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# Mammalian fertilization: the egg's multifunctional zona pellucida

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**ABSTRACT The zona pellucida (ZP) is a specialized extracellular coat that surrounds the plasma membrane of mammalian eggs. Its presence is essential for successful completion of oogenesis, fertilization and preimplantation development. The ZP is composed of only a few glycoproteins which are organized into long crosslinked fibrils that constitute the extracellular coat. A hallmark of ZP glycoproteins is the presence of a ZP domain, a region of polypeptide responsible for polymerization of the glycoproteins into a network of interconnected fibrils. The mouse egg ZP consists of only three glycoproteins, called ZP1, ZP2, and ZP3, that are synthesized and secreted exclusively by growing oocytes. One of the glycoproteins, ZP3, serves as both a binding partner for sperm and inducer of sperm exocytosis, the acrosome reaction. Female mice lacking ZP3 fail to assemble a ZP around growing oocytes and are completely infertile. Sperm bind to the carboxy-terminal region of ZP3 polypeptide encoded by *ZP3* exon-7 and binding is sufficient to induce sperm to complete the acrosome reaction. Whether sperm recognize and bind to ZP3 polypeptide, oligosaccharide, or both remains an unresolved issue. Purified ZP3 self-assembles into long homomeric fibrils under non-denaturing conditions. Apparently, sperm added to ZP3 bind to the fibrils and are prevented from binding to ovulated eggs *in vitro*. These, as well as other aspects of ZP structure and function are addressed in this article.**

KEY WORDS: *sperm, zona pellucida, acrosome reaction, ZP3, ZP domain, sperm combining-site*

*«No theory of fertilization which fails to include the factor of specificity as one of the prime elements can be true.»* Frank Lillie, 1919

## Introduction

The plasma membrane of eggs from eutherian mammals, including those from monotremes and marsupials, is surrounded by a relatively thick extracellular coat called the zona pellucida (ZP), a name derived from the Latin, meaning transparent (*pellucida*) belt or girdle (*zona*) (Fig. 1). The ZP is an elastic structure composed of only a few glycoproteins (Wassarman *et al.,* 1985; Wassarman 1987, 1988, 1999; Conner *et al.,* 2005) each of which contains a relatively large conserved region called the ZP domain (Bork and Sander, 1992; Jovine *et al.,* 2002a, 2005). In addition, the ZP is a porous structure that is permeable to relatively large macromolecules such as antibodies and small viruses (Gwatkin, 1977; Wassarman, 1988).

The structural characteristics of ZP glycoproteins are related to those of extracellular coat proteins of eggs from non-mammalian species that are separated from mammals by as much as 400 million years of evolution (Breed *et al.,* 2002; Monne *et al.,* 2006; Litscher and Wassarman, 2007). In general, the ZP consists of long, interconnected fibrils or filaments that exhibit a structural repeat (Greve and Wassarman, 1985; Wassarman and Mortillo, 1991; Wassarman *et al.,* 1996; Green, 1997). There is evidence to suggest that ZP1, a dimer of identical polypeptides, each with a trefoil or P domain (Bork, 1993), serves as a crosslinker for ZP fibrils (Greve and Wassarman, 1985; Wassarman and Mortillo, 1991; Rankin *et al.,* 1999). For many years it has been known that the ZP can be dissolved by exposure to conditions that do not disrupt covalent bonds, such as low pH, elevated temperature, or low ionic strength buffers (Wassarman, 1988).

The ZP performs a variety of roles before, during, and after

*Abbreviations used in this paper:* AR, acrosome reaction; CFCS, consensus furin cleavage-site; EC, embryonal carcinoma; EHP, external hydrophobic patch; IHP, internal hydrophobic patch; N-linked, asparagine-linked; O-linked, serine/threonine-linked; TMD, transmembrane domain; ZP, zona pellucida.

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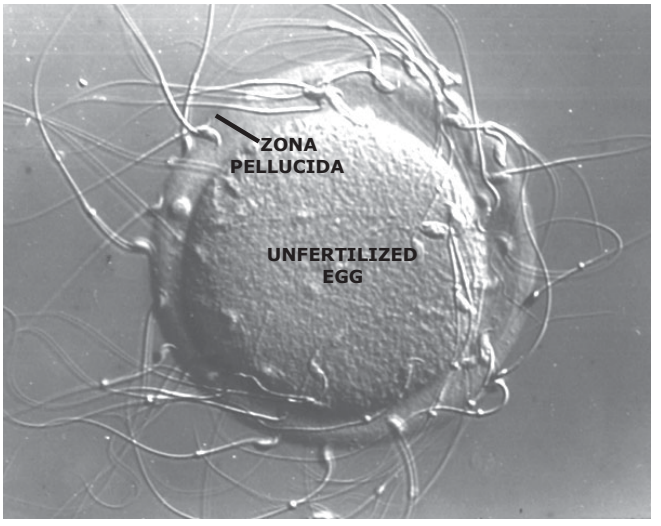



**Fig.1. Binding of free-swimming mouse sperm to the zona pellucida (ZP) of an ovulated mouse egg.** *The light micrograph was taken using Nomarski differential interference contrast optics.*

fertilization of eggs. The ZP is laid down during late stages of oogenesis when non-growing oocytes begin to grow, and it increases in thickness as oocytes increase in diameter (Wassarman and Albertini, 1994). Results of targeted mutagenesis experiments on genes encoding ZP glycoproteins strongly suggest that successful completion of oocyte growth and follicle development is largely dependent on production of a ZP (Liu *et al.,* 1996; Rankin *et al.,* 1996, 2001; Wassarman *et al.,* 1998). Females that fail to produce a ZP during oogenesis are infertile. During fertilization of eggs, sperm bind in a species-restricted manner to the ZP, complete the acrosome reaction (AR), and penetrate through the ZP to reach and fuse with the plasma membrane (Florman and Ducibella, 2006). The AR involves multiple fusions between the sperm's outer acrosomal membrane and plasma membrane overlying the anterior region of the sperm head, formation of hybrid vesicles, and exposure of the inner acrosomal membrane and acrosomal contents. Only acrosome-reacted sperm can penetrate the ZP and fuse with egg plasma membrane to form a zygote. Removal of the ZP from unfertilized eggs virtually eliminates the barrier to fertilization between species *in vitro.*

Several lines of evidence strongly suggest that sperm recognize and bind to a ZP glycoprotein and are therby induced to complete the AR (Wassarman, 1990, 1995, 1999, 2005; Wassarman *et al.,* 2001; Florman and Ducibella, 2006). Following fusion of sperm and egg to form a zygote, the structure of the ZP is changed such that it no longer permits binding of free-swimming sperm and contributes to the prevention of fertilization by more than one sperm (polyspermy) (Gardner *et al.,* 2007). Finally, results of experiments strongly suggest that preimplantation development (McLaren, 1969; Bronson and McLaren, 1970), including specification of a blastocyst axis (Kurotaki *et al.*, 2007), is dependent on the presence of a ZP. Embryos hatch from the ZP just prior to implantation in the uterus. Therefore, the ZP is present only transiently, from initiation of oocyte growth to implantation of the embryo; in mice a period of approximately 4 weeks.

Here we review more than 25 years of research from our laboratory that leads us to conclude that a mouse ZP glycoprotein, called ZP3, is responsible for species-restricted binding of sperm to eggs, as well as for inducing bound sperm to complete the AR. In the interest of brevity and clarity not all experimental evidence available is presented here. However, it should be noted that from the 1970s until today a great many laboratories world-wide have contributed significantly to the conclusions about ZP3 presented here.

## Early observations

### *Identification of ZP glycoproteins*

In the mid-1970's Jeffrey Bleil joined our laboratory as a graduate student and set out to identify the proteins of the mouse egg's ZP. Using ZP removed individually from isolated oocytes or eggs by mouth-pipetting, three proteins were identified and characterized that we named ZP1, 2, and 3 (Bleil and Wassarman, 1980a) (Fig. 2). ZP2 (~120 kDa $M_r$) and ZP3 (~83 kDa $M_r$) behaved as monomers on SDS-PAGE, whereas ZP1 (~200 kDa $M_r$) behaved as a dimer of polypeptides connected by intermolecular disulfides. All three proteins migrated as relatively broad bands on SDS-PAGE and as multiple spots on two-dimensional gel electrophoresis, suggesting to us early on that ZP1-3 were glycosylated proteins. ZP2 and ZP3 are present in roughly equimolar amounts and represent the majority of the mass of the ZP (~3.5 ng). Comparisons of ZP from fertilized and unfertilized eggs in the presence and absence of reducing agent revealed that ZP2 was subjected to limited proteolysis as a consequence of fertilization and was converted to a species we called $ZP2_f$ (Bleil *et al.,* 1981). Subsequent experiments suggested that the proteolytic conversion of ZP2 to $ZP2_f$ was responsible for the so-called hardening of the ZP following fertilization (Moller and Wassarman, 1989). It is likely that hardening of the ZP is attributable to an increase in interactions between the fibrils that constitute the extracellular coat.

In time it was found that mouse ZP1, 2, and 3 are encoded by single-copy genes located on chromosomes 19 (12 exons), 7 (18 exons), and 5 (8 exons), respectively (Epifano *et al.,* 1995). Analyses of the human genome suggest that genes encoding ZP1, 2, and 3 are located on chromosomes 11, 16, and 7, respectively (Chamberlin and Dean, 1990; Van Duin *et al.,* 1992; Liang and Dean, 1993; Hughes and Barratt, 1999). Furthermore, it is now known that the human ZP contains an additional glycoprotein, called ZPB/ZP4 (Lefievre *et al.,* 2004). The polypeptide sequences of ZP2 and ZP3 from mice and humans, two species separated by more than $10^8$ years of evolution, are 56% and 67% identical, respectively. The primary structures of ZP2- and ZP3-related ZP glycoproteins from different mammals are relatively well conserved (~65-98% identity), whereas ZP1-related glycoproteins, possessing a trefoil (P) domain, are conserved to a lesser degree (~40% identity). It is also apparent that ZP1-3 have regions of polypeptide in common, suggesting that these regions may be derived from a common ancestral gene (Spargo and Hope, 2003).

### *Synthesis of ZP glycoproteins*

Having identified ZP1-3, we set out to determine where they were synthesized. Using intact follicles (oocyte-follicle cell complexes), oocytes, and follicle cells isolated from juvenile mice 5-

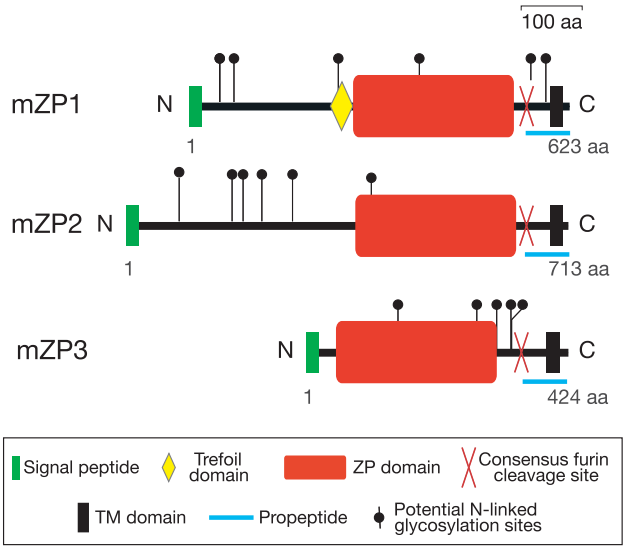



**Fig. 2. Schematic representation of the overall architecture of mouse ZP glycoproteins ZP1, ZP2, and ZP3.** *The polypeptide of each ZP glycoprotein is drawn to scale, with the N- and C-termini indicated. Key features of the polypeptide, including the N-terminal signal peptide (*green*), P or trefoil domain (*yellow*), ZP domain (*red*), CFCS (X), TMD (*black*), and C-terminal propeptide region (*blue bar*) are depicted. Only putative N-linked glycosylation sites, conforming to the strict pattern Asn-X-Ser/Thr-X, where X can be any amino acid other than Pro, are shown. The number of amino acids in the polypeptide of each ZP glycoprotein is indicated.*

21 days-of-age (the source of growing oocytes; Sorensen and Wassarman, 1976) it became clear that only growing oocytes synthesized the three ZP glycoproteins (Bleil and Wassarman, 1980b). Subsequent work from our own (Greve *et al.,* 1982; Salzmann *et al.,* 1983; Roller *et al.,* 1989; Lira *et al.,* 1990, 1993; Schickler *et al.,* 1992) and another (Philpott *et al.,* 1987; Liang *et al.,* 1990; Millar *et al.,* 1991; Epifano *et al.,* 1995) laboratory confirmed that, indeed, mouse ZP glycoproteins are synthesized exclusively by growing oocytes during a 2-to-3 week period and the synthesis is regulated by an oocyte-specific promoter located close to the transcription start-site (i.e., within the proximal 153 nucleotides of the gene's 5'-flanking region) (Lira *et al.,* 1990, 1993). Interestingly, the ZP3 promoter has proven to be extremely useful to control site-specific DNA recombinase Cre in transgenic mice (*ZP3-Cre*) and thereby target specific gene expression (activation/inactivation) in mouse oocytes (Lewandoski *et al.,* 1997; deVries *et al.,* 2000; Kemler *et al.,* 2004; Lan *et al.,* 2004).

### *Functions of ZP glycoproteins*

At this point, we decided to address the potential function(s) of individual ZP glycoproteins. This decision was based in large part on a few publications in the 1970s from Ralph Gwatkin, John Hartmann, and their colleagues. Principally, they had found that when hamster sperm were exposed to solubilized ZP from unfertilized hamster eggs binding of sperm to ovulated eggs was inhibited *in vitro* (Gwatkin and Williams, 1976). Furthermore, solubilized ZP preparations from mouse eggs partially inhibited fertilization of hamster eggs *in vitro* (Gwatkin, 1977). These observations suggested to them and to us that solubilized ZP preparations contained a so-called «sperm receptor» that sperm recognized, bound to, and prevented them from binding to ovulated eggs *in vitro*.

With the results just described in mind, we purified each mouse egg ZP glycoprotein to homogeneity and tested its ability to inhibit binding of mouse sperm to ovulated eggs *in vitro* (a so-called «competition assay»). Surprisingly, of the three glycoproteins, only ZP3 inhibited binding of sperm to eggs at nanomolar concentrations (Fig. 3); results comparable to those of Gwatkin and co-workers with solubilized ZP (Bleil and Wassarman, 1980c). Furthermore, ZP3 purified from fertilized egg or embryo ZP (called $ZP3_f$) failed to inhibit binding of sperm to eggs at equivalent concentrations. These observations first suggested to us that ZP3 was the so-called mouse sperm receptor.

Consistent with the results just described, we found that ZP3 purified from unfertilized egg ZP, but not from fertilized egg ZP ($ZP3_f$), could induce sperm to complete the AR *in vitro* (Bleil and Wassarman, 1983). This reaction involves multiple fusions between sperm plasma and outer acrosomal membranes and exposure of the inner acrosomal membrane (Fig. 4). Bayard Storey and co-workers had reported previously that only acrosome-intact mouse sperm bind to ovulated eggs (Saling *et al.,* 1979) and that solubilized mouse ZP could induce sperm to complete the AR *in vitro* (Florman and Storey, 1982). In view of our results we concluded that ZP3 was the active agent in their preparations of solubilized ZP.

Many later studies provided support for our conclusions about

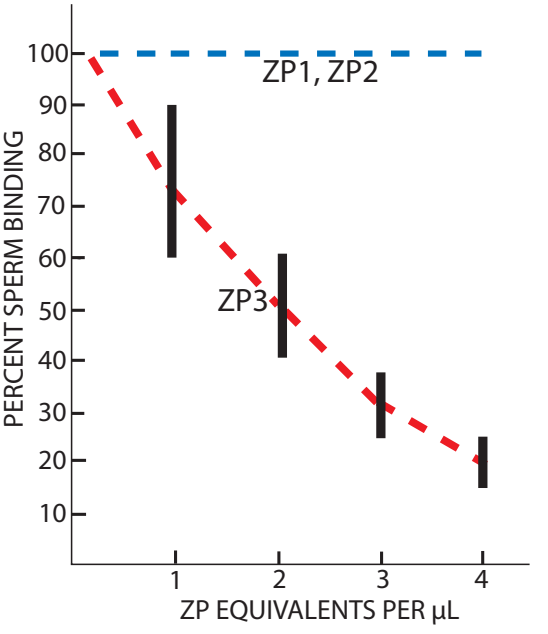
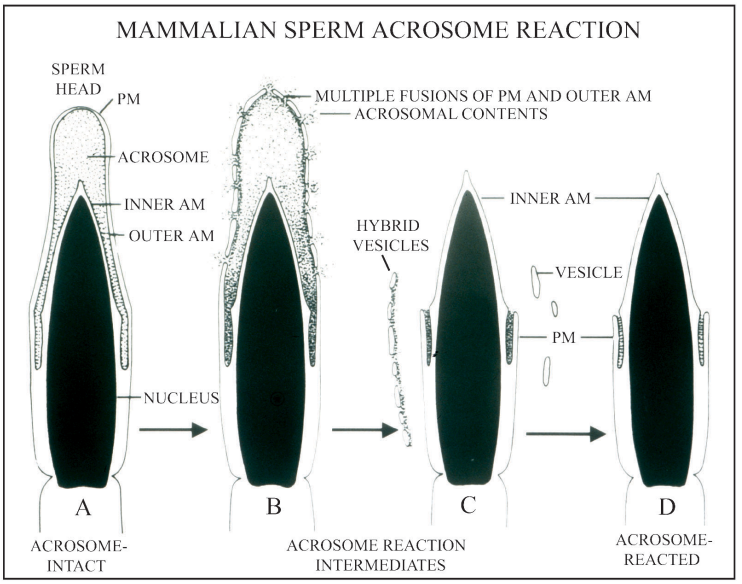



**Fig. 3. Inhibition of binding of mouse sperm to eggs in the presence of purified mouse ZP glycoproteins.** *In these experiments capacitated sperm were incubated in the presence of different concentrations of purified ZP glycoproteins, ovulated eggs were added, and binding of sperm to eggs was determined. The concentration of individual ZP glycoproteins is expressed as ZP equivalents per µl (calculated assuming that each ZP contains ~4 ng protein and that ZP3 represents ~40% of ZP protein). The range of values for percent sperm binding obtained at different concentrations of ZP glycoproteins is indicated by the height of the bar. Only ZP3 inhibited binding of sperm to eggs. For experimental details see Litscher and Wassarman (1996) and Williams* et al. *(2006).*




ZP3 stimulates several signal-transducing components in sperm, including: G proteins, IP3 and IP3 receptors, phospholipase C, voltage sensitive calcium channels.

- Activates G proteins (Gi1, Gi2, Gq/11)
- Depolarizes plasma membrane (-60 to -30 mV)
- Increases intracellular pH (by 0.3 units)
- Increases intracellular [calcium] (from 150 to 400 nM)

**Fig. 4. Features of the mammalian sperm acrosome reaction (AR).** *The AR is shown diagramatically (top) and some of the signal-transducing components are listed (bottom).*

ZP3 and mammalian sperm-egg interactions. For example, in the early 1990s Barry Shur's laboratory reported that β-1,4-galactosyltransferase (GalTase) on the surface of mouse sperm bound to egg coat ZP3 specifically by recognizing ZP3 oligosaccharides and that aggregation of the enzyme induced bound sperm to complete the AR (Macek *et al.,* 1991; Miller *et al.,* 1992). In related studies it was demonstrated that GalTase expressed by *Xenopus* oocytes functions as a ZP3 receptor (ZP2 does not bind) and following aggregation is capable of activating pertussis toxin-sensitive G proteins leading to cellular exocytosis (Shi *et al.,* 2001). Furthermore, Harvey Florman's and Greg Kopf's laboratories went on to establish that the same signaling pathways in sperm bound to eggs that lead to induction of the AR are activated by either solubilized ZP or purified egg ZP3 (Wilde *et al.,* 1992; Ward *et al.,* 1992, 1994; Arnoult *et al.,* 1996; O'Toole *et al.,* 2000; Jungnickel *et al.,* 2001; Florman *et al.,* 2003). The signal-transducing components involved include G proteins, inositol-3,4,5-triphosphate ($IP_3$) and $IP_3$ receptors, phospholipase C, and $Ca^{2+}$ and voltage-sensitive $Ca^{2+}$ channels. For example, it was recently found that purified ZP3 stimulates production of $IP_3$ in sperm membranes (Jungnickel *et al.*, 2007).

Collectively, these results strongly suggest that ZP3 is a binding partner for acrosome-intact sperm and that once bound to ZP3 sperm undergo, or at least complete, the AR. In this context, there is now evidence to suggest that the AR actually is initiated, but not completed, during the process of sperm capacitation (Kim and Gerton, 2003). Although sperm that bind to the ZP appear to be acrosome-intact (e.g., by microscopic examination and/or staining with antibodies or dyes) they probably represent a transitional or intermediate state between acrosome-intact and acrosome-reacted. Consequently, proteins that comprise the acrosomal matrix of acrosome-intact sperm (e.g., sp56 and zonadhesin; Bleil and Wassarman, 1990; Cohen and Wassarman, 2001; Hardy and Garbers, 1995; Bi *et al.,* 2003) may become available at the sperm surface to interact with the egg ZP following capacitation.

## Characteristics of ZP3

Mouse ZP3 is synthesized as a 424-aa polypeptide to which both asparagine- (N-) and serine/threonine- (O-) linked oligosaccharides are added (Wassarman, 1988; Jovine *et al.*, 2007) (Fig. 2). ZP3 has a low isoelectric point (~4.7) due largely to the presence of sialylated and sulfated oligosaccharides (Liu *et al.,* 1997). A 22-aa signal-sequence at the N-terminus and a 71-aa propeptide at the C-terminus (follows a consensus furin cleavage-site; CFCS) of nascent ZP3 are proteolytically removed prior to secretion of mature ZP3 (~37 kDa $M_r$ polypeptide; ~83 kDa $M_r$ glycoprotein) (Litscher *et al.,* 1999; Williams and Wassarman, 2001). The propeptide (Fig. 5) possesses a 22-aa transmembrane domain (TMD) and a conserved 7-aa external hydrophobic patch (EHP; aa-363 to aa-369). We proposed that the TMD anchors ZP3 in oocyte secretory vesicles and plasma membrane (Qi *et al.,* 2002), and the EHP prevents incorporation of ZP3 into fibrils in oocyte cytoplasm by interacting

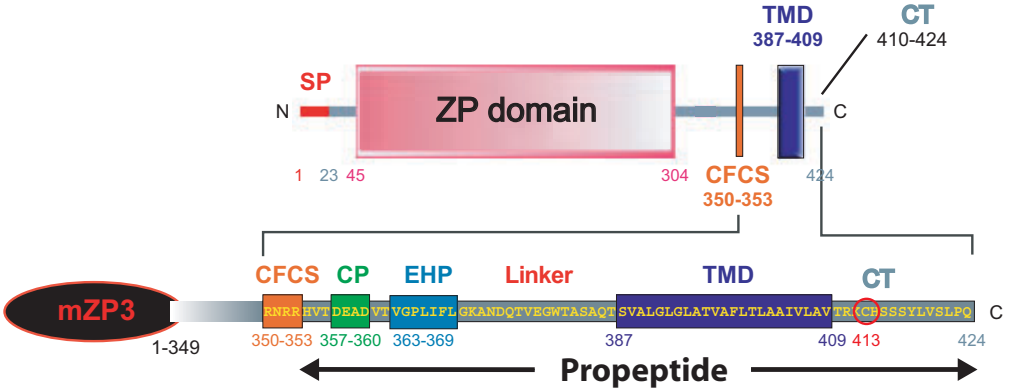



**Fig. 5. Sequence of the C-terminal propeptide of mouse ZP3.** *Polypeptide boundaries are marked by gray bars, with the signal peptide (SP) in red; the ZP domain, CFCS, charged peptide (CP), EHP, and TMD are depicted as pink, orange, green, cyan, and blue rectangles, respectively; the conserved $Cys_{413}$ is circled in red.*

with a conserved 7-aa internal hydrophobic patch (IHP; aa-166 to aa-172) (Jovine *et al.,* 2004) (Fig. 6). Nascent ZP3 (and ZP2) is incorporated into only the innermost layer of the ZP as it thickens around growing oocytes (Qi *et al.,* 2002). This suggests that ZP glycoproteins secreted and assembled at early stages of oocyte growth should be found in the outside layer of the ZP of fully-grown oocytes.

ZP3 also has a large region (~80% of the polypeptide) called the ZP domain (Bork and Sander, 1992) that extends from aa-45 to aa-304 (~260-aa) and contains 8 conserved Cys residues (Fig. 2). All ZP glycoproteins possess a ZP domain, with the ZP domains of ZP1 and ZP2 more homologous to each other (~34% identity) than to ZP3 (~19% identity). ZP domains have been identified in polypeptides from virtually all organisms, from jelly fish (Matveev *et al.,* 2007) to human beings (Jovine *et al.,* 2002a, 2005), and are often glycosylated, modular structures consisting of multiple types of domains (Jovine *et al.,* 2005). For example, the ZP domain is a feature of all ZP glycoproteins and all egg extracellular coat proteins, from echinoderm to human eggs (Jovine *et al.,* 2005).

Several lines of evidence from our laboratory suggest that the ZP domain of ZP glycoproteins, and of many other extracellular proteins, functions as a polymerization module (Jovine *et al.,* 2002b). Apparently, the ZP domain consists of two subdomains, called ZP-N (4 Cys residues) and ZP-C (4 Cys residues), separated by a protease sensitive linker (Jovine *et al.,* 2004; Llorca *et al.,* 2007). Subdomain ZP-N (ZP3 aa-42 to aa-143) on its own forms homodimers that are able to polymerize into fibrils (Jovine *et al.,* 2006, 2007). Furthermore, a recent report suggests that N-terminal extensions of ZP polypeptides consist of single or multiple copies of a domain related to ZP-N (Callebaut *et al.,* 2007). The mechanism used to prevent polymerization of ZP glycoproteins in oocytes (Fig. 6) probably applies to all ZP domain proteins since it relies on sequence elements, EHP and IHP, and events, coupling between proteolytic processing and polymerization, conserved in all ZP domain proteins (Jovine *et al.,* 2005). A similar proteolytic processing mechanism that regulates polymerization has been reported for several other proteins as well (Taylor *et al.*, 1997; Bourne *et al.*, 2000; Handford *et al.*, 2000; Mosesson *et al.*, 2001).

## Targeted mutagenesis of the ZP3 gene

As pointed out in the Introduction, female mice lacking ZP3 have been produced by targeted mutagenesis (homologous recombination) of the *ZP3* gene. Results from our own (Liu *et al.,* 1996) and another (Rankin *et al.,* 1996) laboratory revealed that homozygous null females for ZP3 (*ZP3*$^{-/-}$) fail to have a ZP around their growing oocytes (Fig. 7) and are infertile. That is, although oocytes from *ZP3*$^{-/-}$ females continue to synthesize ZP1 and ZP2, in the absence of ZP3 the glycoproteins are not assembled into a ZP. This finding is consistent with the suggestion that ZP2 and ZP3 form heterodimers and the dimers then polymerize into ZP fibrils (Wassarman *et al.,* 1985; Wassarman, 1987, 1990). The absence of a ZP has deliterious effects on both oocyte growth and follicle development, such that *ZP3*$^{-/-}$ females produce very few, if any, ovulated eggs (Wassarman *et al.,* 1998). This is manifested as reduced ovarian weights, reduced numbers of Graafian follicles, and reduced numbers of fully-grown oocytes. Oocytes from *ZP3*$^{-/-}$ females can undergo meiotic maturation *in vitro* and emit a first polar body (Fig. 7); ovulated eggs lacking a ZP can be fertilized *in vitro* and divide (E.S. Litscher and P.M. Wassarman, unpublished observations).

Heterozygous null female mice (*ZP3*$^{+/-}$) are as fertile as wild-

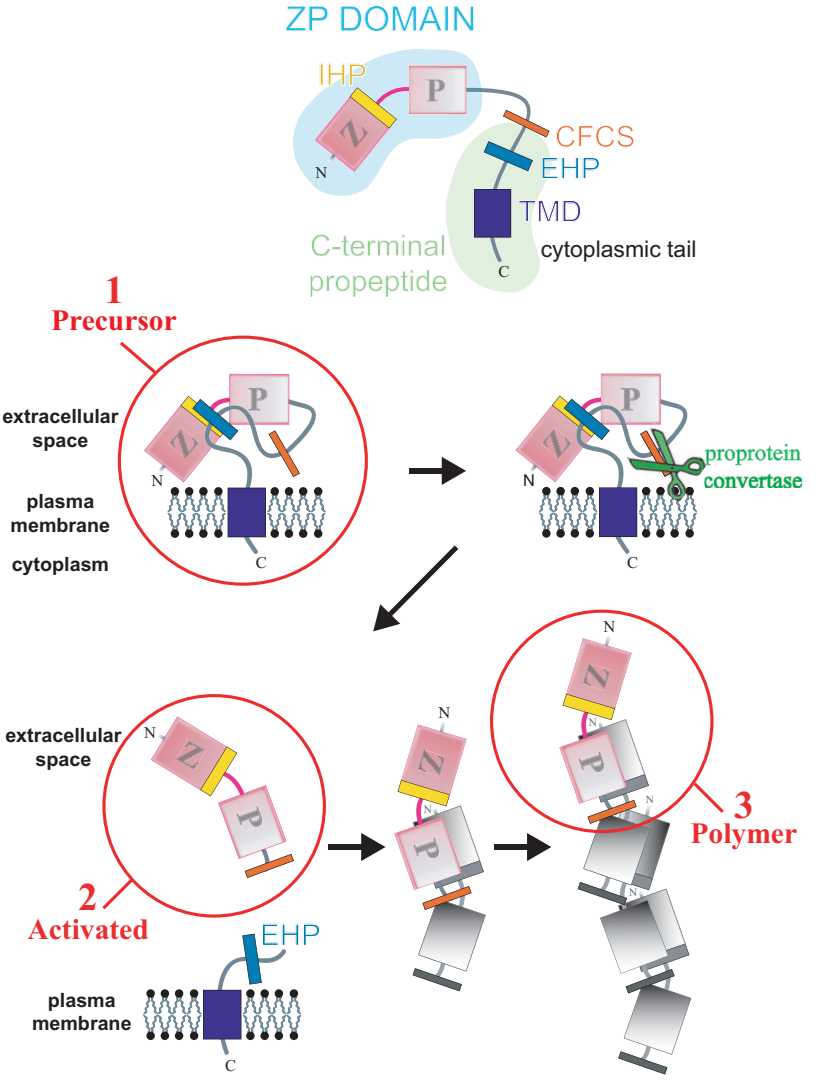



**Fig. 6. A general mechanism for assembly of ZP domain proteins.** *In all ZP domain precursors, the ZP domain is followed by a C-terminal propeptide (CTP) that contains a basic cleavage site (such as a CFCS), and EHP, and, in most cases, a TMD or GPI-anchor* (top panel)*. Precursors do not polymerize within the cell either as a result of direct interaction between the EHP and IHP or because they adopt an inactive conformation dependent on the presence of both patches* (middle left panel)*. C-Terminal processing at the CFCS by a proprotein convertase* (middle right panel) *would lead to dissociation of mature proteins from the EHP* (bottom left panel)*, activating them for assembly into filaments and matrices* (bottom right panel)*. For experimental details that led to this mechanism see Jovine* et al. *(2004).*

type animals, but their eggs have a thin ZP (~2.7 μm) as compared to the ZP (~6.2 μm) of eggs from wild-type animals (Wassarman *et al.,* 1997). These findings suggest that even when the amount of ZP3 is limiting (approximately one-half wild-type levels) it can still participate in ZP assembly with ZP1 and ZP2, yielding a functional, but thinner than normal ZP. In this context, it should be noted that the thickness of the ZP of wild-type eggs varies considerably (6.2 ± 1.9 μm; range 4.3-8.1 μm) and that capacitated sperm bind to growing oocytes (~40-70 μm in diameter) that have ZP of variable widths (Bleil and Wassarman, 1980c). Collectively, these observations suggest that the presence of a ZP of any width can support binding of sperm to eggs.

## Interaction of ZP3 with sperm

As mentioned earlier, mouse egg ZP3 inhibits binding of sperm to eggs and induces sperm to complete the AR *in vitro*. These observations suggest that ZP3 binds selectively to the heads of acrosome-intact sperm. To test this possibility, we examined the binding of either radiolabeled or gold-labeled ZP3 to sperm by either light or electron microscopy, respectively. Whole-mount autoradiography of sperm incubated with radiolabeled ZP3 revealed that ZP3 was localized to the acrosomal cap region of heads of acrosome-intact sperm, but not to the heads of acrosome-reacted sperm (Bleil and Wassarman, 1986). Similarly, transmission electron microscopy of thin sections of sperm incubated with gold-labeled ZP3 revealed that ZP3 was localized preferentially to plasma membrane overlying the acrosomal and post-acrosomal regions of acrosome-intact sperm (Mortillo and Wassarman, 1991). Approximately 4- to 5-times more gold-labeled ZP3 was bound to acrosome-intact than to acrosome-reacted sperm. Results of analogous experimental approaches also suggested that the characteristics of ZP3 binding to sperm were consistent with the proposed biology of sperm-ZP3 interactions (Kerr *et al.,* 2002).

We also carried out solid-phase assays and found that sperm with an intact acrosome (assayed microscopically) could bind to silica beads to which ZP3 was covalently linked (Fig. 8) (Vazquez *et al.,* 1989). The extent of binding of sperm to ZP3-beads was dependent on ZP3 and sperm concentrations, as well as incubation time and temperature. However, it was clear that only acrosome-intact sperm bound to ZP3-beads, not to empty beads or to beads bearing other proteins (e.g., ZP2, fetuin, or BSA), and once bound the sperm could complete the AR and be released from the beads. In addition, sperm bind to EC cells transfected with ZP3 and organized into aggregates about the size of ovulated eggs (Kinloch et al., 1991). Collectively, these results provided direct evidence that ZP3 bound to plasma membrane surrounding heads of apparently acrosome-intact sperm *in vitro*.

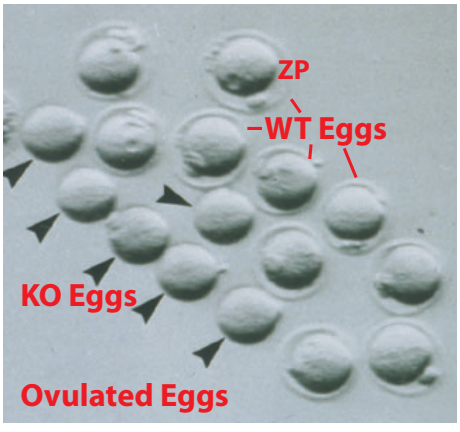
## Sperm combining-site of ZP3

Our investigation of the sperm combining-site of ZP3 has been ongoing since the early 1980s. By using a variety of experimental approaches we have attempted to identify the nature and location of the ZP3 region (i.e., polypeptide, oligosaccharide, or both) to which sperm bind, the so-called sperm combining-site.

### *Nature of the ZP3 sperm combining-site*

The investigations began by extensively degrading purified ZP3 with insoluble Pronase, producing variously sized fragments of the glycoprotein, and assaying the fragments for their ability to inhibit binding of sperm to eggs *in vitro* (Florman *et al.,* 1984). These experiments revealed that small glycopeptides of ZP3 (~1.5-6 kDa $M_r$) were capable of inhibiting binding of sperm to eggs, however, they did not induce sperm to complete the AR. Our results suggested that sperm bound to ZP3 oligosaccharides, rather than to polypeptide, and inhibited binding of sperm to eggs, but that polypeptide was important for inducing sperm to complete the AR. The findings also suggested that the two effects of ZP3 on fertlization were dependent on different molecular features of the glycoprotein. In this context, binding and induction of the AR proved to be distinguishable from one another because of their distinctive time courses (Florman and Storey, 1982; Bleil and Wassarman, 1983), sensitivity to inhibitors (Florman and Storey, 1982), and dependence upon ZP3 concentration (Bleil and Wassarman, 1983), suggesting that they were indeed separate processes.

Direct evidence for participation of oligosaccharides in binding of sperm to ZP3 was obtained by our laboratory when purified ZP3 was exposed to N-glycanase (to remove N-linked oligosaccharides), mild alkaline hydrolysis (to remove O-linked oligosaccharides), or trifluoromethanesulfonic acid (to remove all oligosaccha-



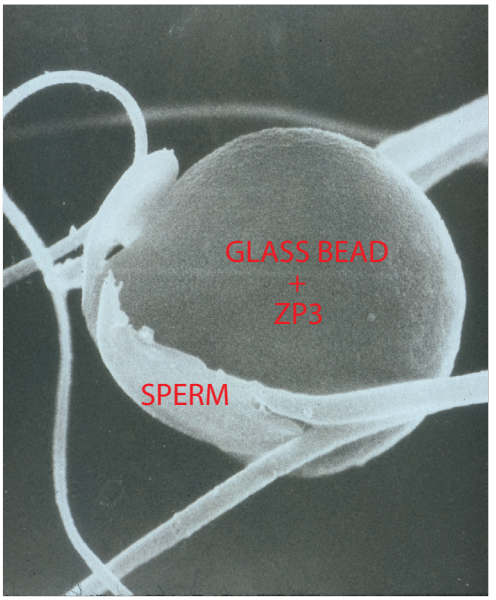




**Fig. 7 (Above). Light micrograph of ovulated eggs from wild-type and *ZP3*$^{-/-}$ female mice.** *Ovulated eggs were obtained from oviducts excised from ~6 week-old superovulated mice. Shown are 6 ovulated eggs recovered from* ZP3$^{-/-}$ *mice (arrowheads; lacking a ZP) and 10 ovulated eggs recovered from wild-type mice. Micrographs were taken using Nomarski differential interference contrast microscopy. For experimental details see Liu* et al. *(1996).*

**Fig. 8 (Right). Scanning electron micrograph of mouse sperm bound to a silica bead containing covalently linked mouse egg ZP3.** *For experimental details see Vazquez* et al. *(1989).*

rides) (Florman and Wassarman, 1985). The latter two treatments destroyed the ability of ZP3 to inhibit binding of sperm to eggs *in vitro* suggesting that sperm bound to ZP3 O-linked oligosaccharides. Furthermore, it was demonstrated directly that a specific size-class of O-linked oligosaccharides released from ZP3 by mild alkaline digestion inhibited binding of sperm to eggs and could be significantly enriched by incubation with sperm. Results of these experiments provided further evidence that mouse sperm recognized and bound to ZP3 oligosaccharides and, in particular, to O-linked oligosaccharides. Subsequently, it was found that digestion of ZP3 by certain glycosidases destroyed its ability to inhibit binding of sperm to eggs (Bleil and Wassarman, 1988) and that incubation of sperm with certain oligosaccharides of defined structure (Seppo *et al.,* 1995) at micromolar concentrations inhibited their binding to eggs, but did not induce sperm to complete the AR (Litscher *et al.,* 1995). For example, in the presence of 10 μM tetraantennary octadecasaccharide with galactose in α-linkage at the nonreducing termini, binding of sperm to eggs was significantly inhibited (~75 %). The latter results were extended in studies of the effects of Lewis X-containing glycans and neoglycoproteins on binding of sperm to eggs and induction of the AR (Kerr *et al.,* 2004; Hanna *et al.,* 2004). Furthermore, a role for ZP3 oligosaccharides in sperm binding is consistent with the unusual stabiltity of the glycoprotein's inhibitory effects following treatment with extremes of temperature or pH, proteases, denaturants, detergents, or fixatives. It is relevant to note that binding of bacteria, animal viruses, parasites, and other pathogens to their cellular hosts, binding of bacteria to plants, binding of pollen to the plant stigma, sexual agglutination in yeast, and binding of amphibian and marine sperm to eggs are all considered to be carbohydrate-mediated events (Varki *et al.,* 1999).

### *Location of the ZP3 sperm combining-site*

To locate the sperm combining-site of ZP3, purified glycoprotein was digested with either papain or V8 protease and the glycopeptides produced were fractionated and assayed for their ability to inhibit binding of sperm to eggs and to induce sperm to complete the AR (Rosiere and Wassarman, 1992; Litscher and Wassarman, 1996a). Each proteolytic digest of ZP3 contained a heavily glycosylated ~55 kDa $M_r$ peptide that was reduced to ~21 kDa $M_r$ after treatment with N-glycanase to remove N-linked oligosaccharides. The ~55 kDa $M_r$ glycopeptide was derived from the carboxy-terminal half of ZP3, possessed 4 or 5 potential N-linked glycosylation sites, and after removal of N-linked oligosaccharides remained quite heterogeneous with respect to both its charge and molecular weight. Both untreated and N-glycanase treated glycopeptide inhibited binding of sperm to eggs and induced sperm to complete the AR *in vitro* to about the same extent as intact ZP3. These findings suggested that the sperm combining-site of ZP3 is located in the carboxy-terminal half of ZP3 and does not involve N-linked oligosaccharides.

In addition to the biochemical approach just described, several molecular genetic approaches were taken by our laboratory to identify the location of the sperm combining-site of ZP3. These approaches were made possible by the successful cloning and sequencing of the mouse ZP3 gene and polypeptide in the late 1980s (Kinloch *et al.,* 1988; Ringuette *et al.,* 1988; Kinloch and Wassarman, 1989). For example, exon swapping and site-directed mutagenesis were carried out on ZP3 (Kinloch *et al.,* 1995;

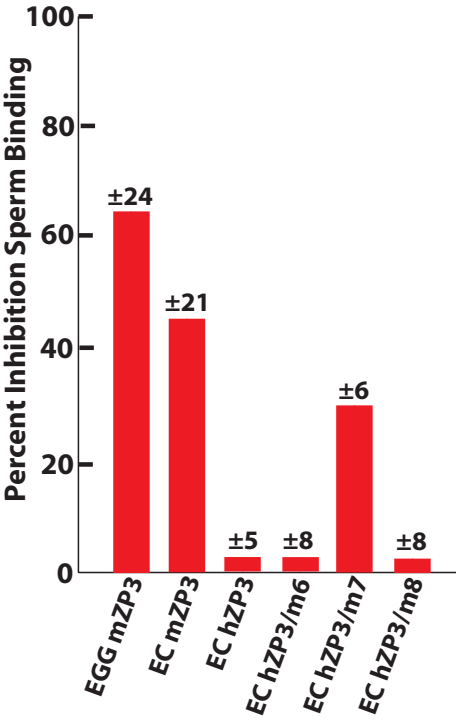



**Fig. 9. Inhibition of binding of mouse sperm to eggs in the presence of wild-type and recombinant mouse and hamster ZP3.** *Shown is the average percent inhibition ± SD of sperm binding to eggs for wild-type and hybrid ZP3. In a control sample sperm were pre-incubated in the presence of M199-M alone (0 % inhibition). In all samples wild-type and recombinant glycoproteins were present at ~5 ng/μl. The values represent the average of 3 or 4 separate experiments with each sample. EC, embryonal carcinoma; m, mouse; h, hamster; m6, mouse exon-6; m7, mouse exon-7; m8, mouse exon-8. For experimental details see Williams* et al. *(2006).*

Chen *et al.,* 1998) using stably transfected embryonal carcinoma (EC) cell lines that synthesized and secreted recombinant ZP3 (Kinloch *et al.,* 1991). Results of such experiments revealed that the sperm combining-site is located in the carboxy-terminal region of ZP3 polypeptide encoded by exon-7 of the ZP3 gene; results consistent with those of the biochemical approach described above. It was noted by us (Kinloch *et al.*, 1995; Wassarman and Litscher, 1995), and later by others (Swanson *et al.,* 2001), that this region of ZP3 polypeptide exhibited considerable sequence divergence during evolution, consistent with a role in species-restricted fertilization.

In a recent study of the sperm combining-site of ZP3, exon-swapping and an IgG(Fc) fusion construct were used to further evaluate whether mouse ZP3 exon-7 is essential for binding of sperm to mouse ZP3 (Williams *et al.,* 2006). In one set of experiments, hamster ZP3 exon-6, -7, and -8 (Kinloch *et al.,* 1990) were individually replaced with the corresponding exon of mouse ZP3. These experiments were possible because, although hamster ZP3 purified from hamster egg ZP inhibits binding of mouse sperm to eggs *in vitro*, recombinant hamster ZP3 made by EC cells has no effect on binding of mouse gametes (Kinloch *et al.,* 1991; Litscher and Wassarman, 1996b). A similar situation (i.e., a shift in sperm binding specificity) has been described by Yonezawa *et al.* (2005) for native and recombinant porcine ZP

glycoproteins and their interaction with porcine and bovine sperm. Stably transfected EC cell lines carrying the recombinant genes were produced and secreted recombinant glycoprotein was purified and assayed for the ability to inhibit binding of sperm to eggs. While EC-hamster ZP3, a recombinant form of hamster ZP3, is unable to inhibit binding of mouse sperm to eggs *in vitro,* the results suggest that substitution of mouse ZP3 exon-7 for hamster ZP3 exon-7, but not mouse ZP3 exon-6 or –8, can impart inhibitory activity to EC-hamster ZP3 with mouse gametes (Fig. 9). In this context, a fusion construct consisting of human IgG(Fc) and mouse ZP3 exon-7 and –8 was prepared, an EC cell line carrying the recombinant gene was produced, and secreted chimeric glycoprotein, called EC-huIgG(Fc)/mouse ZP3(7), was purified and assayed. It was found that the chimeric glycoprotein bound specifically to plasma membrane overlying sperm heads to a similar extent as egg mouse ZP3 and, at nanomolar concentrations, inhibited binding of mouse sperm to eggs *in vitro*.

Collectively, these observations provide further evidence that sperm recognize and bind to a region of mouse ZP3 polypeptide that is encoded by exon-7 and is immediately downstream of its ZP domain. This conclusion is supported by another recent report on the inhibitory effects of the carboxy-terminal region of recombinant mouse ZP3 *in vitro* (Li *et al.,* 2007). Furthermore, mice immunized with a 16 amino acid peptide from this region of mouse ZP3 polypeptide produced antibodies that recognized a 7 amino acid epitope (aa 336-342 encoded by exon-7) and that bound to egg ZP *in vivo* resulting in long-lasting infertility (Millar *et al.,* 1989). Similarly, exposure of eggs to polyclonal antibodies raised against the region of ZP3 polypeptide encoded by exon-7 also inhibited binding of sperm to eggs *in vitro* (Rosiere and Wassarman, 1992). It is of interest that ZP3 is among the 10% most different proteins between rodents and humans (Makalowski and Boguski, 1998; Aagaard *et al.,* 2006). As indicated above, the region of ZP3 polypeptide encoded by exon-7 has undergone a relatively large number of changes during evolution, as compared with the remainder of the polypeptide (Wassarman and Litscher, 1995), and is a proposed site of positive Darwinian selection (Swanson *et al.,* 2001, 2003).

Inspection of the region just upstream of the ZP domain of ZP3 from mammals and related vitelline envelope (VE) proteins from non-mammals, reveals a great deal of conservation. This is especially true of the arrangement of the four closely spaced Cys residues in this region (Fig. 10). For mammals, amphibians, and birds this region has the signature sequence Cys-X-Cys-Cys-X-X-X-X-Cys, whereas for fish the sequence is Cys-X-Cys-Cys-X-X-X-Cys. For the mammalian ZP3 sequences shown (Fig. 10), the first Cys residue is preceeded by an acidic (Asp/Glu) and a hydrophobic (Ileu/Val) residue. Since these Cys residues are present as intramolecular disulfides, this portion of ZP3 exon-7 may be termed a «disulfide knot» that precedes a sequence proposed to participate in species-restricted binding of sperm to eggs. It is possible that this knot may be a structural feature essential for the presentation of the ZP3 combining-site (i.e., polypeptide encoded by exon-7) to sperm.

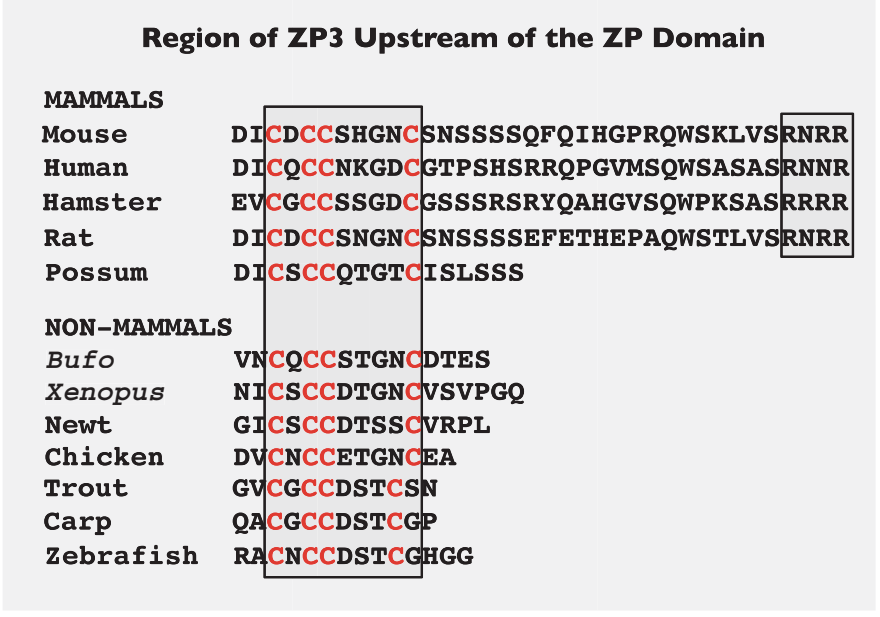



**Fig. 10. Sequences of the region of ZP3-like proteins just upstream of the ZP domain.** *Shown are sequences for mammals, amphibians, birds, and fish using the single letter amino acid code. Boxes surround the 4 conserved Cys residues and the potential furin-like cleavage site.*

## Polymerization of ZP3 into fibrils

Binding of sperm to eggs is carried out under cell culture conditions in Earle's medium 199 in the presence or absence of purified ZP3. In an effort to determine the state of mouse ZP3 when acrosome-intact sperm bind to it, purified ZP3 was analyzed in the absence of either detergents or denaturants by blue native-polyacrylamide gel electrophoresis (BN-PAGE) (Litscher *et al.,* 2008). This technique separates proteins and multiprotein complexes on the basis of molecular weight due to external negative charge provided to proteins by coomassie brilliant blue binding to hydrophobic domains on the surface of proteins (Schagger and von Jagow, 1991; Swamy *et al.,* 2006). Under these conditions, ZP3 migrated, not as a single band, but as several discrete oligomers that gave rise to larger structures that remained at the origin of the gel (Fig. 11). To visualize directly the state of ZP3 under comparable conditions, purified ZP3 was dissolved in water, negatively stained, and subjected to transmission electron microscopy. Relatively large aggregates of long interconnected fibrils composed of contiguous beads, ~13.5 nm in diameter, were observed.

These results suggest that the biological effects of ZP3, inhibition of binding of sperm to eggs and induction of the AR *in vitro*, are due to binding of sperm to homomeric fibrils of ZP3. While the loss of the ability of ZP3 to form long fibrils (e.g., by extensive proteolysis of ZP3) does not interfere with sperm binding, it may result in the failure of ZP3 to induce the AR (Florman *et al.,* 1984; Wassarman *et al.,* 1985; Leyton and Saling, 1989; Litscher and Wassarman, 1996a). It should be noted that, although purified ZP2 also assembles into homomeric fibrils under non-denaturing condi-

tions (Fig. 11), unlike ZP3, ZP2 has no effect on binding of acrosome-intact sperm to eggs. This suggests that ZP2 lacks features present on ZP3 that are recognized by acrosome-intact sperm.

## Summary and final comments

During the past 25 years or so, ZP genes and glycoproteins from a wide variety of mammalian species, ranging from marsupials to human beings, have been sequenced and characterized. A comparison of ZP polypeptides with those of egg VE proteins from a wide variety of non-mammalian species has revealed a surprisingly high degree of conservation over several hundred million years of evolution (Litscher and Wassarman, 2007). Some of this conservation is attributable to the retention of a ~260 amino acid ZP domain, a feature common to all ZP and VE polypeptides that is responsible for polymerization of the proteins into long fibrils (Jovine *et al.,* 2002b, 2005).

Results of several different experimental approaches *in vitro* strongly suggest that acrosome-intact mouse sperm recognize and bind to ZP3, one of three glycoproteins that constitute the mouse egg ZP (Wassarman, 1990, 1999, 2005; Wassarman *et al.,* 2001). Once bound to ZP3, sperm complete the AR and are only then able to penetrate the ZP and fuse with egg plasma membrane. Acrosome-intact sperm (or sperm in a transitional state between acrosome-intact and acrosome-reacted) apparently recognize the region of ZP3 encoded by exon-7 of the *ZP3* gene (i.e., the C-terminal region of the polypeptide), but it remains to be resolved definitively whether sperm recognize polypeptide, oligosaccharide, or both at the sperm combining-site of ZP3. Although there is ample evidence to suggest that sperm recognize ZP3 oligosaccharides, there is no consensus among investigators as to the nature and location of these particular oligosaccharides on ZP3 polypeptide. In this context, a recent report suggests that neither terminal galactose nor N-acetylglucosamine on ZP glycoproteins is required for fertilization in mice (Williams *et al.,* 2007). On the other hand, polypeptide encoded by exon-7 of the *ZP3* gene is considered to have undergone positive Darwinian selection during evolution (Swanson *et al.,* 2001), certainly consistent with its proposed role in species-restricted binding of sperm to eggs.

An extensive literature compiled by many independent laboratories over more than two decades supports the conclusions about ZP3 reported here. However, the role of ZP3 in sperm-egg binding and induction of the AR has been questioned recently and alternative mechanisms not directly involving ZP3 have been proposed to account for mammalian sperm-egg interactions (Rankin *et al.,* 2003; Baibakov *et al.,* 2007). Whether or not these proposals have any merit remains to be determined.

The ZP of mammalian eggs is a rather unique organelle that combines the properties of both the thick jelly coat (AR-inducer) and thin VE (site of sperm receptors) of non-mammalian eggs. In part, it serves to restrict fertilization of eggs to sperm of the same species and to prevent fertilization by more than one sperm (polyspermy). In addition, both egg and preimplantation embryonic development are dependent on the presence of a ZP. Therefore, whatever the specifics of sperm-egg interaction in mammals, the importance of the egg ZP in the successful completion of mammalian oogenesis, fertilization, and preimplantation development cannot be overestimated.

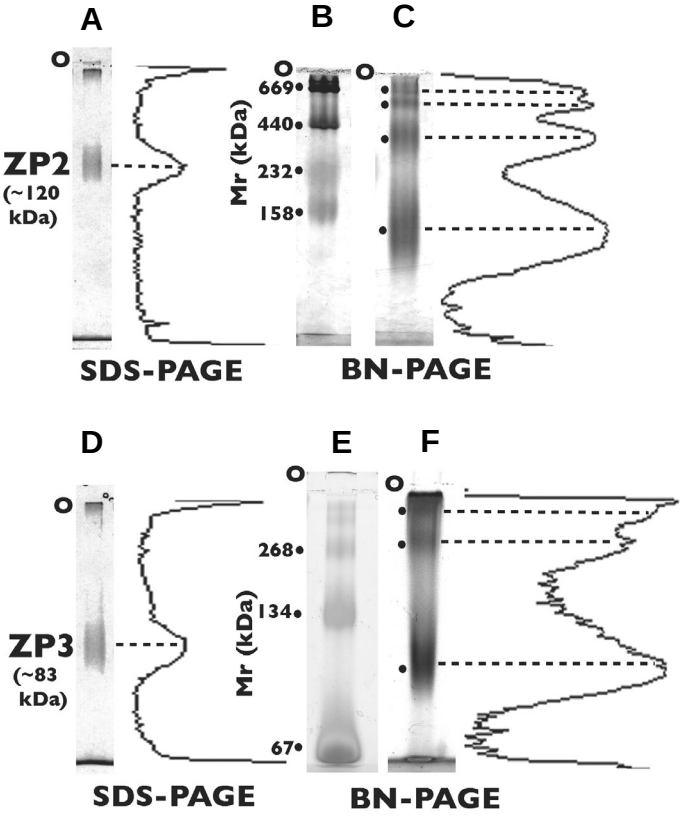



**Fig. 11. Gel-electrophoretic analyses of HPLC-purified mouse ZP2 and ZP3 by SDS-PAGE and BN-PAGE.** *SDS-PAGE analyses of ZP2 and ZP3, together with densitometer tracings of the gels, are shown in lanes A and D, respectively. BN-PAGE analyses of ZP2 and ZP3, together with densitometer tracings of the gels, are shown in lanes C and F, respectively. $M_r$ standards are presented in lanes B and E with apparent $M_r$s (kDa). All lanes were stained with silver nitrate. It should be noted that there is approximately 20-times more glycoprotein present in lanes C and F than in lanes A and D. o, origin. For experimental details see Litscher* et al. *(2008).*

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