

# Chapter 37

## Sperm Proteasome as a Putative Egg Coat Lysin in Mammals

Edward Miles and Peter Sutovsky

**Abstract** During animal and human fertilization, the fertilizing spermatozoon creates and passes through a fertilization slit in the vitelline coat (VC) of the oocyte. It has been hypothesized that the penetration of the mammalian VC, the zona pellucida (ZP), is aided by a proteolytic enzyme capable of locally degrading ZP proteins. This putative “zona lysin” is predicted to reside within the sperm head acrosome and be released or exposed by ZP-induced acrosomal exocytosis. Evidence has been accumulating in favor of the 26S proteasome, the ubiquitin-dependent multi-subunit protease acting as the putative vitelline coat/zona lysin in humans and animals. To confirm this hypothesis, three criteria must be met: (1) the sperm receptor on the ZP must be ubiquitinated, (2) proteasomes must be present, exposed, and enzymatically active in the sperm acrosome, and (3) sperm proteasomes must be able to degrade the sperm receptor on the egg coat/ZP during fertilization. This review discusses recent data from a number of mammalian and nonmammalian models addressing these predictions. These data shed light on the mechanisms controlling sperm interactions with VC and on the evolutionary conservation of the proteasome-assisted fertilization mechanisms.

**Keywords** Egg coat lysin • Fertilization • Oocyte • Proteasome • Proteolysis • Sperm • Ubiquitin • Vitelline coat • Zona pellucida

---

E. Miles • P. Sutovsky (✉)  
Division of Animal Science and Departments of Obstetrics Gynecology and Women’s Health,  
University of Missouri, Columbia, MO 65211-5300, USA  
e-mail: SutovskyP@missouri.edu

## 37.1 Introduction: Zona Lysin and the Elusive Mechanism of Egg Coat Penetration by Sperm

The process of mammalian fertilization has long been studied, but many fundamental questions still remain unanswered. One that continues to be debated, despite decades of research, concerns the mechanism utilized by the fertilizing spermatozoa to penetrate the oocyte vitelline coat (VC), the zona pellucida (ZP). When a capacitated, fertilization-competent spermatozoon binds to the sperm receptor on the ZP, it undergoes acrosomal exocytosis (AE), which causes vesiculation of the acrosomal membrane and exocytosis of the acrosomal cap (Yurewicz et al. 1998; Bleil and Wassarman 1980). This step exposes the acrosome-borne proteolytic enzymes and results in the formation of the acrosomal shroud, which allows the spermatozoa to create a local microenvironment that supports the opening of the fertilization slit and penetration of the sperm through the ZP (Yurewicz et al. 1998). There are two schools of thought about the mechanism of sperm–ZP penetration: (1) the mechanical force of the sperm tail motility is sufficient to fully penetrate the ZP (Green and Purves 1984; Bedford 1998); and (2) an enzyme originating from the acrosome of the fertilizing spermatozoa acts as a putative zona lysin that digests the fertilization slit in the ZP and allows sperm to penetrate through the ZP (Austin and Bishop 1958; Austin 1975). The latter theory was first proposed as early as 1958 by Austin and Bishop (1958).

Several candidates have been proposed for this hypothetical zona lysin. Originally, the acrosomal protease acrosin was favored as the most likely zona lysin, but was ruled out when acrosin knockout mice remained fertile with only delayed ZP penetration (Baba et al. 1994). Acrosin has since been considered to be involved in proteolysis or processing of proteins in the acrosome and on acrosomal membranes (Honda et al. 2002). The serine protease Tesp5/Prss21 on the mouse sperm surface was identified as a plausible candidate. Double-knockout studies have shown reduced fertility *in vitro*, which was rescued by exposure of the spermatozoa to the uterine microenvironment or by treatment with uterine fluids (Yamashita et al. 2008; Kawano et al. 2010). These candidates have since been proposed to be involved in initial sperm–ZP binding (Yamashita et al. 2008; Kawano et al. 2010). Evidence has been accumulating in favor of the 26S proteasome as the candidate zona lysin in mammals, ascidians, and invertebrates (Sakai et al. 2004; Yi et al. 2007a; Sutovsky et al. 2004). This review highlights the significant studies and current research examining the possible role of the 26S proteasome as the putative mammalian zona lysin.

## 37.2 Ubiquitin-Proteasome System

### 37.2.1 Ubiquitin

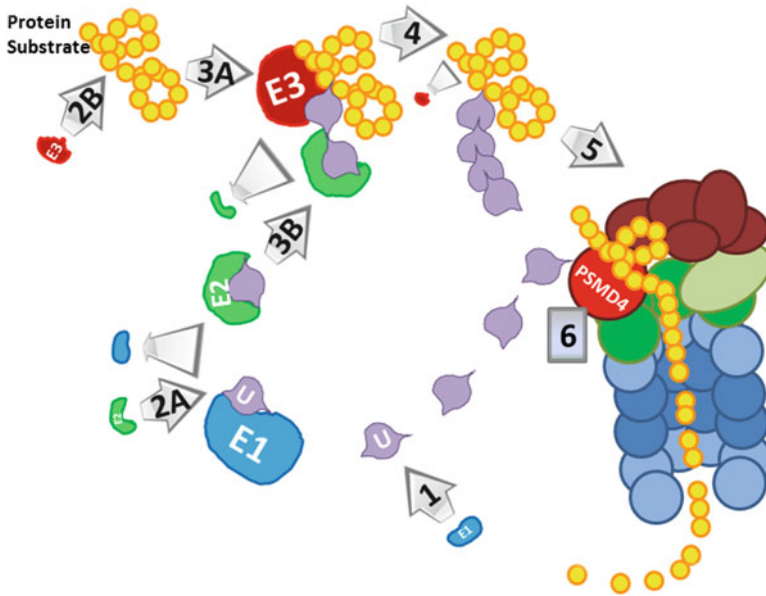
Through a multistep enzymatic process, the ubiquitin-proteasome system (UPS) tags outlived or damaged intracellular proteins with a small chaperone protein ubiquitin. This process, referred to as ubiquitination, typically targets the ubiquitinated

substrates for proteolytic degradation by a multi-subunit protease, the 26S proteasome, (Glickman and Ciechanover 2002). Ubiquitin, an 8.5-kDa, 76-amino-acid protein, was first isolated and purified by the Goldstein laboratory in 1975. They found that this polypeptide induced the differentiation of bovine thymus-derived and bone marrow-derived immunocytes in vitro (Goldstein et al. 1975). Consequently, they named this newly discovered polypeptide ubiquitous immunopoietic polypeptide (UBIP) because of its high degree of evolutionary conservation, exhibiting close structural, functional, and immunological similarity when isolated from species as diverse as protozoans to mammals and plants. In 1976, Etlinger and Goldberg first described a novel ATP-dependent proteolytic system responsible for the rapid degradation of abnormal proteins separate from the lysosomal degradation pathway (Etlinger and Goldberg 1977). The discovery that metabolic energy is required for intracellular protein degradation opposed the commonly accepted idea that cellular proteolysis was an entirely exergonic process occurring in the lysosome. In a joint effort, Aaron Ciechanover, Avram Hershko, and Ervin Rose described the ubiquitin-mediated protein degradation, a discovery for which they shared the Nobel Prize in Chemistry in 2004. Ubiquitin is not just a housekeeping protein that helps recycle outlived or damaged protein molecules. It is also involved in a number of cellular mechanisms and pathologies, including but not limited to antigen presentation in the immune system, apoptosis, Alzheimer's disease, cell-cycle control, endocytosis of membrane receptors, human immunodeficiency virus (HIV) particle internalization, protein quality control in the endoplasmic reticulum, reticulocyte differentiation, signaling, and transcriptional control (Pines 1994; Hershko and Ciechanover 1998; Glickman and Ciechanover 2002).

### 37.2.2 Ubiquitination

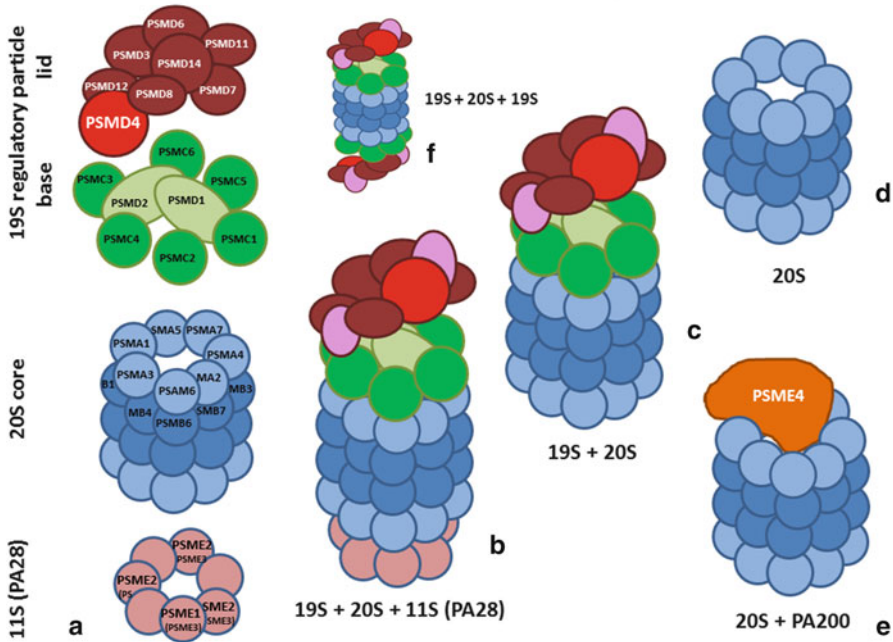
Ubiquitin is typically a cytoplasmic and nuclear protein, but it can be found in the extracellular space in some mammalian, lower vertebrate, and invertebrate systems and is highly substrate specific. Polymerization of substrate-bound ubiquitin molecules into multi-ubiquitin chains serves as a degradation signal for numerous target proteins. The degradation of substrate protein is initiated by the covalent attachment of an isopeptide chain of four or more ubiquitin molecules. These ubiquitin molecules are linked to each other through one of seven lysine residues (K6, K11, K27, K29, K33, K48, K63) to form the poly-ubiquitin chains. All seven of these Lys residues are potential ubiquitin-chain initiation sites, but K48 is the most common linkage site for poly-ubiquitin chains recognized by the 26S proteasome (Walczak et al. 2012; Iwai 2012). The K63 site is the most common site of di-ubiquitination, targeting membrane receptors for lysosomal degradation, chromatin remodeling, and DNA repair (Walczak et al. 2012; Kim et al. 2006).

The formation and ligation of the poly-ubiquitin chain to its target protein occurs through a concerted series of ATP-dependent enzymatic reactions (Fig. 37.1). Ubiquitination starts with the activation of a single ubiquitin molecule with a



**Fig. 37.1** Diagram of protein ubiquitination and degradation by the 26S proteasome. Step 1. Monoubiquitin is activated by a phosphorylated ubiquitin-activating enzyme E1 (*UBA1*). Step 2A. UBE1 is supplanted by ubiquitin carrier enzyme/ubiquitin-conjugating enzyme E2 (*UBE2*). Step 2B. Concurrently, the substrate protein is engaged by an E3-type ubiquitin ligase. Step 3A. Ubiquitin ligase E3 covalently links an activated monoubiquitin to an internal Lys residue of the substrate protein. Step 3B. A second activated ubiquitin molecule is linked to the substrate-bound ubiquitin. Step 4. The ensuing tandem ligation of additional activated ubiquitin molecules results in the formation of a multi-ubiquitin chain. Step 5. Multi-ubiquitin chain of four or more ubiquitin molecules is recognized and engaged by subunit *PSMD4* of the 19S proteasomal regulatory complex. Step 6. The substrate protein is deubiquitinated (liberated ubiquitin molecules re-enter the cycle), unfolded, threaded through the 20S core, and cleaved into small peptides, released from the 20S core lumen. [From Sutovsky 2011, © Society for Reproduction and Fertility (2013). Reproduced by permission]

phosphorylation-dependent ubiquitin-activating enzyme E1 (*UBE1* or *UBA1* in HUGO nomenclature). The *UBA1* is then supplanted by ubiquitin carrier/ubiquitin-conjugating enzyme E2. Simultaneously, an E3-type ubiquitin ligase seeks out and engages the target protein to be ubiquitinated and degraded (Hershko 2005). The E3-type ligases are highly diverse and responsible for the substrate specificity of protein ubiquitination as there are about two E1 proteins, approximately 30 E2 proteins, and more than 500 different E3 proteins in humans (Tanaka 2009). The E3-ligases catalyze the covalent ligation of the C-terminal glycine/Gly residue (G76) of ubiquitin to an internal lysine/Lys residue of the target protein. Next, the ubiquitin chain elongates from the mono-ubiquitinated substrate protein resulting in a poly-ubiquitinated protein. A poly-ubiquitin tail of four or more ubiquitin molecules is needed to signal proteasomal degradation.



**Fig. 37.2** Variations on the subunit composition of the 26S proteasome. **(a)** Subunit makeup of (top to bottom) 19S regulatory particle (lid + base), 20S core, and 11S particle (PA28). **(b)** 20S core capped with 19S particle on one side and an 11S complex/PA28 on the other. **(c)** 20S core capped with one 19S particle. **(d)** Uncapped 20S proteasome. **(e)** 20S core capped with proteasome activator PA200. **(f)** Canonical 26S proteasome, with one 19S particle/cap on each side of 20S core. [From Sutovsky 2011, © Society for Reproduction and Fertility (2013). Reproduced by permission]

### 37.2.3 26S Proteasome

The 26S proteasome is a 2.5-MDa multi-catalytic canonical protease localized in the cell cytosol and nucleus (Fig. 37.2). The 26S proteasome is responsible for degrading ubiquitinated proteins in the cell, although nonubiquitinated proteins could be substrates as well. The typical enzymatically active proteasome consists of two subcomplexes. One is a hollow catalytic 20S core particle (20S CP) capped by one or two terminal 19S regulatory particle(s) (19S RP). The 19S RP is responsible for poly-ubiquitin chain recognition and binding, deubiquitination, and substrate protein priming/linearization/unfolding. The 19S RP consists of at least 17 different subunits between the two subcomplexes: the lid and the base. The lid complex contains 14 regulatory particle non-ATPase subunits (PSMD1-14) (Hanna and Finley 2007). Subunit PSMD4 (Rpn10) is the main 19S subunit responsible for substrate recognition. It binds the poly-ubiquitin chains on the target protein during poly-ubiquitin chain recognition by the 26S proteasome (Yi et al. 2010a, b). Subunit PSMD14 (Rpn11), then deubiquitinates the captured substrate by cleaving the

poly-ubiquitin chain at a proximal site, which is then further cleaved into single ubiquitin molecules by deubiquitinases (DUBs) that can associate with the 19S RP (Verma et al. 2002). The cleaved ubiquitin molecules can be reused for ubiquitination of other protein molecules. New functions are still being discovered for subunits composing the 19S lid. Table 37.1 lists the known 26S proteasomal subunits and their proposed functions by species.

The 19S RP base subcomplex is responsible for (1) capturing target proteins via poly-ubiquitin chain recognition, (2) unfolding the substrate protein, and (3) opening the channel into the 20S CP. The base subcomplex contains six homologous AAA-ATPase subunits (PSMC1–6) and three non-ATPase subunits (PSMD1, -2, -4). These subunits create a narrow gated channel, which only allows unfolded proteins to enter the 20S core. PSMD1 and -2 have been proposed to work together as a functional unit and are required for substrate translocation and opening of the 20S channel. This compartmentalized design of the 26S proteasome separates proteolysis from the cellular milieu and restricts degradation to unfolded and imported proteins. Subunit PSMD2 attaches to the 20S CP, whereas subunit PSMD1 is located on top of PSMD2 and serves as a docking site for substrate recruitment factors (Rosenzweig et al. 2008). Subunit PSMD4 functions as an integral ubiquitin receptor to trap poly-ubiquitinated proteins; PSMD4 does this by its C-terminal ubiquitin-interacting motif (Deveraux et al. 1995). Six ATPase subunits (PSMC1-6) surrounding the base are organized into a hexameric ring that controls the opening of the channel and allows the protein to reach the catalytic sites of the 20S CP. The base ATPases are required not only to open the channel into the 20S CP but to also unfold the substrate protein: this allows the protein to be threaded through the narrow channel of the 20S CP where the catalytic protease subunits are located. The details of the ATP-dependent mechanisms behind these processes are still unknown, but it is certain that these subunits work through a process that requires ATP hydrolysis (Liu et al. 2006).

The 20S CP is a 750-kDa cylindrical protein complex responsible for the proteolysis of the substrate protein. The 20S CP contains 28 subunit molecules of 14 types formed from the stacking of two outer  $\alpha$ -rings and two inner  $\beta$ -rings. Each ring is composed of seven structurally similar  $\alpha$ - and  $\beta$ -subunits, respectively. The rings are heptamerically stacked in an  $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$  pattern. The subunits of the  $\alpha$ -ring (PSMA1-7) connect the 20S CP to the 19S base and act as a gate that opens in the presence of an ubiquitinated protein. The two inner rings are each composed of seven  $\beta$ -type subunits (PSMB1-7) and confer the threonine protease activities of the 26S proteasome. There are three catalytic  $\beta$ -type subunits: PSMB6 ( $\beta$ 1), PSMB7 ( $\beta$ 2), and PSMB5 ( $\beta$ 5). The  $\beta$ 1-subunit is associated with caspase-like/PGPH (peptidylglutamyl-peptide hydrolyzing) activity,  $\beta$ 2 with trypsin-like activity, and  $\beta$ 5 with chymotrypsin-like activities, which confer the ability to cleave peptide bonds at the C-terminal side of acidic, basic, and hydrophobic amino-acid residues, respectively (Tanaka 2009). The 20S proteasome then degrades the target protein into 3 to 15 amino-acid oligopeptides that are released into the cytosol and are further hydrolyzed into single amino acids by cytosolic oligopeptidases or amino-carboxyl peptidases (Tanaka 2009).

**Table 37.1** 26S proteasome subunit function and nomenclature by species

HUGO/GDB symbol	<i>Homo sapiens</i>	<i>Bos taurus</i>	<i>Sus scrofa</i>	<i>Rattus norvegicus</i>	<i>Mus musculus</i>	<i>Caenorhabditis elegans</i>	<i>Saccharomyces cerevisiae</i>	Function
<i>20S α-type</i>								
α1	PSMA6	PSMA6	PSMA6	PSMA6	PsmA6	PsmA6/Pros-27	Sc11/YC7	
α2	PSMA2	PSMA2	PSMA2	PSMA2	PsmA2	PsmA2	Pre8/Y7	
α3	PSMA4	PSMA4	PSMA4	PSMA4	PsmA4	PsmA4	Pre9/Y13	
α4	PSMA7	PSMA7	PSMA7	PSMA7	PsmA7	PsmA7	Pre6	
α5	PSMA5	PSMA5	PSMA5	PSMA5	PsmA5	PsmA5	Pup2/Doa5	
α6	PSMA1	PSMA1	PSMA1	PSMA1	PsmA1	PsmA1/Pros-30	Pre5	
α7	PSMA3	PSMA3	PSMA3	PSMA3	PsmA3	PsmA3	Pre10/YC1	
<i>20S β-type</i>								
β1	PSMB6	PSMB6	PSMB6	PSMB6	PsmB6	PsmB6/Lmp19/ MpnD	Pre3	Caspase-like peptidase activity
β2	PSMB7	PSMB7	PSMB7	PSMB7	PsmB7	PsmB7/MC14	Pup1	Trypsin-like protease activity
β3	PSMB3	PSMB3	PSMB3	PSMB3	PsmB3	PsmB3	Pup3	
β4	PSMB2	PSMB2	PSMB2	PSMB2	PsmB2	PsmB2	Pre1	
β5	PSMB5	PSMB5	PSMB5	PSMB5	PsmB5	PsmB5	Pre2/Doa3	Chymotrypsin-like protease activity
β6	PSMB1	PSMB1	PSMB1	PSMB1	PsmB1	PsmB1	Pre7	
β7	PSMB4	PSMB4	PSMB4	PSMB4	PsmB4	PsmB4	Pre4	
β1i	PSMB9	PSMB9	PSMB9	PSMB9	PsmB9/Lmp2 RING12	PsmB9/Lmp2	–	Caspase-like activity/ immunoprotea- some

(continued)

Table 37.1 (continued)

HUGO/GDB symbol	<i>Homo sapiens</i>	<i>Bos taurus</i>	<i>Sus scrofa</i>	<i>Rattus norvegicus</i>	<i>Mus musculus</i>	<i>Caenorhabditis elegans</i>	<i>Saccharomyces cerevisiae</i>	Function
β2i	PSMB10	PSMB10	PSMB10	PSMB10	Psmb10/Mecl-1/ Lmp7	–	–	Trypsin-like activity/ immunoprotea- some
β5i	PSMB8	PSMB8/ LAMP7	PSMB8	PSMB8/Ring10	Psmb8	–	–	Chymotrypsin-like activity/ immunoprotea- some
<i>19S ATPase</i>								
Rpt1/S7	PSMC2	PSMC2/ MSS1	PSMC2	PSmc2/Mss1	Psmc2/Mss1	RPT-1	Yta3/Cim5	ATPase
Rpt2/S4	PSMC1	PSMC1/p56	PSMC1	PSmc1/S4	Psmc1/P26S4	RPT-2	Yta5/mts2	ATPase, Gate-opening
Rpt3/S6b	PSMC4	TBP7 /S6/p48	PSMC4	PSmc4/Tbp7	Psmc4	RPT-3	Yta2	ATPase, Gate-opening
Rpt4/S10b	PSMC6	p42/CADP44/ SUG2	PSMC6	PSmc6	Psmc6	RPT-4	Sug2/Pcs1/ Cr113	ATPase
Rpt5/S6a	PSMC3	TBP1/S6a	PSMC3/p50	PSmc3/Tbp1	Psmc3	RPT-5	Yta1	ATPase, Gate-opening
Rpt6/S8	PSMC5	p45 /S8/ TRIP1/ SUG1	PSMC5/p45	PSmc5/Sug1	Psmc5/FZA-B/ Sug1/M56	RPT-6	Sug1/Cim3/ let1/Cr13	ATPase
<i>19S non-ATPase</i>								
Rpn1/S2	PSMD2	p97/ TRAP- 2/55.11/S2	PSMD2/p97	PSmd2	Psmd2/Tex190	RPN-1	Nas1/mts4/Hrd2	PIPs scaffolding
Rpn2/S1	PSMD1	p112/S1	PSMD1/ p112	PSmd1	Psmd1/P112	RPN-2	Sen3	PIPs scaffolding



Rpn3/S3	PSMD3	p58/S3/ TSTA2	PSMD3/p58	PSMD3	PsmD3	PsmD3/P91A	RPN-3	Sun2		
Rpn4								Son1/Utd5	Proteasome gene, transcription	
Rpn5/p55	PSMD12	p55	PSMD13/ p55	PSMD12	PsmD12	PsmD12/P55	RPN-5	Nas5		
Rpn6/S9	PSMD11	p44.5/S9	PSMD11/ p44.5	PSMD11	PsmD11	PsmD11/P44.5	RPN-6	Nas4		
Rpn7/S10a	PSMD6	p44/S10a	PSMD6/p44	PSMD6	PsmD6	PsmD6	RPN-7			
Rpn8/S12	PSMD7	p40/S12/ MOV34	PSMD7/p40	PSMD7	PsmD7	PsmD7/Mov-34	RPN-8	Nas3		
Rpn9/S11	PSMD13	p40.5/S11	PSMD13/ p40.5	PSMD13	PsmD13	PsmD13	RPN-9	Nas7/mts1		
Rpn10/S5a	PSMD4	S5a/AF	PSMD4	PSMD4	PsmD4	PsmD4/S5a/ Mcb1	RPN-10	Mcb1/pus1/ Sun1	Ub receptor	
S5b	PSMD5	p50.5/S5b	PSMD5/ p50.5	PSMD5	PsmD5	PsmD5	-	-		
Rpn11/ S13	PSMD14	S13/POH1/ PAD1	PSMD14	PSMD14	PsmD14	PsmD14/Pad1/ Poh1	RPN-11	Mpr1/pad1/mts5	DUB	
Rpn12/ S14	PSMD8	p31/S14/ HIP6/ Nim1p	PSMD8/p31	PSMD8	PsmD8	PsmD8	RPN-12	Nim1/mts3		
Rpn13	ADRM1	ADRM1						DAQ1	Ub receptor, UCH37 recruitment	
p28	PSMD10	p28/S15	PSMD10/ p28	PSMD10	PsmD10/ p28Gank	PsmD10/P28/ gankyrin	-	Nas6		
p27/S15	PSMD9	p27/S15	PSMD10/ p27	PSMD9	PsmD9/Bridge	PsmD9/P27	-	Nas2	PSM modulator	

*HUGO* human genome organization, *GDB* human genome database, *DUB* deubiquitinating enzyme, *Ub* ubiquitin, *PSM* proteasome, *PIP*s proteasome-interacting proteins/proteasome substrates (Voges et al. 1999; Chen et al. 2008; Tanaka 2009)

## 37.3 Sperm Proteasome During Plant, Ascidian, and Echinoderm Fertilization

### 37.3.1 *Plant Fertilization*

The sexual reproduction cycle in higher plants occurs in the reproductive organs of the flower. Similar to animal spermatozoon, pollen, the male gametophyte, is a highly specialized structure, consisting of two or three cells. Pollen is released from the anthers and adheres to the surface of the female stigma, equipped with a cytoplasmic extension known as the pollen tube (Boavida et al. 2005). The pollen tube rapidly grows through the style to the ovules (Boavida et al. 2005). Pollen tube adhesion and growth guides the pollen toward the ovary where it fertilizes the embryo sac, the female gametophyte (Boavida et al. 2005). For a more in-depth account of fertilization in higher plants, please refer to the review by Boavida et al. (2005).

Evolutionary conservation of the components and roles of the UPS appear to reach beyond the animal kingdom to plants, where pollen adhesion and guidance seem to depend on ubiquitin. For example, during maize pollen development, the levels of ubiquitin and ubiquitin–protein conjugates in young pollen precursors, the microspores without vacuoles were 10 to 50 times lower than compared to mature pollen grains (Callis and Bedinger 1994). Treatment with 26S proteasomal inhibitors, MG132 and epoxomicin, significantly reduced pollen tube growth in kiwifruit (*Actinidia deliciosa*) (Speranza et al. 2001). Kim et al. (2006) showed that pollen adhesion to styles of lily (*Lilium longiflorum* Thunb.) can be enhanced in vitro with the addition of exogenous ubiquitin. Furthermore, in *Arabidopsis thaliana* the proper conformation of SCF E3 ubiquitin-ligases that direct ubiquitination is required for male fertility (Devoto et al. 2002). Mutations in genes encoding 26S proteasomal subunits, ubiquitin-specific proteases, deubiquitinating enzymes, and 19S regulatory particle non-ATPases result in infertility from defects in pollen maturation or transmission (Doelling et al. 2007; Book et al. 2009). Several E3s have been shown to play key roles in self/non-self pollen recognition in snapdragons (*Antirrhinum*) (Qiao et al. 2004). In snapdragons, the SCF<sup>FAhSLF2</sup> complex acts as an E3 that targets non-self S-RNases for ubiquitination and destruction by the 26S proteasome; however, it leaves self-S-RNases active during the self-incompatibility response, thus functioning as a cytotoxin that degrades RNA and terminates pollen tube growth (Qiao et al. 2004). The current data strongly suggest a role for the UPS in plant germination and gametophyte development, but more research is needed to elucidate how the UPS is utilized in these processes.

### 37.3.2 *Ascidian Fertilization*

Ascidians are hermaphrodites that reproduce by releasing sperm and eggs simultaneously into the surrounding seawater during the spawning season; thus, large

quantities of readily fertilizable gametes can be easily obtained for research. The exposure to alkaline seawater activates the spermatozoa, very similar to capacitation in mammals, suggesting that the proteasome is then secreted to the sperm surface. The spermatozoa then undergo species-specific binding to the proteinaceous vitelline coat, which they must penetrate via sperm surface proteases that act as a VC-lysin to complete the fertilization process. It has been shown that the proteasomes are exposed on the ascidian sperm surface; proteasomal proteolytic activity was specifically detected in the sperm head region and was clearly increased upon sperm activation (Sawada et al. 2002a, b). Anti-proteasome antibodies, and proteasomal inhibitors MG115 and MG132, inhibit ascidian fertilization (Sawada et al. 2002b).

Studies in two ascidian species, *Ciona intestinalis* and *Halocynthia roretzi*, have shown a strict self-sterility in fertilization (Sawada et al. 2002a). After self/non-self recognition via interactions between the sperm and VC, the sperm-borne egg coat lysin system is activated (Sawada et al. 2002a). Data from Sawada's laboratory have provided evidence that VC penetration by ascidian sperm involves the sequential ubiquitination and proteasomal degradation of the sperm receptor protein HrVC70, a major protein component of the ascidian VC. Ubiquitination of HrVC70 is accomplished by a 700-kDa ubiquitin-conjugating enzyme complex released during sperm activation (Sawada et al. 2002a; Sakai et al. 2003). A 930-kDa proteasome (26S-like proteasome) seems to function as the egg coat lysin that directly degrades the VC. These data suggest that an extracellular, sperm-borne, ubiquitin-conjugating enzyme system is essential for the formation of these poly-ubiquitin chains on HrVC70 and for ascidian fertilization.

The HrVC70 is a 70-kDa component of the VC shown to be a novel sperm receptor that bears significant similarity to components of the mammalian zona pellucida, particularly the proposed mammalian/murine sperm receptor ZPC (Sawada et al. 2002a). Three N-terminal cysteine/Cys residues in the ZP domain of the 120-kDa HrVC70 precursor (HrVC120) share conserved positions within mammalian and frog ZPC (Sawada et al. 2002a). HrVC70 contains 12 EGF (epidermal growth factor)-like repeats that confer its role in sperm binding to VC (Sawada et al. 2002b). This protein has been shown to be degraded by purified ascidian sperm 26S-like proteasomes only in the presence of ubiquitin, ATP, and the ubiquitin-conjugating enzymes purified from a rabbit reticulocyte lysate (Sawada et al. 2002b; Sakai et al. 2004). These results reveal that an extracellular UPS is essential for ascidian fertilization, particularly in the degradation of the proteinaceous vitelline coat.

Recently, Yokota and Sawada investigated an extracellular transport signal and have discovered a novel posttranslational modification of the ascidian sperm proteasome. They found that the 20S core PSMA1/ $\alpha$ 6-subunit of purified sperm proteasomes is distinct from purified egg and muscle proteasomes (Yokota et al. 2011). Tissue specific  $\alpha$ -subunits are not commonplace, but several are expressed in the testis of *Drosophila* ( $\alpha$ 3T,  $\alpha$ 4T2, and  $\alpha$ 6T) (Belote and Zhong 2009). Among these testis-specific  $\alpha$ -subunits,  $\alpha$ 6T is reported to be crucial for spermatogenesis and fertility (Belote and Zhong 2009). The  $\alpha$ 6-subunit of *H. roretzi* contains a cluster of acidic amino acid residues and the removal of this cluster may mimic the state of dephosphorylation of the  $\alpha$ 6-subunit which may affect function and localization of

the sperm proteasome (Yokota et al. 2011). Alternatively, it is also possible that the conserved sequence of the  $\alpha 6$ -subunit in *H. roretzi* and *C. intestinalis* may function as a transport signal to the acrosome during ascidian spermiogenesis. It is not known at present if similar modifications exist in the mammalian sperm proteasome.

### 37.3.3 *Echinoderm Fertilization*

Most echinoderms can reproduce sexually by spawning eggs and spermatozoa into the seawater, much as do ascidians, and asexually by regenerating body parts. For the purpose of this review, only sexual reproduction is examined. It has been confirmed that the proteasomes are present in sea urchin spermatozoa and that the sperm proteasome may be involved in the acrosome reaction; but differing from ascidians, the proteasome is not essential for sperm binding to the echinoderm VC (Yokota and Sawada 2007; Matsumura and Aketa 1991). The role of sperm proteasomes in echinoderm fertilization has been investigated in the sea urchin *Pseudocentrotus depressus* by Yokota and Sawada (2007), who examined the effects of proteasomal inhibitors and synthetic peptide substrates for the proteasome on different steps of the fertilization process. The inhibition of fertilization by proteasomal inhibitors suggests that the echinoderm sperm proteasomes are important in the acrosome reaction and in sperm penetration through the VC, most likely as an egg coat lysin (Yokota and Sawada 2007). Among the examined proteasomal substrates and inhibitors, a caspase substrate (Z-Leu-Leu-Glu-MCA) competitively inhibited the caspase-like catalytic center ( $\beta 1$ -subunit) of the proteasome, which decreased fertilization in a concentration-dependent manner (Yokota and Sawada 2007); this suggests that the caspase-like activity of the proteasome  $\beta 1$ -subunit must play a key role in sea urchin fertilization.

Echinoderm spermatozoa undergo acrosome reaction/acrosomal exocytosis when they enter the jelly coat surrounding the VC. Protein kinase A (PKA) has been implicated in the acrosome reaction in sea urchins (Su et al. 2005), and shown to stimulate the function of mammalian 26S proteasome (Zhang et al. 2007). Therefore, the sperm proteasome may act to degrade certain unknown PKA modulators, which would result in the irreversible activation of PKA during the acrosome reaction. During the acrosome reaction, the acrosomal contents containing an egg coat lysin are exposed and released, allowing spermatozoa to penetrate the VC (Yokota and Sawada 2007). For the sperm proteasome to act as egg coat/VC lysin, the VC must be ubiquitinated before fertilization and extracellular ATP must be present (Yokota and Sawada 2007). Yokota and Sawada used Western blotting with a monoclonal anti-ubiquitin antibody FK2, which recognizes both mono- and multi-ubiquitinated proteins, to reveal a band pattern indicative of VC ubiquitination. The identity of the ubiquitinated VC protein(s) remains to be determined, but current data support the hypothesis that the echinoderm sperm 26S proteasome is released during the acrosome reaction and degrades the ubiquitinated VC proteins in an ATP-dependent fashion.

### 37.4 Proteasome Localization and Activity in Mammalian Spermatozoa

Mammalian fertilization is a unique cellular-recognition event that comprises sequential interactions between the sperm and the oocyte vitelline coat (Yanagimachi 1994). First, a capacitated spermatozoon penetrates the cumulus oophorus, facilitated by a sperm membrane-bound hyaluronidase, and then binds to the zona pellucida, a protective glycoprotein matrix that surrounds the ovulated oocyte. The ZP plays a pivotal role in the species specificity of sperm–oocyte recognition, and binding, as well as in the induction of acrosomal exocytosis (AE), anti-polyspermy defense, and protection of the embryo until implantation. In most of the mammals studied, only spermatozoa with an intact acrosome bind to the ZP, in a species-specific manner. The fertilizing spermatozoan interaction with the zona glycoprotein that serves as sperm receptor (ZP3/ZPC in mouse/human and ZPB/ZPC complex in pig) triggers the AE via signal transduction events in the sperm acrosome, such as the opening of T-type, low voltage-activated  $\text{Ca}^{2+}$  channels, causing an influx of  $\text{Ca}^{2+}$  and the activation of heterotrimeric G proteins (Pasten et al. 2005; Ikawa et al. 2010). An increase in intracellular pH, resulting in sustained  $\text{Ca}^{2+}$  influx, which drives AE, causes the release or exposure of acrosomal enzymes and, when combined with vigorous sperm motility, allows the spermatozoon to penetrate the ZP and fuse with the oocyte plasma membrane, the oolemma (Pasten et al. 2005; Ikawa et al. 2010).

The role of the UPS has been well documented in the reproductive processes of several mammalian species. For example, multi-enzymatic sperm protease complexes with similar sedimentation coefficients as purified proteasomes from other tissues have been isolated from mouse (Pasten et al. 2005), pig (Zimmerman and Sutovsky 2009), and human (Morales et al. 2003; Morales et al. 2004; Baker et al. 2007) spermatozoa. Accordingly, the substrate-specific enzymatic activities (trypsin-like, chymotrypsin-like, and peptidylglutamyl peptide-hydrolyzing activities) of these sperm proteasomes have been recorded in mice (Pasten et al. 2005; Bedard et al. 2011; Rivkin et al. 2009), pigs (Sutovsky et al. 2004; Yi et al. 2007a; Yi et al. 2009), and humans (Morales et al. 2003; Chakravarty et al. 2008; Kong et al. 2009).

The sperm-associated proteasomes are tethered to the acrosomal surface in mice (Pasten et al. 2005), pigs (Sutovsky et al. 2004; Yi et al. 2009), and humans (Morales et al. 2004). The proteasomes probably become associated with the spermatozoa inner and outer acrosomal membranes during acrosomal biogenesis at the spermatid stage (Rivkin et al. 2009). It appears that the extracellular proteasomes exposed on the acrosomal surface of mammalian spermatozoa are involved in the ZP-induced AE and are able to directly interact with the ZP during fertilization (Sutovsky et al. 2004; Yi et al. 2009). The proteasomes located on the cell surface could participate in sperm–ZP binding in some mammalian species, such as humans (Chakravarty et al. 2008; Naz and Dhandapani 2010) and mice (Pasten et al. 2005). Then, the intracellular and extracellular proteasomes would blend into the acrosomal shroud and participate during the AE. Sperm proteasomes have been shown to be essential

for acrosomal function and sperm–zona penetration during fertilization in several mammalian species such as mouse, pig, and human (Pasten et al. 2005; Kong et al. 2009; Zimmerman et al. 2011). It is possible that the proteasomes modulating these cellular events could participate in other steps of fertilization as well.

In addition to proteasomes, other UPS components have been implicated in spermatogenesis, epididymal sperm maturation, and fertilization. For example, ubiquitinated substrates have been detected in the epididymal fluid, the seminal plasma, on the surface of defective epididymal spermatozoa, and on the outer surface of the ZP (Sutovsky et al. 2001, 2004; Zimmerman et al. 2011). Anti-proteasome antibodies have been reported to be present in the seminal plasma of infertile men (Bohring et al. 2001; Bohring and Krause 2003). Addition of the deubiquitinating enzyme inhibitor, ubiquitin-aldehyde, to boar in vitro fertilization assays promotes polyspermy, which might be related to an increase in ubiquitination and degradation of the ZP because this C-terminally modified ubiquitin species accelerates proteasomal proteolysis (Yi et al. 2007b). More recently, sperm deubiquitinating enzymes have been explored by the Wing laboratory in Canada. They reported that the spermatozoa of deubiquitinating enzyme *Usp2* knockout mice are severely subfertile, which appeared to be caused by a defect in ZP binding or penetration, even though the USP2 protein in wild-type mice does not seem to be specifically localized in the acrosome. In elongating spermatids, USP2 was localized perinuclearly in a thin layer between the outer acrosomal membrane and the plasma membrane, but absent from the nucleus (Lin et al. 2000). The *Usp2* null spermatozoa are motile and undergo the AE but fail to penetrate the ZP (Bedard et al. 2011). These data suggest that the deubiquitination of sperm or spermatid proteins by USP2 is an important regulatory mechanism required for the acquisition of fertilizing ability by mammalian spermatozoa. It is not clear if the observed severe subfertility is a result of impaired deubiquitinating activity of the acrosomal USP2 enzyme during fertilization, or if it is rather a result of abnormal assembly of the sperm acrosome during spermiogenesis. Either way, the failure of motile *Usp2* null spermatozoa to penetrate the zona supports the view that sperm motility alone is not sufficient to propel the sperm head through the zona matrix.

The role of UPS in sperm penetration through the ZP has been well documented. Proteasomal inhibitors and anti-proteasome antibodies effectively block mouse, pig, and human sperm ability to penetrate the ZP of their respective species (Morales et al. 2003; Sutovsky et al. 2004; Pasten et al. 2005). The coincubation of pig spermatozoa with proteasomal inhibitors prevents sperm–ZP penetration without affecting sperm motility and binding (Sutovsky et al. 2003, 2004; Yi et al. 2007a; Zimmerman and Sutovsky 2009). However, if the zona is removed before fertilization, fertilization proceeds even with the addition of proteasomal inhibitors (Sutovsky et al. 2004), which suggests a role for the proteasome in sperm–ZP penetration. Chakravarty et al. reported that the human sperm-associated proteasomal activity is not stimulated by binding of recombinant ZP proteins, but that it remains steady after AE, most likely caused by the association of the proteasomes with the inner acrosomal membrane, which is not removed by AE (Chakravarty et al. 2008).

The ZP3/ZPC in mice and humans has been identified as the primary sperm-binding receptor on the ZP and perhaps the inducer of AE (Naz and Dhandapani 2010). The human sperm ubiquitin-associated protein UBAP2L was identified as a ZP-interacting protein by the Naz Lab (Naz and Dhandapani 2010) using a yeast two-hybrid screen (Y2H) and was further tested when UBAP2L antibodies were shown to inhibit sperm–ZP binding when tested *in vitro*. The Y2H procedure is used to identify proteins that interact with a target protein expressed in yeast as a hybrid with a DNA-binding domain and has been widely used to examine protein–protein interactions (Naz and Dhandapani 2010). Human sperm–oocyte recognition, binding, and AE have been proposed to be mediated by sugar residues (*O*- and *N*-linked) and peptide moieties of ZP3 (Chakravarty et al. 2005; Gupta et al. 2009). Results from the Naz lab suggest that ubiquitination, in addition to glycosylation of the ZP proteins, may regulate the sperm–ZP interactions (Naz and Dhandapani 2010).

In agreement with the foregoing study, Aitken and colleagues found that the proteasome is a component of a multimeric ZP-binding complex found in human spermatozoa (Redgrove et al. 2011). They propose that sperm–ZP binding requires the concerted action of several sperm proteins that form multimeric recognition complexes on the sperm surface, which is an alternate and novel view different from the traditional simple lock-and-key mechanism of one receptor, one ligand. The formation of these complexes on the sperm surface is purported to depend upon posttesticular maturation driven by the environmental changes to which the spermatozoa are exposed in the epididymis and within the female reproductive system. They report that human spermatozoa express a number of high molecular weight protein complexes on their surface and that subsets of these complexes display affinity for homologous ZP (Redgrove et al. 2011). Two of these complexes were revealed to be a chaperonin-containing TCP-1 (CCT), which harbors a putative ZP-binding protein, ZPBP2, and several components of the 20S proteasome that were found previously in an analysis of the human sperm proteome (Redgrove et al. 2011; Johnston et al. 2005). The role of these protein complexes in sperm–ZP interactions was further confirmed when antibodies against the individual components of the complex, including proteasomal subunits, inhibited sperm binding to zona-intact oocytes (Redgrove et al. 2011). Because many sperm proteins exhibit ZP-binding affinity, it is possible that complexes containing these ZP-binding proteins may participate in sequential or hierarchical molecular interactions or act synergistically to ensure successful sperm–ZP binding. It is important to note that the proteasome complex was shown as three large bands when examined by blue native polyacrylamide gel electrophoresis. Aitken et al. theorize that this is caused by post-translational modifications of certain subunits of the complex ( $\alpha 1-7$ ,  $\beta 1$ , and  $\beta 4$ ) that displayed charge shift signatures characteristic of tyrosine phosphorylation (Redgrove et al. 2011). This finding is consistent with the proteasomal subunit phosphorylation reported in the acrosome that, according to Diaz et al., modulates the fertilizing capacity of human spermatozoa by inducing AE (Diaz et al. 2007), suggesting that proteasome complexes may be differentially activated during the individual steps of fertilization.



### 37.5 Is the Mammalian Egg Coat Ubiquitinated Before Fertilization?

To confer with the hypothesis that the 26S proteasome acts as the putative egg coat lysin in mammalian fertilization, there must be an ubiquitinated sperm receptor on porcine ZP that is degraded by the sperm acrosome-borne proteasomes during porcine fertilization. These three prerequisites are required: (1) the sperm receptor on the mammalian egg coat is ubiquitinated; (2) proteasomes are present, exposed, and enzymatically active in the sperm acrosomal cap; and (3) sperm proteasomes degrade the sperm receptor on the egg coat during fertilization.

There is evidence that the sperm receptor complex ZPB-ZPC on the porcine ZP is ubiquitinated. In porcine oocytes, the ZPB-ZPC complex has been shown to be responsible for sperm-ZP binding (Yurewicz et al. 1998). Furthermore, the porcine ZPC homologues in mouse (ZP3) and human (ZP3) have been implicated in sperm binding and induction of acrosomal exocytosis (Shur et al. 2006; Gupta et al. 2009). Sutovsky et al. (2004) have shown the presence of ubiquitinated proteins in the unfertilized porcine egg coat. They reported that ubiquitin-immunoreactive proteins can be detected on the outer surface of porcine ZP, visualized ZP digestion with immunofluorescence microscopy with anti-ubiquitin antibodies, and recorded the presence of ubiquitinated proteins in ZP preparations from high-quality metaphase II ova. More recently, they have observed Gly-Gly modifications, a fingerprint of ubiquitinated internal Lys-residues, on all three components of porcine ZP (ZPA, ZPB, and ZPC) using Nanospray LC-MS/MS spectroscopy (Peng et al. 2003; Zimmerman et al. 2011). These findings corroborate the pattern of sequential ubiquitination and proteasomal degradation of the ascidian sperm-receptor protein HrVC70, an analogue of mammalian ZP. Similarly, Yokota and Sawada (2007) reported that the vitelline envelopes of sea urchin eggs are already ubiquitinated before fertilization (Yokota and Sawada 2007).

Enzymatically active proteasomes have been found in the boar sperm acrosome. There is evidence that sperm proteasomes are exposed onto the sperm surface and remain associated with acrosomal membranes during sperm-ZP penetration. Boar sperm proteasomes are associated with acrosomal membranes and matrix before AE and remain associated with the inner acrosomal membrane after AE (Morales et al. 2004; Zimmerman and Sutovsky 2009; Yi et al. 2009; Yi et al. 2010a, b). Proteasomal activity is present in motile boar spermatozoa (Yi et al. 2009). Adenosine triphosphate (ATP) is essential for the integrity of the 26S proteasome, which is maintained by the six 19S ATPase subunits. Depletion of sperm surface ATP by *Solanum tuberosum* apyrase inhibits sea urchin fertilization (Yokota and Sawada 2007) and porcine in vitro fertilization (IVF) (Yi et al. 2009). Enzymatic activity of boar sperm proteasomes was confirmed in whole, motile spermatozoa, sperm acrosomal fractions, and affinity purified proteasomes (Yi et al. 2009; Miles et al. 2013). These purified proteasomes were also tested for functionality via casein degradation (Manandhar and Sutovsky, unpublished data).



### **37.6 Sperm Proteasomes Can Degrade Zona Pellucida Proteins in Solution and In Situ**

Studies in porcine and avian models suggest that sperm proteasomes can degrade the sperm receptor on porcine ZP and on the vitelline coat of Japanese quail egg, respectively (Zimmerman et al. 2011; Sasanami et al. 2012). It is difficult to capture the action of sperm acrosomal enzymes during fertilization, but the Sutovsky lab developed an in vitro assay using live, freshly collected, and capacitated boar spermatozoa coincubated with the ZP proteins solubilized from 100 meiotically mature, fertilization-competent porcine oocytes (Zimmerman et al. 2011). The soluble ZP proteins are thus enabled to bind to sperm acrosomal surface as the zona matrix would during fertilization and induce acrosomal exocytosis, resulting in the formation of acrosomal shrouds similar to those seen on the surface of ZP during IVF. Because there is no solid zona matrix in this assay, the acrosomal shrouds are easily separated from spermatozoa and interrogated for the degradation of the bound ZP-proteins. A distinct degradation pattern of the porcine sperm-receptor component ZPC was observed, similar to the degradation pattern of ZPC with purified sperm proteasomes (Zimmerman et al. 2011). The observed, rapid degradation of ZPC by spermatozoa and purified sperm proteasomes was inhibited by ATP depletion with *S. tuberosum* apyrase and with proteasomal inhibitors (MG132, CLBL, and Epoxomicin); it was accelerated by ubiquitin-aldehyde, a C-terminally modified ubiquitin protein that stimulates proteasomal proteolysis (Zimmerman et al. 2011). Furthermore, they were able to record that purified boar sperm proteasomes can digest intact pig ZP of in vitro maturing ova and reduce the rate of polyspermic fertilization after IVF (Zimmerman et al. 2011). These results were corroborated by a study in Japanese quail model that used a similar in vitro system to demonstrate that the sperm proteasome is important for avian fertilization and helps sperm penetration through the perivitelline membrane, which is homologous to the ZP in mammals. Japanese quail spermatozoa contain proteasomes localized in the acrosomal region and can degrade the ZP1 protein in a fashion similar to the degradation of ZP3/ZPC in the porcine model; this degradation is also inhibited by proteasomal inhibitor MG132 and by extracellular ATP depletion by apyrase (Sasanami et al. 2012).

### **37.7 Recent Advances in the Study of Sperm Ubiquitin Proteasome System**

There is a growing acceptance of the involvement of the ubiquitin proteasome system in the reproductive process in plants, ascidians, echinoderms, and mammals, but many outstanding questions remain. How is sperm proteasomal activity regulated during sperm storage, capacitation, AE, and ZP penetration? How are proteasomes inserted in the acrosome during spermiogenesis? What is the role of

deubiquitinating enzymes in fertilization? Can we target sperm proteasomes for a contraceptive effect? Novel animal models and assays will pave the way to elucidate these mechanisms.

A novel transgenic boar model with green fluorescent protein (GFP) fused to the C-terminus of 20S proteasomal core subunit alpha-type 6 (PSMA1) has been developed through a joint effort between the Sutovsky, Prather, and Wells laboratories at the University of Missouri–Columbia (Miles et al. 2013). Functional GFP-tagged proteasomes have been shown to be incorporated in not only fertilization-competent spermatozoa but in other tissues and cell types (Miles et al. 2013). Using cross-immunoprecipitation experiments, the authors identified various proteins interacting with the GFP-PSMA1 subunit such as lactadherin/MFGE8, spermadhesins, and disintegrins/ADAM metalloproteinases; these proteins may regulate sperm proteasomal activity or may be the substrates of proteasomal proteolysis during fertilization (Miles et al. 2013). They have also proposed a method of isolating enzymatically active GFP proteasomes through GFP affinity purification (Miles et al. 2013). This novel model will be useful for studies of fertilization and wherever UPS plays a role in cellular function or pathology.

A protection assay utilizing proteasomal inhibitors was used as an alternative method of identifying sperm–proteasome interacting proteins. The treatment of porcine spermatozoa with proteasomal inhibitors during coincubation with solubilized ZP proteins led to the accumulation of sperm acrosomal surface-associated proteins that would otherwise be degraded during AE (Zimmerman et al. 2011). The identified proteins that were protected from degradation by proteasomal inhibitors included sperm adhesion molecule 1 (SPAM1), lactadherin/MFGE8, zona pellucida-binding protein 2 (ZPBP2), and fragments of acrosin-binding protein ACRBP (SP32) (Zimmerman et al. 2011). Proteasomal degradation of these acrosomal zona-binding proteins could facilitate the ZP-induced acrosomal exocytosis or serve to terminate primary sperm–ZP binding as the fertilizing spermatozoon starts to move forward and penetrate deeper into the ZP. Furthermore, proteasomal inhibitors suppressed the induction of AE when human spermatozoa were incubated with recombinant human ZP3 and ZP4 (Chakravarty et al. 2008). Current research also implicates the ubiquitin proteasome system in sperm capacitation and AE, and that these sperm transformations may be regulated and reversed by deubiquitinating enzymes that also regulate oocyte anti-polyspermy defense and oocyte maturation. Inhibition of the ubiquitin-activating enzyme UBA1 (E1) during boar sperm capacitation alters proteasomal subunit properties and sperm-fertilizing ability *in vitro* in a dose-dependent manner (Yi et al. 2012). Ubiquitin-conjugating enzyme, the ubiquitin-ligase UBR7, has been detected in the boar sperm acrosome, and *de novo* ubiquitination of UBB+1 has been achieved using sperm acrosomal extract (S.W. Zimmerman and P. Sutovsky, unpublished data). Deubiquitinating enzymes from the ubiquitin C-terminal hydrolase (UCHL) family have been shown to regulate anti-polyspermy defense and oocyte cortex and meiotic spindle formation. Block of sperm UCHL3 increases porcine polyspermy *in vitro* while supplementation of recombinant UCHs to IVF media reduced polyspermy and increased the rate of monospermic fertilization (Yi et al. 2007b). Interference with bovine oocyte

UCHL1 alters cortical granule maturation and causes polyspermy (Susor et al. 2010). Block of murine oocyte UCHL1 and UCHL3 prevents sperm incorporation in the ooplasm and causes meiotic spindle anomalies and polar body extrusion defects (Mtango et al. 2012a, b). Subfertility, *in vitro* polyspermy, and failed morula compaction have been reported in the *gad* mutant mouse expressing an inactive form of UCHL1 (Kwon et al. 2003; Mtango et al. 2012a). These data suggest that ubiquitination plays a key role in sperm function and that the deubiquitinating enzymes (UCHL1 and UCHL3) are important during fertilization and pre-implantation embryo development.

## 37.8 Conclusions

Accumulating evidence suggests that the putative mammalian egg coat lysin is the 26S proteasome. There are data supporting all the prerequisites in the porcine fertilization model: (1) all three pig zona components (ZPA, ZPB, ZPC) are ubiquitinated, (2) enzymatically active proteasomes are present in the boar sperm acrosome and exposed on the acrosomal surface, and (3) motile boar spermatozoa and isolated boar sperm proteasomes are capable of degrading the sperm receptor components of the zona pellucida *in vitro*.

Recent advances have elucidated more ways in which the UPS affects mammalian reproduction that could possibly be manipulated to develop novel forms of nonhormonal contraceptives. Sperm proteasomes have been shown to copurify with various acrosomal sperm membrane-binding proteins. Sperm capacitation and acrosome reaction have been reported to be altered by interference with UPS enzymes responsible for ubiquitin–substrate conjugation, deubiquitination, and proteasomal proteolysis. Acrosomal extracts can ubiquitinate exogenous substrates. Therefore, the properties of sperm-borne proteasomes are consistent with their proposed role of mammalian zona lysin. Evidence from several laboratories, animal models, and human gamete studies supports the participation of sperm proteasomes in multiple aspects of the fertilization process, including sperm capacitation, sperm zona binding, acrosomal exocytosis, and sperm–zona/vitelline coat penetration.

**Acknowledgments** We thank Ms. Kathryn Craighead for editorial and clerical assistance. Our gratitude belongs to our colleagues/associates Dr. Young-Joo Yi, Ms. Miriam Sutovsky, Ms. Wonhee Song, Dr. Peter Vargovic, Dr. Gaurishankar Manandhar, and Dr. Shawn Zimmerman, as well as to our collaborators Drs. Randal Prather, Kevin Wells, Richard Oko, Satish Gupta, and David Miller. Some of the work reviewed in this manuscript was supported by Agriculture and Food Research Initiative Competitive Grants no. 2011-67015-20025 and 2013-67015-20961 from the USDA National Institute of Food and Agriculture, and by the seed funding from the Food for the Twenty-first Century Program of the University of Missouri.

**Open Access:** This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

## References

- Austin CR, Bishop MW (1958) Role of the rodent acrosome and perforatorium in fertilization. *Proc R Soc Lond B Biol Sci* 149(935):241–248
- Baba T, Azuma S et al (1994) Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. *J Biol Chem* 269(50):31845–31849
- Baker MA, Reeves G et al (2007) Identification of gene products present in Triton X-100 soluble and insoluble fractions of human spermatozoa lysates using LC-MS/MS analysis. *Proteomics Clin Appl* 1(5):524–532
- Bedard N, Yang Y et al (2011) Mice lacking the USP2 deubiquitinating enzyme have severe male subfertility associated with defects in fertilization and sperm motility. *Biol Reprod* 85(3):594–604
- Bedford JM (1998) Mammalian fertilization misread? sperm penetration of the eutherian zona pellucida is unlikely to be a lytic event. *Biol Reprod* 59(6):1275–1287
- Belote JM, Zhong L (2009) Duplicated proteasome subunit genes in *Drosophila* and their roles in spermatogenesis. *Heredity (Edinb)* 103(1):23–31
- Bleil JD, Wassarman PM (1980) Mammalian sperm–egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell* 20(3):873–882
- Boavida LC, Vieira AM et al (2005) Gametophyte interaction and sexual reproduction: how plants make a zygote. *Int J Dev Biol* 49(5-6):615–632
- Bohring C, Krause W (2003) Characterization of spermatozoa surface antigens by antisperm antibodies and its influence on acrosomal exocytosis. *Am J Reprod Immunol* 50(5):411–419
- Bohring C, Krause E et al (2001) Isolation and identification of sperm membrane antigens recognized by antisperm antibodies, and their possible role in immunological infertility disease. *Mol Hum Reprod* 7(2):113–118
- Book AJ, Smalle J et al (2009) The RPN5 subunit of the 26S proteasome is essential for gametogenesis, sporophyte development, and complex assembly in *Arabidopsis*. *Plant Cell* 21(2):460–478
- Byrne K, Leahy T et al (2012) Comprehensive mapping of the bull sperm surface proteome. *Proteomics* 12(23-24):3559–3579
- Callis J, Bedinger P (1994) Developmentally regulated loss of ubiquitin and ubiquitinated proteins during pollen maturation in maize. *Proc Natl Acad Sci USA* 91(13):6074–6077
- Chakravarty S, Suraj K et al (2005) Baculovirus-expressed recombinant human zona pellucida glycoprotein-B induces acrosomal exocytosis in capacitated spermatozoa in addition to zona pellucida glycoprotein-C. *Mol Hum Reprod* 11(5):365–372
- Chakravarty S, Bansal P et al (2008) Role of proteasomal activity in the induction of acrosomal exocytosis in human spermatozoa. *Reprod Biomed Online* 16(3):391–400
- Chen C, Huang C et al (2008) Subunit-subunit interactions in the human 26S proteasome. *Proteomics* 8(3):508–520
- Deveraux Q, van Nocker S et al (1995) Inhibition of ubiquitin-mediated proteolysis by the *Arabidopsis* 26 S protease subunit S5a. *J Biol Chem* 270(50):29660–29663
- Devoto A, Nieto-Rostro M et al (2002) COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in *Arabidopsis*. *Plant J* 32(4):457–466
- Diaz ES, Kong M et al (2007) Effect of fibronectin on proteasome activity, acrosome reaction, tyrosine phosphorylation and intracellular calcium concentrations of human sperm. *Hum Reprod* 22(5):1420–1430
- Doelling JH, Phillips AR et al (2007) The ubiquitin-specific protease subfamily UBP3/UBP4 is essential for pollen development and transmission in *Arabidopsis*. *Plant Physiol* 145(3):801–813
- Etlinger JD, Goldberg AL (1977) A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. *Proc Natl Acad Sci USA* 74(1):54–58

- Glickman MH, Ciechanover A (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* 82(2):373–428
- Goldstein G, Scheid M et al (1975) Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proc Natl Acad Sci USA* 72(1):11–15
- Green DP, Purves RD (1984) Mechanical hypothesis of sperm penetration. *Biophys J* 45(4):659–662
- Gupta SK, Bansal P et al (2009) Human zona pellucida glycoproteins: functional relevance during fertilization. *J Reprod Immunol* 83(1-2):50–55
- Hanna J, Finley D (2007) A proteasome for all occasions. *FEBS Lett* 581(15):2854–2861
- Hershko A (2005) The ubiquitin system for protein degradation and some of its roles in the control of the cell division cycle. *Cell Death Differ* 12(9):1191–1197
- Hershko A, Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* 67:425–479
- Honda A, Siruntawineti J et al (2002) Role of acrosomal matrix proteases in sperm-zona pellucida interactions. *Hum Reprod Update* 8(5):405–412
- Ikawa M, Inoue N et al (2010) Fertilization: a sperm's journey to and interaction with the oocyte. *J Clin Invest* 120(4):984–994
- Iwai K (2012) Synthesis and analysis of linear ubiquitin chains. *Methods Mol Biol* 832:229–238
- Johnston DS, Wooters J et al (2005) Analysis of the human sperm proteome. *Ann N Y Acad Sci* 1061:190–202
- Kawano N, Kang W et al (2010) Mice lacking two sperm serine proteases, ACR and PRSS21, are subfertile, but the mutant sperm are infertile in vitro. *Biol Reprod* 83(3):359–369
- Kierszenbaum AL, Rivkin E et al (2011) Cytoskeletal track selection during cargo transport in spermatids is relevant to male fertility. *Spermatogenesis* 1(3):221–230
- Kim ST, Zhang K et al (2006) Exogenous free ubiquitin enhances lily pollen tube adhesion to an in vitro stylar matrix and may facilitate endocytosis of SCA. *Plant Physiol* 142(4):1397–1411
- Kong M, Diaz ES et al (2009) Participation of the human sperm proteasome in the capacitation process and its regulation by protein kinase A and tyrosine kinase. *Biol Reprod* 80(5):1026–1035
- Kwon J, Kikuchi T et al (2003) Characterization of the testis in congenitally ubiquitin carboxy-terminal hydrolase-1 (Uch-L1) defective (gad) mice. *Exp Anim* 52(1):1–9
- Lin H, Keriel A et al (2000) Divergent N-terminal sequences target an inducible testis deubiquitinating enzyme to distinct subcellular structures. *Mol Cell Biol* 20(17):6568–6578
- Liu CW, Li X et al (2006) ATP binding and ATP hydrolysis play distinct roles in the function of 26S proteasome. *Mol Cell* 24(1):39–50
- Matsumura K, Aketa K (1991) Proteasome (multicatalytic proteinase) of sea urchin sperm and its possible participation in the acrosome reaction. *Mol Reprod Dev* 29(2):189–199
- Miles EL, O'Gorman C, Zhao J, Samuel M, Walters E, Yi YJ, Sutovsky M, Prather RS, Wells K, Sutovsky P (2013) Transgenic pig carrying green fluorescent proteasomes. *Proc Natl Acad Sci USA* 110(16):6334–6339
- Morales P, Kong M et al (2003) Participation of the sperm proteasome in human fertilization. *Hum Reprod* 18(5):1010–1017
- Morales P, Pizarro E et al (2004) Extracellular localization of proteasomes in human sperm. *Mol Reprod Dev* 68(1):115–124
- Mtango NR, Sutovsky M et al (2012a) Essential role of maternal UCHL1 and UCHL3 in fertilization and preimplantation embryo development. *J Cell Physiol* 227(4):1592–1603
- Mtango NR, Sutovsky M et al (2012b) Essential role of ubiquitin C-terminal hydrolases UCHL1 and UCHL3 in mammalian oocyte maturation. *J Cell Physiol* 227(5):2022–2029
- Naz RK, Dhandapani L (2010) Identification of human sperm proteins that interact with human zona pellucida3 (ZP3) using yeast two-hybrid system. *J Reprod Immunol* 84(1):24–31
- Pasten C, Morales P et al (2005) Role of the sperm proteasome during fertilization and gamete interaction in the mouse. *Mol Reprod Dev* 71(2):209–219
- Peng J, Schwartz D et al (2003) A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol* 21(8):921–926

- Pines J (1994) Cell cycle. Ubiquitin with everything. *Nature* 371(6500):742–743
- Qiao H, Wang H et al (2004) The F-box protein AhSLF-S2 physically interacts with S-RNases that may be inhibited by the ubiquitin/26S proteasome pathway of protein degradation during compatible pollination in *Antirrhinum*. *Plant Cell* 16(3):582–595
- Redgrove KA, Anderson AL et al (2011) Involvement of multimeric protein complexes in mediating the capacitation-dependent binding of human spermatozoa to homologous zonae pellucidae. *Dev Biol* 356(2):460–474
- Rivkin E, Kierszenbaum AL et al (2009) Rnf19a, a ubiquitin protein ligase, and Psmc3, a component of the 26S proteasome, tether to the acrosome membranes and the head–tail coupling apparatus during rat spermatid development. *Dev Dyn* 238(7):1851–1861
- Rosenzweig R, Osmulski PA et al (2008) The central unit within the 19S regulatory particle of the proteasome. *Nat Struct Mol Biol* 15(6):573–580
- Sakai N, Sawada H et al (2003) Extracellular ubiquitin system implicated in fertilization of the ascidian, *Halocynthia roretzi*: isolation and characterization. *Dev Biol* 264(1):299–307
- Sakai N, Sawada MT et al (2004) Non-traditional roles of ubiquitin-proteasome system in fertilization and gametogenesis. *Int J Biochem Cell Biol* 36(5):776–784
- Sasanami T, Sugiura K et al (2012) Sperm proteasome degrades egg envelope glycoprotein ZP1 during fertilization of Japanese quail (*Coturnix japonica*). *Reproduction* 144(4):423–431
- Sawada H, Sakai N et al (2002a) Extracellular ubiquitination and proteasome-mediated degradation of the ascidian sperm receptor. *Proc Natl Acad Sci USA* 99(3):1223–1228
- Sawada H, Takahashi Y et al (2002b) Localization and roles in fertilization of sperm proteasomes in the ascidian *Halocynthia roretzi*. *Mol Reprod Dev* 62(2):271–276
- Shur BD, Rodeheffer C et al (2006) Identification of novel gamete receptors that mediate sperm adhesion to the egg coat. *Mol Cell Endocrinol* 250(1-2):137–148
- Speranza A, Scoccianti V et al (2001) Inhibition of proteasome activity strongly affects kiwifruit pollen germination. Involvement of the ubiquitin/proteasome pathway as a major regulator. *Plant Physiol* 126(3):1150–1161
- Su YH, Chen SH et al (2005) Tandem mass spectrometry identifies proteins phosphorylated by cyclic AMP-dependent protein kinase when sea urchin sperm undergo the acrosome reaction. *Dev Biol* 285(1):116–125
- Susor A, Liskova L et al (2010) Role of ubiquitin C-terminal hydrolase-L1 in antipolyspermy defense of mammalian oocytes. *Biol Reprod* 82(6):1151–1161
- Sutovsky P (2011) Sperm proteasome and fertilization. *Soc Reprod Fertil* 142:1–14
- Sutovsky P, Moreno R et al (2001) A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. *J Cell Sci* 114(pt 9):1665–1675
- Sutovsky P, McCauley TC et al (2003) Early degradation of paternal mitochondria in domestic pig (*Sus scrofa*) is prevented by selective proteasomal inhibitors lactacystin and MG132. *Biol Reprod* 68(5):1793–1800
- Sutovsky P, Manandhar G et al (2004) Proteasomal interference prevents zona pellucida penetration and fertilization in mammals. *Biol Reprod* 71(5):1625–1637
- Tanaka K (2009) The proteasome: overview of structure and functions. *Proc Jpn Acad Ser B Phys Biol Sci* 85(1):12–36
- Verma R, Aravind L et al (2002) Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* 298(5593):611–615
- Voges D, Zwickl P et al (1999) The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu Rev Biochem* 68:1015–1068
- Walczak H, Iwai K et al (2012) Generation and physiological roles of linear ubiquitin chains. *BMC Biol* 10:23
- Yamashita M, Honda A et al (2008) Reduced fertility of mouse epididymal sperm lacking Prss21/Tesp5 is rescued by sperm exposure to uterine microenvironment. *Genes Cells* 13(10):1001–1013
- Yanagimachi R (1994) Fertility of mammalian spermatozoa: its development and relativity. *Zygote* 2(4):371–372

- Yi YJ, Manandhar G et al (2007a) Mechanism of sperm-zona pellucida penetration during mammalian fertilization: 26S proteasome as a candidate egg coat lysin. *Soc Reprod Fertil Suppl* 63:385–408
- Yi YJ, Manandhar G et al (2007b) Ubiquitin C-terminal hydrolase-activity is involved in sperm acrosomal function and anti-polyspermy defense during porcine fertilization. *Biol Reprod* 77(5):780–793
- Yi YJ, Park CS et al (2009) Sperm-surface ATP in boar spermatozoa is required for fertilization: relevance to sperm proteasomal function. *Syst Biol Reprod Med* 55(2):85–96
- Yi YJ, Manandhar G et al (2010a) Inhibition of 19S proteasomal regulatory complex subunit PSMD8 increases polyspermy during porcine fertilization in vitro. *J Reprod Immunol* 84(2): 154–163
- Yi YJ, Manandhar G et al (2010b) Interference with the 19S proteasomal regulatory complex subunit PSMD4 on the sperm surface inhibits sperm-zona pellucida penetration during porcine fertilization. *Cell Tissue Res* 341(2):325–340
- Yi YJ, Zimmerman SW et al (2012) Ubiquitin-activating enzyme (UBA1) is required for sperm capacitation, acrosomal exocytosis and sperm-egg coat penetration during porcine fertilization. *Int J Androl* 35(2):196–210
- Yokota N, Sawada H (2007) Sperm proteasomes are responsible for the acrosome reaction and sperm penetration of the vitelline envelope during fertilization of the sea urchin *Pseudocentrotus depressus*. *Dev Biol* 308(1):222–231
- Yokota N, Kataoka Y et al (2011) Sperm-specific C-terminal processing of the proteasome PSMA1/alpha6 subunit. *Biochem Biophys Res Commun* 410(4):809–815
- Yurewicz EC, Sacco AG et al (1998) Hetero-oligomerization-dependent binding of pig oocyte zona pellucida glycoproteins ZPB and ZPC to boar sperm membrane vesicles. *J Biol Chem* 273(13):7488–7494
- Zhang F, Hu Y et al (2007) Proteasome function is regulated by cyclic AMP-dependent protein kinase through phosphorylation of Rpt6. *J Biol Chem* 282(31):22460–22471
- Zimmerman S, Sutovsky P (2009) The sperm proteasome during sperm capacitation and fertilization. *J Reprod Immunol* 83(1-2):19–25
- Zimmerman SW, Manandhar G et al (2011) Sperm proteasomes degrade sperm receptor on the egg zona pellucida during mammalian fertilization. *PLoS One* 6(2):e17256