4, that have apparent MWs of ≃200, ≃75, ≃55, and ≃65 kD, respectively (Lefievre et al., 2004; Conner et al., 2005; Gupta, 2018). Mouse and human ZP2-4 are monomers and ZP1 is a dimer held together by a single intermolecular disulfide (Greve and Wassarman, 1985; Nishimura et al., 2019). Mouse and human ZP proteins are glycosylated with both asparagine-linked (N-linked) and serine/threonine-linked (O-linked) oligosaccharides that may be sialylated and sulfated. As a result, the glycosylated proteins are relatively acidic and migrate as broad bands on denaturing gels (Wassarman, 1988).

ZP proteins are synthesized by growing oocytes as polypeptide precursors containing an N-terminal signal-sequence (SS) that targets nascent protein to the secretory pathway and a C-terminal propeptide (CTP) (**Fig. 4,** see below). Mouse ZP1-3 precursors are 623, 713, and 424 aa in length, respectively, and human ZP1-4 precursors are 638, 745, 424, and 540 aa in length, respectively (Gupta, 2018; Wassarman and Litscher, 2018). Both the SS and CTP are removed from nascent ZP proteins by proteases prior to secretion of the proteins into the extracellular space.

The nascent proteins are localized to unusually large secretory vesicles, about 2 μm in diameter, that originate from the swollen Golgi during oocyte growth; oocyte vesicles are about 10-times larger than those of somatic cells (Qi et al., 2002). Secretory vesicle membrane fuses with the oocyte's plasma membrane, nascent ZP proteins are deposited into the extracellular space, and the proteins are incorporated into the innermost layer of the thickening ZP.

ZP proteins from different mammals are well conserved, exhibiting ≃60-98% sequence identity (Litscher and Wassarman, 2014, 2015). Mouse and human ZP1-3 exhibit ≃80-87% sequence similarity and ≃58-68% sequence identity. ZP1 and ZP4 are homologous proteins, their genes are paralogous, and their sequences are ≃72% similar and ≃43% identical.

Domains and Subdomains of ZP Proteins

Nascent ZP proteins possess characteristic domains and subdomains that play specific roles (**Fig. 4**) (Jovine et al., 2005, 2007; Litscher and Wassarman, 2015, 2018). They have a signal-sequence (SS, ≃20-30 aa) at the N-terminus that targets them to the secretory pathway, a zona pellucida domain (ZPD, ≃270 aa) or ZP module (ZPM; Wilburn and Swanson, 2017) consisting of subdomains ZP-N (≃100 aa) and ZP-C (≃145 aa) connected by a linker region (≃25 aa), an internal hydrophobic patch (IHP), and a C-terminal propeptide (CTP) involved in secretion of ZP proteins. The CTP has a consensus furin cleavage-site (CFCS), an external hydrophobic patch (EHP), a hydrophobic transmembrane domain (TMD, ≃20 aa), and a cytoplasmic tail. ZP1 and ZP4 also have a trefoil domain (TD, ≃45 aa), a three-loop structure with three intramolecular disulfides.

Sequence elements present in the CTP and ZPD of nascent ZP proteins regulate their secretion by growing oocytes and polymerization into filaments in the extracellular space (Williams and Wassarman, 2001; Zhao et al., 2003; Jovine et al., 2004; Jimenez-Movilla and Dean, 2011; Litscher and Wassarman, 2018). For example, the two hydrophobic patches, IHP located in the ZPD and EHP located in the CTP, interact with each other and lock nascent ZP proteins in a conformation incompatible with polymerization into filaments. However, proteolytic excision of the CTP at its CFCS results in loss of the EHP, alters protein conformation, and allows secreted nascent ZP proteins to polymerize (**Fig. 5**). If cleavage at the CFCS does not take place, secretion of nascent ZP proteins is severely reduced and they accumulate in the endoplasmic reticulum

(Williams and Wassarman, 2001; Qi et al., 2002; Jovine et al., 2004). This pathway, that involves removal of inhibitory sequences from protein precursors and exposure of polymerization elements, also is found in a variety of other kinds of proteins (e.g., fibrinogen, fibrillin, tau protein, C9, and Cks1).

The ZPD is a bipartite structural element that consists of ≃270 aa and has eight (ZP3) or ten (ZP1, 2, and 4) conserved cysteine residues present as four (ZP3) or five (ZP1, 2, and 4) intramolecular disulfides. ZP-N always has two intramolecular disulfides (ZP1-4) and ZP-C has either two (ZP3) or three (ZP1, 2, and 4) intramolecular disulfides (Jovine et al., 2005; Plaza et al., 2010; Litscher and Wassarman, 2015). Mouse and human ZP2 have three additional ZP-N subdomains at their N-terminus (N1, N2, and N3), and mouse and human ZP1 and human ZP4 have one additional ZP-N subdomain (N1) at their N-terminus. The ZPD is a structural element present in all ZP proteins and is found in hundreds of other proteins that have diverse functions, from receptors to mechanical transducers, in a wide variety of organisms, from jellyfish, flies, and nematodes to humans (about 6 million years of evolution) (Jovine et al., 2005; Litscher and Wassarman, 2015).

ZP1-4 are prototypical ZPD proteins and a comparison of their ZPD sequences reveals that they are ≃90% similar and ≃73% identical. Furthermore, there is strong evidence to suggest that the ZP-N subdomain(s) is used for polymerization of nascent ZP proteins, as well as for polymerization of other ZPD-containing proteins, such as tectorin and uromodulin, into filaments (Jovine et al., 2002, 2006). There is some structural evidence to suggest that both ZP-N and ZP-C are required for polymerization of some ZPD-containing proteins (Stsiapanava et al., 2020). Mutations in genes encoding ZPD proteins can result in severe human pathologies, such as vascular and renal disease, deafness, infertility, and cancer.

Three-Dimensional Structure of ZP Proteins

Much of what is known about the three-dimensional structures of ZP proteins comes from X-ray diffraction studies carried out during the past 15 years (Monné et al., 2008; Han et al., 2010; Raj et al., 2017; Bokhove and Jovine, 2018). Such studies have revealed atomic details about ZP protein structure (**Fig. 7**), including: (1) ZPD subdomains ZP-N and ZP-C adopt immunoglobulin (Ig)-like folds despite the complete absence of sequence identity. ZP-N and ZP-C resemble C-type and V-type Ig-like domains, respectively, but are new Ig superfamily subtype structures. (2)

The ZP-N subdomain consists of an antiparallel sandwich of two β-sheets made up of eight strands of polypeptide that enclose a hydrophobic core; two disulfides clamp both sides of the sandwich. There is an exposed hydrophobic surface between two of the β-strands that could promote successive monomer interactions to generate polymers (filaments). (3) The ZP-C subdomain also consists of a β-sandwich comprising two stacked β-sheets, one with four and the other with six strands of polypeptide. (4) In ZP3 crystals, two molecules are arranged as homodimers in antiparallel orientation to form an asymmetric structure. Their ZPDs are bonded by electrostatic interactions between ZP-N and ZP-C of opposing molecules, ZP-N1: ZP-C2 and ZP-N2:ZP-C1. The strong structural similarity between ZP-N and ZP-C suggests that the ZPD may have arisen by duplication of an ancestral gene encoding a protein containing a single ZP-N.

Structure and Arrangement of ZP Filaments

The mouse and human ZP is composed of long, crosslinked filaments (**Fig. 6**). In mice the filaments are several μm in length, ≃70 Å in width, and have a ZP2:ZP3 dimer located every ≃140 Å along the filaments (Greve and Wassarman, 1985; Wassarman and Mortillo, 1991; Wassarman et al., 1996; Litscher and Wassarman, 2018). ZP1 serves as a crosslinker for the filaments and has a proline-rich N-terminus (≃100 aa, ≃17-21% proline) that may contribute to the elasticity of the ZP (Litscher and Wassarman, 2020a). The surface of the mouse ZP is covered with pores, ≃50 pores/ZP, giving it a spongelike or Swiss cheese appearance (Phillips and Shalgi, 1980). Polarized light microscopy revealed that filaments in the inner and outer layers of the mouse, hamster, and human ZP are oriented perpendicular and parallel, respectively, to the egg's plasma membrane; filaments in the intervening layer are oriented randomly (Keefe et al., 1997; Peletier et al., 2004; Litscher and Wassarman, 2020a). Filaments in the inner layer are more densely packed than those in the outer layer. It is possible that, as the circumference of oocytes increases during growth, about 7-fold in mice, stretching of the outer layer of matrix closest to the surface of the ZP leads to reorientation of filaments from a perpendicular to a parallel orientation with respect to the plasma membrane. It has been proposed that ZP filaments have some structural and physical features analogous to those of amyloids (Litscher and Wassarman, 2020a). However, it should be noted that ZP filaments are heteromeric aggregates (ZP2-ZP3) rather than homomeric aggregates typical of amyloids. Furthermore, there is some recent structural evidence that suggests ZP filaments do not resemble amyloids (Stsiapanava et al., 2020).

Binding of Sperm to the ZP

The acrosome is a lysosome-like vesicle derived from Golgi that sits over the nucleus in the apical region of sperm heads (Toshimori and Eddy, 2015). Both acrosome-intact (AI) and acrosome-reacted (AR) sperm can bind to the egg's ZP (Wassarman and Litscher, 2022b). To penetrate the ZP and fuse with the egg's plasma membrane (fertilization) sperm must undergo the acrosome reaction (Florman et al., 2008; Balbach et al., 2020). This reaction involves multiple fusions between outer acrosomal membrane and plasma membrane at the anterior region of the sperm head resulting in exposure of inner acrosomal membrane and associated acrosomal contents (**Fig. 8**). The reaction may occur as AI-sperm traverse the female oviduct, transit through cumulus cells surrounding ovulated eggs, or following binding to the egg's ZP (Avella and Dean, 2011).

Two ZP proteins, ZP2 and ZP3, have been identified as receptors for binding of either AR- or AI-sperm to the egg's ZP, respectively (**Fig. 9**). For mice, evidence suggests that AI-sperm bind to ZP3 oligosaccharides and undergo the acrosome reaction (Bleil and Wassarman, 1980b; Florman et al., 1984; Florman and Wassarman, 1985; Bleil et al., 1988), whereas AR-sperm bind to to the N-terminal region of ZP2 polypeptide (Bleil et al., 1988; Gahlay et al., 2010; Avella et al., 2014; Tokuhiro and Dean, 2018). It is not surprising that plasma membrane overlying heads of AI-sperm and inner acrosomal membrane exposed on AR-sperm recognize and bind to different epitopes on the egg's ZP (Bleil and Wassarman, 1986; Mortillo and Wassarman, 1991; Wassarman and Litscher, 2022).

Mouse ZP Genes and Female Fertility

Gene targeting in mice has been used to establish lines in which ZP genes *ZP1-3* were inactivated by either homologous recombination or insertional mutagenesis (**Table 2**). Female homozygous nulls for either *ZP2* or *ZP3* produce oocytes and eggs that lack a ZP and the females are infertile due to a scarcity of growing oocytes, developing follicles, and ovulated eggs (Liu et al., 1996; Rankin et al., 1996, 2001). These results are consistent with those of experiments in which antisense oligonucleotides directed against ZP2 or ZP3 mRNAs were injected into growing oocytes and it was that ZP2 and ZP3 are dependent on each other for incorporation into the ZP (Tong et al., 1995). On the other hand, heterozygous *ZP3* null females produce unfertilized eggs that have a thin ZP (≈2.7 μm versus ≈6 μm thick) that contains about one-half the amount of ZP2

and ZP3 found in ZP of wild-type mice, but are fertile (Wassarman et al., 1997). Female homozygous nulls for *ZP1* are fertile, but exhibit reduced fertility due to an insufficiently crosslinked ZP that leads to early loss of preimplantation embryos in the oviduct (Rankin et al., 1999).

Human ZP Genes and Female Fertility

Today it is estimated that about one-in-ten married women worldwide are infertile. Much of the infertility is attributed to either male or female factors and about half has a genetic component (Deshpande and Gupta, 2019). In humans it has been shown that there is a causal relationship between gene sequence variations in ZP genes *ZP1-4* and female infertility (Männikko et al., 2005; Pökkla et al., 2011; Margalit et al., 2012). In more than two dozen cases in *in vitro* fertilization (IVF) clinics it was found that point or frameshift mutations in human ZP genes resulted in failure to assemble a normal ZP around growing oocytes and female infertility (Zhou et al., 2019; Litscher and Wassarman, 2020b; Wassarman and Litscher, 2021; Fei and Zhou, 2022; Liu et al., 2023; Sun et al., 2023). In a number of cases the mutations resulted in synthesis of truncated ZP proteins (i.e., insertion of premature stop-codons in human ZP genes) that lacked sequence elements required for ZP assembly during oocyte growth. Some instances of empty follicle syndrome (EFS) were found among these cases.

Effects of Fertilization on the ZP

Following fertilization of eggs, the physical and biological properties of the ZP change (Evans, 2020; Fahrenkamp et al., 2020) and the changes are collectively called the zona reaction. For example, the ZP becomes significantly less soluble, sperm that had partially penetrated the ZP prior to fertilization can penetrate no further, and free-swimming AI and AR sperm can no longer bind to the ZP. Various methodology has revealed that the stiffness and viscosity of the ZP increase by about 2.6- and 4.4-fold (Kim and Kim, 2013), respectively, and the ZP becomes more resistant to solubilization by various reagents or conditions, such as proteases, reducing agents, or mild acid. The molecular basis of these mechanical changes likely involves proteolytic cleavage of ZP2, changes in ZP fibril and matrix conformation, increased non-covalent interactions between filaments, and partial loss of bound water.

Cortical granules (CG) are membrane bound secretory organelles (0.2-0.6 μm diameter, ≈4,500/mouse egg) that appear during oocyte growth in the cortex of oocytes as a product of the Golgi (Ducibella, 1996; Liu, 2011). CG contain a variety of lectins, proteases, glycosidases, and other macromolecules. Fusion of CG membrane with the egg's plasma membrane following fertilization, the cortical reaction, is Ca^{2+} and G-protein dependent and leads to cleavage of ZP2 and accumulation of Zn^{2+} in the ZP (Zn^{2+}-sparks, 100-500 μM Zn^{2+}/ZP) (Que et al., 2017; Tokuhiro and Dean, 2018); it is known that high concentrations of Zn^{2+} have an inhibitory effect on sperm motility. CG exocytosis results in release of a member of the astacin family of multidomain metallo-endopeptidases, called ovastacin, into the the perivitelline space and ZP. Ovastacin cleaves ZP2 near its N-terminus (mouse ZP2, 166Leu-Ala↓Asp-Glu169) without release of a peptide and inactivates ZP2 as a receptor for AR sperm (Bleil et al., 1981; Burkhart et al., 2012; Körschgen et al., 2017; Tokuhiro and Dean, 2018). ZP3 also is inactivated as a receptor for AI sperm following fertilization, but the molecular basis of inactivation remains unclear.

Summary

The mammalian egg's ZP is a unique ECM that plays vital roles before, during, and after fertilization. The presence of a ZP is required for growth of non-growing oocytes into unfertilized eggs, differentiation and proliferation of ovarian follicle cells, species-restricted fertilization of eggs by a single sperm, prevention of polyspermic fertilization, and protection of cleavage-stage embryos as they traverse the female reproductive tract. Like many other extracellular proteins, nascent ZP1-4 polypeptides possess aa sequences and domains, such as a signal-sequence, ZPD, and CTP essential for secretion and ZP filament formation. Secreted ZP proteins assemble into a viscous, crosslinked, fibrillar ECM that stabilizes oocyte-follicle cell interactions and promotes oocyte growth and follicle development during oogenesis. The ZP provides receptors, ZP2 and ZP3, for binding of free-swimming AR- and AI-sperm, respectively, during species-restricted fertilization of ovulated eggs. The ZP undergoes mechanical and biological changes following fertilization that assist in prevention of polyspermy and help to protect the cleavage-stage embryo as it traverses the female reproductive tract. Interference with production of a normal ZP during oogenesis, by either ZP gene-targeting in mice or ZP gene mutations in humans, can result in female infertility. Further research on the mammalian egg's ZP is bound to reveal many more interesting features of this unique and vital ECM.

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Conflict of Interest Statement

The authors declare that they have no disclosures.

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Figure Legends

Fig. 1. Photographic image of a light micrograph (Nomarski differential interference contrast) of an unfertilized mouse egg incubated in the presence of free-swimming sperm. Sperm are shown bound to the zona pellucida (ZP). Scale bar, ≃1 cm = ≃14 μm.

Fig. 2. Schematic diagram of some steps involved in mammalian oogenesis that culminates in ovulation of eggs, fusion of eggs and sperm (fertilization), and production of zygotes. Primordial germ cells populate the female fetal ovary and give rise to mitotically dividing oogonia. By the time of birth, oogonia have entered meiosis and become non-growing oocytes. During each reproductive cycle, oocytes undergo tremendous growth while arrested in meiosis, become fully-grown oocytes in Graafian follicles, undergo meiotic maturation, and are ovulated as unfertilized eggs. Sperm and eggs fuse (fertilization) to form zygotes that go on to produce adults of the species.

Fig. 3. Schematic diagram of ZP production during oocyte growth in mice. Non-growing oocytes (**a**, ≃12 μm diameter) lack a ZP. As soon as oocyte growth begins they lay down pockets of ZP filaments (**b**) that soon form a discernable ZP (**c**). The ZP continues to thicken throughout the oocyte growth phase (**d-f**, ≃2-3 weeks, ≃300-fold increase in oocyte volume) and results in a 6.2 ± 1.9 μm thick ZP around fully-grown oocytes (**f**, ≃80 μm diameter) and ovulated eggs. The ZP remains around cleavage-stage embryos until the expanded blastocyst stage when the embryo hatches from the ZP and implants in the uterus.

Fig. 4. Schematic representation of the organization of mouse ZP proteins, mZP1-3 (623, 713, and 424 amino acids, respectively), and human ZP proteins, hZP1-4 (638, 745, 424, and 540 amino acids, respectively). In each case, the polypeptide contains a signal-sequence (SS) at the N-terminus (*pink*), a ZP domain (ZPD) consisting of ZP-N (*green*) and ZP-C (*turquoise)* subdomains and a short linker region (*blue*), and a consensus furin cleavage-site (CFCS, arrow), transmembrane domain (TMD, *yellow*), and C-terminal propeptide (CTP). mZP1, hZP1, and hZP4 also have a trefoil domain (*brown*) adjacent to the ZPD. mZP1, mZP2, hZP1, hZP2, and hZP4

have one or three extra copies of the ZP-N subdomain (*green*) between the N-terminus of the polypeptides and the ZPD. The positions of the internal (IHP) and external (EHP) hydrophobic patches are indicated by *red* and *orange* triangles, respectively. The amino acid numbers for each region of the mouse (m) and human (h) ZP polypeptides are indicated above and below the drawings of the polypeptides.

Fig. 5. A general mechanism for assembly of nascent ZP proteins. In all ZPD precursor proteins, the ZPD consists of two subdomains, ZP-N (*green*) and ZP-C (*blue*). The subdomains are followed by a CTP that contains a CFCS (*pink*), an EHP (*purple*), and a TMD (*yellow*). Precursors do not polymerize within the cell, either as a result of direct interaction between the EHP and IHP (*grey*) or because they adopt a conformation dependent on the presence of both hydrophobic patches. Proteolytic processing at the CFCS (marked by a cross) leads to dissociation of mature proteins from the EHP and activation of the ZPD for polymerization into filaments and matrix.

Fig. 6. Scanning electron micrographs of the surface of human and mouse oocytes. (**A**) Human oocyte showing the presence of many pores (9,000-times magnification) on the outer surface of the ZP. (**B**) Higher magnification of a human oocyte showing the fibrillar organization of the ZP (50,000-times magnification), filaments are 0.1-0.4 μm long and 10-14 nm wide. (**C**) Outer surface of a mouse oocyte showing the fibrillar organization of the ZP (50,000-times magnification). Samples were treated with saponin-ruthenium red-osmium-thiocarbohydrazide to reveal ZP filaments. This figure was adapted with permission from G. Familiari (Familiari et al., 2006).

Fig. 7. Three-dimensional structures of ZPD subdomains ZP-N and ZP-C that are related to C-type and V-type Ig-like domains. (**A**) ZP3 subdomain ZP-N and C-type Ig-like domains. β-strands are labeled using Ig terminology, helices are indicated by rectangles. Opposing β-sheets 1 and 2 are *blue* and *green,* respectively, with termini circled. The E’ strand is *orange* and disulfides *magenta*. (**B**) ZP2 ZP-C and V-type Ig-like domains. As in panel A, except for the additional A′ and C′/C″ strands that are *yellow* and *red*, respectively. This figure was adapted with permission from L. Jovine (Bokhove and Jovine, 2018).

Fig. 8. Schematic diagram of some morphological features of a mammalian sperm undergoing and completing the acrosome reaction (AR). The course of the AR is indicated by stages (A)-(D). An

acrosome-intact (AI) sperm head and sperm nucleus (*pale blue*) is shown in (A). In (B), fusion between the sperm's outer acrosomal membrane and plasma membrane is indicated. Hybrid membrane vesicles composed of plasma and outer acrosomal membrane are shown in (C) and (D). pm, plasma membrane; am, acrosomal membrane. This figure is a modified version of Fig. 16 in Yanagimachi, 1994 that appeared as Fig. 5 in Wassarman, 1999.

Fig. 9. Schematic diagram of some steps involved in two different pathways to fertilization in mammals. In the first pathway (*black* arrows), acrosome-intact (AI) sperm bind to ZP3 in the egg's zona pellucida (ZP). As a result of binding to ZP3, AI- sperm undergo the acrosome reaction (AR, multiple fusions between the sperm's outer acrosomal membrane and plasma membrane leading to exposure of the inner acrosomal membrane), and AR-sperm then bind to ZP2. In the second pathway (*grey* arrows), AI-sperm undergo the AR prior to reaching the ZP, perhaps in the *cumulus oophorous* of ovulated eggs, and then bind to ZP2 rather than to ZP3. In both pathways AR-sperm penetrate the ZP and fuse with the egg's plasma membrane (fertilization). Fusion of sperm and egg induces the cortical reaction (CR) in eggs (multiple fusions of cortical granule membrane and the egg's plasma membrane) that results in exocytosis of cortical granule components, including acrosin and Zn^{2+}, into the ZP and induces the zona reaction (ZR). The ZR consists of inactivation of ZP2 and ZP3 as sperm receptors (AI- and AR-sperm cannot bind to the ZP of fertilized eggs) and in a decrease in solubility (hardening) of the ZP.

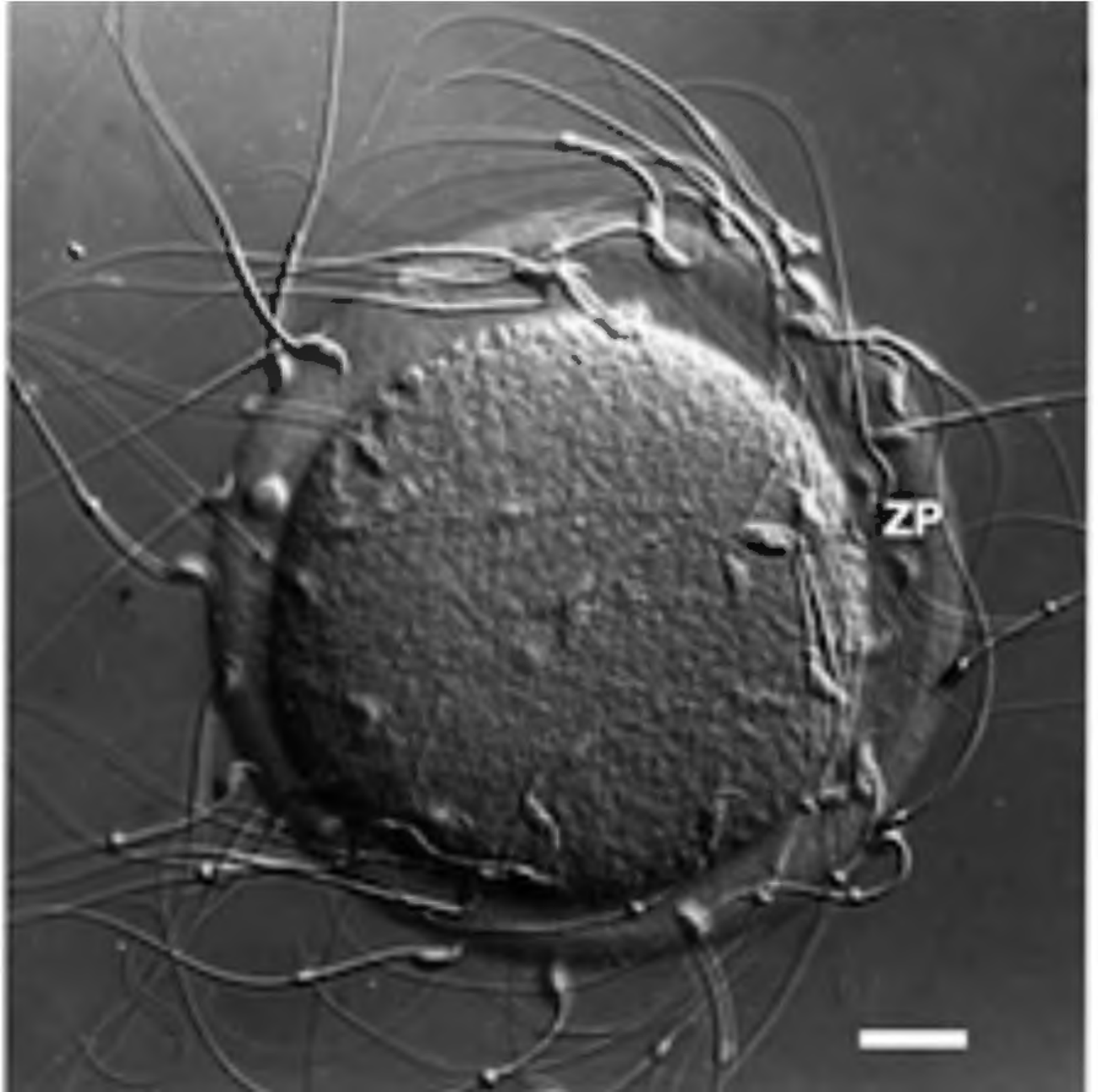
Table 1. Features of Zona Pellucida Genes and Proteins

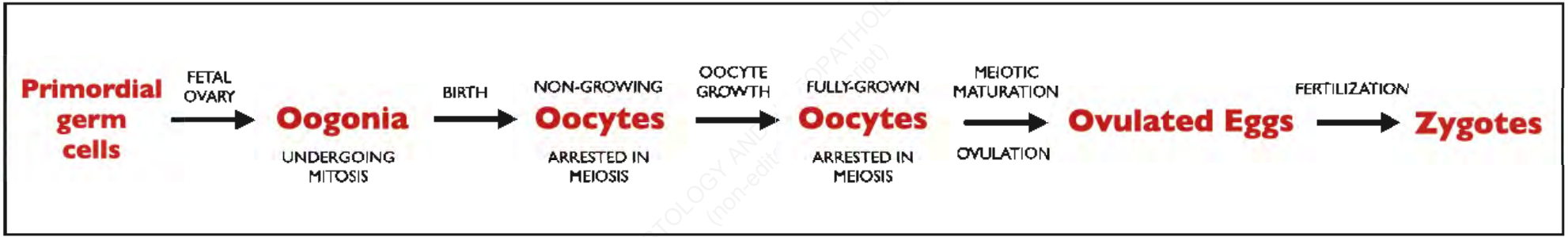
Protein ZP Gene	Chromosome Number	Gene Lenght (kb)	Number Exons	Unprocessed Polypeptide Length (aa)	Processed Polypeptide Lenght (aa)	Glycosylated Apparent MW (kDa)
mZP1 (dimer)	19	6.5	12	623	525	200
hZP1 (dimer)	11	11	12	638	521	200

mZP2 (monomer)	7	12.1	18	713	598	120
hZP2 (monomer)	16	14	19	745	599	120
mZP3 (monomer)	5	8.6	8	424	282	83
hZP3 (monomer)	7	18.3	8	424	281	58
hZP4 (monomer)	1	17	12	540	440	65

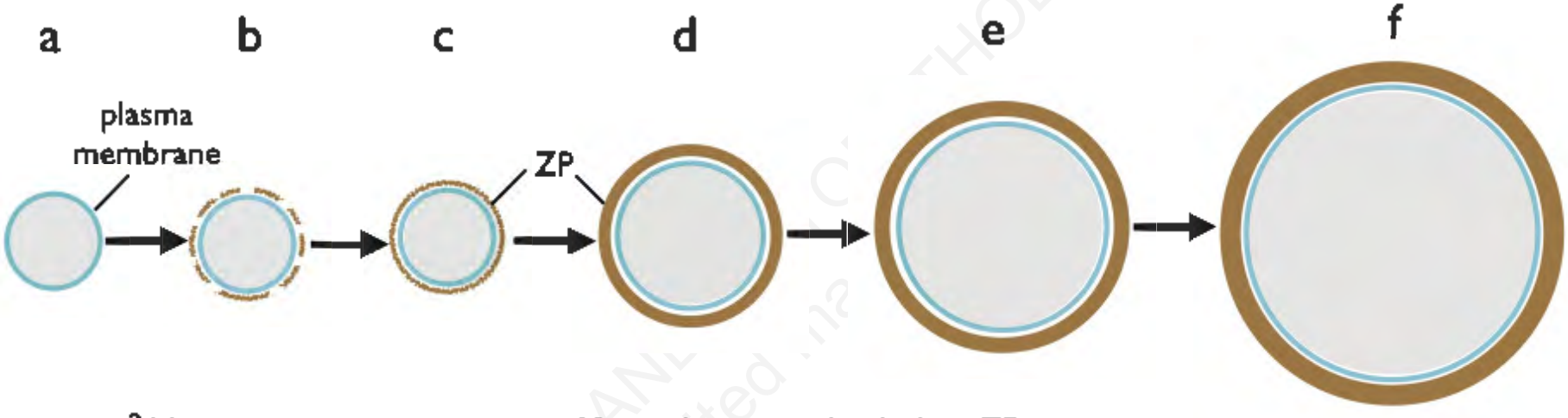
Table 2. Phenotypes of Zona Pellucida Null Female Mice

Genotype	**Fertility**	**ZP**
Wild-type	Fertile	Normal
mZP1 homozygous-null	Low fertility	Abnormal
mZP2 homozygous-null	Infertile	None
mZP3 homozygous-null	Infertile	None
mZP3 heterozygous-null	Fertile	Thin


ZP


Primordial germ cells
FETAL OVARY
Oogonia
UNDERGOING MITOSIS
BIRTH
NON-GROWING
Oocytes
ARRESTED IN MEIOSIS
OOCYTE GROWTH
FULLY-GROWN
Oocytes
ARRESTED IN MEIOSIS
MEIOTIC MATURATION
OVULATION
Ovulated Eggs
FERTILIZATION
Zygotes

Zona Pellucida Appears and Thickens during Oocyte Growth



[a] Non-growing mouse oocyte, ≈12 µm diameter, that lacks a ZP.

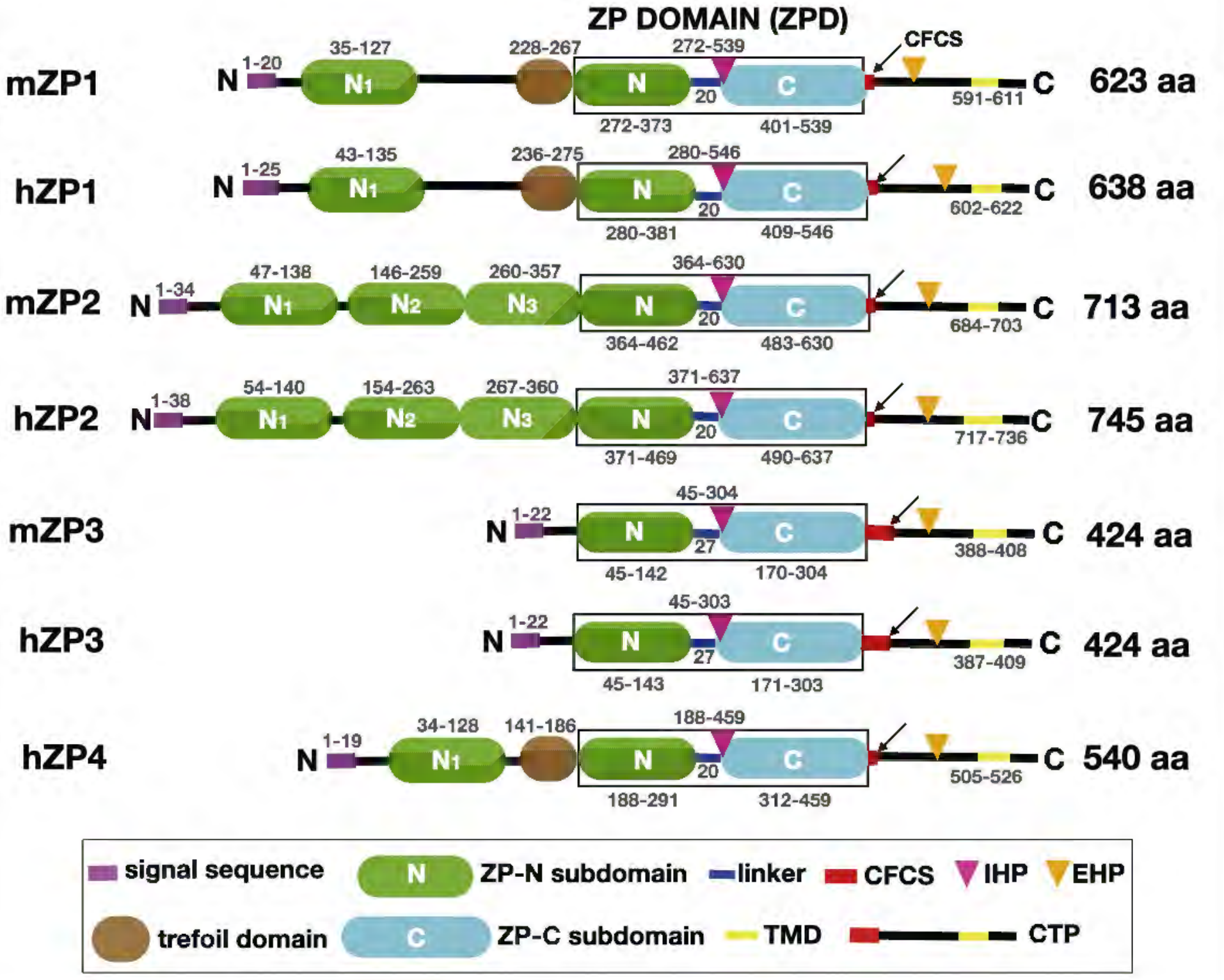
[b] Mouse oocyte that has initiated growth and has several small pockets of ZP fibrils surrounding it.

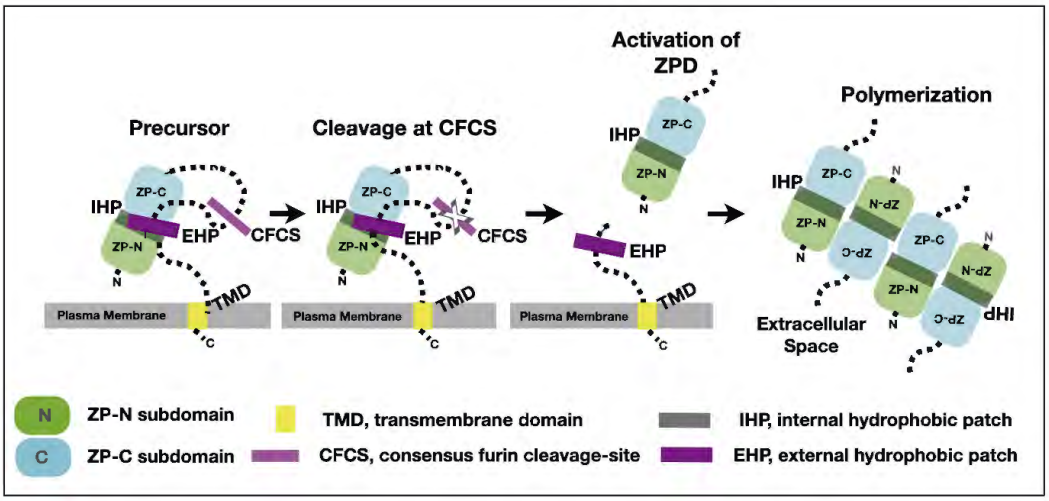
[c] Growing mouse oocyte with a discernible ZP that has formed from small pockets of ZP fibrils.

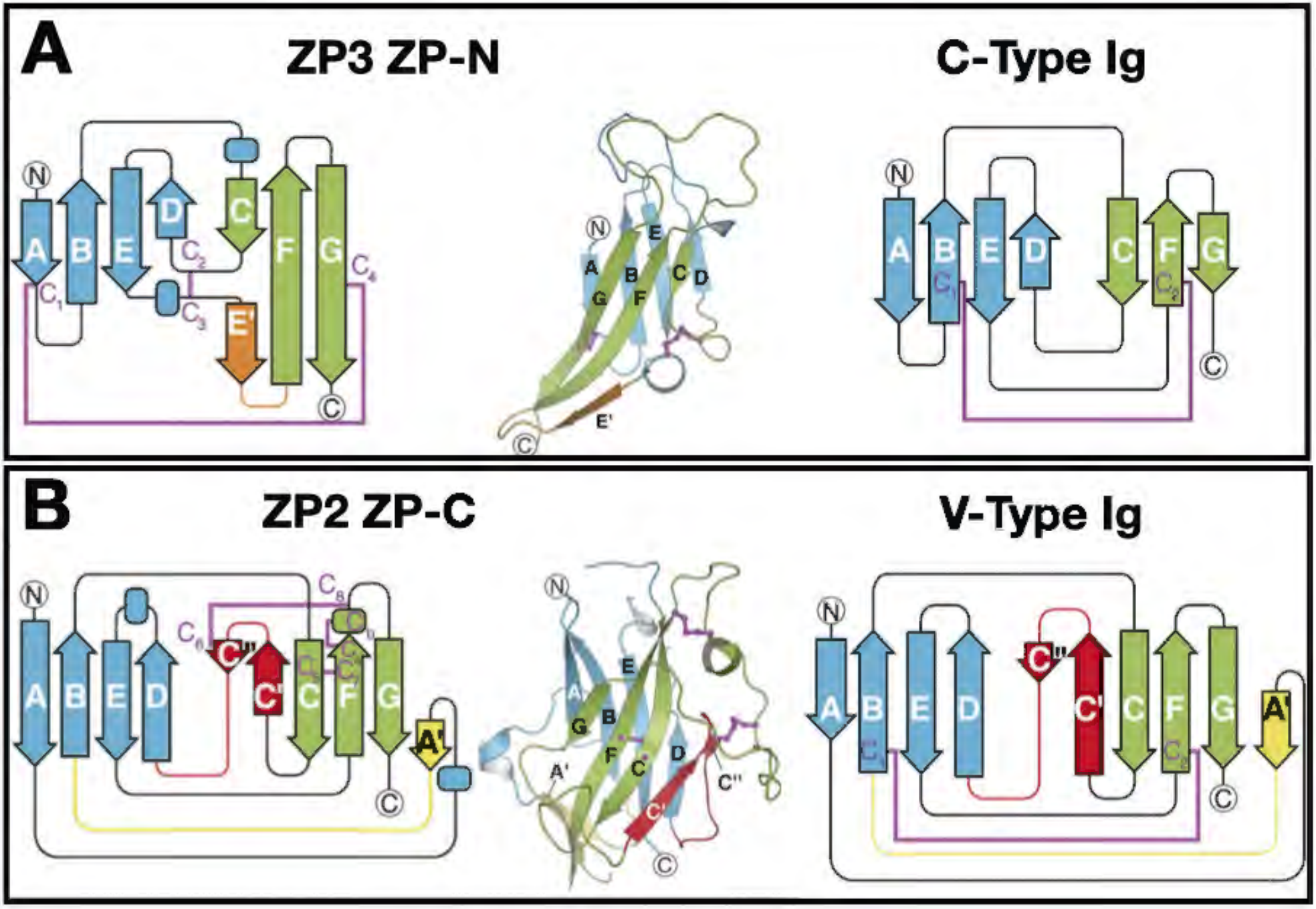
[d] Growing mouse oocyte, ≈40 µm diameter, with a thickening ZP, ≈1.5 µm thick.

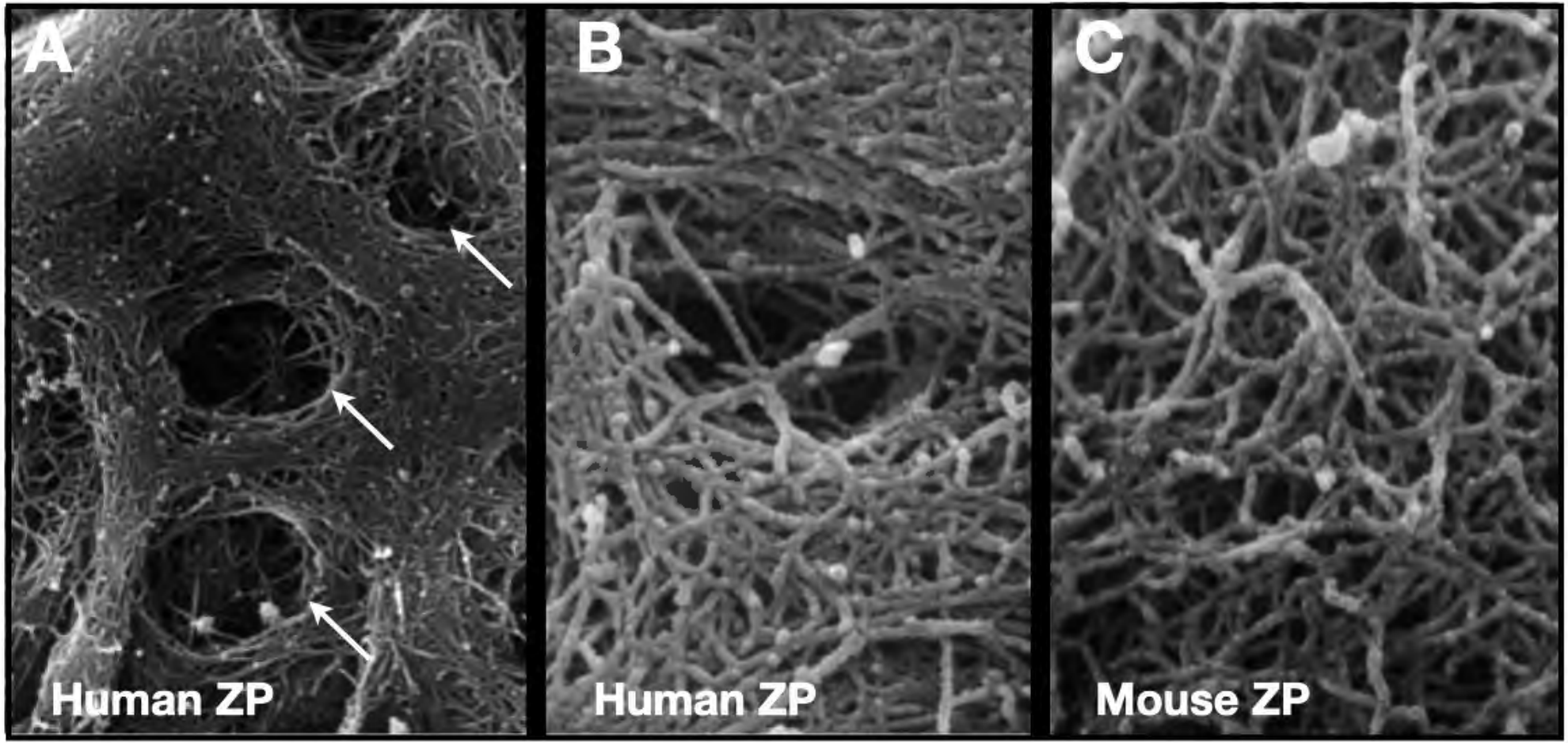
[e] Growing mouse oocyte, ≈60 µm diameter, with a thickening ZP, ≈3.5 µm thick.

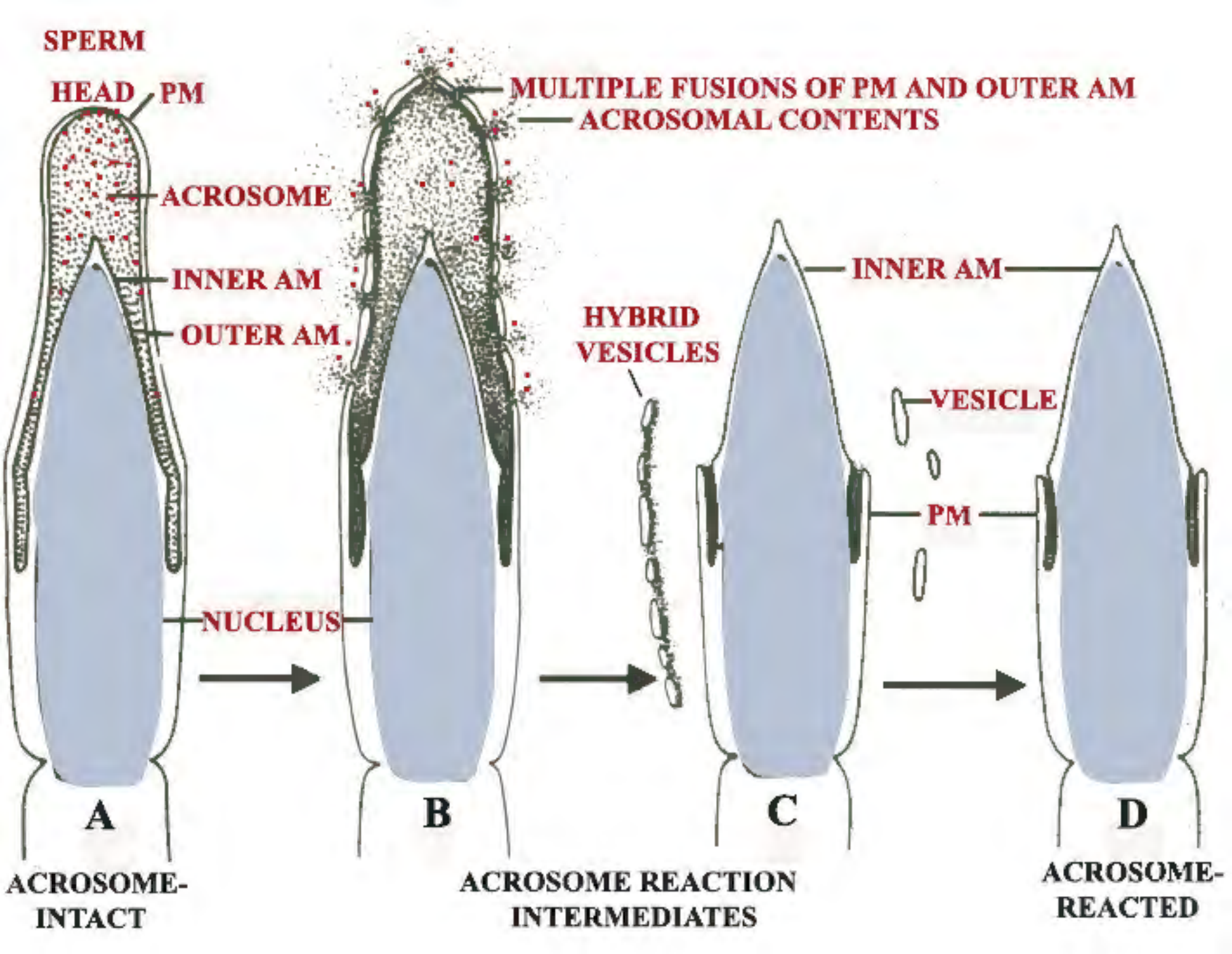
[f] Fully-grown mouse oocyte, ≈80 µm diameter, with a thick ZP, ≈6.2 µm thick.

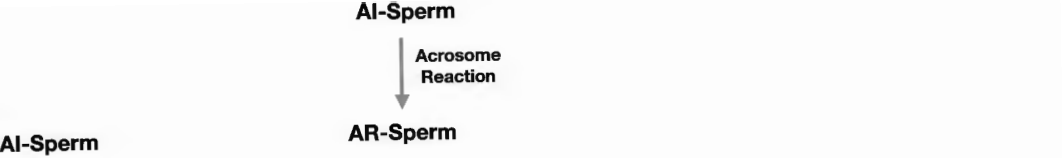

ZP DOMAIN (ZPD)
mZP1
N
1-20
35-127
N1
228-267
272-539
N
20
C
CFCS
C
591-611
272-373
401-539
623 aa
hZP1
N
1-25
43-135
N1
236-275
280-546
N
20
C
C
602-622
280-381
409-546
638 aa
mZP2
N
1-34
47-138
N1
146-259
N2
260-357
N3
364-630
N
20
C
C
684-703
364-462
483-630
713 aa
hZP2
N
1-38
54-140
N1
154-263
N2
267-360
N3
371-637
N
20
C
C
717-736
371-469
490-637
745 aa
mZP3
N
1-22
45-304
N
27
C
C
388-408
45-142
170-304
424 aa
hZP3
N
1-22
45-303
N
27
C
C
387-409
45-143
171-303
424 aa
hZP4
N
1-19
34-128
N1
141-186
188-459
N
20
C
C
505-526
188-291
312-459
540 aa
signal sequence
N
ZP-N subdomain
linker
CFCS
IHP
EHP
trefoil domain
C
ZP-C subdomain
TMD
CTP


Precursor
Cleavage at CFCS
Activation of ZPD
Polymerization
IHP
ZP-C
ZP-N
EHP
CFCS
TMD
Plasma Membrane
N
C
N-dZ
C-dZ
dHI
Extracellular Space
N, ZP-N subdomain
C, ZP-C subdomain
TMD, transmembrane domain
CFCS, consensus furin cleavage-site
IHP, internal hydrophobic patch
EHP, external hydrophobic patch


A
ZP3 ZP-N
C-Type Ig
N
A
B
E
D
C
F
G
C
E'
B
ZP2 ZP-C
V-Type Ig
C''
C'
A'


A
Human ZP
B
Human ZP
C
Mouse ZP


SPERM
HEAD
PM
ACROSOME
INNER AM
OUTER AM.
NUCLEUS
MULTIPLE FUSIONS OF PM AND OUTER AM
ACROSOMAL CONTENTS
HYBRID VESICLES
INNER AM
VESICLE
PM
A
B
C
D
ACROSOME-INTACT
ACROSOME REACTION INTERMEDIATES
ACROSOME-REACTED


AI-Sperm
Acrosome Reaction
AR-Sperm
AI-Sperm
Binding
Binding
Sperm-Egg Fusion
Egg ZP3
AR-Sperm
Egg ZP2
AR-Sperm
Penetration
Fertilization
CR
ZR
Acrosome