Minireview

A Structural View of Egg Coat Architecture and Function in Fertilization[1]

Magnus Monné[3] and Luca Jovine[2]

Department of Biosciences and Nutrition and Center for Biosciences, Karolinska Institutet, Huddinge, Sweden

ABSTRACT

Species-restricted interaction between gametes at the beginning of fertilization is mediated by the extracellular coat of the egg, a matrix of cross-linked glycoprotein filaments called the zona pellucida (ZP) in mammals and the vitelline envelope in nonmammals. All egg coat subunits contain a conserved protein-protein interaction module—the "ZP domain"—that allows them to polymerize upon dissociation of a C-terminal propeptide containing an external hydrophobic patch (EHP). Recently, the first crystal structures of a ZP domain protein, sperm receptor ZP subunit zona pellucida glycoprotein 3 (ZP3), have been reported, giving a glimpse of the structural organization of the ZP at the atomic level and the molecular basis of gamete recognition in vertebrates. The ZP module is divided in two related immunoglobulin-like domains, ZP-N and ZP-C, that contain characteristic disulfide bond patterns and, in the case of ZP-C, also incorporate the EHP. This segment lies at the interface between the two domains, which are connected by a long loop carrying a conserved *O*-glycan important for binding to sperm in vitro. The structures explain several apparently contradictory observations by reconciling the variable disulfide bond patterns found in different homologues of ZP3 as well as the multiple ZP3 determinants alternatively involved in gamete interaction. These findings have implications for our understanding of ZP subunit biogenesis; egg coat assembly, architecture, and interaction with sperm; structural rearrangements leading to postfertilization hardening of the ZP and the block to sperm binding; and the evolutionary origin of egg coats.

crystal structure, egg coat, egg-sperm interaction, fertilization, gamete recognition, vitelline envelope, zona pellucida, ZP module, ZP3, ZP-N domain, ZP-C domain

INTRODUCTION

Approximately 30 years ago, it was discovered that the mouse zona pellucida (ZP) is composed of three glycoprotein subunits—ZP1 (~100 kDa), ZP2 (~120 kDa), and ZP3 (~83 kDa)—that are coordinately synthesized only by growing oocytes over a period of 2–3 wk (Fig. 1A) [1–3]. Based on analysis of solubilized filaments using electron microscopy (EM), it was suggested that ZP2 and ZP3 assemble into micron-long polymers, which are cross-linked into a three-dimensional (3D) matrix by disulfide-linked homodimers of ZP1 (Fig. 1B) [4, 5]. This model is consistent with the molar ratio of the assembled components [2] and has since been confirmed by the phenotype of mice lacking the genes for individual ZP subunits [6–9]. The ZP is thought to have a very similar structure in other mammals, where it often contains an additional ZP1-like subunit, ZP4, the gene for which is not expressed in the mouse [10]. Moreover, proteins homologous to ZPs 1–4 make up the vitelline envelope (VE) of nonmammalian vertebrates [11–14], and related molecules constitute the egg coat of marine invertebrates, such as mollusks and ascidians [15, 16]. Thus, the basic architecture of animal egg coats has been conserved over hundreds of millions of years of evolution [17].

The precursor forms of all ZP/VE proteins contain an N-terminal secretory signal peptide and share a "ZP domain," a C-terminal block of sequence homology that spans approximately 260 amino acids and is distinguished by a few conserved hydrophobic positions as well as either 8 (type I ZP domain, found in ZP3) or 10 (type II ZP domain, found in ZP1/2/4) essentially invariant Cys residues (Fig. 1A) [18, 19]. With the exception of the Cys pair linking the two subunits of the mouse ZP1, all these residues are engaged in intramolecular disulfide bonds [20–24]. The conserved ZP domain, which was suggested to consist of two moieties (ZP-N/ZP-C) [25] and is preceded by a single trefoil/P domain in ZP1 and ZP4 [26], is then followed by a consensus furin cleavage site (CFCS) [27–29]. This separates the mature part of the proteins from a C-terminal propeptide (CTP) that in most cases contains a single-spanning transmembrane domain (TM) and a short cytoplasmic tail (Fig. 1A). A short external hydrophobic patch (EHP), located between the CFCS and the TM, plays a crucial role in the secretion of egg subunit precursors [25, 30]. Dissociation of the EHP upon cleavage of the precursors at the CFCS in the *trans*-Golgi or at the plasma membrane is thought to activate the mature subunits [25], which polymerize into extracellular filaments using their ZP domain [31]. Notably, many different eukaryotic proteins unrelated to fertilization also contain a ZP domain, and parallel studies on some of these systems have provided additional support for the polymerization function of this protein region as well as its control by the EHP-containing CTP [18, 19, 31, 32]. Nevertheless, many questions remain
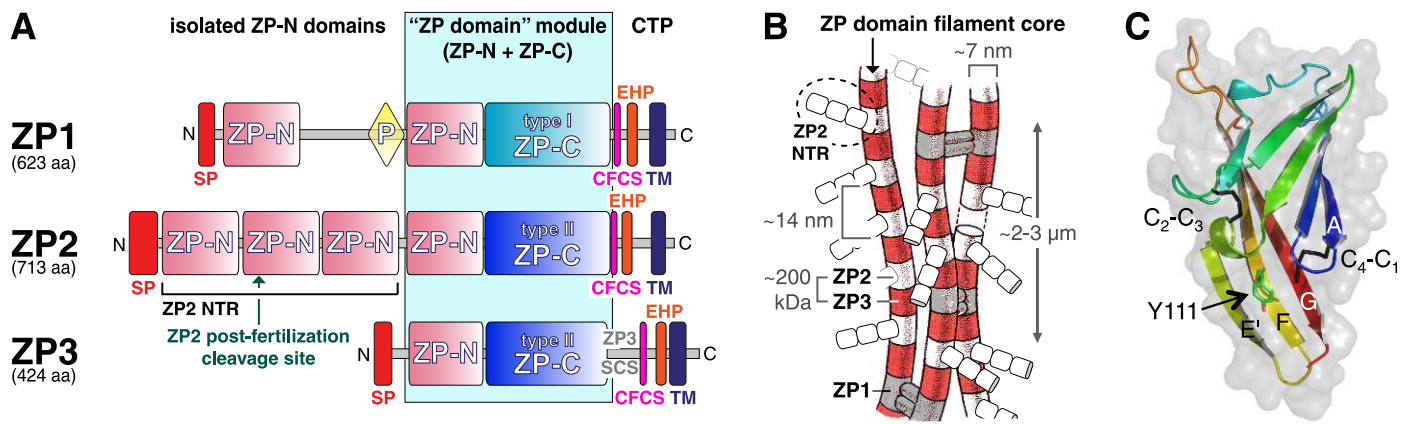
FIG. 1. Structure of the mouse ZP. **A**) Scheme of the domain structure of mouse ZP components ZP1, ZP2, and ZP3. Differences in the number of Cys residues within the ZP-C domains of ZP1/ZP2 and ZP3 give rise to two kinds of ZP modules. aa, amino acids; CFCS, consensus furin cleavage site; CTP, C-terminal propeptide; EHP, external hydrophobic patch; NTR, ZP2 N-terminal region; P, trefoil domain; SCS, sperm combining site; SP, signal peptide; TM, transmembrane domain. Differences in the number of Cys residues within the ZP-C domains of ZP1/ZP2 and ZP3 give rise to two kinds of ZP modules. **B**) Model of overall ZP architecture, modified from Wassarman [90] with permission, to highlight the fact that ZP1 incorporates into ZP filaments using its ZP domain [63], and to show that the three ZP-N domains of the ZP2 NTR protrude from the filament core [4, 44]. **C**) Cartoon diagram and surface representation of the crystal structure of the ZP-N domain of mouse ZP3 [44]. Conserved ZP module Cys residues in this figure and in Figure 2 are indicated by C_n (with n = 1–8, a, or b).

open concerning the mechanism of ZP subunit polymerization as well as the biological function of individual ZP components.

Unlike ZP1-null mice, which produce eggs with a loose ZP and are subfertile [7], the ovulated oocytes of ZP3- and ZP2-knockout mice totally lack a ZP, resulting in complete infertility [6, 8, 9]. Consistent with this observation, both ZP3 and ZP2 bind sperm in vitro, and they have long been considered to act as receptors for non-acrosome-reacted and for acrosome-reacted sperm, respectively [33–36]. Moreover, ZP3 can stimulate the acrosome reaction on sperm in vitro [37, 38], although recent data suggest that cumulus cells might be mainly responsible for inducing this process in vivo [39]. Nevertheless, significant disagreement remains over the precise functions of ZP3 and ZP2 [40, 41], and whether sperm binding to ZP3 is mainly dependent on carbohydrates, the polypeptide, or requires both is unclear [1, 41, 42]. Structural biology could potentially contribute to the solution of this problem, but its application to egg coat subunits—as well as to ZP domain proteins in general—has been hindered by the difficulty of overproducing these molecules in properly folded form at the milligram scale, their very heavy and heterogeneous glycosylation, and their tendency to aggregate at high concentrations. However, x-ray crystallographic structures of both the ZP-N region of mouse ZP3 (Fig. 1C) and full-length chicken ZP3 (Fig. 2) bound to the EHP propeptide have recently been determined [43, 44]. In this review, we discuss the implications that these first atomic-resolution structures of a ZP domain protein have for the architecture, function, and evolution of the ZP.

3D STRUCTURE OF THE ZP DOMAIN

The crystal structures of ZP3 demonstrated that the ZP domain first recognized by pattern-based sequence analysis [19] is in fact a module consisting of two distinct domains, an N-terminal ZP-N domain and a C-terminal ZP-C domain, the latter of which also includes the EHP (Fig. 2) [43, 44]. Although this was not apparent from their sequence, the structure of full-length chicken ZP3 [43] revealed that both of these domains adopt an immunoglobulin (Ig)-like fold, which consists of a sandwich of two β-sheets, the strands A-B-E and

C-F-G of which enclose a hydrophobic core [45]. This accommodates most of the conserved hydrophobic residues originally identified in the ZP domain sequence, the eight invariant Cys residues of which are equally distributed between the two domains [19]. Despite having significantly different primary structure and disulfide bonds, ZP-N and ZP-C share a common basic topology; moreover, structural features both inside and outside their core make them distinct from previously studied Ig-like domains [43].

The ZP-N domain defines a new subtype of the Ig superfamily of proteins by containing a characteristic E′-strand within an E′-F-G β-sheet that extends from the hydrophobic core as well as two almost completely buried disulfide bonds that clamp both sides of the β-sandwich (Figs. 1C and 2A) [43, 44]. These linkages are formed by the first four conserved Cys (C) residues of the ZP module, which adopt a C_1-C_4; C_2-C_3 pattern that is invariant in all ZP subunits so far characterized by mass spectrometry (MS) [20–24]. Mutation of ZP-N disulfide bonds abolishes secretion of ZP3 by transfected CHO cells [43], and their functional importance in vivo is highlighted by the phenotype of substitution Y1870C in the ZP-N domain of inner ear protein tectorin alpha [46]. As discussed below, this mutation causes hearing loss in humans by affecting formation of the canonical C_1-C_4 disulfide [44].

Unlike ZP-N, the ZP-C domain of ZP3 includes an invariant C_5-C_7 disulfide that is located in the hydrophobic core, between residues on neighboring antiparallel β-strands C and F. Previously, such a feature had been found only in the second Ig-like domain of glycoprotein CD4 [47]. Furthermore, two insertions within ZP-C give rise to a ZP3-specific C-terminal subdomain, which contains three additional disulfide bonds and is positioned on the outside of the Ig-like fold (Fig. 2, A and B) [43]. As in the case of their ZP-N counterparts, pairwise mutation of the Cys residues forming the evolutionarily conserved disulfides of ZP-C either severely reduced (ΔC$_5$-C$_7$) or completely abolished secretion of ZP3 [43]. Moreover, the spatial arrangement of these Cys residues provides an explanation to the puzzling observation that different ZP3 homologues can harbor two alternative ZP-C disulfide bond configurations [20–24]: Although chicken ZP3 adopts the same C_6-C_{11}; C_8-C_9; C_{10}-C_{12} pattern as pig ZP3, 3D clustering of the
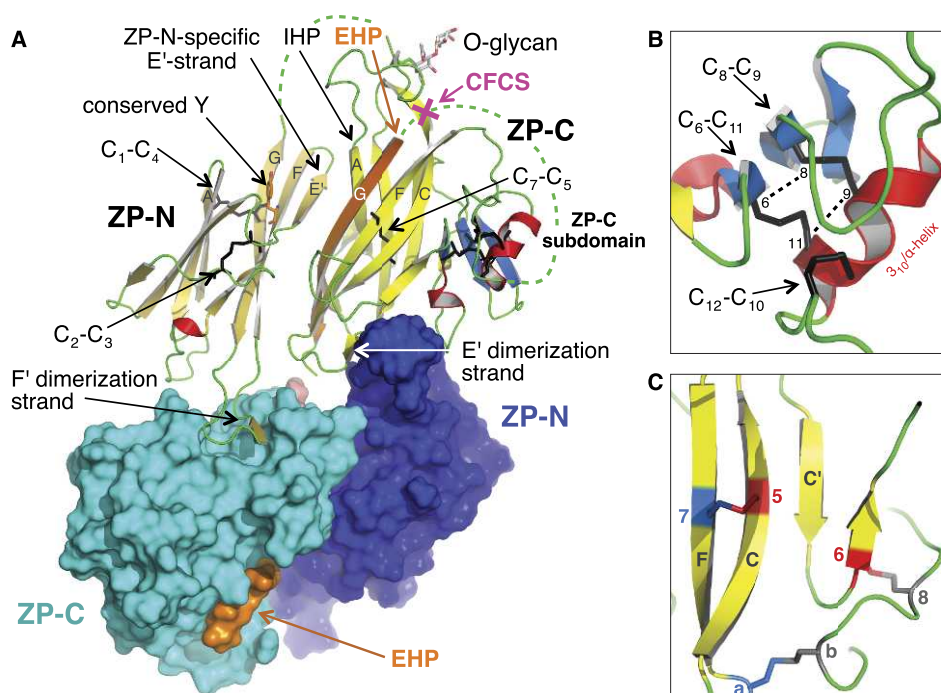
FIG. 2. Crystal structure of the chicken ZP3 homodimer [43]. **A)** View of the protein from the extracellular side, with top and bottom subunits shown in cartoon and surface representation, respectively. **B)** An enlargement of the ZP3-specific ZP-C sub-domain of chicken ZP3, adopting the same disulfide bonds as pig ZP3 [20]. The alternative disulfide bond pattern found in mouse ZP3 and other homologues [21–24] is indicated by dashed lines. **C)** A homology model of the ZP-C domain of mouse ZP2 can be generated on the basis of the chicken ZP3 structure, which is consistent with the experimentally determined disulfide bond linkages of pig ZP4 [20]. To establish the mouse ZP2 disulfide pattern suggested by MS analysis [24], Cys residue pairs highlighted with the same color would have to be engaged in disulfide bonds (C_7-C_a, C_5-C_6, and C_b-C_8) that are incompatible with the fold of ZP3 ZP-C.

Cys could in principle also accommodate the C_6-C_8; C_9-C_{11}; C_{10}-C_{12} connectivity found in fish, mouse, rat, and human ZP3 (Fig. 2B). So what commits the Cys residue of a given ZP3 homologue to its specific disulfide pattern? Lack of secretion of chicken ZP3 constructs, carrying Cys mutations that should result in formation of mouse ZP3-like disulfides, suggests that connectivity is determined by the region around C_9 and C_{11} [43]. In chicken ZP3, these Cys residues are located within a mixed 3_{10}/α-helix having a structure that would not be compatible with a C_9-C_{11} disulfide. Thus, formation of this C-terminal helix likely precedes that of the disulfides in chicken/pig ZP3, whereas in mouse ZP3 and other homologues, a different local conformation of the same polypeptide region presumably favors the alternative disulfide pattern.

Because the overall fold of the ZP-C domain of ZP3 explains the two alternative disulfide bond connectivities of the protein, its structure can be used as a framework to model all other homologues of ZP3 as well as pig ZP4 [20]. However, the fold is not compatible with the disulfide patterns observed in type II subunits, such as mouse ZP1 and ZP2 [24], or fish VEalpha/beta [23]. This can be due only in part to the fact that type II ZP modules lack the ZP-C subdomain specific to ZP3, because formation of these other patterns is hindered by strands F and C′ of the ZP-C β-sandwich (Fig. 2C). Thus, the fold of type II ZP-C domains must be significantly different from that of ZP3, even though it could also cluster conserved Cys residues in space to allow formation of the alternative disulfide patterns of type II subunits.

ZP MODULES OF TWO ZP3 MOLECULES INTERACT TO FORM AN OBLIGATE HOMODIMER

The recombinant chicken ZP3 protein used for structure determination retained a CTP truncated before the TM and was found to be a dimer both in solution and in the crystals (Fig. 2) [43]. At the center of the dimer interface, an antiparallel β-sheet is formed by a short F′-strand in the FG-loop of ZP-N and an E′-strand located below the β-sandwich of the ZP-C domain. This intermolecular interaction is ensured by the high electrostatic complementarity of the two binding partners [43], and it relies on induced fit of the ZP-N moiety of the ZP module, which lacks the F′-strand when crystallized in the absence of ZP-C [44]. As underlined by the high conservation of residues at the interface, the mutation of which abolishes protein secretion [43], formation of the homodimer is essential for biogenesis of ZP3 and, thus, egg coat assembly.

PREVENTION OF PREMATURE POLYMERIZATION AND EVENTS LEADING TO ZP FILAMENT ASSEMBLY

Secretion and incorporation of ZP3 into the ZP are dependent on the EHP, which is conserved in the CTP of all egg coat subunits as well as ZP domain proteins unrelated to fertilization [25, 30]. Analysis of ZP3 proteins mutated in either the EHP or a related internal hydrophobic patch (IHP) region within the ZP module suggested that the presence of both patches prevents premature polymerization of the ZP3 precursor, possibly due to interaction between the patches themselves [25]. This hypothesis was supported by parallel studies on the ZP domain-containing protein uromodulin [32], but other possible functions for the EHP were also suggested [30].

Unexpectedly, the structure of chicken ZP3 revealed that the EHP constitutes the G-strand of the Ig-like fold of ZP-C, so the patch is an integral part of the domain. Moreover, the EHP G-strand is positioned opposite to the IHP, which corresponds to the A-strand of the β-sandwich, and lies at the intramolecular interface between the ZP-C and ZP-N domains (Fig. 2A). The EHP is mainly held together by interactions between hydrophobic residues, the mutation of which abolishes protein secretion [25, 43]. Consequently, four important interfaces exist within the chicken ZP3 structure that both ensure protein dimerization—a prerequisite for secretion—and mask areas that take part into polymerization: two direct intermolecular interfaces between the ZP-N and ZP-C domains of opposite subunits, and two EHP-mediated intramolecular interfaces between the ZP-N and ZP-C domains within each monomer (Fig. 2A) [43]. Although burial of the EHP makes it unlikely that this binds shuttling chaperone(s) important for intracellular trafficking of ZP3 [30], a recent study on ZP domain protein deleted in malignant brain

tumors 1 (also known as hensin) [48] suggests that the essential *cis*-Pro residue in the patch might perhaps be targeted by a secreted peptidyl-prolyl *cis-trans* isomerase during early stages of ZP3 folding. On the other hand, consistent with the finding that ZP subunits traffic independently within oocytes before incorporation into the ZP [49], the position of the EHP does not support the idea that the patch might allow the precursors of ZP subunits to form complexes inside the oocyte [30]; rather, EHP appears to lock the ZP-N and ZP-C domains of the ZP3 precursor in a conformation that is incompatible with polymerization [25, 43]. So what could happen when the CTP, which contains the EHP and the membrane anchor, is cleaved and leaves the dimer? What structural rearrangements could this trigger in ZP3 that lead to interaction with other ZP components and polymerization?

In the structure of chicken ZP3, the CTP is positioned so that the CFCS sits within a surface loop facing the extracellular space. This is followed by the EHP, which penetrates through the core of the homodimer toward the TM on the other side of the molecule (Fig. 2A). Such an arrangement makes the cleavage site highly accessible to protease-mediated hydrolysis, in agreement with the observation that chicken ZP3 can be cleaved by trypsin immediately before the CFCS, which was mutated in the recombinant protein used for crystallographic analysis [43]. Anchoring of the CTP to the plasma membrane suggests that following the initial cleavage event, the EHP must exit the structure in the direction of the TM. Indeed, this directionality is likely to also be maintained in ZP protein precursors that lack a membrane-anchoring element, because processing of ZP3 constructs truncated before the TM is still required for EHP dissociation [43]. Exit of the patch must require significant structural rearrangements (Fig. 2A): The intramolecular interface between ZP-N and ZP-C must open up, and because the EHP is fully incorporated in the ZP-C fold, many hydrogen bonds between the patch and its neighboring F-strand, as well as its interactions with the hydrophobic core, must be broken. Analysis of EHP dissociation after cleavage suggests that this process is slow but can occur spontaneously in vitro [43]; nevertheless, it is possible that interaction between cognate ZP subunits at polymerization facilitates EHP ejection so that the two processes are strictly coordinated in vivo.

POTENTIAL POLYMERIZATION INTERFACES

As a result of EHP dissociation, protein-protein interaction regions must be exposed that trigger polymerization of ZP subunits. Structural analysis of mouse ZP3 ZP-N and full-length chicken ZP3 has identified a number of potential elements that could take part in these contacts. An obvious candidate is the IHP β-strand that has lost its EHP counterpart on the opposite side of the β-sandwich and is therefore exposing its hydrophobic surface. Another potential player could be the EHP neighboring F-strand, which is linked to the C-strand through a C_7-C_5 disulfide bond conserved in all ZP3 homologues so far characterized [20–24, 43]. Interestingly, the analogous disulfide in the second Ig-like domain of extracellular protein CD4 has been suggested to take part in domain swapping and dimerization events [50]. Next to the EHP exit pathway also lies the ZP-N domain E'-F-G extension that contains a highly conserved Tyr residue (Figs. 1C and 2A) implicated in polymerization by human deafness-associated tectorin alpha mutation Y1870C [46]. Introduction of a corresponding Y111C mutation in the ZP-N domain of mouse ZP3 interfered with correct disulfide bond formation, suggesting that tectorin alpha Y1870C acts as a dominant negative by incorporating into filaments of the tectorial membrane—an

important extracellular matrix of the inner ear—and disrupting their further elongation [44]. It is possible that structural changes resulting from EHP ejection are transmitted to the E'-F-G extension through the loops around the C_2-C_3 disulfide of ZP-N, because this adopts different conformations in the structure of isolated mouse ZP3 ZP-N and of full-length chicken ZP3, where it interacts with the ZP-C domain [43, 44].

In addition to the potential interaction interfaces mentioned so far, all of which could be inhibited from taking part in polymerization because of spatial proximity to the EHP, other potential regions could be involved in ZP assembly. Between the A- and G-strands around C_1-C_4 in the ZP-N domain (Figs. 1C and 2A) is a conserved hydrophobic surface that in the structure of mouse ZP3 ZP-N packs against maltose-binding protein (MBP)—a fusion partner that was used to reduce aggregation of ZP-N and facilitate its crystallization [44]. Because the same region is exposed in the crystals of full-length chicken ZP3 [43], it might be available for interaction with other ZP subunits during polymerization. Finally, structural differences reflected by the alternative disulfide bond patterns of the ZP3-specific ZP-C subdomain appear to have a role in determining the specificity of ZP subunit interaction. This is because alternative disulfide connectivities have been found in type II ZP subunits (ZP1/ZP2/ZP4) that seem to correlate with those in type I subunit ZP3 [20]. Accordingly, some experimental evidence suggests that only certain combinations of type I/type II disulfide patterns might be compatible with productive interaction between the respective subunits and incorporation into the ZP [51].

Because no high-resolution structural information is available on the final result of polymerization—the ZP filament—it is still a matter of speculation which of the aforementioned potential interfaces interact with each other during egg coat assembly. Similarly, it remains to be established whether ZP3 incorporates into the filament as a homodimer and if the same quaternary structure also is conserved in other ZP subunits. In this regard, it is notable that the dimensions of the chicken ZP3 dimer approximately match those of the structural repeat found in ZP filaments [5]. Moreover, a recent analysis of the molecular basis of hatching in fish [52] suggests that the dimer interface of chicken ZP3, the structure of which represents a snapshot of the protein after CFCS cleavage but before EHP dissociation (Fig. 2A), could be maintained in the egg coat. However, these data do not exclude the possibility that a comparable heterodimeric interface might be established in the filaments between ZP3 and ZP1/ZP2/ZP4 through domain swapping of activated ZP subunits.

STRUCTURE OF ZP3 RECONCILES APPARENTLY CONTRASTING SPERM-BINDING DATA

A large body of experimental evidence from a number of independent laboratories suggests that the very C-terminal part of mature ZP3 plays a major role in its ability to bind sperm in a species-restricted manner. In particular, this function has been ascribed to the region corresponding to exon 7 of the mouse *Zp3* gene, the sequence of which has relatively high variability between species due to positive Darwinian selection [53]. Following seminal biochemical studies that strongly implicated mouse ZP3 *O*-linked oligosaccharides in sperm binding [54], experiments with recombinant protein expressed in embryonal carcinoma (EC) cells suggested that the functional carbohydrates were linked to S332 and S334, located within a so-called sperm combining site (SCS) in the peptide encoded by exon 7 [55]. However, MS analysis of native mouse ZP material revealed that these sites are not modified in vivo [24], and

replacement of wild-type *Zp3* with a transgene expressing a protein that lacks *O*-glycans at S332 and S334 (as well as *N*-glycans at N327 and N330) was recently reported not to impair mouse fertility [40]. On the other hand, the finding that native mouse ZP3 as well as human ZP3 expressed by transgenic mice carry two conserved *O*-glycosylation sites within the ZP domain (site 1, mouse ZP3 T155; site 2, mouse ZP3 T162/S164/S165) led to the suggestion that these might be the authentic attachment sites of the sperm-binding carbohydrates on ZP3 [56]. As in the case of the alternative disulfide bond patterns of different ZP3 homologues, the crystal structure of the protein provides a possible explanation for these seemingly incompatible observations (Fig. 3).

In the electron-density map of chicken ZP3, clear evidence was found for a single *O*-linked oligosaccharide chain attached to T168, a residue that corresponds to mouse/human ZP3 site 1 [43]. The carbohydrate, which based on both MS and lectin-binding analyses is thought to have a T-antigen (Galα1-3GalNAc) structure, is located in a region of the flexible loop between ZP-N and ZP-C that is ordered in the crystals and packs on top of ZP-C (Fig. 2A). In agreement with the exposed position of the sugar, which does not make significant contacts with the polypeptide moiety of the protein, a chicken ZP3 T168A mutant was indistinguishable from the wild type in terms of secretion and biochemical behavior. However, binding of the mutant protein to avian sperm was reduced by approximately 80% compared with the wild type. Because no other *O*- or *N*-linked carbohydrate chain was attached to the chicken ZP3 protein used for the binding assays, this result strongly suggests that the *O*-glycan attached to the evolutionarily conserved site 1 plays a major role in gamete interaction. At the same time, T168 is located on the same protein surface of the chicken ZP3 residues corresponding to mouse ZP3 T162/S164/S165 (site 2) and S332/S334 (SCS) (Fig. 3). Thus, when properly *O*-glycosylated in other ZP3 homologues, these sites could also be involved in carbohydrate-mediated binding, either together with site 1 or, as in the case of EC cell-derived ZP3, instead of it. Moreover, a conserved *N*-glycosylation site (N159 in chicken ZP3), which was inactivated in the protein used for crystallography, is located in a disordered region of the interdomain loop that precedes T168 by a few residues. As in the case of the *O*-glycan attached to T168, this *N*-glycan is not required for secretion in either chicken ZP3 [43] or mouse ZP3 [49, 57], and it might also contribute to sperm binding, at least in some species [41, 42]. Notably, the structure suggests that this and all other *N*-glycans of mouse and human ZP3 are exposed on the extracellular side of the molecule. Finally, modeling of the hypervariable C-terminal region of mature ZP3, which is not defined in the crystal structure due to high conformational flexibility, suggests that this part of the protein could also be exposed in proximity to the glycosylated sites. As a result, the C-terminal region could modulate the function of the glycans as well as provide polypeptide moieties that might be recognized by sperm. This would explain the residual binding activity of chicken ZP3 T168A as well as data suggesting that core-1-derived *O*-glycans may not be strictly required for fertilization in the mouse [58]. Taken together, these considerations are in line with the hypothesis that multiple distinct binding events might be involved in the recognition of ZP3 by different counterpart proteins on sperm [41, 59].

IMPLICATIONS FOR THE OVERALL DOMAIN ARCHITECTURE OF THE ZP

Threading of sequences N-terminal to the ZP module of other egg coat components onto the 3D structure of the mouse
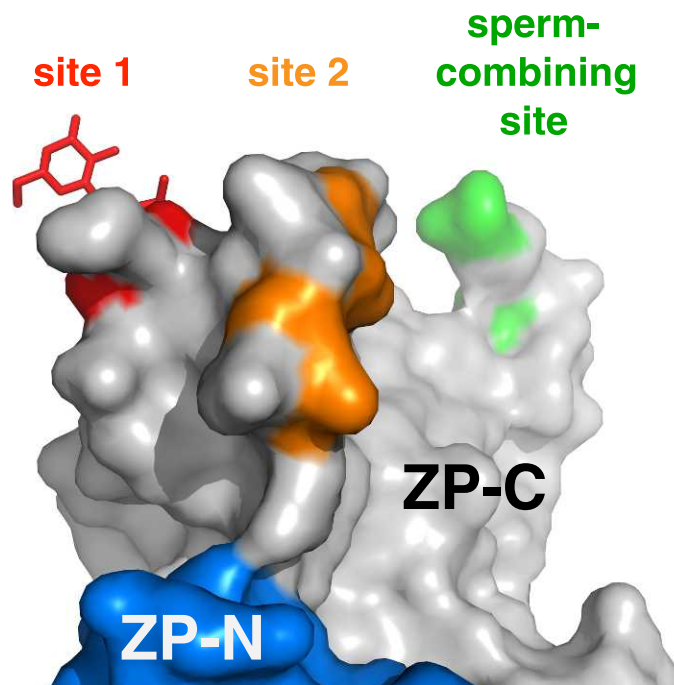

FIG. 3. Different ZP3 regions implicated in binding to sperm are exposed on the same surface of the protein. Chicken ZP3 *O*-glycosylation site 1 (T168) is depicted in red, with the crystallographically resolved carbohydrate [43] shown in a stick representation; chicken ZP3 amino acids corresponding to mouse/human ZP3 site 2 residues [56] are colored in orange. Mouse ZP3 SCS residues S329 to S334 are modeled on top of the structure of the C-terminal part of mature chicken ZP3 (the last visible residue of which, P343, corresponds to S331), with S332 and S334 [55] shown in green.

ZP3 ZP-N domain revealed that in Tetrapoda, ZP1 and ZP4 contain an additional copy of this fold [44]. In the case of ZP1, this is spaced from the central trefoil domain and C-terminal ZP module by a Pro/Thr/Leu-rich region, which in chicken ZP1 is expanded into an approximately 450-residue linker containing a highly repetitive sequence pattern [60]. Moreover, in a subset of ZP1 homologues, the N-terminal ZP-N domain contains an additional unpaired Cys residue within the loop between β-strands C and D, which is presumably responsible for forming an intermolecular disulfide bond that underlies the observed homodimerization of chicken, mouse, and human ZP1 [2, 61, 62].

Similarly, the whole N-terminal region of ZP2 consists of three ZP-N domains (ZP-N1 to ZP-N3) arranged in tandem and connected by short linkers, suggestive of an extended rod-like structure (Figs. 1A and 4A) [44]. Because polymerization of egg coat subunits is mediated by their C-terminal ZP modules [31, 63], and because ZP assembly is not hindered by fusion of large protein tags N-terminal to the ZP module itself [49], the ZP-N repeats of ZP2 likely project out from the ZP module core of ZP filaments (Fig. 1B). Such a supramolecular arrangement is supported by early EM studies of the filaments [4], showing protrusions with a length of approximately 110 Å that matches the span of a homology model of ZP2 ZP-N1 to ZP-N3 (Fig. 4A) [44]. This model, which is consistent with all biochemical data currently available on ZP2, not only visualizes protein regions thought to be important in binding to acrosome-reacted sperm [34] but also provides information on its postfertilization function.

FIG. 4. Postfertilization cleavage of ZP2 and hardening of the ZP. **A**) Homology model of the three ZP-N repeats that constitute the N-terminal region (NTR) of mammalian ZP2, followed by the canonical ZP-N domain of its C-terminal ZP module [44]. The location of the postfertilization cleavage site [40, 70–72] is marked in magenta. **B**) Possible mechanism of ZP hardening. Cleavage of the ZP-N2 domain of ZP2 by an egg cortical granule protease (**left**; green arrows) [64, 65] could cause a conformational change of the ZP2 NTR (**middle**) that leads to interaction between adjacent ZP filaments and compaction of matrix structure (**right**) [66, 67]. For simplicity, ZP subunits within each filament are depicted as monomers.
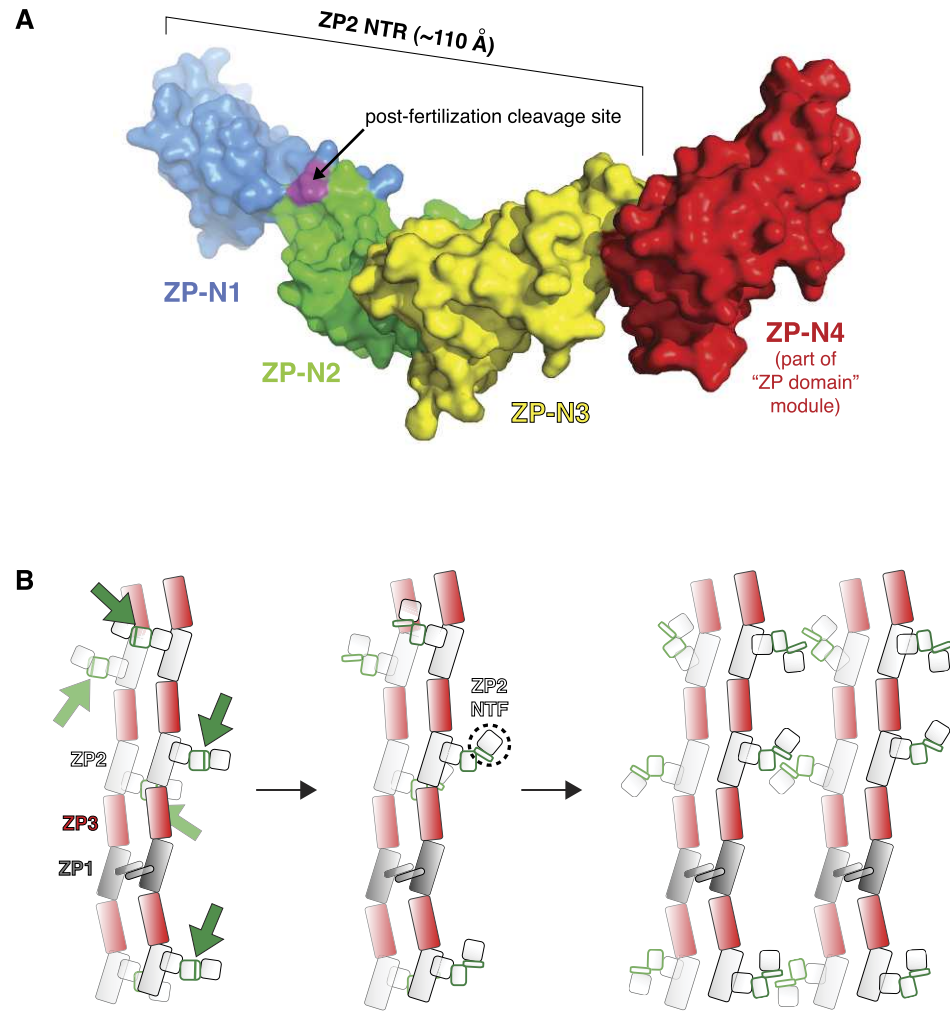
STRUCTURAL INSIGHTS INTO ZP2 CLEAVAGE-ASSOCIATED EVENTS

Upon gamete fusion or artificial activation of unfertilized mouse oocytes with ionophore A23187, ZP2 is specifically cleaved into two proteolytic fragments of approximately 23 kDa (ZP2-N-Terminal Fragment [ZP2-NTF]) and 90 kDa (ZP2$_f$) that remain linked by disulfide bridges [64]. The conversion is catalyzed by an as-yet-unidentified protease that is released into the perivitelline space upon fusion of the egg cortical granules with the plasma membrane [65]. ZP2 cleavage correlates with hardening of the ZP, an alteration of the mechanical properties of the matrix that results from the compaction of filaments in the inner layer of the egg coat and that is thought to contribute to the block to polyspermy by hindering sperm penetration [66, 67]. Experiments in the *Xenopus* system showed that the ZP2-cleaving protease is responsible for hardening of the frog VE [68]. On the other hand, recent transgenesis experiments suggest that processing of ZP2 may not be essential for preventing polyspermy in the mouse but, rather, plays an important role in regulating the interaction between gametes [40, 69]. How can cleavage of ZP2 alter the overall conformation of the egg coat so that this becomes refractory to sperm binding and penetration?

Mapping of the conserved ZP2 cleavage site [40, 70–72] onto the homology model of the protein indicates that the ZP2-specific protease severs the loop that connects β-strands B and C of the second ZP-N repeat (ZP-N2) (Fig. 4A). Thus, ZP2-NTF largely consists of the first ZP-N domain of ZP2, which following cleavage remains attached to the rest of the protein via the canonical C$_1$-C$_4$ disulfide bond of ZP-N2 [44]. Because the ZP-N2 BC loop is at the interface with ZP-N1, cleavage could cause a change in the relative position of the two repeats. This might in turn expose previously shielded protein surfaces, triggering interaction between N-terminal regions of ZP2 molecules that belong to different filaments (Fig. 4B) or, alternatively, between cleaved ZP2 molecules on one filament and the ZP module core of another. By analogy with Ig domain zippers formed by other extracellular matrix proteins [73, 74], the establishment of these new contacts would bring together adjacent ZP filaments and tighten the structure of the egg coat [44]. This may both shield recognition elements on the ZP, causing loss of sperm binding upon ZP2 cleavage [40], as well as contribute to creating a physical block to sperm penetration. Moreover, it would explain the recent observation that ZP2-NTF remains associated with the ZP of two-cell embryos even upon reduction of disulfide bonds [40].

ORIGIN OF THE ZP AND PARALLELS WITH REPRODUCTIVE PROTEINS FROM MOLLUSK AND YEAST

The structure-based identification of isolated ZP-N domains lacking a ZP-C counterpart within ZP subunits of Tetrapoda strongly supports a suggestion derived from hydrophobic cluster analysis [75]. Together with the presence of single

isolated ZP-N domains in several non-egg coat proteins [76], this finding provides further evidence that—as implied by its 3D structure [44]—ZP-N should be considered a domain of its own. Moreover, considering the aforementioned structural similarity between ZP-N and ZP-C, it raises the possibility that the current domain architecture of the egg coat results from duplication events that involved an ancestral ZP-N protein-encoding gene [43]. Interestingly, with the exception of some homologues of the amphibian ZPAX VE subunit [11], ZP-N repeats are not detected in VE proteins from fish, which have a structural role comparable to that of their counterparts from Tetrapoda but are not involved in sperm binding. Similarly, despite the fact that a C-terminal ZP module is also conserved in hundreds of extracellular eukaryotic proteins unrelated to fertilization [18], none of these molecules appears to contain ZP-N repeats. These considerations suggest that—in agreement with data available on ZP2 [34, 35, 40]—the repeats have functional roles specific to fertilization. This is highly reminiscent of the mollusk and ascidian egg coat proteins VERL and VC70, which also contain a C-terminal ZP module preceded by repeats that have been implicated in sperm binding [77–80]. Whereas the 12 repeats of VC70 could be easily recognized as epidermal growth factor (EGF)-like domains [78], the 22 tandem repeats of abalone VERL did not display obvious sequence similarities to known protein domains [79]. However, a recent threading analysis of VERL repeats suggested that just like their ZP2 counterparts, these probably also adopt a ZP-N fold, consistent with the absolute conservation of four Cys residues within each repeat [81]. The presence of a single VERL-like ZP-N domain within the N-terminal region of VEZP14, another abalone egg coat protein thought to be involved in gamete fusion [15], subsequently acted as a bridge toward the identification of a further putative ZP-N domain within yeast protein alpha-agglutinin, Sag1p [81]. This is consistent with significant experimental data available on Sag1p, which was already known to contain a functionally essential and structurally unusual Ig-like domain (Ig III) [82–84]. The surprising aspect of this finding comes from the fact that Sag1p Ig III/ZP-N is directly involved in the interaction between yeast haploid cells at mating [85]—a process that mirrors the encounter of egg and sperm in higher eukaryotes. These considerations raise the possibility that despite being separated by 1 billion years of evolution, reproductive processes like mammalian gamete recognition and yeast mating share common structural features [81]. At the same time, they imply that the biological role of the ZP fold in fertilization is not limited to polymerization, but can also extend to the specific recognition of molecules that do not contain a ZP module. Indeed, this is consistent with findings from a different biological system, showing that the ZP module of transforming growth factor (TGF)-beta receptor III (TGFBR3; also known as betaglycan) is directly responsible for its binding to both TGF-beta and inhibin [86–88].

CONCLUSIONS AND FUTURE DIRECTIONS

The recently reported crystal structures of ZP3 have provided information that goes well beyond the function of this particular molecule: Within just a few years, we have moved from a situation in which the fold of ZP proteins was completely unknown to one in which, due to the presence of ZP-N repeats in subunits ZP1/2/4, we can generate approximate models of all egg coat components. Of course, this does not obviate the need for additional experimental structures, but it does allow us to start interpreting a large amount of available biochemical data in structural terms. At the same time, many important questions remain to be answered. How do the ZP-C domains of type II subunits differ from that of ZP3? How do type I and type II subunits interact, and what is their relative arrangement within ZP filaments? How do ZP components bind to their sperm counterparts at the atomic level, and what is the structural basis of the species-specificity of gamete recognition? What conformational changes do ZP filaments undergo during postfertilization hardening, and how are they unraveled at hatching? Future structural studies addressing these issues have the potential to make fundamental contributions to our understanding of fertilization and early development. Moreover, structural information on ZP3 and other ZP subunits could be used to inform the rational design of targeted nonhormonal contraceptives as well as to interpret the effect of mutations in human ZP genes associated with infertility. In this regard, it is notable that human ZP3 sequence variation P221R, which was very recently identified in a single in vitro fertilization patient with an anomalous ZP [89], affects a residue located next to ZP-C β-strands E′ and F. Although the significance of this variant remains to be established using larger sample sets, P221R might destabilize dimer formation or impair the ability of strand F to take part in polymerization following EHP ejection.

REFERENCES

1. Wassarman PM, Litscher ES. Mammalian fertilization: the egg's multifunctional zona pellucida. Int J Dev Biol 2008; 52:665–676.
2. Bleil JD, Wassarman PM. Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. Dev Biol 1980; 76:185–202.
3. Bleil JD, Wassarman PM. Synthesis of zona pellucida proteins by denuded and follicle-enclosed mouse oocytes during culture in vitro. Proc Natl Acad Sci U S A 1980; 77:1029–1033.
4. Wassarman PM, Mortillo S. Structure of the mouse egg extracellular coat, the zona pellucida. Int Rev Cytol 1991; 130:85–110.
5. Greve JM, Wassarman PM. Mouse egg extracellular coat is a matrix of interconnected filaments possessing a structural repeat. J Mol Biol 1985; 181:253–264.
6. Rankin TL, O'Brien M, Lee E, Wigglesworth K, Eppig J, Dean J. Defective zonae pellucidae in Zp2-null mice disrupt folliculogenesis, fertility and development. Development 2001; 128:1119–1126.
7. Rankin T, Talbot P, Lee E, Dean J. Abnormal zonae pellucidae in mice lacking ZP1 result in early embryonic loss. Development 1999; 126:3847–3855.
8. Rankin T, Familari M, Lee E, Ginsberg A, Dwyer N, Blanchette-Mackie J, Drago J, Westphal H, Dean J. Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile. Development 1996; 122:2903–2910.
9. Liu C, Litscher ES, Mortillo S, Sakai Y, Kinloch RA, Stewart CL, Wassarman PM. Targeted disruption of the mZP3 gene results in production of eggs lacking a zona pellucida and infertility in female mice. Proc Natl Acad Sci U S A 1996; 93:5431–5436.
10. Conner SJ, Lefievre L, Hughes DC, Barratt CL. Cracking the egg: increased complexity in the zona pellucida. Hum Reprod 2005; 20:1148–1152.
11. Hedrick JL. Anuran and pig egg zona pellucida glycoproteins in fertilization and early development. Int J Dev Biol 2008; 52:683–701.
12. Litscher ES, Wassarman PM. Egg extracellular coat proteins: from fish to mammals. Histol Histopathol 2007; 22:337–347.
13. Smith J, Paton IR, Hughes DC, Burt DW. Isolation and mapping the chicken zona pellucida genes: an insight into the evolution of orthologous genes in different species. Mol Reprod Dev 2005; 70:133–145.
14. Hyllner SJ, Westerlund L, Olsson PE, Schopen A. Cloning of rainbow trout egg envelope proteins: members of a unique group of structural proteins. Biol Reprod 2001; 64:805–811.

15. Aagaard JE, Vacquier VD, MacCoss MJ, Swanson WJ. ZP domain proteins in the abalone egg coat include a paralog of VERL under positive selection that binds lysin and 18-kDa sperm proteins. Mol Biol Evol 2010; 27:193–203.

16. Yamada L, Saito T, Taniguchi H, Sawada H, Harada Y. Comprehensive egg coat proteome of the ascidian *Ciona intestinalis* reveals gamete recognition molecules involved in self-sterility. J Biol Chem 2009; 284:9402–9410.

17. Monné M, Han L, Jovine L. Tracking down the ZP domain: from the mammalian zona pellucida to the molluscan vitelline envelope. Semin Reprod Med 2006; 24:204–216.

18. Jovine L, Darie CC, Litscher ES, Wassarman PM. Zona pellucida domain proteins. Annu Rev Biochem 2005; 74:83–114.

19. Bork P, Sander C. A large domain common to sperm receptors (Zp2 and Zp3) and TGF-β type III receptor. FEBS Lett 1992; 300:237–240.

20. Kanai S, Kitayama T, Yonezawa N, Sawano Y, Tanokura M, Nakano M. Disulfide linkage patterns of pig zona pellucida glycoproteins ZP3 and ZP4. Mol Reprod Dev 2008; 75:847–856.

21. Boja ES, Hoodbhoy T, Garfield M, Fales HM. Structural conservation of mouse and rat zona pellucida glycoproteins. Probing the native rat zona pellucida proteome by mass spectrometry. Biochemistry 2005; 44:16445–16460.

22. Zhao M, Boja ES, Hoodbhoy T, Nawrocki J, Kaufman JB, Kresge N, Ghirlando R, Shiloach J, Pannell L, Levine RL, Fales HM, Dean J. Mass spectrometry analysis of recombinant human ZP3 expressed in glycosyl-ation-deficient CHO cells. Biochemistry 2004; 43:12090–12104.

23. Darie CC, Biniossek ML, Jovine L, Litscher ES, Wassarman PM. Structural characterization of fish egg vitelline envelope proteins by mass spectrometry. Biochemistry 2004; 43:7459–7478.

24. Boja ES, Hoodbhoy T, Fales HM, Dean J. Structural characterization of native mouse zona pellucida proteins using mass spectrometry. J Biol Chem 2003; 278:34189–34202.

25. Jovine L, Qi H, Williams Z, Litscher ES, Wassarman PM. A duplicated motif controls assembly of zona pellucida domain proteins. Proc Natl Acad Sci U S A 2004; 101:5922–5927.

26. Bork P. A trefoil domain in the major rabbit zona pellucida protein. Protein Sci 1993; 2:669–670.

27. Williams Z, Wassarman PM. Secretion of mouse ZP3, the sperm receptor, requires cleavage of its polypeptide at a consensus furin cleavage-site. Biochemistry 2001; 40:929–937.

28. Litscher ES, Qi H, Wassarman PM. Mouse zona pellucida glycoproteins mZP2 and mZP3 undergo carboxy-terminal proteolytic processing in growing oocytes. Biochemistry 1999; 38:12280–12287.

29. Yurewicz EC, Hibler D, Fontenot GK, Sacco AG, Harris J. Nucleotide sequence of cDNA encoding ZP3α, a sperm-binding glycoprotein from zona pellucida of pig oocyte. Biochim Biophys Acta 1993; 1174:211–214.

30. Zhao M, Gold L, Dorward H, Liang LF, Hoodbhoy T, Boja E, Fales HM, Dean J. Mutation of a conserved hydrophobic patch prevents incorporation of ZP3 into the zona pellucida surrounding mouse eggs. Mol Cell Biol 2003; 23:8982–8991.

31. Jovine L, Qi H, Williams Z, Litscher E, Wassarman PM. The ZP domain is a conserved module for polymerization of extracellular proteins. Nat Cell Biol 2002; 4:457–461.

32. Schaeffer C, Santambrogio S, Perucca S, Casari G, Rampoldi L. Analysis of uromodulin polymerization provides new insights into the mechanisms regulating ZP domain-mediated protein assembly. Mol Biol Cell 2009; 20:589–599.

33. Bansal P, Chakrabarti K, Gupta SK. Functional activity of human ZP3-primary sperm receptor resides towards its C-terminus. Biol Reprod 2009; 81:7–15.

34. Tsubamoto H, Hasegawa A, Nakata Y, Naito S, Yamasaki N, Koyama K. Expression of recombinant human zona pellucida protein 2 and its binding capacity to spermatozoa. Biol Reprod 1999; 61:1649–1654.

35. Bleil JD, Greve JM, Wassarman PM. Identification of a secondary sperm receptor in the mouse egg zona pellucida: role in maintenance of binding of acrosome-reacted sperm to eggs. Dev Biol 1988; 128:376–385.

36. Bleil JD, Wassarman PM. Mammalian sperm-egg interaction: identifica-tion of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. Cell 1980; 20:873–882.

37. O'Toole CM, Arnoult C, Darszon A, Steinhardt RA, Florman HM. Ca²⁺ entry through store-operated channels in mouse sperm is initiated by egg ZP3 and drives the acrosome reaction. Mol Biol Cell 2000; 11:1571–1584.

38. Bleil JD, Wassarman PM. Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. Dev Biol 1983; 95:317–324.

39. Jin M, Fujiwara E, Kakiuchi Y, Okabe M, Satouh Y, Baba SA, Chiba K, Hirohashi N. Most fertilizing mouse spermatozoa begin their acrosome

40. Gahlay G, Gauthier L, Baibakov B, Epifano O, Dean J. Gamete recognition in mice depends on the cleavage status of an egg's zona pellucida protein. Science 2010; 329:216–219.

41. Shur BD. Reassessing the role of protein-carbohydrate complementarity during sperm-egg interactions in the mouse. Int J Dev Biol 2008; 52:703–715.

42. Clark GF. Molecular models for mouse sperm-oocyte binding. Glycobi-ology 2011; 21:3–5.

43. Han L, Monné M, Okumura H, Schwend T, Cherry AL, Flot D, Matsuda T, Jovine L. Insights into egg coat assembly and egg-sperm interaction from the x-ray structure of full-length ZP3. Cell 2010; 143:404–415.

44. Monné M, Han L, Schwend T, Burendahl S, Jovine L. Crystal structure of the ZP-N domain of ZP3 reveals the core fold of animal egg coats. Nature 2008; 456:653–657.

45. Halaby DM, Poupon A, Mornon J. The immunoglobulin fold family: sequence analysis and 3D structure comparisons. Protein Eng 1999; 12:563–571.

46. Legan PK, Lukashkina VA, Goodyear RJ, Lukashkin AN, Verhoeven K, Van Kamp G, Russell IJ, Richardson GP. A deafness mutation isolates a second role for the tectorial membrane in hearing. Nat Neurosci 2005; 8:1035–1042.

47. Wang JH, Yan YW, Garrett TP, Liu JH, Rodgers DW, Garlick RL, Tarr GE, Husain Y, Reinherz EL, Harrison SC. Atomic structure of a fragment of human CD4 containing two immunoglobulin-like domains. Nature 1990; 348:411–418.

48. Peng H, Vijayakumar S, Schiene-Fischer C, Li H, Purkerson JM, Malesevic M, Liebscher J, Al-Awqati Q, Schwartz GJ. Secreted cyclo-philin A, a peptidylprolyl *cis-trans* isomerase, mediates matrix assembly of hensin, a protein implicated in epithelial differentiation. J Biol Chem 2009; 284:6465–6475.

49. Hoodbhoy T, Aviles M, Baibakov B, Epifano O, Jiménez-Movilla M, Gauthier L, Dean J. ZP2 and ZP3 traffic independently within oocytes prior to assembly into the extracellular zona pellucida. Mol Cell Biol 2006; 26:7991–7998.

50. Sanejouand YH. Domain swapping of CD4 upon dimerization. Proteins 2004; 57:205–212.

51. Hasegawa A, Kanazawa N, Sawai H, Komori S, Koyama K. Pig zona pellucida 2 (pZP2) protein does not participate in zona pellucida formation in transgenic mice. Reproduction 2006; 132:455–464.

52. Yasumasu S, Kawaguchi M, Ouchi S, Sano K, Murata K, Sugiyama H, Akema T, Iuchi I. Mechanism of egg envelope digestion by hatching enzymes, HCE and LCE, in medaka, *Oryzias latipes*. J Biochem 2010; 148:439–448.

53. Litscher ES, Williams Z, Wassarman PM. Zona pellucida glycoprotein ZP3 and fertilization in mammals. Mol Reprod Dev 2009; 76:933–941.

54. Florman HM, Wassarman PM. *O*-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. Cell 1985; 41:313–324.

55. Chen J, Litscher ES, Wassarman PM. Inactivation of the mouse sperm receptor, mZP3, by site-directed mutagenesis of individual serine residues located at the combining site for sperm. Proc Natl Acad Sci U S A 1998; 95:6193–6197.

56. Chalabi S, Panico M, Sutton-Smith M, Haslam SM, Patankar MS, Lattanzio FA, Morris HR, Clark GF, Dell A. Differential *O*-glycosylation of a conserved domain expressed in murine and human ZP3. Biochemistry 2006; 45:637–647.

57. Roller RJ, Wassarman PM. Role of asparagine-linked oligosaccharides in secretion of glycoproteins of the mouse egg's extracellular coat. J Biol Chem 1983; 258:13243–13249.

58. Williams SA, Xia L, Cummings RD, McEver RP, Stanley P. Fertilization in mouse does not require terminal galactose or N-acetylglucosamine on the zona pellucida glycans. J Cell Sci 2007; 120:1341–1349.

59. Ikawa M, Inoue N, Benham AM, Okabe M. Fertilization: a sperm's journey to and interaction with the oocyte. J Clin Invest 2010; 120:984–994.

60. Bausek N, Waclawek M, Schneider WJ, Wohlrab F. The major chicken egg envelope protein ZP1 is different from ZPB and is synthesized in the liver. J Biol Chem 2000; 275:28866–28872.

61. Takeuchi Y, Cho R, Iwata Y, Nishimura K, Kato T, Aoki N, Kitajima K, Matsuda T. Morphological and biochemical changes of isolated chicken egg-envelope during sperm penetration: degradation of the 97-kilodalton glycoprotein is involved in sperm-driven hole formation on the egg-envelope. Biol Reprod 2001; 64:822–830.

62. Moos J, Kalab P, Kopf GS, Schultz RM. Rapid, nonradioactive, and quantitative method to analyze zona pellucida modifications in single mouse eggs. Mol Reprod Dev 1994; 38:91–93.

63. Sasanami T, Ohtsuki M, Ishiguro T, Matsushima K, Hiyama G, Kansaku N, Doi Y, Mori M. Zona pellucida domain of ZPB1 controls specific binding of ZPB1 and ZPC in Japanese quail (*Coturnix japonica*). Cells Tissues Organs 2006; 183:41–52.

64. Bleil JD, Beall CF, Wassarman PM. Mammalian sperm-egg interaction: fertilization of mouse eggs triggers modification of the major zona pellucida glycoprotein, ZP2. Dev Biol 1981; 86:189–197.

65. Moller CC, Wassarman PM. Characterization of a proteinase that cleaves zona pellucida glycoprotein ZP2 following activation of mouse eggs. Dev Biol 1989; 132:103–112.

66. Papi M, Brunelli R, Sylla L, Parasassi T, Monaci M, Maulucci G, Missori M, Arcovito G, Ursini F, De Spirito M. Mechanical properties of zona pellucida hardening. Eur Biophys J 2010; 39:987–992.

67. Familiari G, Heyn R, Relucenti M, Sathananthan H. Structural changes of the zona pellucida during fertilization and embryo development. Front Biosci 2008; 13:6730–6751.

68. Lindsay LL, Hedrick JL. Proteolysis of *Xenopus laevis* egg envelope ZPA triggers envelope hardening. Biochem Biophys Res Commun 2004; 324:648–654.

69. Rankin TL, Coleman JS, Epifano O, Hoodbhoy T, Turner SG, Castle PE, Lee E, Gore-Langton R, Dean J. Fertility and taxon-specific sperm binding persist after replacement of mouse sperm receptors with human homologues. Dev Cell 2003; 5:33–43.

70. Tian J, Gong H, Lennarz WJ. *Xenopus laevis* sperm receptor gp69/64 glycoprotein is a homologue of the mammalian sperm receptor ZP2. Proc Natl Acad Sci U S A 1999; 96:829–834.

71. Noguchi S, Yonezawa N, Katsumata T, Hashizume K, Kuwayama M, Hamano S, Watanabe S, Nakano M. Characterization of the zona pellucida glycoproteins from bovine ovarian and fertilized eggs. Biochim Biophys Acta 1994; 1201:7–14.

72. Hasegawa A, Koyama K, Okazaki Y, Sugimoto M, Isojima S. Amino acid sequence of a porcine zona pellucida glycoprotein ZP4 determined by peptide mapping and cDNA cloning. J Reprod Fertil 1994; 100:245–255.

73. Garrod D, Kimura TE. Hyperadhesion: a new concept in cell-cell adhesion. Biochem Soc Trans 2008; 36:195–201.

74. Aricescu AR, Jones EY. Immunoglobulin superfamily cell adhesion molecules: zippers and signals. Curr Opin Cell Biol 2007; 19:543–550.

75. Callebaut I, Mornon JP, Monget P. Isolated ZP-N domains constitute the N-terminal extensions of zona pellucida proteins. Bioinformatics 2007; 23:1871–1874.

76. Jovine L, Janssen WG, Litscher ES, Wassarman PM. The PLAC1-homology region of the ZP domain is sufficient for protein polymerization. BMC Biochem 2006; 7:11.

77. Ban S, Harada Y, Yokosawa H, Sawada H. Highly polymorphic vitelline-coat protein HaVC80 from the ascidian, *Halocynthia aurantium*: structural analysis and involvement in self/nonself recognition during fertilization. Dev Biol 2005; 286:440–451.

78. Sawada H, Tanaka E, Ban S, Yamasaki C, Fujino J, Ooura K, Abe Y, Matsumoto K, Yokosawa H. Self/nonself recognition in ascidian fertilization: vitelline coat protein HrVC70 is a candidate allorecognition molecule. Proc Natl Acad Sci U S A 2004; 101:15615–15620.

79. Galindo BE, Moy GW, Swanson WJ, Vacquier VD. Full-length sequence of VERL, the egg vitelline envelope receptor for abalone sperm lysin. Gene 2002; 288:111–117.

80. Swanson WJ, Vacquier VD. The abalone egg vitelline envelope receptor for sperm lysin is a giant multivalent molecule. Proc Natl Acad Sci U S A 1997; 94:6724–6729.

81. Swanson WJ, Aagaard JE, Vacquier VD, Monné M, Sadat Al Hosseini H, Jovine L. The molecular basis of sex: linking yeast to human. Mol Biol Evol 2011; 28:1963–1966.

82. de Nobel H, Lipke PN, Kurjan J. Identification of a ligand-binding site in an immunoglobulin fold domain of the *Saccharomyces cerevisiae* adhesion protein α-agglutinin. Mol Biol Cell 1996; 7:143–153.

83. Chen MH, Shen ZM, Bobin S, Kahn PC, Lipke PN. Structure of *Saccharomyces cerevisiae* α-agglutinin. Evidence for a yeast cell wall protein with multiple immunoglobulin-like domains with atypical disulfides. J Biol Chem 1995; 270:26168–26177.

84. Cappellaro C, Hauser K, Mrsá V, Watzele M, Watzele G, Gruber C, Tanner W. *Saccharomyces cerevisiae* a- and α-agglutinin: characterization of their molecular interaction. EMBO J 1991; 10:4081–4088.

85. Dranginis AM, Rauceo JM, Coronado JE, Lipke PN. A biochemical guide to yeast adhesins: glycoproteins for social and antisocial occasions. Microbiol Mol Biol Rev 2007; 71:282–294.

86. Mendoza V, Vilchis-Landeros MM, Mendoza-Hernández G, Huang T, Villarreal MM, Hinck AP, López-Casillas F, Montiel JL. Betaglycan has two independent domains required for high affinity TGF-β binding: proteolytic cleavage separates the domains and inactivates the neutralizing activity of the soluble receptor. Biochemistry 2009; 48:11755–11765.

87. Wiater E, Harrison CA, Lewis KA, Gray PC, Vale WW. Identification of distinct inhibin and transforming growth factor β-binding sites on betaglycan: functional separation of betaglycan co-receptor actions. J Biol Chem 2006; 281:17011–17022.

88. Esparza-Lopez J, Montiel JL, Vilchis-Landeros MM, Okadome T, Miyazono K, Lopez-Casillas F. Ligand binding and functional properties of betaglycan, a co-receptor of the transforming growth factor-β superfamily. Specialized binding regions for transforming growth factor-β and inhibin A. J Biol Chem 2001; 276:14588–14596.

89. Pökkylä RM, Lakkakorpi JT, Nuojua-Huttunen SH, Tapanainen JS. Sequence variations in human ZP genes as potential modifiers of zona pellucida architecture. Fertil Steril 2011; 95:2669–2672.

90. Wassarman PM. Fertilization in mammals. Sci Am 1988; 259:78–84.