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MINI-REVIEW

Dicalcin, a zona pellucida protein that regulates fertilization competence of the egg coat in *Xenopus laevis*

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Abstract Fertilization is a highly coordinated process whereby sperm interact with the egg-coating envelope (called the zona pellucida, ZP) in a taxon-restricted manner, Fertilization triggers the resumption of the cell cycle of the egg, ultimately leading to generation of a new organism that contains hereditary information of the parents. The complete sperm-ZP interaction comprises sperm recognition of the ZP, the acrosome reaction, penetration of the ZP, and fusion with the egg. Recent evidence suggests that these processes involve oligosaccharides associated with a ZP constituent (termed ZP protein), the polypeptide backbone of a ZP protein, and/or the proper three-dimensional filamentous structure of the ZP. However, a detailed description of the molecular mechanisms involved in sperm-ZP interaction remains elusive. Recently, I found that dicalcin, a novel ZP protein-associated protein, suppresses fertilization through its association with gp41, the frog counterpart of the mammalian ZPC protein. This review focuses on molecular aspects of sperm-ZP interaction and describes the fertilization-suppressive function of dicalcin and associated molecular mechanisms. The amount of dicalcin in the ZP significantly correlates with alteration of the lectin-staining pattern within the ZP and the orientation pattern of ZP filaments, which may assist in elucidating the complex molecular mechanisms that underlie sperm-ZP interaction.

Keywords Fertilization · Egg-coating envelope · Sperm · ZP protein

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Abbreviations

ZP	Zona pellucida
VE	Vitelline envelope
AR	Acrosome reaction
Gal/GalNAc	Galactose/N-acetylgalactosamine
RCA-I	*Ricinus communis* agglutinin I

Introduction

Oocytes are surrounded by an extracellular envelope that is called either the zona pellucida (ZP) in mammals or the vitelline envelope (VE) in non-mammals [1]. This extracellular matrix, with a thickness of several micrometers, plays multiple roles in zygote generation and development, including taxon-selective interaction between gametes, induction of the acrosome reaction (AR), polyspermy block, and protection of the developing embryo from physical damage [2]. The ZP contains three-dimensional filaments formed by polymerization of ZP proteins and other components, including hyaluronan and ZP protein-associated proteins. ZP proteins of the egg coats comprise three to four ZP proteins, including ZP1–4 in humans, ZP1–3 in mice, and gp120, gp69/64, gp41, and gp37 in *Xenopus laevis*. ZP proteins are mainly secreted by growing oocytes and are modified post-translationally by glycosylation [3]. These ZP proteins associate with other ZP proteins via a conserved ~260-amino-acid motif called the ZP domain, creating micrometer-long filaments, pairs of which are interconnected to form a three-dimensional meshwork [4]. Note that following their biochemical identification, the name of each ZP protein has still been used interchangeably (e.g., ZPA or ZP2 or gp64/69, ZPB or

ZP1 or gp37, ZPC or ZP3 or gp41). Here, I refer to ZPA, ZPB, and ZPC throughout this review, as suggested by others [5], to prevent possible confusion. Successful fertilization requires the proper sperm-ZP interaction, beginning with sperm recognition of the ZP. Early biochemical studies in mice initially proposed a model of sperm-ZP interaction wherein sperm recognize oligosaccharides attached to ZPC [6]. However, another biochemical study in frogs predicted an alternative model emphasizing the binding of sperm to oligosaccharides attached to ZPA [7]. Meanwhile, recent evidence from experiments using genetically engineered mice supports the notion that sperm recognize an N-terminal amino acid region of ZPA and/or the proper three-dimensional structure of the ZP [8]. Thus, molecular mechanisms of sperm-ZP interaction are not consistent across different experimental approaches and species. These various models (i.e., carbohydrate-mediated sperm-ZP interaction and ZP structure-associated interaction) could both be important mechanisms for the initiation of fertilization. Recently, I found that dicalcin, a novel ZP protein-associated protein present in the intact egg coat of unfertilized mature frog eggs, suppresses fertilization through its association with gp41, the frog counterpart of mammalian ZPC [9]. I also discovered that dicalcin regulates the oligosaccharide distribution pattern within the ZP, as well as the orientation pattern of ZP filaments, providing novel clues critical for a more complete understanding of fertilization.

Fertilization overview

A detailed description of the molecular events occurring during fertilization is beyond the scope of this review, but they are excellently summarized elsewhere [1]. Briefly, for example in mammals, a large number of sperm (e.g., tens of millions in humans) is deposited in the lower female reproductive tract upon ejaculation, but only a small proportion (e.g., thousands of sperm in humans) enters the oviduct. Subsequently, even fewer reach the ampulla region of the oviduct during the appropriate time window for fertilization, and only one spermatozoon ultimately fertilizes the egg. During this behavior of sperm in the female reproductive tract, the sperm membrane undergoes various physiological changes, called capacitation, which confers susceptibility to further sperm alterations (for reviews see [10, 11]). Although many factors, including glucose, cholesterol, bicarbonate, and intracellular Ca^{2+} are involved in capacitation, the precise molecular mechanisms underlying these processes remain to be clarified. Once capacitated, sperm are receptive to progesterone-dependent activation involving Ca^{2+} influx through the sperm Ca^{2+} channel, Catsper [12]. Calcium influx leads to a vigorous swimming pattern called hyperactivation, a movement that confers a strong thrusting power to facilitate progression to egg-surrounding structures, including the cumulus layer (the outer egg-coating structure) and the ZP (the inner one). Following a species-restricted interaction with these structures, sperm then undergo an exocytotic event called the AR, during which a variety of lytic enzymes and ZP-binding proteins are released. The AR facilitates sperm-penetration of the ZP, ultimately enabling sperm to reach the plasma membrane of oocytes and fuse with them, thereby initiating the resumption of the arrested cell cycle. In the external fertilization in *X. laevis*, sperm initiate their motility by osmotic shock during ejaculation into pond water [13], move toward eggs in response to a concentration gradient of allurin, a chemoattractant secreted from the oviduct and attached onto the jelly layers, an outer coat of the oviposited frog egg [14]. Following penetration of the jelly layers, sperm then interact with the ZP (specifically vitelline envelope) that has undergone the limited processing of ZPC (gp43–gp41) by oviductin, a serine protease secreted from the oviduct during oviduct passage [15, 16], and subsequently undergo the AR assisted by ARISX, an AR-inducing substance [17], penetrate the ZP and fuse with the egg plasma membrane (for a review see [18]).

ZP properties and altered fertility

Few reports have demonstrated a potential correlation between ZP properties and fertility in mature unfertilized eggs, with the exception of one immunohistochemical examination [19]. Talevi et al. found that *Maclura pomifera* agglutinin (MPA), a Gal/GalNAc-sensitive lectin, reacts with the fertilization-failed human ZP in varied patterns, as follows: (1) restricted labeling to the outer surface of the ZP (33 % of total eggs); (2) restricted labeling of the outer layer (non-surface) of the ZP (17 %); (3) uniform labeling (50 %) [19]. Intriguingly, these results suggest that the distribution of oligosaccharides within the ZP and/or the ZP ultrastructure could be related to fertilization competence of mature eggs and reduced fertility during aging [20].

Protein chemistry of dicalcin

Isolation and distribution of dicalcin

Dicalcin was originally identified in frog (*Rana catesbeiana*) olfactory cilia as an intracellularly expressed Ca^{2+}-binding protein [21]. Since its original identification, this protein has also been observed in other tissues, including the egg and the lung [22]. The previous immunohistochemical study using *X. laevis* eggs revealed that dicalcin

is localized uniformly within the ZP as well as in the intracellular marginal region of the egg. These findings suggest that dicalcin is released from the egg and retained within the ZP via its binding to ZP constituent(s). Dicalcin lacks an N-terminal leader sequence; therefore, the secretory pathway of dicalcin could be distinct from the classic ER-Golgi pathway. N-terminal leaderless secretion has been observed for other proteins, including interleukin-1β and fibroblast growth factor-2 [23].

Structure of dicalcin

Dicalcin is an S100-like Ca^{2+}-binding protein present in the frog ZP [9, 24]. The S100 protein family comprises small (10–14 kDa) calcium binding proteins that regulate various extra- and intracellular processes (for reviews see [25, 26]). The primary structure of dicalcin consists of two S100-like regions connected by a linker region, which features this protein as a dimeric form of S100 calcium binding protein. Since S100 proteins are known to function as dimers, monomeric dicalcin could exert functions similar to those of other S100 members. Indeed, the three-dimensional structure of dicalcin is reasonably represented by the folding pattern of the dimeric form of S100B [27]. Extensive biochemical analyses have revealed the Ca^{2+} binding mechanism of dicalcin: (1) four Ca^{2+}-binding motifs (called EF-hands) in the dicalcin sequence are functional, therefore dicalcin is capable of binding to four Ca^{2+} per protein; (2) the first and second Ca^{2+} binding to the higher-affinity EF-hands induce a major conformational change accompanied by an increase in the α-helical content, as measured using circular dichroism [28].

Target proteins of dicalcin

Dicalcin displays no enzymatic activities in and of itself, and instead, through binding to target proteins, it regulates various cellular events. In *X. laevis* eggs, dicalcin interacts with several egg proteins, including ZPC (gp41) and ZPB (gp37) of the ZP [9]. Binding of dicalcin to ZPC and ZPB is mediated via interaction between the protein cores, but not via interaction between dicalcin and oligosaccharides attached to the ZP protein. The external Ca^{2+} concentration surrounding the frog egg is high, so that dicalcin is retained by ZP proteins in the ZP, as confirmed by my previous immunohistochemical study. Through this interaction with ZP proteins, dicalcin plays an important role in fertilization (see below). My recent study successfully identified the interactive amino acid regions between dicalcin and its target ZPC [29]. In addition to the egg, dicalcin also binds to some ciliary proteins, including annexins and a β-adrenergic receptor kinase-like protein [30, 31], regulating the ciliary functions of olfactory neurons such as olfactory signaling and/or ciliary membrane repair [32].

Function of dicalcin at fertilization

Suppressive action of dicalcin on fertilization in *X. laevis*

The amount of dicalcin in the ZP exerts a substantial effect on the fertilization rate in *X. laevis*: preincubation of unfertilized eggs with a dicalcin-specific antibody increases the fertilization rate, whereas preincubation with recombinant dicalcin inhibits fertilization and sperm binding to the ZP, as well as in vitro sperm-penetration through the ZP protein layer [9]. It should be noted that this suppressive action occurs in unfertilized eggs at fertilization, which precedes the polyspermy block observed post-fertilization. Furthermore, dicalcin treatment reduced sperm binding to the ZP only to ∼77 % of the control value, whereas treatment inhibited sperm-penetration in vitro to ∼50 % of that of the control, indicating that dicalcin preferentially affects the sperm-penetration process, rather than the initial sperm-ZP binding. Through these actions, dicalcin inherently suppresses fertilization in *X. laevis* [9].

Regulation of the distribution pattern of oligosaccharides within the VE by dicalcin

Carbohydrate-dependent recognition has been shown to play an important role in the establishment of an appropriate sperm-ZP interaction [33]. Indeed, changes in the staining patterns of lectins have been observed in human ZP of fertilization-failed oocytes [19]. Furthermore, I have discovered that pretreatment with dicalcin increases the in vivo reactivity of the frog ZP to the Gal/GalNAc-sensitive lectin, *Ricinus communis* agglutinin I (RCA-I). Quantification of RCA-I immunosignals revealed that pretreatment with dicalcin increased the intensity of the RCA-I signal in the outermost region of the ZP and broadened the RCA-I reactivity within the ZP of both hemispheres (i.e., animal and vegetal) [9]. Since dicalcin binds to ZPC, and RCA-I solely reacts with the oligosaccharides of ZPC, dicalcin regulates the oligosaccharide distribution pattern within the ZP through its binding to ZPC. Dicalcin-dependent modification of RCA-I reactivity of the ZP is unique, as there are no reported examples of molecules that change the interactive affinity between proteins and carbohydrates. In future studies, it would be of interest to determine the true biological benefit of varied levels of exposure of oligosaccharides on ZPC in the presence of dicalcin.

Regulation of ZP structure by dicalcin

I have identified the interactive regions between dicalcin and ZPC, and also demonstrated that pretreatments of unfertilized eggs with synthetic peptides corresponding to the interactive regions of these proteins markedly affected the fertilization rate [29]. These synthetic peptides will allow me to control the status of the unfertilized ZP to establish either fertilization competence or incompetence. For example, if unfertilized eggs were treated with a peptide corresponding to the ZPC-binding region of dicalcin (named dcp11), the action of dicalcin could be enhanced, thereby rendering the ZP fertilization incompetent. In contrast, if unfertilized eggs were treated with a different peptide corresponding to the dicalcin-binding region of ZPC (named gpp2), the action of dicalcin could be inhibited (or masked), enabling the ZP status to be fertilization competent. On the basis of this consideration, I examined the ultrastructure of ZP filaments of both fertilization statuses by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM observation of unfertilized eggs revealed that each ZP filament had a globular structure with a width of $\sim$100 nm (Fig. 1a, control); this size was thought to be greater than its actual size, possibly because of osmium coating of the entire lumen [34]. Surprisingly, the VE structure following dcp11 treatment was markedly distinct from that following gpp2 treatment (Fig. 1a): dcp11-treated VE (i.e., excessive action of dicalcin) exhibited a highly flattened structure, whereas gpp2-treated VE (i.e., deficiency of dicalcin) resembled a rugged structure. This marked difference suggested that ZP exhibited a well-organized and sheet-like structure in the presence of dcp11; conversely, a randomly disorganized structure was observed in the presence of gpp2. Our TEM analysis revealed that ZP filaments pretreated with dcp11 were arranged parallel to the egg plasma membrane, exhibiting the appearance of a "pin-stripe" pattern, whereas ZP filaments treated with gpp2 were randomly organized with many filaments arranged obliquely relative to the egg plasma membrane, forming a zigzag or occasionally "herring-bone" pattern (Fig. 1b) [29]. These results indicate that there is a striking difference between fertilization competent and incompetent ZP. This ultrastructural difference likely underlies the suppressive action of dicalcin on sperm-binding and sperm-penetration processes in frogs [9] as discussed below.

Possible molecular mechanisms of dicalcin

At fertilization, sperm initially recognize and bind to the ZP in a largely species-specific manner, and subsequently undergo hyperactivation and the exocytotic AR, both of which are believed to be essential for penetration through the ZP [35]. As described above, dicalcin preferentially affected the sperm-penetration process rather than the initial sperm-ZP binding. Electron microscopy analyses showed that the fertilization rate was high under the random (or disorganized) nanoscale ZP structure, suggesting that a disorganized ZP structure could induce the AR with a high probability. How a disorganized structure promotes AR induction is an open question. One possible explanation is that sperm is likely to be trapped by this structure, generating shearing forces on its plasma membrane and activating mechanosensory (MS) transduction, which results an increase in Ca^{2+} and consequently the occurrence of the AR. Although the AR is known to be induced or enhanced by several soluble factors, including progesterone and hyaluronan, the potential effects of physical stress on the AR have been poorly characterized with the exception of one study, which showed an increased AR induction in vitro when sperm penetrated a polycarbonate filter having a 3-μm diameter [36]. Among several MS ion channel families, including the mechanosensitive channel (Msc), the transient receptor potential (TRP) channel and the degenerin/epithelial sodium channel (DEG/ENaC) [37, 38], and certain MS channels, only TRP members are known to be expressed in sperm. While gene deletion studies in mice showed that polycystin-1 (a distantly classified homolog of TRP channels) possibly reinforces a distinct set of AR pathways, these mice are still fertile. In the sea urchin, polycystin-1 binds to its cognate polycystin-2 to form a functional complex that is involved in the AR; however, the molecular mechanisms whereby these proteins induce the AR remain unknown. The properties of MS channels have been most studied in Msc of bacteria. The MscL (mechanosensitive channel of large conductance) protein, a prototype Msc in *Escherichia coli*, is activated to open within a range of $\sim$100 mmHg, equal to $\sim$13 nN/μm^2 [39]. Hyperactivated sperm are considered to generate $\sim$45 pN at maximum, which is converted to 2.2 pN/μm^2, assuming that the surface area of the acrosomal cap (the cylinder with a radius of 0.25 μm radius and a height of 2 μm) is 20.4 μm^2 [40]. Applying these results to sperm, the maximum force of sperm ($\sim$2.2 pN/μm^2) is thought to be 1/2000 of that ($\sim$13 nN/μm^2) necessary to activate the MS channel, and therefore sperm hyperactivity alone is not sufficient to open the MS channel. Notably, the composition and viscosity of the sperm membrane are modified during capacitation such that the stretch tension on the membrane, but not the pressure, could vary at passage through the ZP. Interestingly, depletion of cholesterol in mouse muscle cells uncouples the cytoskeleton from the lipid bilayer and increases the tension to the MS channel, allowing it to open at a lower threshold [41]. Further characterization of the membrane tension of capacitated sperm may provide novel insights into the stress-induced

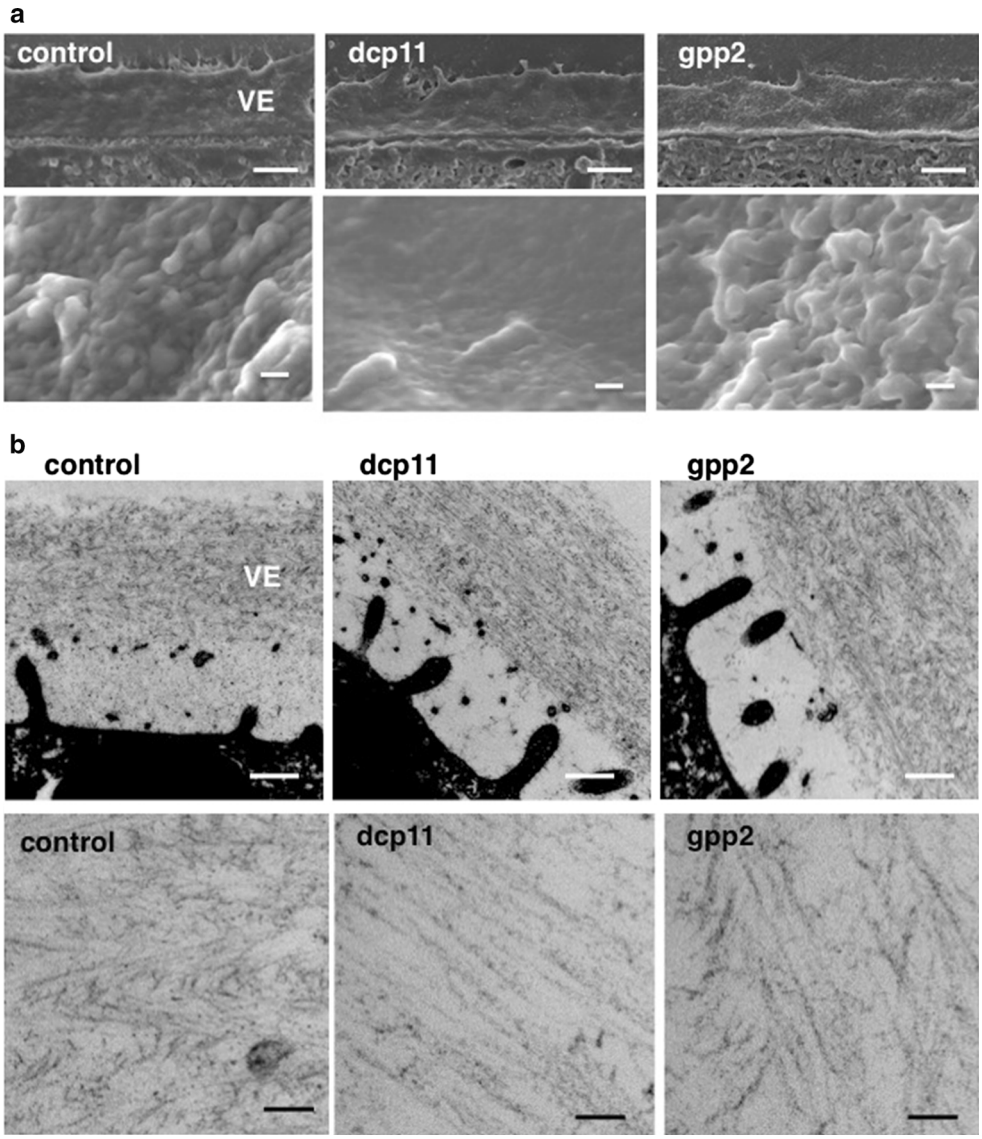

Fig. 1 Dicalcin- and gp41-derived peptides induce distinct nanoscale ZP meshworks. **a** SEM analysis of the VE treated with dcp11 and gpp2. *Upper* low magnification SEM images of paraffin-sectioned VE treated with peptides (dcp1 as a control, dcp11 and gpp2; 4 μM; $n = 3$; for a detailed description of the peptides, see [25]). *Scale bar* 1 μm. *Lower* higher magnification images. *Scale bar* 100 nm. **b** TEM analysis of the VE treated with dcp11 and gpp2. *Upper* low magnification images of the VE treated with peptides (dcp1 as a control, dcp11 and gpp2; 4 μM; $n = 3$). *Scale bar* 500 nm. *Lower* higher magnification images. *Scale bar* 30 nm. Reproduced from Miwa et al. (2015) [29] with permission from the publisher

AR mechanism. Alternatively, a shearing force could activate sperm membrane proteins that are not considered to sense membrane tension, with the assistance of extracellular oligosaccharides attached to the ZP meshwork, resulting in AR induction. Indeed, recent studies in endothelial cells have shown that G protein-coupled receptors, such as the bradykinin B_2 receptor (B2R) and α_1-adrenergic receptor (α_1-AR) [42, 43], sense fluid shear stress in the endothelial cell membrane of the coronary artery. In these examples, the degree of cellular responses to ligands changes in the presence of oligosaccharides of the extracellular endothelial surface layer (ESL) [44]. The interactions between receptor proteins and extracellular oligosaccharides in the ESL have been shown to be specific, such as the interaction between lectin and oligosaccharides, which specifies the cellular response among diverse cardiac functions (e.g., oxygen usage, constriction), vessel tone, and release of multiple bioactive agents [45, 46]. Assuming that this specific interaction is also the case in sperm, this working model could become a fundamental mechanism underlying species-restricted AR. Although receptors for progesterone and hyaluronan have not been reported to be modulated by force, hyaluronidase treatment has been shown to change the ligand-gated properties of B2R and α_1-AR in the endothelial cells [43], suggesting that activating properties of sperm membrane protein(s) (e.g., progesterone or hyaluronan receptors, TRP channels) could be modulated by oligosaccharides within the ZP, and could be activated by lower shearing forces, thereby initiating AR signaling (for reference, see Fig. 2). This working model appears to support my observed results quite well: the three-dimensional structure and oligosaccharides of the ZP were both involved in successful sperm-penetration through the ZP, and may integrate currently

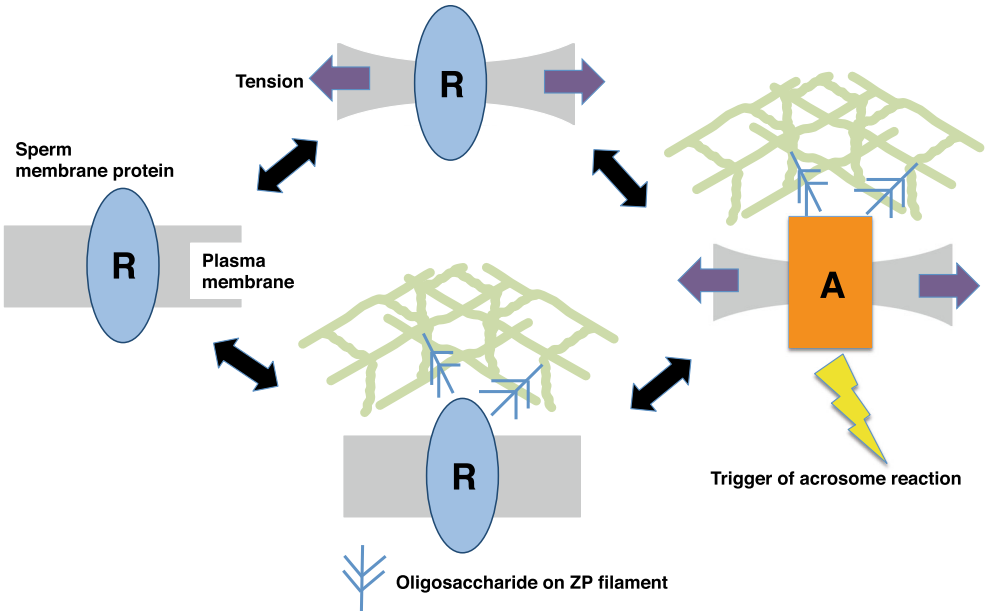

Fig. 2 Hypothesized model of the activation of the sperm membrane protein receptor by concomitant stimulation with mechanical stress and oligosaccharide binding. An unidentified sperm membrane protein responsible for the initiation of the acrosome reaction (AR) may be localized on the sperm membrane at the resting (non-activated) state (designated by R, *left*). Mechanical stress generated at sperm passage through the ZP was not sufficient for its activation (*upper middle*). On the other hand, the extracellular oligosaccharides on ZP glycoproteins bound to the membrane protein in a specific manner, as observed frequently for oligosaccharide and lectin; however, the oligosaccharide binding alone was not sufficient to activate the membrane protein (*lower middle*). When the mechanical stress induced by the proper structure of the ZP meshwork and oligosaccaride binding occurred concomitantly, the sperm protein could essentially be activated (A, *right*), triggering intracellular AR signaling (for details, see text). My previous results demonstrate the importance of the proper three-dimensional structure of the ZP meshwork and oligosaccharides within the ZP, and fit well to this model

incompatible molecular models of fertilization, including carbohydrate-mediated sperm-ZP interaction and ZP structure-mediated interaction.

Biological significance of dicalcin at fertilization

Dicalcin suppresses fertilization by regulating the distribution pattern of oligosaccharides within the ZP and the three-dimensional ultrastructure of ZP filaments. The true biological benefit of dicalcin-induced fertilization suppression is unfortunately unknown at present. However, several mechanisms are also known to impede fertilization, including fertilization inhibition by oviductin [47], osteopontin [48], and glycodelin-A [49]. There is also a female sperm reservoir in the mammalian oviduct in which the more competent sperm are arranged (for a review see [50]), which suggests that sperm-selection mechanisms could be involved in ensuring that only the most competent sperm can reach the egg plasma membrane [51]. On this basis, I speculate that dicalcin binds to ZPC and regulates ZP properties, forming a functional barrier that creates a challenging microenvironment for sperm to reach the egg plasma membrane, which may favor selection of more competent sperm. In conclusion, dicalcin is a key regulatory protein involved in mediating fertilization competence of the egg coat in *X. laevis*.

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Compliance with ethical standards

Conflict of interest The author declares no conflict of interest.

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