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Peter Sutovsky *Editor*

Posttranslational Protein Modifications in the Reproductive System

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Preface: Posttranslational Modifications—When the Sperm Cell Meets the Egg

By various accounts, the human genome includes up to 25,000 genes transcribed into messages from which a staggering one million of proteins and protein variants are derived that constitute the human body proteome. While some of this increase between gene and protein is due to alternative transcription and posttranscriptional processing of mRNA by alternative splicing, most of the alternative gene products at the protein level are due to posttranslational modifications (PTM) of nascent proteins. Within the focus area of the present book, reproductive biology, the genomic era armed us with knowledge about transcripts that appear at distinct stages of reproductive process. However, the abundance of a transcript does not directly translate into an abundance of a protein and that in turn is not a direct indicator of biological activity, which is regulated by PTM. The goal of the present book is to increase the awareness of a great variety of PTM observed in gonads, gametes, embryos, and in the male and female reproductive system in general. While far from all-encompassing, some of the most intriguing reproductive strategies, mechanisms, and pathways involving PTM are discussed, with an added angle of evolutionary conservation and diversity.

Posttranslational protein modifications by ubiquitin and ubiquitin-like proteins (e.g., SUMO, NEDD4, NEDD8, and ISG15) involve stable, covalent ligation of one or more molecules of a small chaperone protein that predestines the substrate for proteolytic degradation or alters its function. Protein ubiquitination is important for all phases of spermatogenesis, including germ cell renewal and proliferation, meiosis and post-meiotic differentiation into spermatozoa, reviewed by *Rohini Bose, Gurpreet Manku, Martine Culty, and Simon S. Wing.*

Adding a comparative/evolutionary aspect, *Long Miao and Steven W. L'Hernault* discuss the roles that protein phosphorylation, proteolysis, ubiquitination, and palmitoylation play in the spermatogenesis and sperm function of nematode worms *Caenorhabditis elegans* and *Ascaris suum.* Spermatozoa of both of these species use a unique form of amoeboid motility termed crawling, and many posttranslational-modification-controlled aspects of nematode spermatogenesis and motility are different from mammals.

Even though they are morphologically fully differentiated, the testicular spermatozoa in mammals are not yet competent to fertilize an oocyte. For this to happen, mammalian spermatozoa have to travel through epididymis where they undergo a

complex change referred to as epididymal sperm maturation. This involves posttranslational modifications of structural sperm proteins, as well as modification of sperm surface by addition or removal of sperm surface proteins via apocrine protein secretion and targeted proteolytic processing. While this complex transformation into a fertilization competent spermatozoon is not yet fully understood, some of the recent work on epididymal function has been focused on PTM of sperm and epididymal luminal fluid proteins, reviewed by *Gail A. Cornwall.*

Mammalian vitelline coat, the zona pellucida (ZP) is a unique, specialized extracellular matrix composed of three or four heavily glycosylated proteins, some of which have the ability to bind spermatozoa and induce sperm-acrosomal exocytosis. Zona protein glycosylation and proteolysis determine sperm–oocyte interactions and anti-polyspermy defense. While often reviewed from the point of view of murine model or human ZP data, the wealth of data from ungulate models is seldom a subject of a comprehensive review. This gap is now filled by *Naoto Yonezawa*, discussing protein glycosylation and other posttranslational modifications of porcine, bovine, murine, and human ZP proteins.

While traditionally thought to be primarily confined to cell cytoplasm and nucleus, the ubiquitin-proteasome system (UPS) has been revealed in a surprising extracellular context in several systems. Among them, the functioning of ubiquitin-conjugating machinery and that of the sperm borne 26S proteasome, the endpoint protease of UPS, plays a surprisingly well conserved role in sperm penetration through the oocyte vitelline coat. While relevant to mammals including humans, some of the earliest studies of gametic extracellular UPS were conducted in ascidian and echinoderm animal models, and are reviewed in the chapter by *Hitoshi Sawada, Masako Mino, and Mari Akasaka.*

The intracellular balance of polymeric and free, unconjugated ubiquitin is maintained by deubiquitinating enzymes, which also have the ability to reverse protein ubiquitination. Among them, the ubiquitin C-terminal hydrolase (UCH) family enzymes are some of the most abundant proteins found in mammalian oocytes and embryos. *Namdori R. Mtango, Peter Sutovsky, and Keith E. Latham* discuss the recent evidence for the involvement of UCHs in the regulation of oocyte cortex and meiotic spindle during oocyte maturation, fertilization, and preimplantation embryo development.

Besides studying embryo development after natural or assisted (*in vitro*) fertilization, somatic cell nuclear transfer (SCNT) offers an intriguing alternative model for understanding early development. Donor cell nuclear remodeling is a central event during embryo reconstruction by SCNT, which is, together with the subsequent reprogramming of donor cell genome, tightly regulated by PTM of the DNA-packaging histone proteins, such as acetylation and ubiquitination. Additionally, establishment of the first mitotic spindle differs with regard to protein content, localization, and PTM between IVF and SCNT zygotes. These and other aspects of PTM influence on embryo development after SCNT are reviewed by *Keith E. Latham*.

Protein phosphorylation is paramount to cellular signaling in every system, including the reproductive one. Strategies for success of species led to evolution of unique protein kinases within the phylogenetic tree. Chapter 3 by *William H. Kinsey*

provides an evolutionary insight into the role of protein kinases, and tyrosine kinases in particular, in the regulation of cellular signaling during oocyte maturation and fertilization in animal models ranging from insects to mammals.

Cytoskeleton provides the structural scaffold for meiosis, fertilization, and early embryo polarization and differentiation during preimplantation development. Furthermore, cytoskeletal tracks and cytoskeleton organizing centers are indispensable for cellular signaling and cargo trafficking in germ cells, gametes, and embryos. *Heide Schatten and Qing-Yuan Sun* discuss how the phosphorylation of cytoskeletal and cytoskeleton-associated proteins and structural modifications of cytoskeletal proteins such as the acetylation, glycosylation, ubiquitination, tyrosination, polyglutamylation, poly-glycylation, sumoylation, and palmitoylation regulate microtubules, microfilaments, and intermediate filaments in gametes and embryos.

Following fertilization and pre-embryo development, the success of mammalian pregnancy depends on the remodeling of the uterine lining, the endometrium, and the modifications of intrauterine environment that favor embryo implantation and development to term. Chapter 2 by *Thomas R. Hansen and James K. Pru* summarizes the contribution of PTM to these crucial steps of mammalian reproductive process, with particular focus on protein ISGylation, a ubiquitination-like protein modification that is significantly upregulated as a part of maternal response to the developing conceptus.

As is the case in all areas of biology and medicine, the knowledge of posttranslational protein modifications and their outcomes will continue gathering interest of reproductive biologists and clinical practitioners of assisted reproduction. In addition to better understanding of life, this learning process will lead to new or optimized assisted reproductive therapies for infertile couples, to improvement of reproductive health of our population, to the optimized strategies for stem cell derivation and animal transgenesis, and for increased reproductive performance of livestock animals.

Columbia, Missouri Peter Sutovsky

Acknowledgments

This book was inspired by the minisymposium on *Posttranslational Protein Modifications in Gametes, Embryos, and the Reproductive System* at the 44th Annual Meeting of the Society for the Study of Reproduction (SSR), held on July 31 to August 4, 2011 in Portland, OR. I had the honor of chairing and co-organizing this minisymposium with Dr. Janice Bailey, whom I would like to acknowledge along with the members of Program Committee of the SSR. My sincere thanks belong to Janice and two presenters in that session who kindly accepted my invitation to contribute to this book, to Drs. Thomas Hansen and Hitoshi Sawada, as well as to all contributing authors who invested their valuable time and effort for the success of this project. The idea to assemble this tome came from Ms. Samantha Lewis at Springer Science & Business Media in New York, whose help and dedication to this project, along with that of Development Editor Mr. Michael Griffin, is sincerely appreciated. Further to Springer's involvement with this book, the Springer journal *Cell & Tissue Research* offered to sponsor the minisymposium, through generous efforts of Coordinating Editor Dr. Klaus Unsicker and Senior Publishing Editor Dr. Meran Owen. My ability to devote time and effort to this volume was greatly enhanced by my seed funding from the Food for The twenty-first century Program of the University of Missouri as well as by extramural funding from the USDA National Institute of Food & Agriculture and NIH National Institute of Child & Human Development, all of which is gratefully acknowledged.

The publication of this tome coincides with my promotion to "full" professor at the University of Missouri, a personal and professional milestone worthy of reflecting on my scientific career so far. Thus I would like to dedicate it to all my past teachers, mentors, colleagues, and collaborators who had encouraged and guided me on my journey in reproductive biology.

Last but not least, my heartfelt thanks go to my wife and lab mate Miriam and our son Martin, for their unconditional love and support, as well as to my parents, my brother, and my family at large. In particular, I would like to dedicate this work to the memory of my recently departed and dearly missed aunt Olinka who was my great fan and supporter since my pupil years.

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Sperm Proteases and Extracellular Ubiquitin–Proteasome System Involved in Fertilization of Ascidians and Sea Urchins

1

Hitoshi Sawada, Masako Mino, and Mari Akasaka

Abstract

Ascidians (primitive chordates) are hermaphroditic animals that release spermatozoa and eggs almost simultaneously, but some species, including *Halocynthia roretzi*, show strict self-sterility. In *H. roretzi*, a 70-kDa vitelline coat (VC) protein consisting of 12 EGF-like repeats, named HrVC70, appears to be a promising candidate for the self/nonself-recognition (or allorecognition) system during gamete interaction. After spermatozoon recognizes the VC as nonself, sperm 700-kDa extracellular ubiquitin-conjugating enzyme complex appears to ubiquitinate Lys234 of HrVC70, and the ubiquitinated HrVC70 is degraded by the sperm 26S proteasome that is located on the sperm head surface. This novel ubiquitin–proteasome system enables spermatozoa to penetrate through the VC. Sperm trypsin-like proteases, acrosin and spermosin, also participate in fertilization, probably as sperm-side 'movable' binding proteins to the VC.

Keywords

Ubiquitin • Proteasome • Sperm • Fertilization • Extracellular • Ascidian

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Introduction

In order to accomplish successful fertilization, it is essential for spermatozoa to specifically bind to and penetrate through the proteinaceous egg coat, referred to as the vitelline coat (VC) in marine invertebrates and the zona pellucida (ZP) in mammals [1–4]. Species-specific recognition and adhesion between spermatozoa and the VC of eggs seem to be critical for marine invertebrates. Self-sterility or self-incompatibility is also beneficial for hermaphroditic organisms, including ascidians, to avoid self-fertilization. Most of these recognition processes are carried out in the interaction between spermatozoa and the VC of eggs, since VC-free eggs are self-fertile and occasionally cross-fertilizable between different species.

In mammals, an acrosomal trypsin-like protease, named acrosin [EC 3.4.21.10], had long been believed to be a lytic agent, lysin, which makes a small hole for sperm passage through the ZP of the oocyte [5–7]. However, since mouse spermatozoa lacking the *acrosin* gene can penetrate through the ZP [8], it is currently believed that acrosin is not essential for the penetration of spermatozoa through the ZP, and that it is involved in the dispersal of acrosomal contents during acrosome reaction [9] and in the secondary binding of spermatozoa to the ZP [10, 11]. However, the physiological substrates of sperm trypsin-like proteases are still debated.

Ascidians (tunicates; primitive chordates) are marine invertebrates occupying a phylogenetic position between vertebrates and 'true' invertebrates. Generally, ascidians are hermaphrodites, most of which release spermatozoa and eggs almost simultaneously, but many species show self-sterility or preference for nonself-fertilization rather than self-fertilization. Since VC-free eggs are self-fertile, it is thought that allorecognition takes place in the process of interaction between spermatozoa and the VC of eggs. Therefore, sperm lysin appears to be activated or exposed to the VC after spermatozoon recognizes the VC as nonself.

Ascidians are useful animals for fertilization studies, since fertilization experiments can be easily carried out and also since large quantities of gametes are easily obtained by controlling the seawater temperature and light conditions from the aquacultured species *Halocynthia roretzi* [4]. By using this species, it has been elucidated that the sperm ubiquitin–proteasome system (UPS) plays a key role in the penetration of spermatozoa though the VC, most probably as a lysin. In the present review, we summarize the sperm proteases including the novel extracellular UPS, which are involved in ascidian fertilization, from the viewpoint of posttranslational modification of gamete proteins.

Roles of Sperm Proteases in Fertilization

Structures and Functions of Sperm Trypsin-Like Proteases

Hoshi et al. reported that of various protease inhibitors, trypsin inhibitors such as leupeptin and soybean trypsin inhibitor (proteinaceous inhibitor) and chymotrypsin inhibitors such as chymostatin potently inhibited the fertilization of intact eggs of

H. roretzi in a concentration-dependent manner [12]. The strong inhibition of fertilization by leupeptin and chymostatin was markedly reduced in the case of VC-free eggs, suggesting that sperm trypsin-like and chymotrypsin-like proteases are involved in the process of sperm binding to and penetrating through the VC [12]. Sawada and his colleagues examined the effects of various fluorogenic peptide substrates on fertilization and found that Boc-Val-Pro-Arg-MCA and Suc-Leu-Leu-Val-Tyr-MCA were the strongest inhibitors of fertilization among trypsin substrates and chymotrypsin substrates, respectively [13, 14]. Two trypsin-like proteases called acrosin and spermosin were then purified from *H. roretzi* sperm using the above trypsin substrate [15]. *H. roretzi* acrosin (HrAcrosin) showed a relatively broad substrate specificity toward peptidyl-Arg-MCAs, but *H. roretzi* spermosin (HrSpermosin) showed a narrow substrate specificity. Boc-Val-Pro-Arg-MCA appears to be a specific substrate among peptidyl-MCA substrates for spermosin. It was suggested that both of these proteases participate in fertilization by comparing the effects of various leupeptin analogs (peptidyl-argininal) on fertilization and enzymatic activities [16, 17], and also by examining the inhibitory effect of anti-spermosin antibody on fertilization [18]. However, the purified enzymes hardly degraded the VC proteins, which are insoluble proteins under physiological conditions (unpublished data). In connection with this, it should be noted that it is still debatable whether mammalian acrosin has the ability to digest the ZP from the same species (see review [3]).

From the structural bases, biological functions of ascidian sperm trypsin-like proteases have been proposed. Both HrProacrosin (precursor of HrAcrosin) and HrSpermosin possess several candidate regions for protein–protein interaction, *i.e.*, two CUB domains in the C-terminus of HrProacrosin and a Pro-rich region in the N-terminus of the light chain of HrSpermosin. Two VC proteins (25-kDa and 30-kDa VC proteins) were identified as binding proteins to these proteases: The 25-kDa VC protein was adsorbed to CUB1-peptide-immobilized agarose beads and HrSpermosin Pro-rich-region-immobilized agarose, while the 30-kDa VC protein was capable of binding to CUB1-peptide-immobilized agarose and GST-CUB1 recombinant protein [19–21]. By cDNA sequencing, it was revealed that the 25-kDa and 30-kDa proteins correspond to the C-terminal region of high-molecular-mass vitellogenin, which belongs to a family of lipid transfer proteins [21, 22]. The 25-kDa protein, which corresponds to a von Willebrand factor D domain, was connected to the N-terminal side of the 30-kDa protein, which corresponds to a 'C-terminal coding region' of vitellogenin [23]. Since the hepatopancreas is a major organ expressing vitellogenin, we proposed that vitellogenin is expressed in the hepatopancreas and transferred to the oocytes via bloodstream, during which its C-terminal region might be cut off by a protease(s) and attached to the VC, which in turn may play a role in gamete interactions [21]. Two mRNA species weakly expressed in the ovary, which correspond to the C-terminal regions of vitellogenin called vitellogenin S1 and S2, were recently isolated and sequenced [22]. Vitellogenin S1 and S2 appear to be expressed in oocytes and probably also in test cells, and located at the boundary of the oocyte plasma membrane and test cells. During oocyte maturation, these proteins appear to be released to the perivitelline

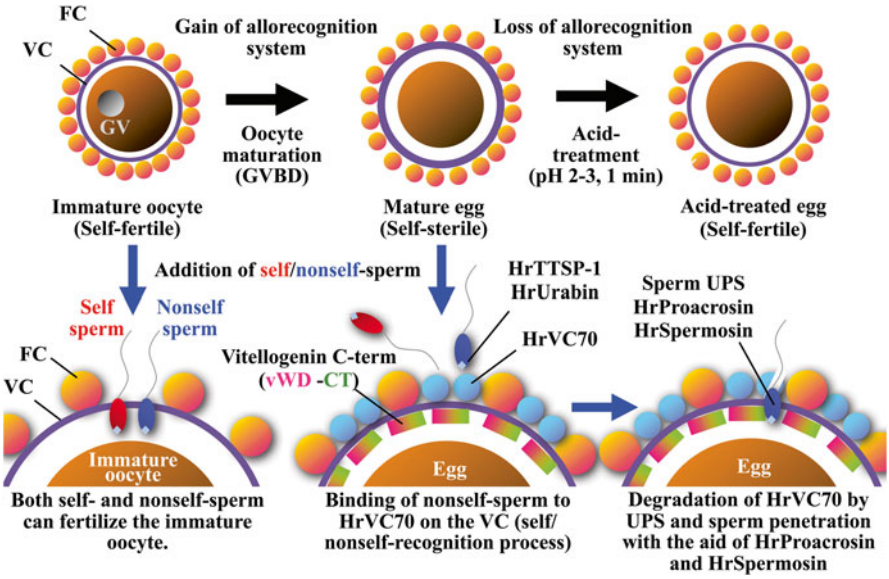

Fig. 1.1 Working hypothesis for the roles of sperm proteases as a lysin and allorecognition system in the ascidian *Halocynthia roretzi* (Modified from [48]). In the ascidian *H. roretzi*, immature oocytes (*upper left*) are self-fertile, but mature eggs (*upper middle*) are strictly self-sterile. When mature eggs are treated with acidic seawater (pH 2–3) for 1 min, self-sterile eggs become self-fertile. This appears to be because HrVC70 consisting of 12 EGF-like repeats, which is an allo-recognizable sperm receptor, is attached to the vitelline coat (VC) during oocyte maturation and detached from the VC when treated with 1–10 mM HCl (~pH 2–3). If nonself-spermatozoa recognize the VC as nonself, the sperm-side novel extracellular ubiquitin–proteasome system (UPS) must be activated or exposed to the sperm cell surface, which enables spermatozoa to penetrate through the VC. In this process, sperm trypsin-like proteases HrProacrosin and HrSpermosin may support the movable sperm binding to the VC (for details, see text)

space and eventually attach to the VC from inside. Since these proteins are capable of interacting with sperm proteases HrProacrosin and HrSpermosin, these proteins may participate as scaffold proteins to assist binding and movement of spermatozoa during sperm passage through the VC, although it is presently unclear whether these proteins are derived from oocytes or the hepatopancreas.

In connection with this, we have a working hypothesis that spermatozoa bind to the C-terminal fragments of vitellogenin located on the VC, this process being mediated by the sperm-side HrProacrosin CUB domain and HrSpermosin Pro-rich region, and then sperm proteases may degrade these VC proteins or process the precursor regions, enabling spermatozoa to detach and penetrate the VC. These sequential actions may explain the phenomena of sperm binding to and penetrating through the VC (see Fig. 1.1).

Extracellular Ubiquitin–Proteasome System Functions as a Vitelline-Coat-Lysin in Ascidians

As described above, since the purified preparations of ascidian sperm trypsin-like proteases were unable to degrade the VC, an attempt was made to purify a chymotrypsin-like protease from *H. roretzi* spermatozoa using Suc-Leu-Leu-Val-Tyr-MCA as a substrate. The main proteases that hydrolyze this synthetic substrate were identified as the 20S and 26S (or 26S-like) proteasomes [24–26].

The 26S proteasome is one of the most important intracellular protein-degradation machineries in eukaryotic cells [27, 28]. In this pathway, the intracellular short-lived and aberrant proteins are tagged with ubiquitin by sequential actions of ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin-ligase E3, and then degraded by the 26S proteasome in an ATP-dependent manner [27–29]. The 26S proteasome is made up of the 20S proteasome, a barrel-shaped protease complex consisting of four stacked heptameric rings, α7β7β7α7, and the 19S regulatory particle (19S RP)/PA700, consisting of 19 subunits, including 6 ATPase subunits and a Ub-recognizing subunit S5a, which caps one or both sides of the cavities of the 20S proteasome [27, 28]. The 20S proteasome has three protease activities, *i.e.*, caspase-like (β1), trypsin-like (β2) and chymotrypsin-like (β5) activities [28].

The 26S proteasome-containing fraction partially purified from activated *H. roretzi* spermatozoa showed a weak VC-degrading activity [24]. It was also found that a 70-kDa main VC component, HrVC70, is degraded by the purified sperm 26S proteasome in the presence of ATP and ubiquitin [25, 26]. HrVC70 consists of 12 EGF-like repeats [25] and appears to be specifically expressed in oocytes [30] within the gonad as a 120-kDa precursor protein HrVC120, which contains a single truncated EGF-like domain and one ZP domain in its C-terminal region [25]. There are several lines of evidence showing the participation of extracellular UPS in ascidian fertilization. First of all, *H. roretzi* fertilization was inhibited by proteasome inhibitors such as MG115 and MG132 and also by an anti-proteasome antibody and the anti-multi-ubuiquitin chain-specific monoclonal antibody FK2 [25, 26]. Secondly, Suc-Leu-Leu-Val-Tyr-MCA-hydrolyzing proteasome activity, which was specifically inhibited by MG115, was detected in the sperm head region under a fluorescence microscope when activated by alkaline seawater [26]. Thirdly, sperm proteasomes, as well as HrVC70-ubiquitinating enzyme, ATP and ubiquitin, appear to be partially released from sperm when activated by alkaline seawater (unpublished data) [31]. Fourthly, HrVC70 on the VC appears to be ubiquitinated upon insemination on the basis of Western blotting and immunocytochemistry using the monoclonal antibody FK2 [25, 31]. Although the sorting mechanism of the proteasome to the surface of the sperm head is not known, it is notable that only the sperm proteasome possesses an α6 subunit lacking the C-terminal 16 residues. This specific processing may be involved in the functions and localization of the sperm proteasome [32].

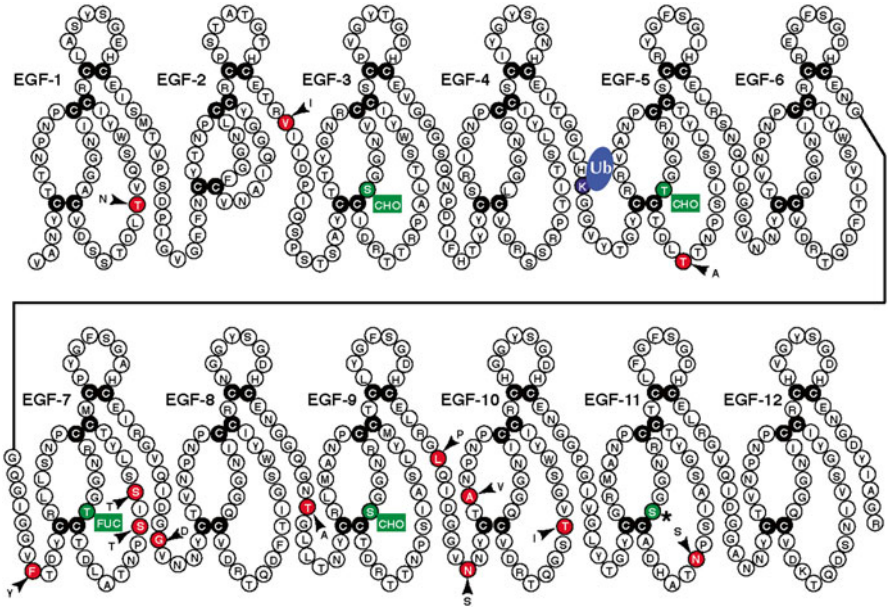

Fig. 1.2 Schematic drawing of HrVC70 [33, 45]. HrVC70 comprises 12 epidermal growth factor (EGF)-like repeats, and shows polymorphisms among individuals. The polymorphic regions indicated by *arrowheads* and *(red)* letters are restricted to the region between the third and fourth Cys residues of each EGF domain and EGF-domain connecting regions. Ubiquitination occurs at Lys234, which is catalyzed by a 700-kDa ubiquitin-conjugating enzyme complex released from spermatozoa upon activation. There are five potential *O*-fucosylation sites (*green*), among which Ser450 (*asterisk*) seems unlikely to be fucosylated as revealed by LC/MS/MS analysis (Sawada et al., to be published)

HrVC70-ubiquitinating enzyme was purified from sperm exudate, a fraction released from spermatozoa activated by alkaline seawater, by DEAE-cellulose chromatography, ubiquitin–agarose chromatography, and 10–40 % glycerol density gradient centrifugation [31]. The molecular size of the enzyme was estimated to be approximately 700 kDa by glycerol density gradient centrifugation [31]. The purified enzyme exhibited activity in artificial seawater and required a high concentration (~10 mM) of Ca^{2+} for its activity. These enzymatic features also support our idea that the purified enzyme functions extracellularly in seawater. Furthermore, apyrase, which depletes ATP and inhibits the HrVC70-ubiquitinating activity, inhibited the fertilization when added to the surrounding seawater. These results indicate that a novel extracellular 700-kDa HrVC70-ubiquitinating enzyme complex plays a pivotal role in ubiquitination of HrVC70. There are two Lys residues in HrVC70, Lys234 and Lys636, but only Lys234 was identified as a ubiquitination site, as revealed by a ubiquitin-conjugation assay using several site-directed Lys-to-Arg mutant recombinant proteins of HrVC70 [33] (see Fig. 1.2). Since it is widely believed that only one molecular species of E1 is committed to every ubiquitination reaction, the existence of the extracellular UPS may give us a new insight in the ubiquitin system.

Involvement of Sperm Proteasome in the Acrosome Reaction and Sperm Penetration of the Vitelline Coat in Sea Urchins

It has been shown that sperm chymotrypsin-like protease is involved in sea urchin fertilization, most probably as a VC lysin, by examining the effects of various protease inhibitors on fertilization of intact and VC-impaired eggs [34]. A chymotrypsin-like protease was then purified from sea urchin spermatozoa, and it was proposed that this enzyme is a VC lysin [35]. However, a high concentration (more than 100 μM) of chymostatin was necessary for inhibiting fertilization, whereas the purified enzyme was very susceptible to chymostatin at lower concentrations. Taking into account the participation of ascidian sperm proteasome in fertilization, we examined the effects of various protease inhibitors, including proteasome inhibitors, on sea urchin fertilization [36, 37]. The results showed that the proteasome inhibitors MG132, MG115 and lactacystin had inhibitory effects on fertilization at a concentration of 100 μM, whereas leupeptin or chymostatin showed no or less inhibition at the same concentration. Proteasome substrates also inhibited fertilization: Among the substrates tested, Z-Leu-Leu-Glu-MCA, a substrate for caspase-like activity, showed the strongest inhibitory effect on fertilization. Proteasome activity was detected in the acrosomal content, a fraction released from acrosome by exocytosis, where the proteasome antigen was detected by Western blotting using an anti-proteasome antibody [37]. MG132 showed no apparent inhibition toward the sperm binding to the VC, but it showed significant inhibition toward fertilization of dejellied eggs using acrosome-reacted spermatozoa. Among three catalytic sites, the caspase-like activity of the proteasome appears to be involved in sea urchin fertilization as revealed by comparing the effects of various protease inhibitors on fertilization and three proteasomal proteolytic activities [37].

It has been proposed that the sperm proteasome is involved in the acrosome reaction (AR) in sea urchins [38]. However, the effects of proteasome inhibitors on the AR had not been studied in detail. Therefore, we re-examined the effects of MG132 on the AR, and we found that MG132 inhibited egg-jelly-induced AR but not Ca^{2+} ionophore-induced AR. From these results, it seems likely that the sperm proteasome plays a key role in the AR, particularly in a certain process leading to the increase in intracellular Ca^{2+} concentration. Taken together, the proteasome is involved not only in the process of sperm penetration though the VC as a lysin but also in the AR before increase in intracellular Ca^{2+} concentration in sea urchin spermatozoa.

Sutovsky and his colleagues showed several lines of evidence indicating that the proteasome is located in an acrosome and involved in the penetration of sperm through the ZP as a lysin in mammals [39–41]. Recently, they succeeded in generating transgenic pigs that express a GFP-PSMA1 (α6) subunit of the proteasome [42]. They reported that the fluorescent proteasome was detected in the acrosome of boar spermatozoa [42]. These results unambiguously demonstrate that the proteasome is localized on the acrosome, although the sorting mechanism of the proteasome into an acrosome is an important issue that remains to be solved. In any case, it should

be emphasized that the sperm proteasome plays an important extracellular role in fertilization, as a lysin, and this lysin system might be commonly utilized in deuterostomes.

Allorecognition in Ascidian Fertilization

It is well known that self-sterile ascidian eggs become self-fertile when the eggs are treated with acidic (pH 2–3) seawater for a short period (~1 min) [43, 44]. It is also known that immature or VC-free eggs are self-fertile [43, 44]. These phenomena led us to speculate that a certain allorecognition factor may be attached to the VC during oocyte maturation and that such a putative factor may be detached from the VC or irreversibly denatured by weak acid. To test this possibility, VCs were isolated from immature and mature eggs and subjected to SDS-PAGE. The results clearly showed that HrVC70 is attached to the VC during oocyte maturation. It was also revealed that HrVC70 is easily solubilized from the isolated VC by 1–10 mM HCl and that spermatozoa are capable of binding to HrVC70 immobilized on agarose beads. It is notable that the number of sperm bound to HrVC70 from nonself-eggs was significantly larger than the number of spermatozoa bound to HrVC70 from self-eggs. In addition, HrVC70 isolated from nonself-eggs more efficiently inhibited the fertilization than did that from self-eggs [45]. From these results, together with the fact that HrVC70 shows high polymorphisms among individuals and that even a single amino-acid substitution in EGF-like repeat regions in Notch protein is sufficient to cause Notch-signaling diseases [46], it is thought that HrVC70 is a promising candidate for allorecognition in fertilization of *H. roretzi*. Although it is still unclear whether the amino-acid substitution in HrVC70 is actually responsible for allorecognition during gamete interaction in *H. roretzi*, all of the biochemical data so far obtained support the idea that HrVC70 is a key protein involved in allogeneic recognition.

As sperm-side binding partners of HrVC70, HrTTSP-1 (Type-II transmembrane serine protease) and HrUrabin (*u*nique *RA*FT-derived *bin*ding partner for HrVC70: a GPI-anchored CRISP-family protein) have been identified by yeast two hybrid screening [30] and Far-Western blot analysis, respectively [47]. HrTTSP1 has an estimated molecular mass of 337 kDa and it contains 23 CCP/SCP/Sushi-domains, 3 ricin B domains and 1 CUB domain in its extracellular region. Although HrTTSP-1 contains several putative interesting domains, its precise function is still unknown. In contrast, HrUrabin appears to play a key role in allorecognition since anti-HrUrabin antibody can inhibit fertilization and also allorecognizable sperm binding to HrVC70-agarose beads. However, HrUrabin had little polymorphism among individuals and showed no difference in its binding ability to HrVC70 from self-eggs and nonself-eggs. Therefore, it is currently thought that HrUrabin is unable to directly distinguish self- and nonself-HrVC70 but that it participates in the allorecognition process since the antibody against HrUrabin potently inhibited the allorecognizable sperm binding to HrVC70.

Conclusions and Perspective

Whereas ascidians are hermaphroditic animals, several ascidians, including *H. roretzi*, show strict self-sterility. In *H. roretzi*, after spermatozoon recognizes the VC as nonself, a novel 700-kDa extracellular ubiquitin-conjugating enzyme complex, which ubiquitinates the Lys234 residue of HrVC70 on the VC, must be activated or exposed to the sperm surface, resulting in sperm penetration of the VC. During this sperm penetration process, it is likely that the sperm HrProacrosin C-terminal CUB-domains and HrSpermosin Pro-rich region are responsible for the sperm binging to the 25-kDa and 30-kDa VC proteins, which correspond to the C-terminus of vitellogenin on the VC. After sperm binding to the above VC proteins, these VC proteins themselves or the binding domains of sperm proteases might be hydrolyzed by sperm HrAcrosin and/or HrSpermosin, which allow sperm movement in the process of sperm penetration of the VC (see Fig. 1.1) [48]. Further studies are necessary to evaluate this working hypothesis.

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ISGylation: A Conserved Pathway in Mammalian Pregnancy

2

Thomas R. Hansen and James K. Pru

Abstract

Successful pregnancy includes remodeling and differentiation of the endometrium in response to sex steroid hormones, development of maternal immunotolerance to the implanting embryo, and modification of the local uterine environment by the embryo to suit its own needs. The major signal released by the ruminant conceptus during establishment of pregnancy is interferon-tau (IFNT) that stimulates the expression of many genes in the endometrium and ovary. One of these genes is called interferon stimulated gene 15 (ISG15), which encodes a ubiquitin homolog with a C-terminal Gly that becomes covalently attached to Lys residues on targeted proteins through an ATP-dependent multi-step enzymatic reaction called ISGylation. The conceptus-derived induction of endometrial ISGs also occurs in mouse and human deciduas and placenta, in response to pregnancy presumably through action of cytokines such as interleukins and type I IFN. Described herein is evidence to support the concept that ISGylation is a maternal response to the developing conceptus, implantation and placentation that is conserved across mammalian pregnancy. Although the precise role for ISG15 remains elusive during pregnancy, it is clear that up-regulation in response to pregnancy may impart a pre-emptive defense to infection or other environmental insults, and protection of the conceptus against inflammatory insults across species.

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Conceptus • Decidua • Endometrium • Evolutionary conservation • Interferon • ISG15 • Pregnancy • Ubiquitin

Introduction

The most abundant protein secreted from cultured ovine conceptuses was isolated during the peri-implantation period [1]. Through adding radiolabeled leucine in leucine-deficient medium, this conceptus-derived secretory product was demonstrated to be released by using a 2D-PAGE approach [2]. Large-scale purification of this acidic protein, subsequently called trophoblast protein 1 or TP-1, led to studies demonstrating that it bound to endometrial receptors [2, 3], induced upregulation of many endometrial proteins [2, 4–7], altered release of luteolytic prostaglandin $F_2\alpha$ (PGF) [8, 9] and resulted in extended estrous cycles when infused into the uterine lumen of cyclic ewes. The inferred amino acid sequence from cloned cDNAs revealed homology with ovine [10] and bovine [11] type I IFN cDNAs. TP-1 was subsequently found to be encoded by several genes [12, 13] and was later renamed IFNT.

The general IFN response, characterized by increased expression of ISGs observed in ruminant species during early pregnancy, is conserved across species with different modes of implantation. Mice and humans lack the IFNT gene; however, embryo-derived factors other than IFNT are known to activate an IFN-like response. For example, many other Type I IFN (α, β, δ, ε, ω) and cytokines such as interleukins also activate JAK/STAT signal transduction and increase expression of ISGs. In humans, there is strong evidence for induction of ISGs in endometrial decidual cells when cultured with conditioned human trophoblast cell medium [14]. One candidate for induction of ISGs in mice and in humans might be interleukins [15]. This chapter describes the identification of ISG15 as a maternal response to pregnancy in many animal models including humans. It also presents data to support a functional role for ISGylation and post-translational modification of proteins based on negative impact of gene deletion on both establishment of pregnancy and liter size in mice.

IFNT Functions as an Anti-Luteolytic Signal

PGF is released from the luminal epithelium of the endometrium and is the luteolytic factor in ruminants [16–18]. Oxytocin enhances cyclooxygenase-2 (*COX2*) transcription, which increases pulse amplitude of PGF production by the endometrium [19]. In turn, PGF promotes both functional (i.e., decline in progesterone biosynthesis) and structural (i.e., programmed cell death) regression of the corpus luteum, thereby initiating a new estrous cycle. Maternal recognition of pregnancy in ruminant species is successful only when PGF synthesis and release from the endometrium is attenuated. Luteolysis is averted during pregnancy by conceptus secreted IFNT.

Early pregnancy is maintained in ruminants through the actions of conceptus-derived IFNT on the endometrium. IFNT alters uterine release of PGF, which

results in rescue of the corpus luteum and continued secretion of progesterone. In sheep, cumulative PGF is not altered during early pregnancy [20]. Rather, IFNT suppresses up-regulation of estrogen receptor gene expression [21], which is in turn necessary for up-regulation of oxytocin receptor (*OTR*) in the absence of pregnancy [22]. At least in the ewe, IFNT exerts its inhibitory actions, at the proximal end of the signaling pathway mediating PGF secretion (i.e., OTR). This may help explain how IFNT disrupts pulsatile rather than total PGF release in the ewe. Unlike the promoter for the *OTR* gene of the ewe, the bovine *OTR* gene lacks a classical palindromic estrogen response element [23] and no change in ESR1 expression has been observed [24]. Using in situ hybridization, Robinson et al. showed that despite the slight decrease in OTR mRNA in endometrium from Day 16 pregnant cows when compared with estrous cycling cows, estrogen receptor mRNA does not change [24]. Thus, pregnancy can apparently alter OTR mRNA expression exclusive of the estrogen receptor in cows. Because IFNT acts in paracrine fashion directly on the endometrium, several groups have sought to establish transcriptional responses to IFNT exposure in the endometrium [25, 26], as well as endocrine responses in the ovary [25, 27–29]. One subset of genes that become upregulated in response to IFNT in reproductive tissues are the interferon stimulated genes (ISGs).

Interferon-Stimulated Genes: Identification of ISG15 in the Bovine Uterus

Studies were initiated on endometrial response to pregnancy and conceptus-derived IFNT in the early 1990s. In 1995, a ~16 kDa protein was described that was released into media by endometrial explants cultured from day 18 pregnant cows and endometrial explants from non-pregnant cows that were treated in vitro with recombinant bovine IFNT (rboIFNT) [5]. A protein of similar size, ~14.4 kDa, was previously described following culture of Ehrlich ascites tumor cells with other type 1 IFN [30]. About five years later, the same protein was described in human fibroblasts and MDBK cells when treated with IFNA or IFNB [31]. The function and identity of this ISG remained unknown until Dr. Haas examined regulation of intracellular ubiquitinated proteins in response to viral infection [32]. When completing western blots of intracellular proteins with antibody against ubiquitin following viral infection, a 15 kDa protein was up-regulated that immunoreacted with antibody against ubiquitin. This apparent 15 kDa protein, subsequently named ubiquitin cross-reactive protein (UCRP), maintained amino acid sequence identity with a tandem ubiquitin repeat and was upregulated following exposure to IFNA and IFNB, and to a lesser extent following culture with IFNG. By using affinity-purified antibody against UCRP that did not cross-react with ubiquitin, these investigators described conjugation of UCRP to proteins in response to IFN [33] which utilized distinct activating and conjugating enzymes, and resulted in UCRP—protein conjugates that were distinct from those conjugated to ubiquitin [34].

By using anti-ubiquitin antibody and anti-UCRP antibody from Dr. A.L. Haas, the ~16 kDa endometrial protein shown to be upregulated and secreted in response

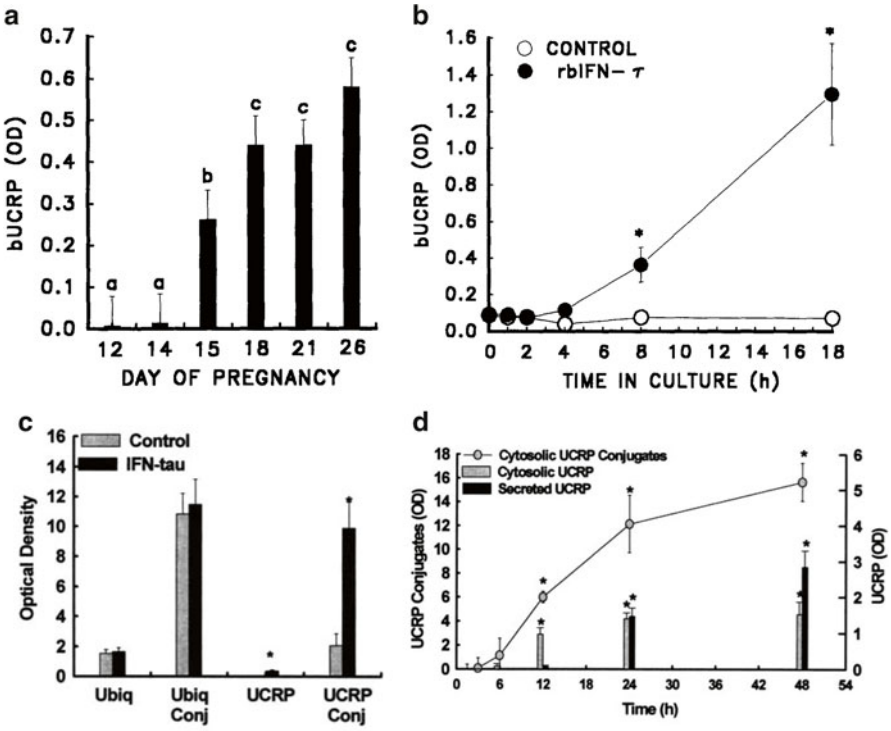


Fig. 2.1 Temporal endometrial release and intracellular endometrial concentrations of UCRP in response to pregnancy and time in culture in response to IFNT treatment. Panel **a** describes release of ^{3}H-Leu-UCRP from cultured endometrial explants collected on days 12–26 of pregnancy (*a*, *b*: $P<0.05$; *b*, *c*: $P<0.01$; *c*, *d*: $P<0.001$). Panel **b** shows the increase in UCRP released into the media following culture of endometrial explants collected on day 12 of the estrous cycle with 25 nM rboIFNT for times specified (*, $P<0.05$). Panels **a** and **b** are from [35]. By using anti-UCRP antibody and anti-ubiquitin antibodies, intracellular UCRP and its conjugates were shown to be up-regulated in response to IFNG and pregnancy [37]. Free ubiquitin and its conjugates did not change in cultured endometrial explants in response to rbIFNT (Panel **c**). This is in contrast to induction of cytosolic UCRP and its conjugates by 12 h followed by release of UCRP into the media by 24 h after culture with rboIFNT (Panels **c** and **d**)

to pregnancy and IFNT in bovine endometrium [5] was found to be the same ubiquitin-like paralog described by the Haas and Knight groups [35] (Fig. 2.1). UCRP increased in endometrium in response to pregnancy from days 14 to 15 and remained detectable through day 26 of pregnancy (Fig. 2.1A). It was inducible in endometrium from non-pregnant cows within 8 h of culture with recombinant bovine IFNT (Fig. 2.1B). Examination of ubiquitin and its conjugates revealed no induction by pregnancy or treatment with IFNT [36, 37] (Fig. 2.1C). Interestingly, ISG15 was not only found as a cytosolic protein in culture endometrial explants, but it was also identified as a secretory product in culture medium (Fig. 2.1D). The presence of secreted ISG15 in uterine flushings from early pregnancy was also

later confirmed, suggesting an extracellular function for ISG15. These data were interpreted to suggest that ISG15 production was a pregnancy- and IFNT-dependent endometrial response.

A bovine endometrial cDNA expression library was constructed and then screened to isolate and sequence ubiquitin and UCRP cDNA [38]. Nucleotide sequence of bovine UCRP was 70 % identical to hUCRP and 30 % identical to a tandem ubiquitin repeat. Use of radio-labeled bUCRP cDNA when screening northern blots revealed that UCRP was detected in bovine endometrium by day 15 and increased to greatest concentrations by days 17–21 of pregnancy [39]. The inferred bUCRP amino acid sequence confirmed retention of the C-terminal Leu-Arg-Gly-Gly amino acids known to be required for conjugation of ubiquitin and ubiquitin paralogues to target proteins [38]. Generation of an anti-bUCRP peptide antibody and use in western blotting of endometrial explants revealed induction of free and conjugated UCRP in response to pregnancy and rbIFNT [37].

Since these early studies, UCRP has been called: ISG15, Ubiquitin-Like Modifier, UCRP1, G1P2, Interferon-Induced 17-kDa/15-kDa Protein, ISG17, Interferon-Stimulated Protein 15 kDa, IFI15, Ubiquitin-Like Protein ISG15, Interferon Alpha-Inducible Protein (Clone IFI-15 K), Interferon-Induced 15 kDa Protein, Ubiquitin Cross-Reactive Protein, and Interferon-Induced 17 kDa Protein. Although the molecular weight more closely approximates 17 kDa across mammalian species, the official protein name is ISG15 and the Entrez Gene name is *ISG15* (Isg15 or *Isg15*; respectively in mice), which will be used from this point forward in this chapter.

Bovine ISG15 and Pregnancy

The b*ISG15* cDNA was used to screen a bovine genomic library and to isolate and sequence the b*ISG15* gene [40]. The b*ISG15* gene, similar to the cDNA, shared about 30 % identity with a tandem ubiquitin repeat and 70 % identity with h*ISG15*. The bISG15 gene promoter has a tandem IFN-stimulated response element (ISRE), which was demonstrated to interact with IFN regulatory factor 1 (IRF1) following culture of bovine endometrial (BEND) cells [41] with rbIFNT. The interaction of this ISRE with BEND cell extracts containing IRF1 corresponded with timing of up-regulation of phosphorylated STAT1 and STAT2 [40].

Because bISG15 was released from cells and also had intracellular ubiquitin-like function when conjugating to cellular proteins, recombinant bISG15 was generated using a *Pichia pastoris* yeast expression system [42] so that it could be more extensively studied. This yeast expression system was used to preclude problems reported when generating ISG15 in bacteria by inherent bacterial carboxypeptidases that cleaved the C-terminus, resulting in removal of active Gly residues. After production and purification of rbISG15 in yeast, C-terminal amino acid sequencing confirmed retention of C-terminal Leu-Arg-Leu-Arg-Gly-Gly residues. Also, the ability of bISG15 to induce up-regulation of IFNG mRNA and protein was demonstrated in cultured peripheral bovine mononuclear cells [42].

A second generation of antibodies against recombinant boISG15 (rboISG15) was developed which included rabbit polyclonal and mouse monocolonal anti-rboISG15 antibodies. Use of a mouse anti-ISG15 monoclonal antibody called 5F10 in immunohistochemistry revealed localization throughout the endometrium from cows on days 18–23 of pregnancy [36]. The most abundant expression of ISG15 localized to the subluminal glandular epithelial cells with lesser, but significant staining in surrounding stromal tissue. There was no staining for ISG15 in endometrium from cows during the estrous cycle and although ubiquitin staining was present in all endometrial sections, ubiquitin was not upregulated in response to pregnancy. A more extensive examination of ISG15 in bovine endometrium on day 18 of pregnancy using transmission electron microscopy and immunogold labeling with anti-bISG15 antibody revealed localization in endometrial glandular epithelium throughout the nucleus, mitochondria, smooth endoplasmic reticulum and plasma membrane [36].

Similar studies in sheep also demonstrated induction of ISG15 in endometrium by pregnancy and IFNT and localization of ISG15 mRNA to endometrial subluminal glandular epithelium and stromal tissue, as well as myometrium [43, 44]. Also, *ISG15* mRNA concentrations have been described to be greater in circulating blood cells during early pregnancy in both sheep and cattle [45, 46]. This prompted studies designed to examine endocrine induction of ISG15 in other ovine tissues during early pregnancy, such as the corpus luteum (CL) [29]. Both ISG15 mRNA and protein were up-regulated in CL in response to early pregnancy. ISG15 localized primarily to the steroidogenic large luteal cells, but also to the gonadotropin-dependent small luteal cells. Recent studies suggest that the inducer of ISG15 in the CL is IFNT that derives from the conceptus, enters uterine vein drainage [28] and then reaches the CL to activate type I IFN receptors, signal transducers such as the STATs and IRFs, and ISG15 gene expression. ISG15 may confer resistance of the CL to PGF through protecting the integrity and steroidogenic machinery and/or attenuating apoptosis in the CL of pregnancy [25, 27].

Structural Features of bISG15

ISG15 interacts with activating enzymes (E1), conjugating enzymes (E2) and ligases (E3) in a manner that is similar, but distinct from that described for ubiquitin [34] (Fig. 2.2A). A ubiquitin activating E1-like (UBE1L) protein was initially described as an enzyme specific for ubiquitin, but later was shown to be specific for ISG15 [47]. Recombinant glutathione S-transferase (GST)-bISG15 was generated using a baculovirus expression system in insect cells, and used to affinity purify interacting proteins from IFNT-treated BEND cells. A 110 kDa protein that bound to GST-ISG15 was purified using this approach [48]. This 110 kDa protein was digested with trypsin and resulting peptides were purified and then submitted to mass spectroscopy determination of mass, which revealed 43–100 % identity to human UBE1L. Bovine UBE1L was localized and upregulated in the endometrium

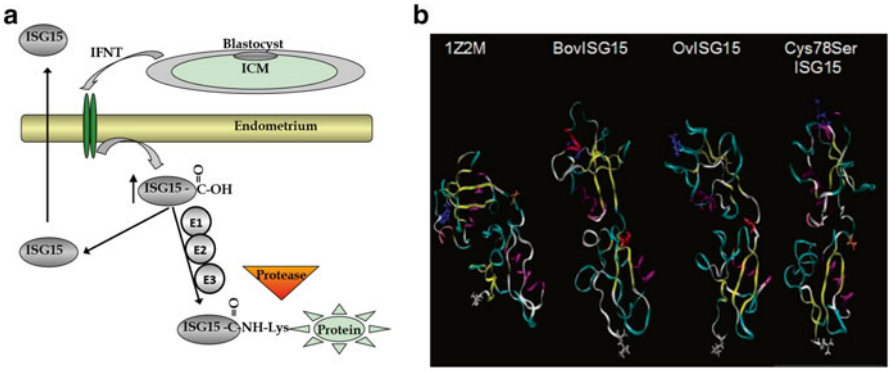


Fig. 2.2 ISG15 conjugation pathway and protein structure. Panel **a** describes release of IFNT from the conceptus, activation of type I IFN receptors on endometrial membranes and synthesis of free ISG15 that can either be released from the cells or can be conjugated to intracellular targeted proteins in an ATP- and multiple enzyme-dependent series of reactions (E1, E2 and E3 activating and conjugating enzymes). After being conjugated to target proteins, ISG15 can be removed through the actions of a de-isgylating enzyme UBP43. Panel **b** shows molecular dynamic simulation of boISG15, ovISG15, and mutant Cys78/Ser ovISG15 based on secondary structure and modeling of human ISG15:1Z2M (from [50]). Note that discrete changes in the hinge region impact structure of both ubiquitin-like domains of ISG15. Bond mode colors represent *blue* for residue 3 in 1Z2M and residue 1 in other ISG15 models, *silver* for residue 154 in 1Z2M protein, 149 in boISG15, 152 in Cys78/Ser ovISG15, *red* for Cys78 and *orange* for Ser 78. *Ribbons* indicate secondary structures as follows: α-helix—*purple*; β-sheet—*yellow*; turn—*cyan*; β-bridge—*tan*; 3–10 α- helix—*mauve*; π-helix coil—*white*

in a manner similar to ISG15 in response to pregnancy and treatment with IFNT. Interestingly, over-expression of GST-ISG15 killed the insect (SF9) cells. Insects do not have an ISG15 ortholog. Thus, the lethal consequences of overexpression of ISG15 might be related to interference by ISG15 with the ubiquitin-dependent pathways that are critical for survival of SF9 cells.

Bovine ISG15 was found to lose stability and three-dimensional structure in solution over time. Intracellular conjugation of ISG15 to target proteins occurs under reducing conditions, and this is consistent with the cytoplasmic environment in general. A critical cysteine residue exists at position 80 in both bovine and ovine ISG15 proteins, and this corresponds to the hinge region between the two ubiquitin-like domains. Cys80 is conserved across mice, humans, bovines and sheep but causes destabilization in hISG15 (actually Cys78 in hISG15) through disulfide bond formation [49]. Modeling of bovine and ovine ISG15 structures was based on human ISG15 (1Z2M) and is shown in Fig. 2.2B. Recombinant rboISG15, roISG15 and corresponding Cys80 → Ser mutants were generated with a C-terminal Arg cap in *Escherichia coli* [50] to protect the C-terminal Gly from endogenous bacterial carboxy-peptidase activity during synthesis [49]. GST-ISG15 was then purified from *E. coli* using affinity chromatography. The C-terminal Arg was removed using carboxy-peptidase B and then correctly processed ISG15 terminating in a C-terminal

Gly was removed from the column using thrombin. Site directed mutagenesis of bISG15 and chemical modification of Cys80 stabilized rboISG15 [50]. The Cys80Ser oISG15 also was more stable and interacted more efficiently with UBE1L. These studies were interpreted to suggest that the hinge region Cys, spacing in the hinge region between the two ubiquitin-like domains and slightly reducing environment are essential to ISG15 structure and are similar to earlier findings by Narasimhan et al. [49]. For example, the hinge region Cys has been shown to form a disulphide bridge with ubiquitin-conjugating enzyme Ubc13 [51]. This non-traditional form of conjugation can be blocked with reducing agents and temporally precedes formation of ISGylated proteins via classical C-terminal Gly-target protein Lys interaction. Other modification to the hinge region Cys may also impact stability and function of ISG15. Post-translational nitrosylation of Cys residues is a recognized cellular response to infection with virus or bacteria. The hinge region Cys of ISG15 may become nitrosylated in response to innate immune responses by nitric oxide, thereby preventing Cys dimerization of ISG15 or interaction of the Cys with other proteins, thus allowing for more free, monomeric ISG15 to interact with targeted proteins via C-terminal ISGylation [52].

Immunoregulatory Action of ISG15

ISG15 is released from human monocytes and lymphocytes in response to culture by Type I IFN [53] and induces the release of IFNG by T lymphocytes, but not natural killer (NK) cells. Bovine ISG15 was first identified in the endometrium as a protein released into the medium following culture of endometrial explants from pregnant cows, as well as from explants or BEND cells from non-pregnant cows treated with bIFNT [5, 35, 41]. For this reason, ISG15 was tested for its ability to induce IFNG in cultured bovine peripheral blood mononuclear cells. While induction of IFNG after culture with ISG15 was detectable, it also was quite variable. Attempts to radioiodinate the single Tyr in bISG15 in radioreceptor assays resulted in very low specific activity which contributed to a lack of sensitivity and problems when trying to demonstrate specific binding to endometrial or PBMC membranes. Likewise, addition of an N-terminal ^{35}S-Met residue also resulted in a protein that had insufficient specific activity for use in receptor binding studies. However, other groups have also suggested an extracellular role of ISG15 in that it: (1) is released from human lymphocytes and monocytes [54] to induce up-regulation of IFNG [53, 55]; (2) induces E-cadherin when cultured with tumor-infiltrating dendritic cells [56]; (3) induces neutrophil-mediated immune responses [57]; and (4) combats mycobacterial infection in humans, presumably through induction of IFNG [58, 59]. This later study implicates an extracellular role for ISG15 in mediating mycobacterial diseases through IFNG, based on a human condition of inherited ISG15 deficiency. A receptor for ISG15 and signal transduction downstream from action of ISG15 on cells has yet to be described. Also, the release of ISG5 from cells is not mediated through a signal peptide; however, non-conventional release might be facilitated through interactions with myxovirus resistance protein MX1 [60].

Mouse ISG15: Studies Relevant to Pregnancy

Because a functional role for ISG15 had not been established in ruminants, our studies of this ubiquitin homolog transitioned to the mouse model, which offered a powerful genetic approach to test gene function. *Isg15* mRNA expression was first examined in the mouse uterus in response to the implanting conceptus [61]. *Isg15* mRNA concentrations increased in the decidualizing stromal compartment (i.e., decidua) between 4.5 and 7.5 days post coitum (dpc) in pregnant mice, and 7.5 and 9.5 dpc mice had significantly greater endometrial *Isg15* mRNA concentrations when compared to pseudopregnant mice (Fig. 2.3A and B). In situ hybridization revealed that *Isg15* mRNA was more extensively localized to antimesometrial decidua on 7.5 dpc and that expression was specific to the pregnancy-induced decidua when compared to the artificially induced deciduoma (Fig. 2.3C) [61, 62]. This confirmed that *Isg15* mRNA was localized to the antimesometrial decidua on 7.5 dpc and is a pregnancy-induced response, rather than a general response to an artificially-induced deciduoma.

Using a cDNA membrane array containing 83 targets, *Isg15* (aka: *G1p2*) was found to be one of five ISGs where steady-state levels in the decidualized uterus depended on the presence of a conceptus [62]. A more extensive screen utilizing the entire mouse genome [63] also identified several up-regulated ISGs in 7.5 dpc decidua when compared to artificially-induced deciduoma. Microarray data were confirmed using RT-PCR for genes known to have biological importance in the endometrium. Semi-quantitative RT-PCR was used to show an increase ($P < 0.05$) in mRNA expression for interferon regulatory factor-8 (*Irf-8*, 1.6-fold), interferon

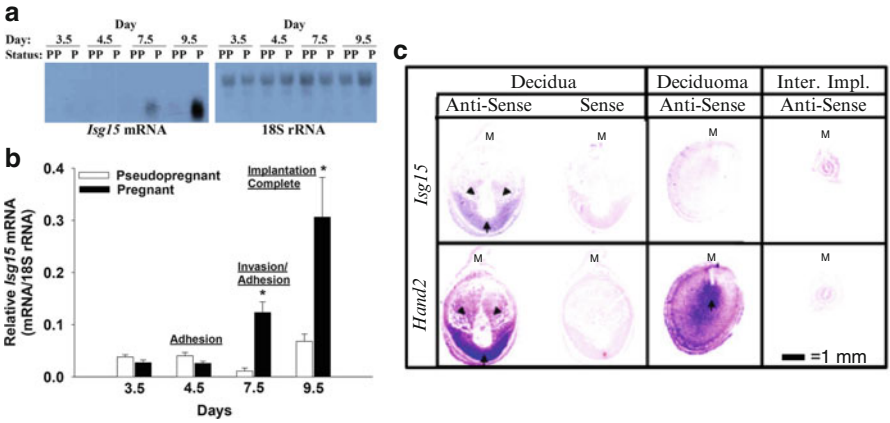


Fig. 2.3 Upregulation of mouse *Isg15* mRNA concentrations in decidua in response to pregnancy. Northern blot revealed induction of ISG15 in mouse uterus in response to pregnancy (P) by day 7.5 post coitum, but not pseudopregnancy (**a** and **b**; *, $P < 0.05$). In situ hybridization of *Isg15* mRNA revealed upregulation in anti-mesometrial decidua in response to pregnancy, but not in response to artificially induced deciduoma (**c**). This is in contrast to up-regulation of *Hand2* mRNA in both decidua and deciduoma. Data are from [61]

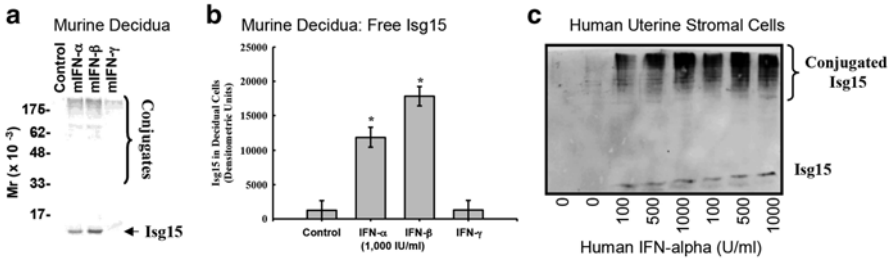


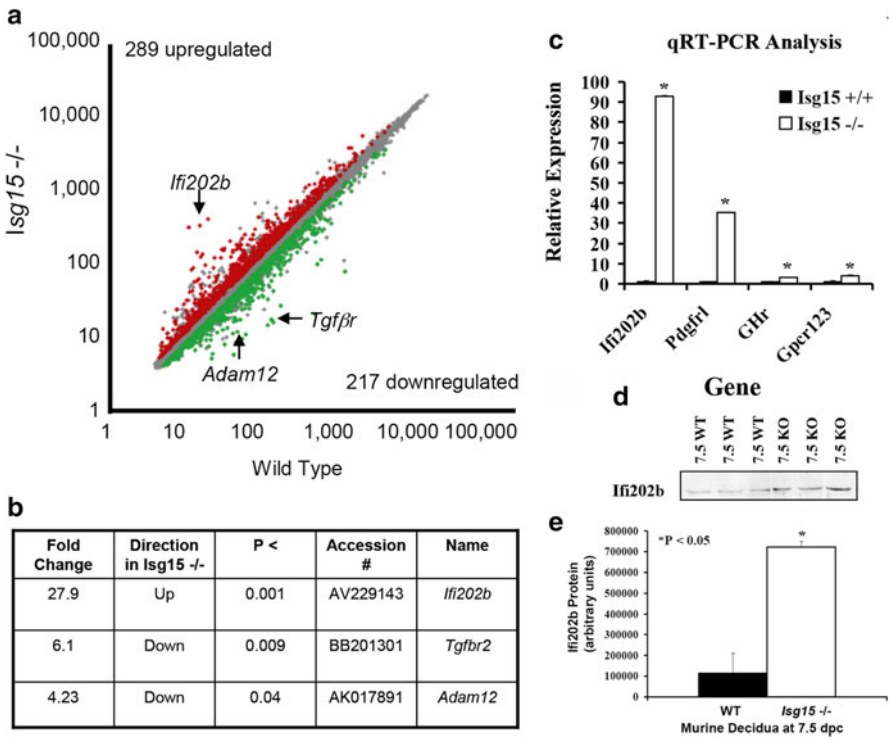
Fig. 2.4 Induction of ISG15 in murine (**a**, **b**) and human (**c**) endometrial stromal cells (Hansen and co-workers, unpublished data). Free ISG15 and its conjugates were up-regulated following 24 h culture of 7.5 dpc murine decidua with IFN-α and IFN-β, but not IFN-γ (Western blot—Panel **a**; Quantitation—Panel **b**; *, P = 0.05). Likewise, human uterine stromal cells responded to culture with IFN-α with a concentration-dependent increase in free ISG15 and ISG15 conjugates

alpha responsive protein (*Iarp*, 2.0-fold), interferon activated gene 202B (*IFI202b*, 1.9-fold), interferon gamma-induced GTPase (*Iigp1*, 1.6-fold), and the interferon stimulated genes *Isg12* (2.5-fold) and *Isg15* (1.6-fold). Uterine ISG15 protein concentrations increased from 4.5 through 7.5 dpc, and then further increased in concentration by 9 dpc. Also culture of murine decidua with type I IFN induced free ISG15 and its conjugated proteins (Fig. 2.4A and B). As was observed in ruminant species, ISGs, including ISG15, are induced in murine endometrium as part of a general interferon/cytokine response to pregnancy.

ISG15 Mouse Mutagenesis Studies

Preliminary study using mutant mice in which the *Isg15* gene was globally deleted (i.e., *Isg15*$^{-/-}$) [64], reported no reproductive phenotype, no impact on antiviral response to vesicular stomatitis virus and lymphocytic chriomenengitis virus, and no apparent impact on IFN signaling through the JAK/STAT signaling pathway. After this primary publication, subsequent studies described *Isg15*$^{-/-}$ mice to be much more susceptible to other viral insults. For example, *Isg15*$^{-/-}$ mice die when challenged with influenza A/WSN/33 and influenza B/Lee/40 [65] viruses. *Isg15*$^{-/-}$ mice also are more susceptible to herpes simplex virus type 1, gammaherpes virus 68 and Sindbis virus infection when compared to WT mice.

Studies in our laboratory using *Isg15*$^{-/-}$ mice resulted in litter sizes at birth that were 50 % smaller when compared to litters from wild type dams (Henkes and Hansen, unpublished results). Despite the fact that surviving *Isg15*$^{-/-}$ pups weighed less at birth, ponderal development following birth was similar when compared to +/+ pups. Loss of embryos in *Isg15*$^{-/-}$mice might be caused by a disruption in gene expression in the maternal decidua. Because *Isg15*$^{-/-}$ embryos died between 7.5 and 12.5 dpc, decidual gene expression was examined on 7.5 dpc. In addition to displaying peak stromal cell decidualization, this stage of early pregnancy was chosen because it represented a time of less trophoblast cell invasion and consequently, less



b

Fold Change	Direction in Isg15 -/-	P <	Accession #	Name
27.9	Up	0.001	AV229143	*Ifi202b*
6.1	Down	0.009	BB201301	*Tgfbr2*
4.23	Down	0.04	AK017891	*Adam12*

Fig. 2.5 Changes in gene expression in decidua from wild type and *Isg15*$^{-/-}$ mice. Scatter plot of 289 up-regulated (*red dots*) and 217 down-regulated (*green dots*) decidual genes that changed 1.5-fold or greater (P<0.05) in *Isg15* null compared to wild type mice. *Arrows* help identifying the *IFI202b*, *Tgfβr* and *Adam12* genes. Panel **b** describes fold-change in *Isg15*$^{-/-}$ mice, P value, accession number and gene name. RT-PCR (Panel **c**) and Western blotting (Panels **d** and **e**) were used to confirm up-regulation of IFI202b in *Isg15*$^{-/-}$ when compared to wild type (WT) 7.5 decidua. Primary pathways impacted because of *Isg15*$^{-/-}$ were signal transduction, immune cell function and vascularization/angiogenesis. Figures adapted from [15]

chance of contamination of uterine decidual tissue mRNA pool with conceptus-derived trophoblast mRNA when compared with later stages of pregnancy. Also, 7.5 dpc was selected because all *Isg15*$^{-/-}$ embryos were found to be alive at this time and it represented a time just prior to loss of pregnancies observed after this time. Following a screen of the Affymetrix GeneChip® Mouse Genome 430 2.0, it was concluded that ISG15 deficiency caused 506 genes to be differentially expressed in decidual tissue, with 289 upregulated (e.g., *Ifi202b*) and 217 down-regulated (e.g., *Adam12* and *Tgfβ2r*) genes [15] (Fig. 2.5A and B).

Ifi202b was identified as the greatest up-regulated gene (28-fold) in the Isg15$^{-/-}$ decidua at 7.5 dpc. The IFI202 family functions in cell-survival and cell cycle regulation. IFI202 is up-regulated by type I IFNs, lipopolysaccharide, and poly-rI:rC treatment [66, 67]. The increase in *Ifi202b* may be a cell-survival mechanism that compensates for some, but not all, of the responses that are mediated through ISG15

for proper decidualization and embryo survival. Down-regulation of decidual *Adam* genes may also be relevant because ADAM8 and ADAM12, localized in mouse implantation sites, are important mediators of uterine remodeling during implantation [68]. Also, low serum ADAM12 concentrations have been associated with pre-eclampsia [69] and intrauterine growth restriction in humans [70]. Whether changes in transcription of these genes are mediated by ISG15 and contribute to establishment of normal litter size and pregnancy remains to be determined.

Ubp43$^{-/-}$ Mouse Studies

ISG15 contains two ubiquitin-like domains connected in tandem. Similar to ubiquitin, ISG15 becomes covalently linked to substrate proteins through cooperative activities of at least three classes of enzymes [71], including an ATP-dependent E1-like enzyme responsible for activation of ISG15 [72], an E2 (UbcH8) conjugating enzyme [73], and an E3 (HERC5 in human and HERC6 in mouse) ligase enzyme [74, 75] (see Fig. 2.2A). ISG15 is removed from targeted proteins through actions of UBP43. A balance of free and conjugated ISG15 is maintained through actions of UBP43, which is up-regulated in response to viral infections, type I IFN and early pregnancy.

Dr. Dong-Er Zhang's laboratory generated and characterized Ubp43$^{-/-}$ mice [76–80]. When *Ubp43* is deleted, dysregulation of the ISG15 system occurs through an abnormal accumulation of conjugated ISG15. The first report using these mice revealed that over-abundance of conjugated ISG15 resulted in live offspring that developed brain cell injury, hypersensitivity to IFN and a 50 % mortality rate by 6 weeks of age [80]. The *Ubp43*$^{-/-}$ brain contained abnormally increased levels of conjugated ISG15, which may have caused ependymal cells to undergo necrosis prior to the development of hydrocephalus. We received these mice from Dr. Zhang and never developed *Ubp43*$^{-/-}$ offspring. By 17.5 dpc, 100 % of null embryos were dead [81]. Examination of *Ubp43*$^{-/-}$ implantation sites on 12.5 dpc revealed disruption of the junctional zone and spongiotrophoblast cells and implantation sites with less vascularization, based on lectin B4 staining, and with greater *Isg15* mRNA and VEGF-A protein concentrations when compared to wild type (WT) placenta. It was concluded that ISG15 and its conjugates were present in implantation sites during mid to late gestation and that deletion of *Ubp43* caused abnormally high concentrations of free and conjugated ISG15 at the feto-maternal interface, which resulted in embryonic death. Because the *Ubp43*$^{-/-}$ mice are from different genetic backgrounds, there may be strain specific genetic modifiers that potentiate lethality of deletion of *Ubp43*. The experiments by Rempel et al. [81], in addition to more recent experiments in Dr. Zhang's laboratory at the Scripps Institute, revealed that under some genetic backgrounds (e.g., backcrossing *Ubp43*$^{+/-}$ into C57 or Balb/c at the F9–F10 generation), *Ubp43*$^{-/-}$ mice were not born. Our studies support the suggestion that dysregulation of ISGylation through abnormal accumulation/over-expression of conjugated ISG15 results in embryo mortality. When combined with the *Isg15*$^{-/-}$ studies, the UBP43 studies also suggest that the ISG15 conjugation

pathway likely plays essential roles on both the maternal and embryonic sides of the implantation interface. Exactly why *Ubp43*$^{-/-}$ results in disrupted implantation sites and embryonic death is unclear, but the consequence of loss of UBP43 is certainly over-accumulation of ISG15 that is conjugated to targeted proteins. This over-accumulation of ISGylated proteins most likely disrupts the balance of free vs. conjugated ISG15, which is maintained by UBP43.

Human and Non-human Primate ISG15 in Decidua in Response to Pregnancy

As with many other mammalian species [4, 35, 36, 39], an early endometrial response to pregnancy in humans and non-human primates [82, 83] is induction of ISG15. ISG15 is induced by IFNs and other cytokines as part of anti-proliferative, antiviral, and inflammatory responses. Localization of ISG15 and ISGylated proteins revealed lack of staining in endometrium from non-pregnant women [82]. However, in response to pregnancy, proteins immune-reactive to anti-ISG15 antibodies were strongly localized to decidual cells. This was apparent in tissues collected from pregnant women on weeks 7–10 and 12 of pregnancy. A similar up-regulation of ISGylated proteins was observed in response to pregnancy in baboon tissues.

Because interleukin 1β (IL1β) induces the decidualization response in primates [84–87], we tested whether ISG15 could be up-regulated in human uterine fibroblast cells (HuF cells) cultured with IL-1β. Treatment of HuF cells with IL-1β resulted in up-regulation of ISG15 and its conjugates. Likewise, culture of HuF cells with type I IFN also resulted in up-regulation of ISG15 and its conjugates (Fig. 2.4C). Other data to support the concept that ISG15 and ISGylation are induced in human decidua in response to pregnancy are provided in a microarray study by Hess et al. where human endometrial stromal cells were decidualized with progesterone and then cultured with media conditioned by human trophoblast [14]. Following microarray analysis, several ISGs, including ISG15 (12-fold up-regulated), were found to be up-regulated following endometrial culture with human trophoblast-conditioned medium. In addition to ISG15, its ISGylation enzymes including initiating enzyme (Ube1l), E2 enzyme (Ubch8) and E3 ligase enzyme (Herc5) were all up-regulated in human uterine stromal cells in response to IL-1β. Exactly why ISG15 is up-regulated in uterine decidua in response to pregnancy and cytokine such as IL and IFN is the focus of future studies.

Summary

Why is ISGylation a conserved response to pregnancy across mammalian species with very divergent forms of embryo implantation and placentation? Many proteins become conjugated with ISG15; yet, a clear role for ISGylation remains to be determined [79, 88–93]. Many of the proposed ISGylated proteins have been tested and

some have been confirmed to be conjugated to ISG15, but the functional roles for ISGylated targets remains to be determined. The amount of targeted protein that becomes ISGylated is low (less that 5 % in some cases), which provides challenges when addressing functional relevance. However, mouse genetic models and human disease conditions with mutations in genes encoding the ISGylation system provide great insights into overall importance of this system to health and well-being. There is no consensus amino acid sequence, to our knowledge, that will allow prediction of proteins that might become ISGylated. Also the massive number of proteins suggested to be conjugated to ISG15 is extensive and these potential targeted proteins have intracellular functions that span the nucleus, cytoplasm, cytoplasmic organelles and plasma membrane.

It is very clear that type I IFNs induce ISGgylation, and this may be important in many species in the endometrium during pregnancy. However, viruses also induce up regulation of ISG15 and ISGylation. If ISGgylation is a general stress response, or subtle innate immune response, perhaps up-regulation in the endometrium helps provide a promp, but not fully activated immune response. This would allow for rapid innate immune response by the mother in the event that infection was approaching the placenta and fetus. Signals from the conceptus, whether IFNT in ruminants or other related cytokines in primates and mice, may coordinate local and systemic immune-modulatory responses to equip the mother when protecting the pregnancy from infection, while also curbing more aggressive adaptive immune responses that could harm the embryo/fetus. This was recently suggested in ruminants [25] and may also have relevance to murine and primate pregnancy.

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Src-Family Tyrosine Kinases in Oogenesis, Oocyte Maturation and Fertilization: An Evolutionary Perspective

3

William H. Kinsey

Abstract

The oocyte is a highly specialized cell poised to respond to fertilization with a unique set of actions needed to recognize and incorporate a single sperm, complete meiosis, reprogram maternal and paternal genomes and assemble them into a unique zygotic genome, and finally initiate the mitotic cell cycle. Oocytes accomplish this diverse series of events through an array of signal transduction pathway components that include a characteristic collection of protein tyrosine kinases. The *src*-family protein kinases (SFKs) figure importantly in this signaling array and oocytes characteristically express certain *SFKs* at high levels to provide for the unique actions that the oocyte must perform. The *SFKs* typically exhibit a distinct pattern of subcellular localization in oocytes and perform critical functions in different subcellular compartments at different steps during oocyte maturation and fertilization. While many aspects of *SFK* signaling are conserved among oocytes from different species, significant differences exist in the extent to which *src*-family-mediated pathways are used by oocytes from species that fertilize externally vs those which are fertilized internally. The observation that several oocyte functions which require *SFK* signaling appear to represent common points of failure during assisted reproductive techniques in humans, highlights the importance of these signaling pathways for human reproductive health.

Keywords

Meiosis • Maturation • Fertilization • Oocyte • SRC • FYN • YES • FGR • FAK • PYK2 • Protein kinase

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Introduction

Oogenesis represents a transformation of the female primordial germ cell through intermediate growth stages during which the oocyte becomes specialized with the capacity to store metabolic precursors sufficient for the initial stages of embryo development together with a combination of meiotic cell cycle machinery, chromatin remodeling factors, and pre-assembled mRNAs (maternal factors) that encode enzymes critical for fertilization and early zygote development. Once fully grown, the oocyte may remain relatively inert for some period maintaining gap junction communication with associated follicle cells, and awaiting endocrine and paracrine signals that signal the time for meiotic maturation and subsequent fertilization. The necessity of the oocyte to respond to the diverse signals for nuclear and cytoplasmic maturation as well as fertilization is met by preassembly of signal transduction networks. These networked pathways sense inputs from the follicle cells as well as the fertilizing sperm and, in turn, control expression of maternal genes as well as the mechanical processes involved in cytoplasmic and nuclear maturation, sperm incorporation and ultimately oocyte activation. Many of these signal transduction pathways involve protein kinases at key regulatory steps and the orderly assembly and maintenance of these signaling proteins is a critical aspect of oocyte quality [1]. The protein kinases involved in meiotic maturation have been the focus of intense study for at least 30 years and that body of work propelled the oocyte into a prominent place in the cell biology field providing an important complement to the yeast system in the field of cell cycle control. For the most part, meiosis is controlled by protein kinases that phosphorylate serine or threonine (Ser/Thr kinases) and these elements have been reviewed elsewhere [2–6]. In addition, several protein tyrosine kinases (PTKs) have been found to play significant roles in the processes of oocyte maturation and fertilization [7–11] and the purpose of this chapter is to review the current knowledge in this aspect of oocyte biology.

Protein Tyrosine Kinases: Significance

The phosphorylation of proteins as a stable form of secondary modification occurs mostly at serine, threonine and tyrosine residues (phospho-histidine being relatively unstable). To be functionally significant as a signaling mechanism, a phosphorylation pathway must have enzymes to both add and remove the phosphate in order to control the level of signal. In addition, a substrate or product binding domain adds targeting specificity as opposed to non-specific phosphorylation. Such sophisticated mechanisms were developed for Ser/Thr phosphorylation early on and are present in all prokaryotic and eukaryotic cells. While some level of Tyr phosphorylation occurs in bacteria, plants, and single cell eukaryotes, the kinases involved are either BY kinases in bacteria [12] or are structurally related to P-loop nucleotide triphosphatases, or dual specificity kinases (TKL kinases [13, 14]) and are not related to eukaryotic tyrosine kinases. The appearance of an efficient P-Tyr signaling pathway with tyrosine specific kinases, phosphatases and binding domains is thought to

occur just prior to evolution of metazoans as exemplified by the choanoflagellates [15]. The appearance of this pathway has been described as a 'game changing innovation' [16] which provided the ability to establish new signaling pathways without the possibility of interference with pre-existing Ser/Thr kinase pathways which were by that time critical for success. The unique tyrosine specificity provided by the catalytic domain together with the P-Tyr binding specificity of the SH2 domain and opposing phosphatases with their own unique specificity enabled a vast array of signaling possibilities which could facilitate the evolution of multicellular communication and specialized tissue physiology [17]. The diversification of PTK families into cytoplasmic and transmembrane receptor classes is apparent even in these premetazoans [18] although the rapid expansion of the receptor PTKs among metazoans suggests that these receptors, which are thought to initially have functioned as environmental sensors, were very successfully applied in cell–cell communication.

Src-Family PTKs: Structure and Capabilities

The *src*-family of PTKs (SFKs) appears to have evolved its characteristic domain structure in choanoflagellates (*Monosiga brevicollis*) where four Src kinase homologs were reported [19]. These 57–60 kDa proteins consist of five conserved domains, the N-terminal unique domain, the SH3 and SH2 protein interactions domains, the catalytic domain, and the C-terminal regulatory domain (Fig. 3.1) and this structure is retained in all metazoan species. The N-terminal unique (U or SH4) domain exhibits the most sequence divergence among Src-family members (thus the name 'unique'). This domain contains two fatty acid acylation sites that typically become derivatized with palmitate and myristate which function in targeting the kinase to the plasma membrane rafts [20]. Phosphorylation sites at Ser17, Thr37, and ser75 are thought to antagonize membrane localization [21]. This domain also contains a lysine that is often methylated and seems to play a role during cell spreading [22]. Other functions of this domain include binding of filamentous actin bundles [23] and direct interaction with PLCγ [24, 25].

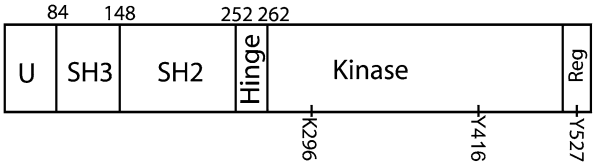

Fig. 3.1 Domain structure of *Src*-family protein kinases. *Src*-family PTKs contain a small N-terminal unique (U or SH4) domain followed by the adjacent SH3 domain and SH2 domain. A short hinge region separates the N-terminal domains from the kinase domain which contains the catalytic site with its critical lysine 296 which is in the ATP binding pocket. An important tyrosine phosphorylation site (Y416) in the A loop. The C-terminal regulatory domain contains the second regulatory phosphorylation site (Y527)

The SH3 and SH2 domains are important for protein–protein interactions that relate to modification of kinase activity as well as targeting specific signal transduction pathways. The SH3 domain binds proline-rich sequences and is involved in docking interactions with other signaling proteins such as PI-3 kinase, BLK, Shc, WASP [24, 26–28], and BLK [29]. Other diverse functions including interactions with voltage gated sodium channels also occur via this domain [30]. Lastly, the integrin B3 protein binds to and is thought to activate Src though interaction between a RGT sequence and the SH3 domain [31]. The SH2 domain of SFKs exhibits binding specificity for phosphorylated tyrosine within the following motif (Y-E-E-I/L/V/P) [32]. A primary significance of this domain is that in multicellular organisms, it functions in an intra-molecular binding interaction with a C-terminal tyrosine to stabilize a 'tight' or 'closed' tertiary structure that obscures the catalytic domain and renders the kinase inactive [33, 34]. The SH2 domain also participates in interactions with other proteins such as the TRPC6 channel protein [35], growth factor receptors such as the PDGF receptor and discoidin domain receptor DDR1 [36, 37], receptor guanyl cyclase C [38], other kinases such as FAK and FLT3 [39, 40], as well as cytoskeletal elements such as cortactin [41].

The catalytic domain of Src-family PTKs is based on a catalytic structure and mechanism common among most protein kinases including serine and threonine kinases such as protein kinase A. The domain exhibits an N-terminal lobe (N-lobe), a C-terminal lobe (C-lobe) and a central activation loop (A-loop). The catalytic site where the gamma phosphate of ATP is transferred to substrate tyrosine is located between the N and C lobes and the configuration of the A-loop controls access to the active site [42, 43]. Phosphorylation of tyrosine 416 in the A-loop [44] favors a conformational change of the N and C-lobes that increases access to the catalytic site and increases catalytic activity.

The C-terminal domain of SFKs contains a number of Serine residues that can be phosphorylated as well as a phosphorylation site at tyrosine 527. While the size of the C-terminal domain might suggest several important functions, the observation that the C-terminal domain can be replaced with the syntropin PDZ domain and its ligand to produce a functional SFK [45] suggested that the predominant requirement is that the domain function to establish the 'closed' configuration typical of the inactive state.

Regulation

The SFKs are held in an inactive configuration in most cells and are only activated transiently by a multi-step process involving dephosphorylation of the C-terminal tyrosine (Y^{527}) and activation-loop phosphorylation [44]. In the absence of stimuli, the inactive configuration is maintained by phosphorylation of Y^{527} by CSK kinase and, once phosphorylated, the flexibility of the SH2-linker region allows the SH2 domain to bind Y^{527}, locking the kinase into a 'closed' configuration. Appropriate stimulation by growth factors, cell–cell contact, or cell cycle events causes a phosphatase such as rPTPα [46] to dephosphorylate Y^{527} of the C-terminal domain

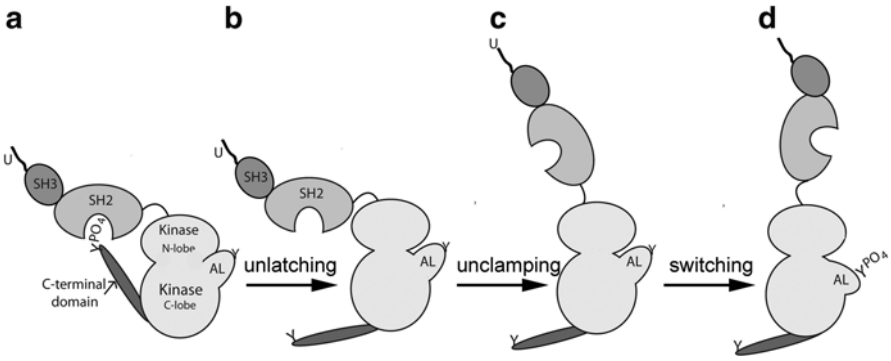

Fig. 3.2 Src-family kinase activation mechanism. *Src*-family PTKs are held in a predominantly inactive form in most cells and are activated only in response to stimuli via a common mechanism. The inactive kinase (*left*) is maintained in the closed conformation by the binding interaction between SH2 domain and the phosphorylated C-terminal tyrosine (**a**). Dephosphorylation of the C-terminal tyrosine 'unlatches' the protein (**b**) allowing it to assume an open configuration which allows catalytic activity 'unclamping' (**c**). Autophosphorylation of the tyrosine phosphorylation site within the activation loop (AL) stabilizes the open configuration 'switching' the enzyme to full catalytic activity (**d**) (after Roskoski [44])

('unlatching' shown in Fig. 3.2) allowing the kinase to unfold into the 'open' configuration exposing the catalytic site ('unclamping ' as in Fig. 3.2). The catalytic site itself requires the proper orientation of the N and C-lobes as well as the activation loop (A-loop). The activation loop alternates between the active configuration, where all elements of the active site are in the correct position, and an inactive configuration that does not support phosphorylation of tyrosine. The active conformation of the A-loop is stabilized by a second phosphorylation event, trans-autophosphorylation of Y^{416} in the A-loop, which switches the kinase into full catalytic activity.

Expression

SFKs are expressed in oocytes of species as diverse as *Drosophila melanogaster* [47], *Asteria miniata* [48, 49], *S. purpuratus* [50], *Cerebratulus* [51], *Xenopus* [52, 53] and mammals. While most of the typical Src-family domains are reasonably well conserved, significant sequence differences among the insect and marine invertebrate species that have complicated classification of individual SFKs in these species. For example, while early immunoprecipitation assays using antibodies directed against vertebrate *src*-family members detected PTK activities resembling Src and Fyn [54, 55] later sequence analysis of individual SFKs cloned from the sea urchin and starfish [48, 50] identified several novel PTKs which were clearly members of the SRC-family based on homology, but which could not be directly linked to known vertebrate SFKs (reviewed in [11]). The number of Src-family members expressed in these marine invertebrate oocytes suggests that these oocytes face requirements that are best suited by a diversity of protein tyrosine kinase properties and functional

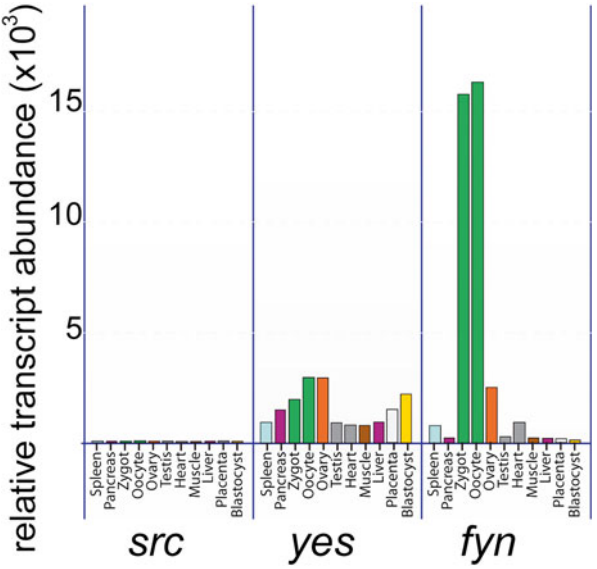

Fig. 3.3 Oocytes express *fyn* and *yes* transcripts are high levels relative to somatic cells. The relative abundance of the most common *Src*-family kinase mRNAs reported for mouse oocytes in the BioGPS expression array database (Novartis BioGPS, http://biogps.gnf.org; [149]) is presented for several different tissues and cell types. Values in *green* represent oocyte and zygote material

studies have demonstrated that the different Src-family members do perform different roles during fertilization. The presence of SFKs in vertebrate oocytes was initially demonstrated by cDNA cloning of mRNA isolated from *Xenopus laevis* oocytes [56] and was later confirmed at the protein level in *X. laevis* where the Xyk kinase was purified and described [57, 58]. Proteomic analysis revealed that three *src*-family members (Src1, Src2, and xSrc) are expressed in proteins in the *Xenopus* oocyte [59]. In the zebrafish system, FYN was detected by immune-complex assay and subsequently cloned and sequenced [60, 61]. The first demonstration of SFK expression in mammalian oocytes was performed on rat and mouse oocytes [62–65] and was greatly facilitated by the availability of well-characterized antibodies specific for the different *src*-family members which made possible western-blot and immune-complex assays as well as immunofluorescence techniques. Later, array-based mRNA expression data of different stage mouse oocytes revealed that most members of the SFKs (fgr, hck, lyk, lyn, blk) are barely detectable in murine oocytes while *fyn* and *yes* [66] are expressed at very high levels in oocytes (Fig. 3.3). This result seems to conflict with the situation in marine invertebrate oocytes where a number of different *src*-family members are expressed and play different roles during fertilization. It suggests that the mammalian oocyte does not need the diversity of SFK signaling mechanisms that are critical for marine invertebrates either because of the evolutionary distance or because of the fact that species that fertilize externally face unique challenges that the mammalian oocyte does not experience. Given the fact that *fyn* expression levels are much higher in oocytes than even neurons and T-cells, one might even refer to FYN kinase as an 'oocyte-specific kinase'. At least it is clear that the oocyte is highly specialized biochemically with a large commitment to signaling pathways involving the FYN kinase. The biology of the oocyte is

such that it must establish and maintain a pool of the protein kinases in order to remain ready for signals to begin meiotic maturation and later for fertilization which will trigger rapid zygote development. FYN appears to be an essential component of the oocyte signaling machinery and proper subcellular localization must be an important aspect of oocyte quality. Once the blastula stage has been reached, the high levels of FYN kinase appear to be no longer required as evidenced by the relatively low expression levels typical of the blastocyst (Fig. 3.3).

Subcellular Localization

The membrane targeting (U) and protein interaction domains (SH3, SH2) of *src-family* PTKs direct physical association of these kinases with the subcellular structures where they function. Specific targeting of FYN and YES kinases to structures within the oocyte has been detected with antibodies specific for individual kinases and the activation state of SFKs can be detected with phosphorylation site-specific antibodies such as the clone 28 mouse monoclonal which recognizes the dephosphorylated form of the C-terminal regulatory tyrosine (Y^{527}) [67]. Also, antibodies specific for the phosphorylated and non-phosphorylated regulatory tyrosine located in the catalytic domain (Y^{416}) of SFKs can differentiate between active and inactive forms but cannot distinguish among the different family members due to the highly conserved sequence of the phosphorylation site. Early subcellular fractionation studies demonstrated that Fyn and possibly other SFKs were concentrated in the plasma membrane and cortex of sea urchin and zebrafish oocytes [61, 68, 69]. Confocal immunofluorescence analysis has been used to examine the subcellular localization of SFKs in oocytes from sea urchins, zebrafish, and mice. In the sea urchin, the SpSFK1/7 kinase was detected in the cortical actin layer and in the subadjacent cortical cytoplasm which is typically enriched in endoplasmic reticulum [50]. A similar distribution of active SFKs was detected in the zebrafish oocyte [70] and mouse oocyte [71] suggesting that this kinase family plays some role in regulation of cortical cytoskeletal components. This supposition is supported by the fact that SpSFK1/7 [50] and activated SFKs detected with the clone 28 antibody were found to be highly concentrated in the fertilization cone of sea urchin and zebrafish oocytes [72]. In the mammalian oocyte, a characteristic localization pattern of SFKs has been demonstrated as early as the germinal vesicle (GV) stage where FYN has been reported to preferentially localize in the cortical region while YES was distributed uniformly in the ooplasm [62, 71, 73]. During the GV stage, a subpopulation of FYN is also concentrated within the nuclear envelope raising the possibility that it might play some role in chromatin remodeling or nuclear envelope dynamics in addition to having functions within the oocyte cortex. This property was unique to FYN, as the YES kinase did not concentrate in the GV of wild type oocytes [11]. After germinal vesicle breakdown (GVBD), FYN and YES were both localized to the cortex of the mature oocyte in zebrafish, rat, and mouse systems [62, 64, 66, 72]. Once the meiotic spindle was formed, FYN and possibly other active SFKs detected with the clone 28 antibody, were found to be highly concentrated in close

association with microtubules of the meiotic spindle or residual body [10, 64, 74–76]. The tight association of FYN with spindle components has also been observed in studies of somatic cells [77, 78] at least some fraction of the spindle-associated SFKs are in the active state as demonstrated with the clone 28 antibody [67, 79, 80]. A separate antibody to (Y^{416}) of SFKs has also been used to detect activated SFKs that were closely associated with the meiotic spindle of mouse oocytes [76, 81].

Functions During Oocyte Maturation

The first analysis of PTK activation during oocyte maturation was done with the starfish *Marthasterias glacialis* where induction of oocyte maturation with 1-methyl adenine induced activation of PTK activity detected via accumulation of P-Tyr-containing proteins in the oocyte [82]. This study also detected a 68 KDa PTK activity in autophosphorylation assays performed on purified cortex preparations suggesting a possible role of *src*-family PTKs during oocyte maturation. However, the first definitive proof that a *src*-family PTK was involved in oocyte maturation was revealed in *X. laevis* oocytes where SFK activation represents one of the earliest responses to progesterone treatment of the oocyte [83, 84]. The progesterone receptor is known, in some cases, to activate SRC kinase activity through an SH3 displacement interaction [85] which raises the possibility that the progesterone receptor in the oocyte or in tightly associated follicle cells might be a key element of meiosis regulation. The potential function of SRC during oocyte maturation was shown by injection of active SRC kinase into GV stage *Xenopus* oocytes which resulted in accelerated progesterone-induced GV breakdown [83] and MAPK activation [84].

In mammalian oocytes, progesterone or LH stimulation of GV stage oocytes has not been associated with elevated SFK activity, however significant changes in the subcellular distribution of active Src-family PTKs has been reported [10].

GV stage oocytes are characterized by concentration of active SFKs at cytoplasmic microtubule arrays and in the region surrounding the nucleus [10]. After GVBD, active kinase was detected only on the meiotic spindle of the MI and MII oocyte. The function of SFK members during oocyte maturation has been studied with chemical inhibitors such as SKI-606, PP2 and SU6656 as well as by siRNA knockdown, dominant-negative constructs, and single gene knockout models. Each approach has its own drawbacks. The chemical inhibitors cannot distinguish among different Src-family members very well and usually inhibit the closely related Abl kinase [86–89]. Dominant-negative constructs provide better specificity toward SFKs and can block scaffolding interactions, but require injection of cRNA and adequate expression in the oocyte to exert their effect. Knockdown and gene knockout studies provide better specificity toward individual SFKs but allow compensation due to increased expression of other SRC-family members [90]. As might be expected given the limitations of the different methods used, experimental analysis of the role of SFKs in initiation of meiosis (GVBD) has produced conflicting results. Inhibitors such as PP2 [76] and SU6656 [73] block GVBD in culture, while SKI-606 does not block GVBD, but instead stimulates GVBD even in the presence of

phosphodiesterase inhibitors [10]. Functional studies in which the role of FYN was tested by injection of cRNA encoding a dominant-negative FYN construct, partially blocked GVBD in culture [73], while siRNA knockdown and *fyn*-null oocytes revealed no inhibition of GVBD [91]. This type of conflict might result from compensation by other SFKs if chemical inhibitors and dominant-negative constructs were more effective against compensation by other kinases due to the similarity of the catalytic sites or the protein interaction domains. Single gene knockdown or knockout would leave the possibility of compensation entirely open as demonstrated by up-regulation of Yes kinase in the *fyn*-null oocyte [91].

While SFK activity may or may not be required for initiation of GVBD, suppression of SFK activity with SKI-606 or by siRNA knockdown or knockout of *fyn* caused significant disruptions in the spindle and chromatin organization that resulted in failure to complete MI and reach normal metaphase-II [10, 74, 90, 91]. In addition to the effects on spindle function, suppression of SFKs (particularly FYN) had additional effects on the cortical actin layer polarity. During oocyte maturation, the metaphase-I spindle must remain in close proximity to the egg cortex while the spindle rotates and the polar body is extruded at telophase-I. However, chemical inhibition by SKI-606 or knockout of *fyn* leads to abnormal cortical polarity seen as reduced filamentous actin content near the spindle and enlarged polar bodies which correlated well with a failure of the meiotic spindle to maintain a position close to the oocyte cortex [91]. It is likely that FYN and other SFKs act directly on the cortical actin layer since injection of active viral-SRC into oocytes was shown to have dramatic effects on the cortical cytoskeleton [92], and moesin, a known component of the oocyte cortex, was identified as a SFK target in *Xenopus* oocytes [93]. In any case, the suppression of Fyn kinase through gene knockout, knockdown, or chemical inhibition resulted in loss of developmental competence with high failure rates during MI and MII stages of maturation [10, 90].

Functions During Fertilization

SFKs function in a variety of signaling pathways including several that have found application in the oocyte during fertilization. As mentioned above, highly reactive cells such as platelets and T-cells use SFKs such as SRC and FYN to stimulate phospholipase Cγ activation either through direct phosphorylation or through PI-3 kinase activation [94, 95] as part of a rapid IP3-mediated calcium signal and or mitogenic signals. SFKs also play an important cell cycle control events at the G2/M transition [96, 97] and, together with FAK, can function during actin remodeling events [23, 98–100]. Since the biology of fertilization involves some basic functions that are shared between invertebrate and vertebrate animals (ex. gamete binding-fusion, block to polyspermy, metabolic activation, and cell cycle resumption) it is not surprising that most oocytes studied rely heavily on a similar complement of protein kinases. However, since fertilization strategies can differ significantly depending on the environment where reproduction occurs (ex. external fertilization vs. internal fertilization) the relative importance of different pathways can also vary.

Early Events During External Fertilization

Among species that fertilize externally, the functional significance of SFKs during fertilization has been found to center primarily in the area of regulating PLCγ mediated calcium signaling during the earliest phases of fertilization. Marine invertebrate, amphibian, and fish species fertilize large numbers of oocytes externally which facilitated biochemical analysis of large numbers of synchronously fertilized eggs. As a result, SFK activity could be measured directly by immune-complex assays and autophosphorylation assays which were not generally feasible for mammalian oocytes. The most thoroughly studied species include sea urchins, starfish, frogs and zebrafish which typically exhibit rapid activation of SFK activity within the oocyte cortex or plasma membrane compartment [55, 57, 58]. The significance of the SFKs to fertilization in externally fertilizing species is particularly obvious in the sea urchin and starfish systems since these oocytes expresses multiple different SFKs simultaneously. In the sea urchin *S. purpuratus*, four SRC-family members SpFRK, SpSFK1, 3, and 7 are expressed as proteins in oocytes [50], while in the starfish *A. miniata*, AmSFK1, AmSFK2, and AmSFK3 are expressed as proteins [48]. SpSFK1 and SpSFK7 as well as AmSFK 1, 2, and 3 are known to be concentrated in the plasma membrane or cortex of oocytes as determined by subcellular fractionation or immunofluorescence microscopy. Upon fertilization, AmSFK1 was activated transiently within 20–40 s post fertilization followed by AmSFK3 which exhibited a slower time course [48]. The timing of kinase activation correlates well with the high amplitude fertilization-induced calcium transient typical of marine invertebrate oocytes and functional studies using recombinant-SH2 domain fusion proteins specific for AmSFK1 and 3 demonstrated that these two kinases play a role in initiation or amplification of this important calcium signaling event. Given the fact that PLCγ-mediated IP3 production was known to play a major role in the fertilization-induced calcium transient of oocytes which were fertilized externally [101–103] the potential role of specific SRC-family PTKs to stimulate PLCγ-mediated IP3 production was of intense interest. Additional mechanistic analysis demonstrated that SpSFK1 binds directly to the SH2 domain of PLCγ, suggesting that this kinase might be primarily responsible for activating PLCγ during fertilization [50]. This work culminated a series of studies from several different labs which demonstrated a functional relationship between SFKs and PLCγ-mediated calcium signaling at fertilization [104–107] reviewed in [108, 109]). This has led to a model for the initiation of calcium release at fertilization in sea urchins where sperm-egg binding or fusion leads to the activation of SpSFK3 which quickly triggers activation of SpSFK1. SpSFK1 directly interacts with and activates PLCγ, leading to the production of diacylglycerol and IP_3. Calcium would then be released from the endoplasmic reticulum through the IP_3 receptor as occurs in a wide variety of cells types. Interestingly, evidence also supports an alternative mechanism involving NAADP-mediated calcium signaling during the initial 'cortical flash' that precedes the high amplitude calcium transient in marine invertebrate oocytes. The 'cortical flash' is then thought to be amplified by the above IP3-mediated mechanism and prolonged by cADPR signaling [110, 111]. In fact, both a plasma membrane and an endoplasmic reticulum associated ADP-ribosyl cyclase was demonstrated in the sea

urchin egg [112] indicating that cADPr may play a role in calcium flux across the plasma membrane as well as the endoplasmic reticulum or other intracellular vesicles. Taken together, it is clear that the PLCγ/IP3 mechanism plays a dominant role in producing the high amplitude calcium transient that occurs in most externally fertilizing species and appears to rely on SRC-family PTKs for activation, but the significance and regulation of the NAADP, and cADPR-mediated calcium events needs to be resolved before a full understanding of fertilization in externally fertilizing species can be achieved.

Evidence that SFK activation of PLCγ plays a role in the fertilization-dependent calcium transient other external fertilization models has been developed in *X. laevis* and in *Danio rerio* where pharmacological and dominant-negative suppression of SFKs was also found to suppress the fertilization-induced calcium transient [101, 113, 114]. In *Xenopus*, the *src*-related kinase XYK was shown to associate with PLCγ and stimulate calcium release when injected into mature oocytes [7, 115, 116]. In *Xenopus*, cADPr is thought to promote IP3-mediated calcium signaling through its effect on SERCA pumps which maintain calcium stores in the endoplasmic reticulum [117]. As yet, a direct effect of cADPR or NAADP pathways on the fertilization-induced calcium transient has not been demonstrated. The zebrafish oocyte is characterized by a large central yolk mass that is surrounded by a more active cortical cytoplasm and extensive imaging studies have documented that IP-3 mediated calcium release is primarily responsible for the fertilization-induced calcium transient in this oocyte [118, 119]. The cortical cytoplasm is enriched in SFKs and in IP3r channels relative to the central cytoplasm and confocal-based calcium imaging indicates that the fertilization-induced calcium transient propagates through the cortical cytoplasm faster than through the central cytoplasm [72]. The importance of SFK signaling in the fertilization-induced calcium transient through the cortical cytoplasm was shown by injection of a FYN-SH2 domain fusion construct which suppressed the calcium response in the cortex, but had little effect in the yolk mass.

The fact that the extracellular coat or chorion of fish oocytes is specialized to exclude sperm except for a single pore (the micropyle) through which sperm can pass presented the opportunity to reliably predict where the fertilizing sperm would contact the oocyte. This feature enabled detection of the initial effect of sperm-oocyte contact or fusion on SFK activation which revealed that activation of SFKs (as well as PYK2 kinase activation) occurred initially in the immediate vicinity of sperm-oocyte contact/fusion [120]. Kinase activation then progressed through the oocyte cortex from animal pole to vegetal pole eventually involving the entire oocyte cortex. The fact that the progression of SFK activation through the egg cortex correlated with the progress of the high amplitude calcium transient raised the possibility that SFK activation might function to amplify and propagate PLCγ mediated calcium release through the relatively large distances required to traverse the fish oocyte, which in the case of zebrafish, represents a diameter of 700 μm. This was supported to some extent by the fact that the central yolk mass (largely devoid of FYN kinase and IP3r exhibited a slower calcium response to fertilization. However, additional work to demonstrate that a propagated wave of PLCγ phosphorylation occurred coincident with the SFK activation would further strengthen this model.

Early Events During Internal Fertilization

The characteristics and role of SFKs in fertilization of the mammalian oocyte appear significantly different from that in species which fertilize externally. Mouse and rat oocytes express FYN, YES, and perhaps SRC proteins [62, 63]. FYN is concentrated in the oocyte cortex [62, 64] as were some of the SFKs expressed in marine invertebrate oocytes described above. However, while SFK activation could be demonstrated in the cortex of sea urchin, frog, and zebrafish oocytes by biochemical analysis of membrane fractions [55, 58, 61] or through the use of phosphorylation site-specific antibodies [70], mammalian oocytes were nearly impossible to analyze biochemically and immunofluorescence failed to detect activated SFKs in the mouse egg cortex [81, 121]. While a negative result proves nothing, the fact that exogenous Fyn-eGFP constructs modified to remain in the 'open' configuration preferentially localized at the oocyte cortex suggests that if fertilization had resulted in activation of endogenous FYN, it would have become localized in the cortex as well [122]. The failure to detect endogenous activated SFKs in the cortex of fertilized mouse oocytes by immunofluorescence may indicate that it does not normally occur and can be detected only by over-expressing the FYN protein. In any case, functional studies using chemical inhibitors, dominant-negative constructs, and single gene knockout have consistently demonstrated that suppression of SFKs in the mammalian oocyte has, at best, a minor impact on the fertilization-induced calcium oscillations [49, 64, 65]. The subtle changes in oscillation frequency that do occur in *fyn*-null oocytes seem more likely to reflect disruption of the normal distribution of IP3 receptors and cortical cytoskeletal organization which results from *fyn* suppression rather than any effect of phospholipase C activity [90]. In mammalian oocytes, SFK activity seems to be important before fertilization to maintain organization of cytoskeletal components in a fertilizable state and during the later stages of fertilization including pronuclear congression and the entry into mitosis.

Other Functions of SFKs During Fertilization

Additional aspects of egg activation that involve SFKs activity include maintenance of meiotic spindle and cortical actin cytoskeleton integrity. The role of SFKs in these cytoskeletal functions seems to be conserved among externally fertilizing and internally fertilizing species. SFKs are known to become concentrated in the region of both mitotic and meiotic spindle microtubules [74, 80, 81, 123] so it is not surprising that suppression of this kinase family has an impact on spindle organization. Early experiments in the sea urchin system revealed that SFKs inhibitors caused disruption of the spindle microtubules [124] which was interpreted to indicate a role in microtubule dynamics. Later work in the mouse oocyte revealed that chemical inhibition of SFKs and knockout of *fyn* caused disorganization of the MII spindle with loss of the metaphase plate structure, as well as frequent displacement of individual chromosomes from the spindle, and failure to maintain the spindle in close proximity to the oocyte cortex [91]. The phenotype resulting from SFK suppression

in oocytes appears more severe than that which occurs in somatic cells where chemical SFK suppression and triple *src*/*fyn*/*yes* (SYF cells) resulted in a reduced rate of spindle assembly and a high frequency of mis-oriented spindles, but loss of chromosomes from the spindle were not reported [125]. The fact that oocytes rely very heavily on SFK activity for the assembly and maintenance of the meiotic spindle is particularly significant since errors in spindle function during meiosis lead to chromosomal abnormalities that will impact the new embryo and subsequent generations. The question of whether SFK suppression of microtubule function is responsible for the observed high rate of pronuclear congression failure [64, 90, 124, 126, 127] remains open at present.

The potential role of SFKs in regulation of the cortical actin cytoskeleton was first demonstrated in *Xenopus* oocytes where constitutively active mutant forms of SRC and FYN were found to cause patches of enhanced actin filament density that co-localized with accumulation of phosphotyrosine-containing proteins and cortical pigment granules [92]. Later work in the mouse oocyte demonstrated the role of FYN kinase in maintenance of the polarity of the cortical filamentous actin layer with a thickened actin cap overlying the meiotic spindle and polarized distribution of cortical secretory granules and microvilli [90, 91]. Suppression of FYN activity by chemical inhibition or *fyn* knockout resulted in a less polarized actin layer, disorganized cortical granule distribution, failure to maintain the normally close relationship between the spindle and the actin layer, as well as displacement of chromosomes from the spindle as mentioned above. These changes in the filamentous cortical actin layer correlated well with the morphological defects exhibited by *fyn*-null oocytes (Fig. 3.4). Oocytes from *fyn*-null mice incorporated sperm at normal rates but exhibited reduced developmental competence which likely resulted from several mechanisms. For example, mouse oocytes normally maintain a microvilli-free zone over the MII spindle which is thought to reduce the possibility of gamete fusion near the site of second polar body emission. Suppression of SFK activity chemically or by *fyn* knockout allowed microvilli to form near the spindle and an increased frequency of gamete fusion near the MII spindle. This occasionally resulted in the sperm nucleus being expelled as another polar body as the cytokinesis machinery was unable to differentiate between maternal and paternal chromatin during polar body emission. In addition, SFK knockout caused disorganization of the cortical granules that normally undergo exocytosis at fertilization and subsequently establish a slow block to polyspermy as well as modifying the oocyte surface for subsequent development. Detachment of these secretory granules from the oocyte cortex likely impaired the post-fertilization wave of exocytosis although the significance of that for development is not understood at present. Other potential consequences of impaired cortical cytoskeletal structure could include disruption of the subcortical maternal complex important during post-transcriptional gene regulation prior to the maternal to zygotic transition [128] and reduced ability to establish cell–cell communication at compaction even when fertilized by wild-type sperm.

Another of the events that require SFK activity during the later stages of fertilization is pronuclear congression and entry into mitosis. Once meiosis has been completed and sperm incorporation is complete, the maternal and paternal nuclei

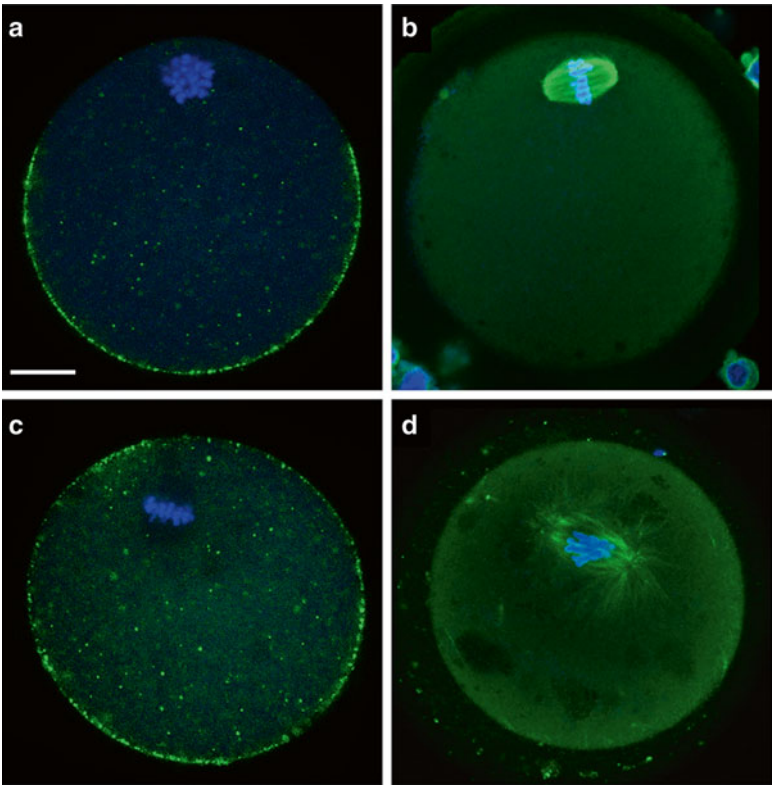

Fig. 3.4 Cytoskeletal changes in oocytes as a result of *fyn* knockout. Oocytes from wild type (**a**, **c**) and *fyn*-null mice (**b**, **d**) were labeled with FITC-tagged wheat germ agglutinin to demonstrate the distribution of cortical granules (**a**, **c**) or with an antibody to tubulin to label spindle components (**b**, **c**) and imaged by confocal immunofluorescence. Chromatin was labeled with ethidium homodimer (*blue*). Magnification is indicated by the *bar* which represents 10 μm. Images by Jinping Luo

become surrounded by nuclear envelopes forming the male and female pronuclei. These pronuclei enter S phase and proceed through DNA replication. After a short G2 interval, the pronuclear envelopes break down and both maternal and paternal chromosomes commingle for the first time, a process termed pronuclear congression. The chromosomes align on the mitotic spindle and the zygote enters mitosis marking the end of the fertilization process. The formation and disassembly of the pronuclear envelope is under control of MAPK [129]. Fertilization triggers reduction of both MPF and MAPK in the egg which is necessary to allow the pronuclear envelopes to form [130]. As DNA synthesis nears completion, MAPK and MPF activities increase again and the MAPK activity triggers pronuclear envelope breakdown while MPF activity promotes entry into mitosis. SFK inhibition by chemical inhibitors, dominant-negative constructs, or *fyn* knockout results in a high developmental failure rate at the late pronuclear stage [64, 90, 124, 127, 131].

The specific events that require SFK activity are unknown but include cell cycle control mechanisms that may require SFK activity. For example, SFK activity is stimulated at G2/M [97] and Src-family PTK activity is required for the *G2/M* transition in fibroblasts [96, 123]. The P62 GAP-associated protein associates with SRC via SH3 and SH2 domains and is the major SRC substrate at this point of the cell cycle [27, 132] although the relationship between p62 phosphorylation and pronuclear congression has not been demonstrated. The role of SFKs during pronuclear congression could be very important relative to ART procedures as failure of pronuclear congression is reported to account for 19.2 % of the failed oocytes in human IVF and 22.6 % in human ICSI procedures [133].

In summary, SFK signaling plays an important role in assembly and/or maintenance of the cortical actin layer and the meiotic spindle in oocytes from externally and internally fertilizing species. In addition, oocytes from externally fertilizing species make use of an SFK-mediated pathway to drive PLCγ-mediated IP3 production while mammalian oocytes rely instead on the sperm-borne PLZζ to initiate IP3 production [134]. We have proposed that this difference may reflect the need for very rapid establishment of the block to polyspermy during external fertilization. As mentioned above, SFK-induced PLCγ signaling often occurs in highly reactive cells such as platelets where a rapid, high amplitude calcium transient is used to trigger secretory granule release in response to some stimulus, a feature that oocytes share. Species that fertilize internally would likely have less need of such a rapid polyspermy block and could rely on the slower plasma membrane block to polyspermy [135] or the even slower zona reaction that follows cortical granule release in mammals.

Mechanism of SFK Activation at Fertilization

The above functional consequences of SFK signaling in oocytes highlight the obvious question of how these kinases are activated at different points during the response to fertilization. As described above, SFKs are activated in a multistep process that is initiated by removal of a phosphate from the C-terminal tyrosine by a phosphatase. However, the signals that trigger this dephosphorylation event and the phosphatase that accomplishes it may be different at different points in the oocyte life. The question is complicated by the fact that many other non-*src*-related PTKs are regulated by PTPases including Wee1 kinase which is regulated by Cdc25 to activate cyclin-dependent kinase [136]. In other, well-studied tissues, receptor-type PTPases such as CD45 [137], the leukocyte antigen receptor family (LAR) of PTPases, rPTPepsilon, and the orphan receptor phosphatase rPTPα are implicated in regulation of Fyn [138–140] and other SFKs. Cytosolic PTPases such as PTP1B [141] have been shown to regulate SFKs in many somatic cells.

The importance of PTPase activity in activation of SFK activity in the zebrafish oocyte was demonstrated with PTPase inhibitors that delayed the cortical reaction, cleavage, and FYN kinase activation [142]. The rPTPα phosphatase was observed to complex with FYN in zebrafish oocyte plasma membranes, but our subsequent unpublished studies indicate that rPTPα is not critical for successful fertilization in

that species. More functional information regarding the role of PTPases during fertilization has been developed in the *Caenorhabditis elegans* oocyte where the EGG3 phosphatase [143], EGG 4 and 5 [144, 145] have been shown to play a role in cortical actin cytoskeletal organization. These related phosphatases share a complex interdependence with EGG-4 and EGG-5 being required to properly coordinate redistribution of EGG-3 away from the cortex during meiotic anaphase I and are thought to link events during egg activation with the advancing cell cycle. This fertilization system has obvious advantages for the study of egg activation, but at present, the possible role of the EGG PTPases in PTK activation is entirely unknown.

An alternative mechanism for the activation of SFKs at fertilization makes use of Focal Adhesion Kinase which can complex with SFKs resulting in conformational changes in both kinases. This interaction occurs between phosphorylated tyrosine 397 of FAK and the SH2 domain of SFKs. This SH2 displacement mechanism forces the C-terminal negative regulatory domain of the SFK into the open configuration rendering it available for dephosphorylation. The end result is that the active configuration of the SFK is favored resulting in increased SFK activity [146, 147]. Interestingly, SFK activity in turn promotes the activity of FAK or PYK2 [148] leading to a reciprocal activation cascade involving both kinases. This mechanism may prove to be relevant to oocytes of externally fertilizing species where rapid activation of SFKs and the FAK family kinase PYK2 (PTK2b) occurs in response to fertilization [120]. In the zebrafish oocyte, PYK2 can be activated by IP3-mediated calcium release and it is possible that activated PYK2 could induce or accelerate SFK activation in the oocyte cortex. However, at present, there is no convincing data supporting any of the above mechanisms of SFK activation in fertilized oocytes.

Summary

The Src-family PTKs perform multiple functions in oocytes as they do in most highly specialized cell types. Oocytes express a characteristic subset of this protein kinase family including FYN, YES, and FGR with the other SFKs being underrepresented at the protein level. This array of kinases provides unique signaling capabilities that meet the requirements of this germ cell during meiotic maturation and fertilization. Oocytes from all species examined appear to require SFKs for establishment or maintenance of the cortical actin cytoskeleton structure as well as spindle organization and events at the G2/M transition. Oocytes that are fertilized externally also exhibit a requirement for SFKs during the fertilization-induced calcium transient which plays such an important role in rapidly establishing a permanent block to polyspermy. Since the ovulated oocyte must make the proper response to fertilization and carry out a pre-programmed egg activation process until the zygotic genome can direct operations, it must be prepared during maturation by preassembly of the signal transduction components needed to complete fertilization and egg activation. The SFKs must be properly expressed, undergo important post-translational modifications and protein–protein interactions, and be appropriately localized within the oocyte in order to prepare the oocyte for the responses to

fertilization which occur fairly rapidly even in mammalian oocytes. This state of readiness must then be maintained for some period of time until fertilization occurs. The development of Assisted Reproduction Techniques and their application to human health have presented us with a compelling need to understand how the oocyte signaling pathways can be maintained at a proper state of readiness in the culture environment, and the biochemistry of SFK regulation in oocytes is an important aspect of this question.

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4 Posttranslationally Modified Tubulins and Other Cytoskeletal Proteins: Their Role in Gametogenesis, Oocyte Maturation, Fertilization and Pre-implantation Embryo Development

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Abstract

The cytoskeleton, mainly consisting of microtubules, intermediate filaments and microfilaments, along with cytoskeleton associated and interconnecting proteins as well as the centrosome, plays enormously important roles in all stages of embryogenesis and undergoes significant changes to accommodate a diversity of cellular functions during gametogenesis, oocyte maturation, fertilization and pre-implantation embryo development. The varied functions of the cytoskeleton can be accomplished on many different levels, among which are a diversity of different posttranslational modifications (PTMs), chemical modifications that regulate activity, localization and interactions with other cellular molecules. PTMs of the cytoskeleton, including phosphorylation, glycosylation, ubiquitination, detyrosination/tyrosination, (poly)glutamylation and (poly)glycylation, acetylation, sumoylation, and palmitoylation, will be addressed in this chapter. Focus will be on (1) Microtubules, microtubule organizing centers (centrosomes), intermediate filaments, microfilaments and their PTMs; (2) Cytoskeletal functions and cytoskeletal PTMs during gametogenesis and oocyte maturation; and (3) Cytoskeletal functions and cytoskeletal PTMs during fertilization and pre-implantation embryo development.

Keywords

Posttranslational modifications • Cytoskeleton • Microtubules • Centrosomes • Meiosis • Sperm • Fertilization • Mitosis • Cell division • Embryo development

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Introduction

The cytoskeleton plays enormously important roles in all stages of embryogenesis and it undergoes significant changes to accommodate a diversity of cellular functions during gametogenesis, oocyte maturation, fertilization and pre-implantation embryo development. Three major components comprise the cytoskeleton that are discussed in more detail below and include microtubules, intermediate filaments and microfilaments. The present chapter will also include the centrosome, a major microtubule organizing center that plays an essential role in the organization of the interconnected cytoskeleton and significantly impacts structural and metabolic functions through its microtubule organizing capabilities. The varied functions of the cytoskeleton can be accomplished on many different levels, among which are a diversity of different posttranslational modifications (PTMs), chemical modifications that regulate activity, localization and interactions of cytoskeletal proteins with other cellular molecules. PTM modifications of the cytoskeleton include phosphorylation, glycosylation, ubiquitination, detyrosonation/tyrosination, (poly)glutamylation and (poly)glycylation, acetylation, sumoylation, and palmitoylation, among others, as will be discussed in more detail below. These modifications are important for regulated cellular functions and they also allow dynamic changes in response to different stimuli.

Cytoskeletal reorganization and remodeling occurs throughout embryo development and allows for the establishment of the germ cell lineage and the migration of primordial germ cells (PGCs) from the hindgut region to the genital ridges in the post-implantation embryo. Oocyte maturation depends on cytoskeletal functions for the formation of the meiotic spindle at the oocyte center and its migration to the oocyte periphery followed by meiosis I and meiosis II, to separate chromosomes and achieve haploidy in preparation for fertilization. The process of fertilization triggers a cascade of cytoskeletal reorganizations to form the sperm aster, zygote aster, mitotic apparatus and subsequent symmetric and asymmetric cell divisions, and cellular polarization in the developing pre-implantation embryos. These processes will be addressed in more detail in the specific sections below, with focus on (1) Microtubules, microtubule organizing centers (centrosomes), intermediate filaments, microfilaments and their PTMs; (2) Cytoskeletal functions and cytoskeletal PTMs during gametogenesis and oocyte maturation; and (3) Cytoskeletal functions and cytoskeletal PTMs during fertilization and pre-implantation embryo development.

Microtubules, Microtubule Organizing Centers (Centrosomes), Intermediate Filaments, Microfilaments and Their Posttranslational Modifications

As mentioned above, the cytoskeleton consists of a complex network of fibers primarily composed of three families of protein molecules that are assembled to form three main types of filaments: microtubules, intermediate filaments and microfilaments. Hundreds of accessory proteins link these filaments to each other as well as

to different cellular components that allow intra- and intercellular communications and signal transduction to fulfill specific cellular functions.

Microtubules are composed of α/β subunit heterodimers that typically are linked into 13 protofilaments to compose one single complete microtubule. The lateral association of protofilaments forms a cylindrical microtubule with an outer diameter of 25 nm. The number of protofilaments to form one microtubule may differ in different systems and the regulation of protofilament number may be linked to tubulin acetylation [1]. Various and different α and β-tubulin isotypes are expressed within one cell to form microtubules with clearly differentiated and specific cellular functions, although the isoforms may to some extent be functionally interchangeable.

Microtubules are polarized structures with a dynamic plus end and a minus end that can be stabilized by attachment to cellular structures such as microtubule organizing centers (MTOC; centrosome). Individual microtubules undergo highly dynamic changes within a cell which is referred to as 'dynamic instability', allowing for phases of growth and rapid depolymerization. In addition to α- and β-tubulin subunits, four more tubulins have been discovered in more recent years which are delta (Δ)-, epsilon (ε)-, zeta (ζ)- and eta (η)-tubulins [2], whose functions are linked to eukaryotic centrioles and/or basal bodies. The γ-tubulin is a member of the tubulin family whose functions include microtubule nucleation and organization; it is primarily found at MTOCs (centrosomes) but also can be localized to other cellular compartments such as the plasma membrane where microtubule nucleation can take place.

Microtubules are heterogeneous in length and fulfill a variety of different functions in cells either alone or by interacting with different cellular components. The plus-end directed microtubule motor protein kinesin and minus-end directed microtubule motor protein dynein are important for transport of cargo along microtubules to their functional destinations and therefore also play a role in cellular and intercellular signal transductions. The regulation of microtubule dynamics and stability includes participation of a heterogeneous group of numerous non-motor microtubule-associated proteins (MAPs) and microtubule-interacting molecules that provide additional functional microtubule diversity.

Microtubules are important for maintenance of cell shape, cellular transport of membrane vesicles, macromolecules and organelles such as mitochondria, for cell motility, meiosis, mitosis and cell division, and for the formation of centrioles, immotile primary cilia, and motile cilia and flagella.

Drugs that interfere with microtubule polymerization include colcemid, colchicine, nocodazole, podophyllotoxin, and griseofulvin, among others; the best-known drug that promotes tubulin polymerization and interferes with tubulin depolymerization is taxol [3–5], a drug that is used for cancer treatment, as it prevents depolymerization of mitotic microtubules, therefore preventing cancer cells from rapid cell division.

Biological structures that are distinctly enriched in microtubules include cilia, primary cilia, centrioles, the meiotic apparatus, and the mitotic apparatus that are briefly described as follows.

Cilia: Cilia or flagella are microtubule-based structures with a cytoskeletal core referred to as the axoneme that is important for sperm tail motility, for functions of

cells in the Fallopian tubes to move the egg from the ovary to the uterus, and for a diversity of ciliated cell functions throughout development. Motile cilia are found in all stages of embryo development and they are distinguished from non-motile primary cilia by their microtubule organizations and by the presence of dynein. Motile cilia are known for their 9 + 2 microtubule arrangement which refers to their composition of nine outer doublet microtubules and a central pair of single microtubules. Each outer microtubule doublet contains a complete A tubule and an incomplete B tubule that is fused with the complete A tubule.

Primary cilia: The primary cilium is a specialized non-motile cilium that protrudes as one single cilium from almost all cells in our body [6]. It originates from a basal body that develops from the mother (elder) centriole of the cell's centrosome complex and becomes coordinated with cell cycle regulation. Primary cilia contain 9 outer microtubule doublets but no central microtubule pair (9 + 0), and no dynein. These sensory cellular antennas with a receptor-rich membrane coordinate a large number of signaling pathways that are coupled with nuclear activation and cell division. A close relationship exists between primary cilia and the centrosome complex, as primary cilia undergo cell cycle-specific assembly and disassembly and share mother centriole components during this process. Typically, in the G1 stage, primary cilia are assembled when the distal end of the centrosome's mother centriole becomes reorganized to form the basal body for the primary cilium's axoneme that directly assembles onto the microtubules of the modified mother centriole. Primary cilia are disassembled at the entry into mitosis when fully matured centrioles become located at the mitotic poles; they are reassembled during exit from mitosis (reviewed in [7–10]). Primary cilia play important roles in embryo development and are critical for signal transduction pathways [11, 12]. Primary cilia dysfunction has been implicated in a variety of diseases and disorders (reviewed in [13]).

Centrioles: Centrioles are cylindrical structures composed of nine outer triplet microtubules, each consisting of a complete A, an incomplete B, and an incomplete C microtubule. Centrioles do not contain central microtubule pairs. In mitotic cells, a typical centriolar duplex consists of two centrioles representing mother and daughter centriole that are organized perpendicular to one another. The mother (older) centriole is structurally and functionally distinguished from the daughter (younger) centriole by characteristic distal and subdistal appendages. Both centrioles are connected through interconnecting fibers.

Meiotic apparatus: The meiotic apparatus (meiotic spindle) consists of acentriolar centrosome material, chromosomes, and two classes of microtubules that connect both meiotic spindle poles (pole-to-pole or interpolar microtubules) and those that connect one pole with the kinetochores of chromosomes (kinetochore microtubules). The meiotic apparatus is formed at the oocyte center during oocyte maturation, following germinal vesicle breakdown (GVBD). The fully formed spindle moves to the oocyte periphery to become the MI spindle followed by formation of the MII spindle. Two asymmetric cell divisions following meiosis I and meiosis II take place, resulting in two polar bodies and a haploid oocyte.

Mitotic apparatus: The mitotic apparatus is formed to separate chromosomes equally into two new daughter cells. A typical mitotic apparatus consists of centrosomes containing a pair of perpendicularly oriented centrioles, chromosomes, and two classes of microtubules (pole-to-pole or interpolar microtubules and kinetochore microtubules) that along with numerous accessory proteins provide the complete machinery to separate chromosomes into the dividing daughter cells. In the mouse preimplantation embryo, no centrioles have been observed during mitotic cell divisions up to the blastula stage, while in non-rodent mammalian systems, the sperm-derived centrioles are present during mitosis that participate in symmetric and asymmetric cell divisions throughout pre-implantation embryo development (reviewed in [7, 9]).

Microtubule Organizing Centers (*MTOCs; Centrosomes*): In addition to the above-mentioned microtubule-enriched structures, the microtubule organizing centers (MTOCs; centrosomes) are important for cytoskeletal coordination and functions (reviewed in more detail in [8, 9]). The functional units of MTOCs can have different molecular compositions that organize various microtubule formations and include spindles of the meiotic and mitotic spindle apparatus as well as cytoplasmic asters (reviewed in [7–9]). MTOCs may or may not contain centrioles. Most scientists in the field define MTOCs as structural units that contain γ-tubulin and numerous other proteins that are important for cell cycle-specific nucleation and organization of microtubules (reviewed in [14]). The best-studied MTOCs are the centriole-containing centrosomes in somatic cells that are composed of a large number of centrosomal proteins, with at least 60 of them being directly associated with the interphase centrosome structure, and numerous others that are associated with centrosomes to perform cell cycle-specific functions (reviewed in [14]). Details on centrosome structure and functions are not included in the present chapter but have been reviewed previously [7–9, 15–17].

Intermediate Filaments are ropelike fibers with a diameter of ~10 nm. These fibers are important for cellular structure and organization, serving as mechanical scaffolds in many capacities. They are composed of intermediate filament proteins that comprise a large and heterogeneous family of over 50 different proteins being subcategorized into six different types or classes in vertebrates. One type of intermediate filaments is organized into the nuclear lamina, a meshwork of filaments underlining the inner nuclear envelope. Other types play a role in intra- and intercellular communications and include vimentin and cytokeratin, among others (reviewed in [18]). Intermediate filaments are more stable to experimental treatments compared to microtubules and microfilaments.

Microfilaments (*actin filaments*) are composed of actin subunits that form two-stranded helical polymers resulting in filaments with a typical diameter of ~7–8 nm. Microfilaments are important for a great variety of cellular functions including cellular motility, membrane trafficking and shape changes, among numerous others. Actin filaments can be highly dynamic or they can be anchored, such as in muscle tissue. Cellular microfilaments can be organized in linear bundles, two-dimensional networks, and three-dimensional gels. Similar to microtubules, different actin isoforms allow different actin filament functions.

A large number of microfilament-associated and microfilament-interacting molecules have been identified that assure varied microfilament functions. Of the actin-based cell motility proteins, several have been well studied while others are still being investigated in numerous laboratories. The Arp2/3 (actin-related protein 2/3) complex is a well-known actin nucleation complex that is important for the formation of new actin filaments off the sides of existing microfilaments (reviewed by [19]).

Posttranslational Modifications of Cytoskeletal Proteins with a Focus on Tubulins

PTMs allow for significant diversity, complexity, and heterogeneity of gene products on qualitative and quantitative levels and for spatio-temporal control of protein activities and dynamics in biological processes. As mentioned above, PTMs modulate molecular interactions, protein localization, and protein stability which are important for cellular functions, while dysfunctions have been implicated in various diseases including cancers, diabetes, dysmetabolic syndrome, neurological disorders and others for which onset may be traced back to PTM disorders in early embryo development.

PTMs are chemical alterations to protein structure that frequently involve substrate-specific enzymes and may include more than 300 different types of modifications. Gene products can be modified in various combinations resulting in a large heterogeneity of the protein population. Several methods have been employed to determine PTMs including mass spectrometry, two-dimensional gel electrophoresis (2DE), immunological probes, and others (reviewed in [20]). The following will provide an overview of PTMs and will highlight PTMs of the cytoskeletal proteins including tubulins, intermediate filament proteins, actin, and centrosomes.

Of the PTMs referred to in the introduction, the most generally studied are phosphorylation, ubiquitination, and sumoylation [21–23], for which functions have been established, although a number of functions associated with these PTM changes for tubulin are not yet fully understood. The best understood PTMs for tubulin (microtubules) include detyrosination and the related Δ2 modification, (poly)glutamylation, (poly)glycylation, and acetylation [20, 21, 23, 24], which will be addressed below. Most of the PTMs occur on formed microtubules and provide specific characteristics for specific functions. In general, PTM takes place at amino acid side chains or peptide linkages with the majority of them being enzyme-mediated. PTMs can alter the functional capabilities, dynamics and biophysical properties of microtubules and their interacting molecules.

Protein phosphorylation most frequently occurs on serine, threonine or tyrosine residues and plays significant roles in the fine-regulation of cell cycle stages and in a number of different signal transduction pathways.

Ubiquitination is important for the degradation of proteins and plays a role in numerous specific cell cycle regulation events, as will be discussed below. Ubiquitination and acetylation are the best characterized lysine modifications of cytoskeletal proteins.

Glycosylation affects protein folding, distribution, stability, and activity.

Glutamylation and glycation refer to the addition of glutamate (glutamylation) or glycine (glycylation) residues onto glutamate residues in the C-terminal tails (CTTs) of both α-and β-tubulin [21, 24]. Glycylation is mainly associated with tubulin incorporated into axonemes (cilia and flagella) while glutamylation is seen in neuronal cells, centrioles, axonemes, and the mitotic spindle.

Glycylation and glutamylation can take place on either α-tubulin or β-tubulin, forming long or short side chains. These PTM modifications can occur on a single microtubule or on different microtubules, but the selectivity for different modification sites on tubulin tails or their spatial and temporal regulation is not yet known. Both PTMs can overlap and compete with each other for modification sites [25, 26].

Tubulin polyglutamylation and polyglycylation: Polyglutamylation refers to progressive addition of Glu residues onto the γ-carboxyl group of one or more Glu residues near the C-terminus of polymerized tubulin [27–29]. Hydrolysis of linear Glu chains removes polyglutamylation from tubulin and it also generates Δ2-tubulin from detyrosinated tubulin, as addressed below. Enzymes of the TTLL (tubulin Tyr ligase-like) family carry out the polyglycylation PTM of tubulin which plays a role in the dynamics and stability of axonemes. The TTLL enzyme family comprises glutamylating [30–32] and glycylating [25, 26, 33] enzymes.

Sumoylation refers to the reversible addition and removal of SUMO (small ubiquitin-related modifier) polypeptides on lysine residues that play a role in cell cycle regulation as well as in other cellular processes and in the DNA damage response (DDR). Sumoylation is associated with changes in stability and function of various cytoskeletal structures.

Palmitoylation: Tubulin has been shown to incorporate radioactively labeled [^{3}H] palmitate, predominantly on the α-subunit at cysteine 376. Palmitoylation has been studied in mutants of the budding yeast *Saccharomyces cerevisiae* in which mitosis occurred but aster microtubules showed defects and may have affected microtubule interactions with the cortex (reviewed in [21]).

Detyrosonation/tyrosination: *Detyrosination* of microtubule polymers is based on the removal of the gene-encoded C-terminal tyrosine of α-tubulin by a yet to be identified carboxypeptidase [21, 24, 34, 35]. The removal of the C-terminal Glu residue on detyrosinated tubulin results in Δ2-tubulin [36]. The Δ2-tubulin PTM is irreversible and catalyzed by deglutamylase enzymes of the CCP family [37]. Historically, detyrosinated tubulin was referred to as Glu-tubulin, and it was renamed to avoid confusion when glutamylated tubulin was discovered [27]. *Tyrosination* on the other hand involves the addition of a tyrosine residue to the C-terminal glutamate residue of α-tubulin within the soluble tubulin heterodimers that is catalyzed by tubulin tyrosine ligase (TTL) [21, 24]. Tyrosination refers to the enzymatic addition of Tyr to α-tubulin; the reversible tyrosination–detyrosination cycle is initiated by the removal of a Tyr functional group (detyrosination), whereas re-addition of Tyr (tyrosination) returns tubulin to its nascent state. The detyrosination/tyrosination PTM cycle plays a role in the recruitment of two types of microtubule-binding proteins, molecular motors and plus-end tracking proteins (+TIPs). For example, the microtubule motor protein kinesin-1 binds preferentially

to detyrosinated microtubules in neuronal cells [38] while kinesin selectively binds to detyrosinated tubulin, therefore allowing selective interactions with other cellular components such as vimentin ([39]; reviewed in [20]).

Acetylation is a reversible PTM that plays a major role in tubulin modifications and is also an important modification for cross-talk with other PTMs including phosphorylation, ubiquitination and methylation, all of which can modify the biological function of acetylated proteins [40].

Acetylation of Lys-40 on α-tubulin takes place on the luminal face of the microtubule polymer [41]; newer studies identified acetylation on Lys252 of β-tubulin that preferentially takes place on non-polymerized tubulin [42]. While the enzyme for acetylation has not yet been identified, two tubulin deacetylating enzymes are known: the histone deacetylase 6 (HDAC6) [43, 44] to reverse acetylation of Lys40, and sirtuin 2 (SIRT2) [45] which shows preferential activity towards a tubulin peptide substrate as compared to a histone peptide substrate.

So far, PTMs have mainly been described for α- and β-tubulins. Most α-tubulins are acetylated on the ε-amino group of a conserved lysine residue at position 40 in the N-terminus. Acetylation is frequently associated with stable microtubules as seen in axonemes, and it takes place after microtubule assembly; however, acetylation does not necessarily cause stabilization, which has been shown in several studies and includes the finding that the HDAC6 protein, itself, can inhibit microtubule growth [46]. PTMs on the other hand, can protect microtubules from severing enzyme activities such as that of Katanin and Spastin [47]; on the other hand, tubulin modification by detyrosination allows the microtubule-depolymerizing kinesins from the kinesin-13 family (mitotic centromere-associated kinesin (MCAK; also known as KIF2C) and KIF2A) to preferentially depolymerize the now tyrosinated microtubules [48]. The studies by Peris et al. [48] were the first to demonstrate a mechanism explaining how detyrosination can stabilize microtubules, as it was shown that tyrosinated microtubules are better substrates of depolymerizing kinesins.

It is also important that microtubule motor activity can be regulated by PTMs which may serve as an effective mechanism to spatially and temporally segregate a range of subcellular transport activities. For example, tubulin detyrosination regulates the binding and motor activity of the ubiquitous Kinesin-1 (the 'conventional' kinesin, KIF5) to microtubules [39, 49–51]. Polyglutamylation and polyglycylation may further play a role in the fine-tuning of microtubule motor activities (reviewed in [23]).

The above-mentioned studies and others show that PTMs create marks on microtubules that allow targeting of cytoskeletal structures for their destination during specific cellular functions.

The intermediate filament proteins vimentin and cytokeratin are acetylated on lysine residues which has been reported to destabilize the polymer [52, 53], thereby destabilizing intermediate filament functions, which differs from microtubule and microfilament acetylation.

In regard to microfilaments, six of the seven ARP2/3 complex subunits are acetylated on lysine residues [54]. A regulator of the ARP2/3 complex, cortactin, is

important for invadopodia functions [55]; it is acetylated along with the actin/ARP2/3-interacting proteins cofilin and coronin [54, 56]. Cortactin can be acetylated on nine different lysine residues, which results in decreased actin-binding capacity and decreased translocation to the periphery (reviewed in [57].

The three major isoforms of actin, α, β, and γ-actin all can be acetylated [54, 58]. Acetylation of γ-actin has been implicated in stabilization of stress fibers. Formins participate in the microfilament-mediated formation of the contractile ring at the end of mitosis which may involve ubiquitin-mediated degradation of formin to complete cell division.

Posttranslational Modifications of Centrosomes, Basal Bodies, Cilia and Flagella

As mentioned above, posttranslational modifications can define the location, interactions, and function of proteins which also holds true for centrosomal proteins and centrosome protein complexes.

While relatively little is known about PTMs in centrosomes, various data are available on some aspects that in part are related to the entire centriole-centrosome complex; a non-catalytic subunit of the TTLL1 complex, polyglutamylase complex subunit 1 (PGS1) [59], was specifically localized to the centrosome when overexpressed in cultured cells [60], perhaps implying a role for polyglutamylation in centrosome functions.

Several other TTLL enzymes have been associated with the centrosome complex and basal bodies in overexpression studies [32]. However, as mentioned above, centrosomes are communication centers for various cellular processes and it is not clear which enzymes are responsible for the modification of centrioles and which enzymes accumulate on the centrosome or basal body to assume other functions for related cellular activities such as the modification of the mitotic spindle or for cilia-related functions.

Perhaps the best-understood posttranslational modifications of centrosomes are phosphorylations that modulate the functions of centrosome proteins. Recent proteomic screens and computational analysis identified a variety of substrates for centrosome-associated kinases [61–63]. Phosphorylation-dependent functions of specific centrosome proteins have been described and include CPAP, the human homologue of Sas-4 involved in centriole duplication, which has been identified as a PLK2 substrate. The PLK2-phosphorylated CPAP localizes to procentrioles and plays a role in procentriole elongation [64]. Other posttranslational modifications include Sumoylation and ubiquitination that play important roles in regulating centrosome functions. Ubiquitination of the centrosomal protein CP110 during the G2 phase of cell cycle, and its subsequent degradation is required for centrosome and spindle integrity [65–72]. Several investigators have shown that ubiquitin-dependent proteolysis is important for centrosome duplication, procentriole formation and control of daughter centriole length [69, 70, 73].

The centriolar protein SAS6 is required in human cells for procentriole nucleation and formation which is regulated through the ubiquitin ligase APCCdh1 that targets SAS-6 for degradation [74]. The E3 ubiquitin ligase complex SCF-FBXW5 ubiquitinates SAS6 and is negatively regulated by the Polo kinase PLK4 [75]. Autophosphorylation of PLK4 results in ubiquitin and proteasome-dependent degradation of PLK4 which then causes a block of centriolar reduplication by releasing the activity of the SCF-FBXW5 complex. These molecular cascades use cycles of phosphorylation and proteolytic processes for centriolar replication [75].

In addition to phosphorylation and ubiquitination, Sumoylation of centrosomal proteins is important for regulation of the nuclear localization of centrin-2, which otherwise resides in mammalian centrosomes as core centrosomal protein [76]. While these data provide some insights into PTMs of centrosomes, this aspect of centrosome biology is only partially understood and more studies are required to better understand mechanisms, regulation, and functions of PTMs of centrosomal proteins.

Proteins of the NEK family are important for centrosome functions as well as for basal body, motile cilia, and primary cilia functions [77, 78]. NEK49, a NIMA (never in mitosis gene A)-related kinase localizes to the basal body and shows a decreasing gradient along the flagellum which correlates with the glutamylation profile of the axoneme. The human genome encodes at least 11 NEKS [79] and disruption of the *Nek1* gene in mice causes pleiotropic effects, including male sterility and polycystic kidney disease [80], which is caused by primary cilia dysfunction and potentially consistent with a role for NEK1 in tubulin modification.

Detyronisation is unique in motile cilia in that the central pairs of microtubules are mostly detyrosinated, while the B-tubule of the outer microtubule doublets is more heavily tyrosinated compared to the A-tubule which has been studied in *Chlamydomonas* flagella (reviewed in [23]). In axonemes, it has been shown that Δ2-tubulin is enriched on the B-tubule [81] which may indicate irreversible detyrosination to provide stability.

Acetylation is significantly enriched in axonemal microtubules [82, 83] but it is not clear whether or not it affects the assembly and function of cilia; this relates to other findings in mice lacking HDAC6 which did not affect axonemal functions such as male fertility. Nevertheless, developmental defects were seen in HDAC6-knockout mice which included accelerated bone growth that may be the result of dysfunctional signal transductions in primary cilia with increased acetylation. Functional studies [84, 85] revealed that αTAT1 (enzyme MEC-17) that carries out acetylation of Lys40 on α-tubulin decreased acetylation and slowed ciliary assembly, affecting primary cilia functions. Other strong enrichment of PTMs in cilia and flagella include polyglutamylation and polyglycylation [86].

PTMs may be developmentally regulated. For example, in *Drosophila melanogaster* testes, the assembly of sperm axonemes was not affected by depletion of a key glycylase while it was affected in later stages, in which loss of glycylation resulted in complete disassembly of sperm axonemes and total sterility of male flies [25].

While there are evolutionary differences in cilia, in most animal species polyglutamylation is required for ciliary assembly and functions and it may be involved in intraflagellar transport (IFT). Beating asymmetry in cilia was prevented in

Ttll1-knockout mice in which the levels of polyglutamylation on both α- and β-tubulin were reduced. Airway cilia and sperm flagella were most affected in these animals [87, 88].

Glutamylation and glycylation may allow different functions in axonemes and they may regulate each other. Glycylation allows structural stabilization while glutamylation is important for the regulation of beating behavior. Nearly complete loss of cilia was shown in zebrafish with double knockdown of the glycylase *Ttll3* and the polyglutamase *Ttll6*.

Centrioles and basal bodies are enriched in PTMs with the highest enrichment of detyrosinated [89], acetylated [90], and polyglutamylated tubulin and Δ2-tubulin [81]. Polyglutamylation is distinguished by long Glu side chains [89, 91]. In HeLa cells, injection of the glutamylation-specific antibody GT335 [92] resulted in disassembly of centrosomes [93], possibly as a result of centriole destabilization during the G2/M stage of cell cycle [94].

Posttranslational Modifications in Cycling Cells

In cultured cells, detyrosination [95], acetylation [90], and polyglutamination [91] are enriched during cell division in the central mitotic spindle and in the midbody, but not in astral mitotic microtubules. The PTMs shown in the central mitotic spindle may play a role in stabilizing kinetochore microtubules. Stabilization of microtubules by tyrosination may regulate the activity of the depolymerizing kinesin MCAK [48], which becomes important for chromosome segregation during anaphase [96] and may serve as regulator for chromosome segregation. Also, polyglutamylation induces enzymatic microtubule severing [97], thereby controlling the length of the mitotic spindle via katanin-mediated microtubule severing [98, 99]. Further, by controlling spastin-dependent severing [100], polyglutamylation may ensure timely abscission during cytokinesis.

Temporal and spatial control of tubulin PTMs may allow for specific functions of multiple microtubule-interacting proteins during cell division.

Cytoskeletal Functions and Cytoskeletal PTMs During Gametogenesis and Oocyte Maturation

The stages of male and female gametogenesis and the role of the cytoskeleton in these processes have been described in detail in previous papers and reviews [101–107], as well as in several chapters of this book, and are not specifically addressed in this section. Briefly, gametogenesis refers to a complex developmental process resulting in the production of male of female germ cells. During spermatogenesis, mature spermatozoa are produced in a well-organized sequence of events in which cell proliferation, meiosis and differentiation take place. To accomplish this process, the proliferating primordial germ cells (PGCs) migrate from their site of origin to the future gonad position to associate with somatic gonadal precursor cells for gonad formation.

PGCs then differentiate in a sex-specific manner while undergoing a distinct program of proliferation and quiescence. In the male genital ridge, the PGCs become enclosed by precursor somatic Sertoli cells followed by seminiferous cord formation during which PGCs and Sertoli cells form solid strands of cells which later become seminiferous tubules when the cords form a lumen. PGCs enclosed in seminiferous cords undergo morphological changes resulting in gonocytes that proliferate for several days before being arrested at the G0/G1 stage of cell cycle. In rats and mice, gonocytes resume proliferation within a few days after birth to give rise to spermatogonial stem cells (SSCs) which initiate the first round of spermatogenesis to produce spermatozoa at the onset of reproductive age [108].

Centrioles and centrosomes undergo important developmental processes during gametogenesis. As reviewed in detail by Manandhar et al. [109] and Sun and Schatten [110], when spermatids transform into mature spermatozoa, most of the centrosomal material is lost. However, the proximal centriole is completely retained and becomes localized close to the nucleus to perform critically important functions after fertilization, as will be discussed in section "Cytoskeletal Functions and Cytoskeletal PTMs During Fertilization and Pre-implantation Embryo Development". The distal centriole becomes partially reduced, and it becomes associated with the sperm axoneme in the midpiece and tail after being restructured to lose the triplet microtubule organization while forming a central pair of microtubule doublets, as is characteristic for the axoneme.

Of all the microtubule-containing structures that play a role in spermiogenesis, we mainly have data on the transient caudal manchette and on the stable axoneme (reviewed in [111]). Both structures contain PTMs but only the PTMs of the axoneme have been studied in detail.

Oocyte development begins during fetal growth and is completed in the adult. During oogenesis, centrioles that are present in oogonia until the pachytene stage of oogenesis [112] (reviewed in [109]) become lost, and the mature oocyte is devoid of centrioles in most species. However, reduced amounts of centrosomal components are present in the cytoplasm (reviewed in [109]) that can be visualized in parthenogenetically activated oocytes [15, 16, 113] and become associated with the sperm centriole after fertilization.

Mammalian oocytes are arrested in diakinesis of meiotic prophase I at birth and are located within the primordial follicle pool. A large nucleus (germinal vesicle; GV) forms during follicle growth. Stimulation by gonadotrophins induces GV-breakdown (GVBD) and meiotic resumption followed by the first meiotic cell cycle stages of prometaphase I, metaphase I, anaphase I, and telophase I to accomplish chromosome separation and extrusion of the first polar body (PB1). The second meiotic cell cycle continues up to the metaphase II stage (MII), at which the oocyte becomes arrested until fertilization or parthenogenetic activation takes place. The MII oocyte is the end result of a complex process of oocyte maturation during which the oocyte becomes fertilization-competent and achieves developmental potential. In most species except in the mouse, the MII spindle is localized perpendicular to the cell surface, and it displays a barrel-shaped to pointed spindle morphology (it is parallel to the egg surface in the mouse which represents one of the

features that are different in mouse oocytes compared to other non-rodent mammalian oocytes). The MII spindle takes a special place in the reproductive cell cycle as this is the stage at which fertilization occurs in most mammalian species. Although it appears static in immunofluorescence and transmission electron microscopy (TEM) images, the MII spindle is a highly dynamic structure whose integrity is actively maintained by a complex set of regulatory kinases and other regulatory proteins. Loss of spindle integrity includes alterations in regulatory kinases as has been shown in aging oocytes (reviewed in [114, 115]). Failure in MII spindle function can lead to aneuploidy and subsequent cell and developmental abnormalities resulting in abortion, disease, or developmental defects (reviewed in [114–116]).

Posttranscriptional regulation of pre-existing maternal mRNA and posttranslational modification of proteins is critical for meiotic progression from the primordial stage to the zygote and several key proteins such as CDK1/cyclin B are posttranslationally modified to precisely control meiotic progression. Others include the kinases PKA, AKT, MAPK, Aurora A, CaMKII, the phosphatases CDC5, CDK14s and others that participate in the meiotic process. The posttranscriptional and posttranslational modifications of proteins other than cytoskeletal proteins have recently been reviewed for mouse oocytes by Kang and Han [117] and are not addressed in this chapter that is focused on cytoskeletal proteins.

Compared to our knowledge of PTMs in somatic cells, the information for cytoskeletal PTMs in germ cells is still sparse despite the fact that microtubule organization and centrosome functions are among the most important processes for oocyte maturation with consequences for successful fertilization and embryo development. In this section, cytoskeletal functions will be correlated to their known PTMs keeping in mind that the information on PTM-related cytoskeletal functions is incomplete, as only sporadic data are available for selected aspects. While the best data for PTMs come from studies of mature sperm tails, only scarce data are available for maturing oocytes from the GV stage to the formation of the central, MI, and MII meiotic spindles.

The mechanisms for spindle formation and migration in different systems have previously been reviewed in detail [8, 116, 118, 119] and are not specifically addressed here. Significant reorganization of the microtubule network takes place during oocyte maturation which is shown in Fig. 4.1, highlighting microtubule organization and centrosome formation at the meiotic spindle poles that are critical for accurate chromosome segregation. Two asymmetric divisions take place in oocytes after first and second meiosis, when the first and second polar bodies are extruded, respectively, to remove half of the chromosome complement and excess centrosomal material.

The acentriolar centrosome formation at the two meiotic spindle poles includes participation of the centrosomal proteins γ-tubulin, pericentrin, centrin, and the nuclear mitotic apparatus protein, NuMA. The specific participation of these proteins may differ in different systems on both qualitative and quantitative levels. The acentriolar centrosomes play important roles in microtubule stabilization and in maintenance of functional meiotic spindles. As mentioned above, spindle integrity is lost in aging oocytes which includes centrosome and microtubule instabilities,

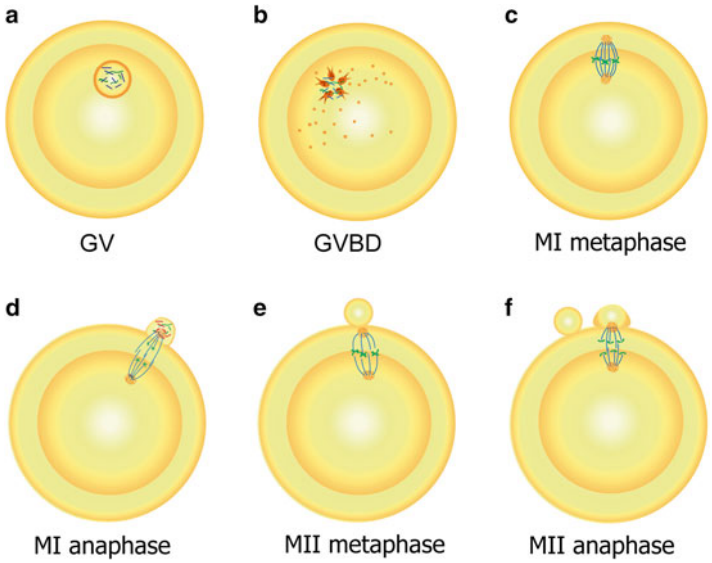

Fig. 4.1 Schematic diagram of oocyte maturation stages. (**a**): Germinal vesicle (GV) stage; (**b**) germinal vesicle breakdown (GVBD); (**c**) first meiotic spindle in metaphase; (**d**) first meiotic spindle in anaphase and first polar body extrusion; (**e**) meiosis II (MII) metaphase II spindle; (**f**) anaphase II spindle with extruded second polar body. *Blue* = microtubules; *green* = chromatin/chromosomes; *red* = centrosomal components

with consequences for chromosomal mis-segregation resulting in female infertility and developmental abnormalities. It is not known whether PTMs are altered in microtubules of aging oocytes.

While we do not yet have a complete understanding of PTMs in MII oocytes, some data have emerged in recent years that have mainly been generated in the mouse system. In the unfertilized mouse oocyte, it was shown that the acetylated form of α-tubulin is predominantly localized at the poles of the arrested MII spindle, as detected with a monoclonal antibody to acetylated α-tubulin and analyzed with immunofluorescence and immuno high-voltage electron microscopy (immuno HVEM) using colloidal gold [120]. Microtubules in the cytoplasmic cytasters that are present in the unfertilized mouse oocyte [121, 122] were not acetylated. The meiotic spindle became labeled with acetylated α-tubulin at meiotic anaphase; by telophase and during second polar body formation, the acetylated α-tubulin was only detected at the meiotic midbody. After perturbing microtubule dynamics with cold, colcemid, or griseofulvin treatment, the remaining stable meiotic spindle microtubules showed positive staining for acetylated α-tubulin; however, taxol stabilization of microtubules did not alter tubulin acetylation patterns. These results show that acetylated microtubules are present during meiosis and display a cell-cycle-specific pattern of acetylation, with acetylated microtubules associated with microtubules at the centrosomal

area in meiotic metaphase, an increase in spindle microtubule acetylation at anaphase, and selective deacetylation at telophase with acetylated microtubules in the midbody during the asymmetric meiotic cell division prior to polar body extrusion.

Cytoskeletal Functions and Cytoskeletal PTMs During Fertilization and Pre-implantation Embryo Development

Preimplantation embryo development includes all stages from fertilization to implantation and it is a well-orchestrated program that includes symmetric and asymmetric cell divisions, morula and blastocyst formation. Preimplantation embryo development is regulated genetically, epigenetically and posttranslationally.

In mammals, exit from MII arrest and meiotic resumption is typically achieved by the fertilizing spermatozoon, which evokes in the oocyte a cascade of calcium signaling followed by significant cytoskeletal reorganization and remodeling. During non-rodent mammalian (including human) fertilization, the spermatozoon contributes the proximal centriole as major microtubule nucleating and organizing center that is important for the aggregation of oocyte centrosomal components and the enlargement of the sperm aster into the zygote aster and mitotic apparatus. The components involved in sperm centrosomal functions have previously been reviewed in detail [7, 15, 16, 109] and will not be discussed in the present chapter. For successful fertilization and sperm aster organization, the sperm-derived centriole must first disengage from the sperm tail connecting piece; release of the proximal centriole is facilitated by sperm (and oocyte) proteasomes [123], which then allows rapid nucleation and accurate formation of microtubules into sperm aster, zygote aster and mitotic apparatus; failure in accurate centrosome, and microtubule organization can lead to infertility or to disorders that are manifested later in life (reviewed in [15, 16]). Centriole duplication occurs during the pronuclear stage (Fig. 4.2) but our knowledge of PTMs during these fertilization stages is sparse. We also still do not fully understand how the centriole-centrosome complex becomes duplicated during the pronuclear stage, although we understand from centriole duplication in some model organisms and in somatic cells that duplication of this complex is under cytoplasmic control and driven by cyclin-dependent kinase 2 (CDK2) complexed with cyclin E or cyclin A (reviewed by [124]).

The sperm centrioles duplicate during the pronuclear stage (in subsequent cell cycles during the G1/S phases) and starts with procentriole growth from the existing centriole, a process termed semiconservative centriole duplication. The procentriole grows into the daughter centriole that is oriented perpendicular to the older (mother) centriole, resulting in two pairs of centrioles that also indicate duplication of centrosomal material. The duplicated centrioles separate and migrate around the zygote nucleus to form the opposite poles of the first mitotic spindle.

Remodeling of the centrosome complex is critically important for proper development. While the centriole complex itself does not change its structure during development this complex has the capability to attract centrosomal material that surrounds the centriole complex and allows centrosomal plasticity throughout

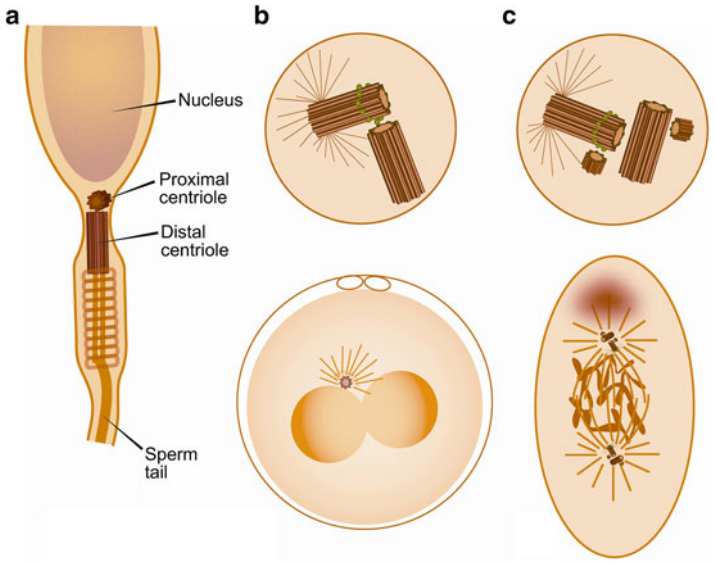

Fig. 4.2 (**a**) Schematic diagram representing spermatozoa in non-rodent mammalian species displaying nucleus, proximal and distal centrioles, and sperm tail; (**b**) while the distal centriole deteriorates, the proximal centriole serves as microtubule organizing center (MTOC) to form the sperm aster during the pronuclear stage after fertilization; (**c**) formation of the procentrioles from the older centrioles that form the mitotic poles and organize the mitotic apparatus

development for embryo-specific functions. Phosphorylation and other posttranslational modifications are important aspects in centrosome regulation required for cell cycle-specific changes (reviewed in [125, 126]).

In most systems, the non-membrane bound centrosome organelle of ~1 μm in size (Fig. 4.3) consists of a large number of centrosomal proteins embedded in a centrosomal matrix that typically surround a pair of perpendicularly oriented cylindrical centrioles; however, it is important to note that centrioles are not present in the centrosome complex during mitosis and cell divisions in mouse embryos up to the blastula stage. Details on mouse and non-rodent mammalian centrosomes have been presented in several recent review papers [7–9, 14–17] and are not included in the present chapter.

Centrosome functions play major roles in cell cycle regulation (reviewed in [14]), symmetric and asymmetric cell divisions, in cellular differentiation, in stem cell maintenance and stem cell differentiation, and in embryo development. Centrosomal composition varies in different cell cycle stages, in order to perform cell cycle-specific functions including controlling the length and amount of microtubule organization. The centrosome complex plays a major role during preimplantation embryo development. Through its microtubule organizing capabilities, the centrosome facilitates many cellular activities including cell motility, polarity, maintenance of cell shape, cell division, transport of vesicles, distribution of cell fate determinants, and targeting of a variety of signaling molecules.

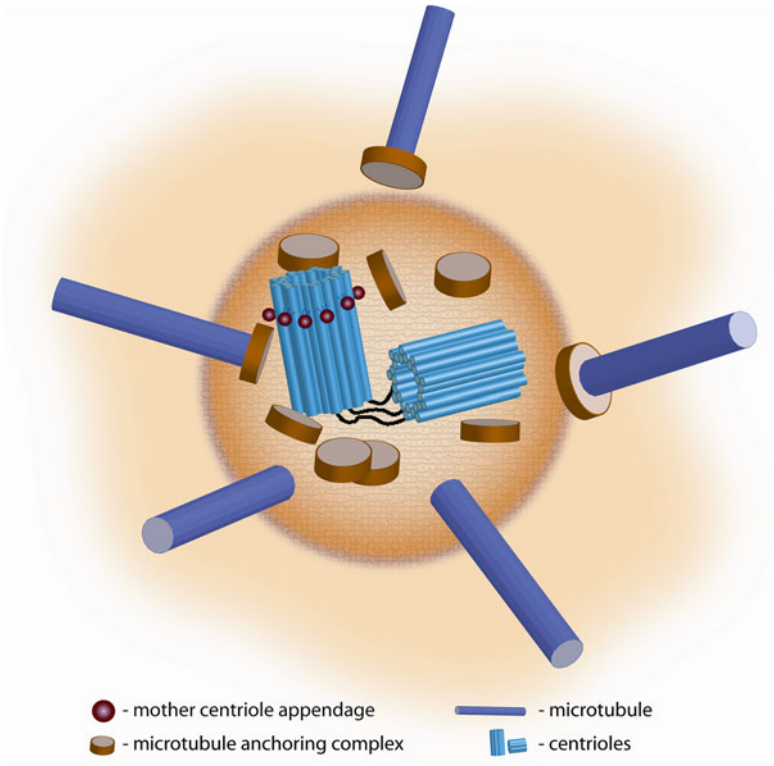

Fig. 4.3 Schematic diagram of a typical somatic cell centrosome composed of two centrioles, termed mother and daughter centrioles, that are connected to each other by interconnecting fibers and surrounded by centrosomal material (also termed pericentriolar material; PCM). The mother centriole is distinguished from the daughter centriole by distal (shown) and subdistal (not shown) appendages. Microtubules are nucleated by the gamma-tubulin ring complex (γ-TuRC) and accessory proteins, and anchored at their minus ends by the microtubule anchoring complex within the centrosomal core structure. Microtubule growth is regulated by distal plus-end addition of tubulin subunits

Differential accumulation of cellular components is critical for proper development and assures targeted distribution of cellular components during subsequent cell divisions. It includes cytoplasmic factors such as transcripts of developmental genes, which allows for cell type-specific gene activity.

In the developing embryo, centrosome duplication occurs during the S phase and is tightly synchronized with DNA replication. After duplication during each embryonic cell cycle, centrosomes separate toward the opposite poles, establishing the bipolar mitotic apparatus that contributes to cellular differentiation. During each cell cycle, the maturation of interphase centrosomes into mitotic centrosomes includes acquisition of mitosis-specific centrosome proteins such as the Nuclear Mitotic Apparatus protein (NUMA) that moves out of the nucleus during the cell's exit from interphase and associates with the centrosomal core structure during

mitosis. At this stage, centrosomal material can separate symmetrically or asymmetrically. Molecular centrosome asymmetry will have consequences for microtubule organization and transport of cargo, generating unequal inheritance of cell fate altering molecules in different cells during subsequent development.

Most of our knowledge about centrosomes comes from somatic cells and other studies derived from the *Drosophila* or *C. elegans* model systems, both of which may provide information applicable to some extent to mitotic cells during preimplantation embryo development in mammals, which has not been studied extensively.

Several studies have focused on specific aspects of PTM by acetylation and tyrosination during preimplantation embryo development. It was shown that α-tubulin in microtubules of mouse embryos is acetylated in a specific spatial and temporal sequence during preimplantation embryo development [120]. Furthermore, the sperm axoneme retains its acetylation after incorporation while interphase, oocyte-derived microtubules are not detected with an antibody to the acetylated form of α-tubulin. Similar to meiosis, results for mitosis show the presence of acetylated mitotic microtubules and demonstrate a cell-cycle-specific pattern of tubulin acetylation, with acetylated microtubules found at the centrosomes at metaphase, an increase in spindle labeling at anaphase, and the selective deacetylation of all but midbody microtubules at telophase. First mitosis follows a pattern similar to that observed during second meiosis, as described in section "Cytoskeletal Functions and Cytoskeletal PTMs During Gametogenesis and Oocyte Maturation"; only the mitotic midbodies are acetylated and no other acetylated microtubules are detectable in the interphase daughter cells. As described for meiosis in section "Cytoskeletal Functions and Cytoskeletal PTMs During Gametogenesis and Oocyte Maturation", after treatment with cold, colcemid, or griseofulvin, the remaining stable microtubules showed staining for acetylated microtubules (Fig. 4.4).

The first embryonic cell cycle is completed when the zygote chromosomes become separated during first mitosis. Centrosomes organize microtubules equally in both spindle halves during mitosis and up until recently it was also thought that they receive equal amounts of centrosome material. However, newer studies show that cellular asymmetry can already occur when centrosomal material becomes distributed unequally or when it becomes differentially modified [127]. Differences in centrosome quantity and quality in the two dividing daughter cells may set up the pattern for differentiation and polarization of the preimplantation embryo.

Stages of preimplantation development have been explored in great detail in mouse embryos, relative to gene expression patterns and specific phases of development with phase I spanning fertilization to the 2-cell stage; phase II spanning the 4-cell to the 8-cell stage; and phase III spanning the 8-cell embryo to the blastocyst stage.

As mentioned above, the mouse preimplantation embryo does not contain centrioles and the formation of the preimplantation mitotic and division stages is acentriolar (reviewed in [7–9, 116]), which is an important difference compared to other mammalian systems including humans.

Preimplantation development has also been studied in vivo and in vitro in several suitable animal models that do have sperm-derived centrioles in all stages of embryo

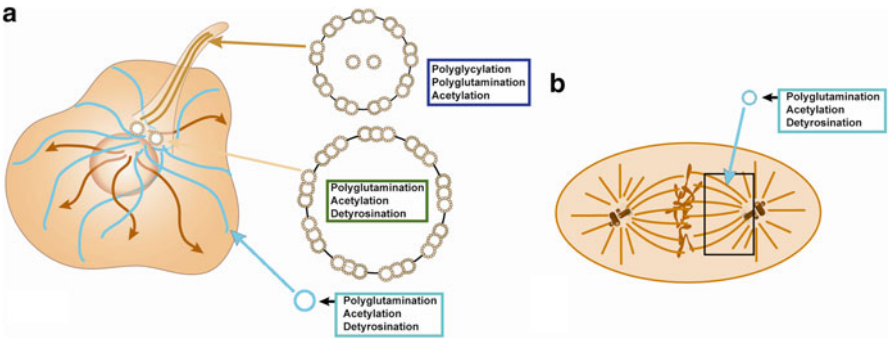

Fig. 4.4 Localization of microtubule structures and PTMs in typical mammalian somatic cells. (**a**) In interphase cells, dynamic (more labile; *orange*) and stable microtubules (*blue*) are present. While dynamic microtubules lack PTMs, stable microtubules accumulate PTMs in a cell cycle-specific progression pattern as described in the text. Microtubules in motile cilia contain PTMs displaying high accumulation on the B tubule of the outer microtubule doublets. While motile cilia shown here contain the 9 + 2 arrangement of nine doublet microtubules with dynein arms surrounding a central pair, primary cilia (not shown) lack the central pair of microtubules (9 + 0) and such primary non-motile cilia do not contain dynein. PTMs are also accumulated in centriolar/basal body triplet microtubules. (**b**) In mitotic cells, spindle microtubules (kinetochore and/or interpolar) show a high amount of PTMs, while astral microtubules are mostly unmodified. The centrioles within the mitotic centrosomes at the spindle poles display PTMs as described in the text and are distinguished by high levels of polyglutamylation. Differentiated microtubule structures, such as centrioles, cilia and flagella are highly polyglutamylated, acetylated and detyrosinated, and show specific accumulation of Δ2-tubulin. Glycylation is highly specific for cilia and flagella, and can appear as monoglycylation and polyglycylation. Combinations of different PTMs play a role in the control of stability and movement of cilia and flagella. Modified from [20]

development, including sheep, bovine and porcine embryos, and human embryos that had been discarded because of poor embryo quality but provided valuable research material.

In all mammalian species studied so far, the first preimplantation development stages are characterized by a synchronous doubling of cell numbers until the 8-cell stage as shown in the representative diagram in Fig. 4.5. Asynchronous cell divisions take place after morula compaction. At the 8- to 16-cell stage, the embryo starts developing into a blastocyst, at which time the first events of cellular differentiation are observed. At the blastocyst stage, the embryo hatches from the zona pellucida and implants in the uterus.

Several changes are associated with the development of the preimplantation embryo and include changes in protein synthesis and changes in energy requirements that correlate with morphological changes [128, 129], resulting in compaction that clearly indicates cellular differentiation.

Species-specific differences are observed during these stages in the mouse and other mammalian species. In the mouse, development from the 1-cell stage to the blastocyst stage containing 32 or more cells takes about three and a half days, with the first cleavage from one into two cells taking 16–20 h and the second cleavage from 2 to 4-cell stage taking 18–22 h. Embryonic genome activation occurs during

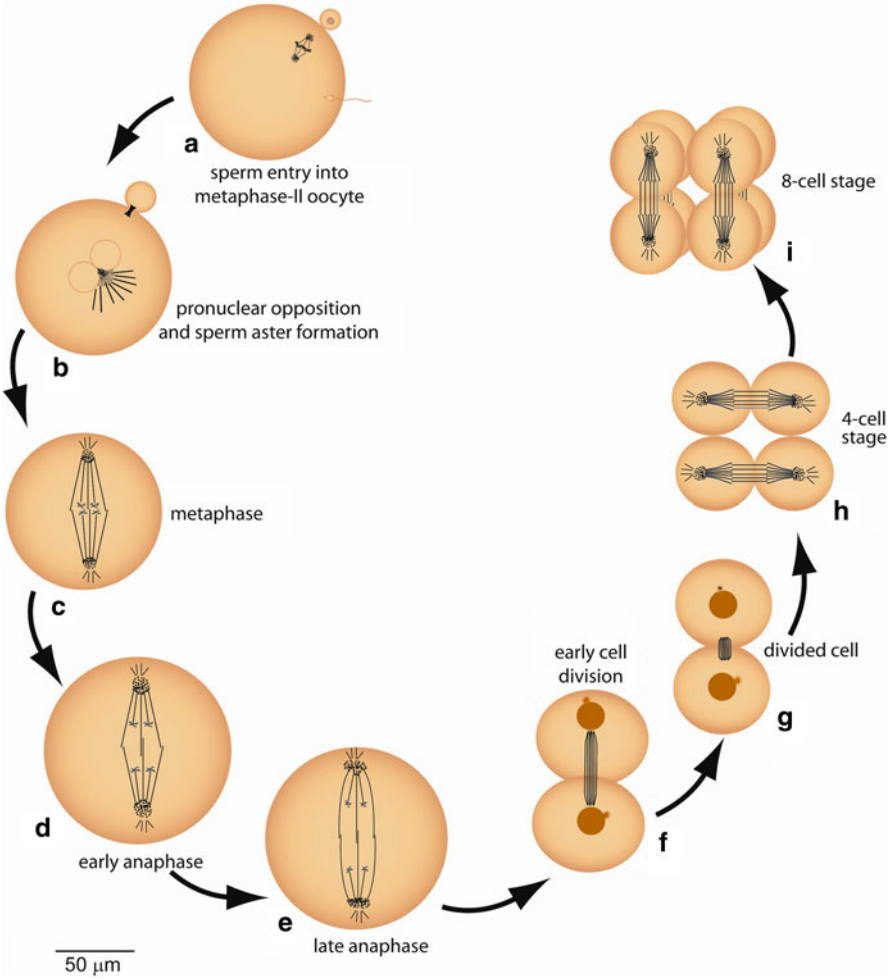

Fig. 4.5 Schematic diagram of early preimplantation development from fertilization to the 8 cell stage. Spermatozoon enters the MII-stage oocyte (**a**), followed by pronuclear apposition (**b**), metaphase (**c**), early anaphase (**d**), late anaphase (**e**), early cell division (**f**), divided, 2 cell stage (**g**), 4-cell stage (**h**), and 8-cell stage (**i**). PTMs take place in different stages and include different types of modifications in different subcellular microtubule organizations. Subcellular distribution of tubulin PTMs takes place in the MII spindle and in mitotic spindles as described in the text for developing embryo cells during the first cell cycles. Subsequent preimplantation PTMs are likely to follow those described for somatic cells although specific analysis for embryo cells is not yet available except for limited data for TE and ICM cells. We know from somatic cells that in undifferentiated cells, PTM modification levels are low in interphase as depicted in Fig. 4.3; detyrosination (Δ1), acetylation on Lys40 (Ac) and glutamylation are increased at the inner mitotic spindle and on the midbody that also shows a slight increase in polyglutamylation. The high levels of polyglutamylation of centrioles have been linked to centrosomal stability

a lengthened cell cycle from the 2- to 4-cell stage in the mouse. During the earliest developmental changes, mouse embryos are under post-transcriptional maternal control, relying on changes in the translation of mRNAs synthesized during oocyte growth, and/or post-translational protein modifications. New transcription takes place during the late 4- and 8-cell stage to prepare the embryo for compaction. During the morula-blastocyst transition stage, an increase in the rate of protein synthesis is observed leading to the formation of the inner cell mass (ICM) and trophectoderm (TE) at the blastocyst stage.

As indicated above, compaction indicates the first morphological changes in the preimplantation embryo when cellular differentiation takes place. During this stage, two distinct cell populations are produced. The blastomeres remaining in contact with the outside of the preimplantation embryo will differentiate into the trophectodermal (TE) lineage while the blastomeres differentiating inside the embryo will form the inner cell mass (ICM). Cellular polarization takes place during the 8-, 16- and 32-cell stages, with specific cells changing their morphological and functional phenotype to a polarized phenotype. Specifically, divisions of the 8-cell embryo will result in an average of 9 cells located on the outside and 7 cells located on the inside of the developing embryo. The outer cells become polarized and larger while the inner cells remain apolar. The polarization process of the outer cells is apparent by the basal migration of the nucleus and the apical accumulation of actin, clathrin, endosomes and microvilli.

While we do not have complete information on PTMs during these stages, we have some information obtained on mouse preimplantation embryos and we do have information on the shift in PTMs during polarization in somatic cells.

As has been reported by Houliston and Maro [130], posttranslational modification of distinct microtubule subpopulations takes place during cell polarization and differentiation in the mouse preimplantation embryo. These studies showed that during the process of cellular differentiation (the time of compaction) into trophectoderm cells and inner cell mass, tyrosinated α-tubulin was detected by immunofluorescence microscopy in subsets of microtubules within and between cells. All microtubules contained tyrosinated α-tubulin but acetylated α-tubulin was only detected in a subpopulation located predominantly at the cell cortices. It was shown that during development one population of cytoplasmic microtubules containing tyrosinated α-tubulin redistributed toward the apex of cells during the 8-cell stage, while at the same time a population of cortical microtubules displaying acetylated α-tubulin accumulated near the intercellular contact zone at the basal part of the cell. This finding indicates differential microtubule dynamics between apical and basal regions during this stage of preimplantation development. Such a pattern of microtubule PTM was also seen during the 16-cell stage. Cortical microtubules were preferentially acetylated in the cortex which correlates well with the PTM pattern described for polarized epithelial cells [131], discussed below. The inside cells of the developing embryos contained more acetylated microtubules than the outside cells. Most acetylated microtubules could be depolymerized by nocodazole but some remained in the outside cells confirming that acetylation does not necessarily relate to microtubule stability.

As mentioned above, while the trophectodermal cells are polar, the ICM cells are adhesive and compact. During blastocyst formation, the trophectodermal cells acquire characteristics of epithelial cells and may display PTMs as described below for polarization of tissue culture cells. However, we do not yet have detailed information on the sequential acquisition of PTMs that play a role in the polarization process during preimplantation embryo development.

Most of our knowledge regarding preimplantation development comes from the mouse and many of the events that occur during mouse preimplantation development have also been observed in other mammalian embryos. Nevertheless, it is important to point out that significant species-specific differences exist, with major differences in the duration of cell cycle stages and timing of specific events. Common to most mammalian species are the synchronous cell divisions for the first few cell cycles followed by asynchronous divisions, typically after the 8-cell stage. Significant differences exist during the blastocyst stage when various embryos undergo long periods of blastocyst expansion which is especially characteristic for farm animals, including the sheep and pig in which increases in cell number and size take place [128, 129]. Differences are also significant in the timing of embryonic genome activation.

In porcine embryos, pre-implantation embryonic development has been described in detail [128, 132] including stages of blastula formation and cellular differentiation into trophectoderm and inner cell mass. In porcine embryos ICM, formation takes place on day 5 and continues with differentiation into epiblast and hypoblast at around the time when the embryo hatches from the zona pellucida [133, 134]. ICM and TE cell lineages are vital and essential for embryonic and fetal survival. TE cells and ICM-derived extra-embryonic membranes form the fetal placenta during later development.

The preimplantation stages and cell cycle timing has been reviewed by Niakan et al. [129] and time-lapse imaging studies have provided excellent data on dynamic behavior of human embryos during the first week of in vitro development [135]. These studies showed that human embryos undergo cytokinesis within 14.3 ± 6.0 min and complete second division within 11.1 ± 2.2 h after completion of first cytokinesis. In humans, the implantation occurs at ca. day 7 of development.

PTMs during cellular polarization: Using immunocytochemistry and immunoblotting techniques in somatic cells, Quinones et al. [131] reported that PTM of tubulin undergoes a switch from detyrosination to acetylation as epithelial cells become polarized. By using Madin-Darby Canine Kidney (MDCK) epithelial cells, the authors showed that the composition and distribution of modified microtubules change as the cells undergo morphogenesis associated with polarization. They showed that two-dimensionally spreading cells contain more detyrosinated microtubules with orientations toward the leading edge. In contrast, three-dimensionally polarized cells contain more acetylated microtubules that are oriented toward the apical domain, which correlates well with the data reported for polarizing cells in the developing mouse embryo [130]. While the functional aspects of these modifications await further clarification, it may be assumed that such modifications allow

directional transport of cargo along microtubules. Data by these authors also revealed that microtubules are not necessarily acetylated along their entire length but that only short segments of microtubules may be acetylated rather than the entire microtubule. Polyglutamylation was seen stochastically on tubulin of most microtubules but further studies are needed to characterize functional aspects correlated to this observation. Interestingly, when the authors used nocodazole to depolymerize microtubules, they found that in subconfluent cells, detyrosinated microtubules initially appeared more stable compared to acetylated microtubules; after treatment for one hour, few acetylated microtubules remained while detyrosinated microtubules were still present. Based on these results, the authors propose that there are different classes of stable microtubules that are affected differently by drug treatment. Corresponding results were also obtained for confluent cells in which a small number of acetylated microtubules persisted after treatment with nocodazole for one hour while no polyglutamylated microtubules were resistant to drug treatment. In polarized cells, more microtubules were resistant to drug treatment, with most of them being acetylated. Detyrosinated and polyglutamylated tubulin was mostly detected in primary cilia. Taken together, the authors were able to demonstrate that there are different degrees of microtubule stability which may not directly correlate to PTMs, but PTMs are important for functional diversity in polarized cells. Further studies by Zink et al. [136] using depletion and overexpression approaches revealed that tubulin detyrosination promotes monolayer formation and apical trafficking in epithelial cells. These studies also identified alternating stretches of detyrosinated and tyrosinated tubulin. In detyrosination-depleted cells, premature polarization of cells could be induced. The authors propose that the detyrosinated tubulin-enriched microtubules may serve as cytoskeletal tracks to guide membrane cargo in polarized MDCK cells. These recent findings are most informative as to the possible functions of PTMs in cellular polarization and may be extended to preimplantation embryo in which cellular polarization is crucial for successful development. It may also be useful for studies on embryonic stem cells and their differentiation into various tissues.

Conclusions and Perspectives

The present chapter has provided an overview of our current knowledge on cytoskeletal PTMs during gametogenesis, oocyte maturation, fertilization and preembryo development with focus on the microtubule cytoskeleton. The importance of PTMs in cytoskeletal functions has been well recognized, and significant new information has been gained by studying PTMs in neuronal cells and in a variety of somatic cell systems. However, data on reproductive system cells are still incomplete and further investigations are needed, particularly since the cytoskeleton plays critically important roles in fertilization, symmetric and asymmetric cell divisions, stem cell maintenance and differentiation, cellular polarization, cilia and primary cilia formation, and various other aspects that are important for maintaining and reorganizing cell and tissue architecture to accommodate a diversity of cellular functions during embryo development. Investigations of specific cytoskeletal PTMs

during fertilization and all subsequent stages of embryo development are important to address the role of cytoskeletal PTMs in allowing functional differences of this complex network of interconnected fibers. PTM abnormalities may be among the underlying reasons for cytoskeletal dysfunctions with consequences for infertility and developmental abnormalities.

While not yet included in the present chapter it should be noted that septins have been called a fourth novel unconventional component of the cytoskeleton [137]. Septins are a family of proteins that can form non-polar filaments or rings and can interact with the actin and microtubule cytoskeleton. Septins play a role in cytokinesis by recruiting different proteins to the contractile ring; by doing so they are important for cell division and for budding in yeast as is known for *Saccharomyces cerevisiae* in which septins had originally been discovered. In mouse oocytes, it has been shown that Septin2 is posttranslationally modified by SUMOylation and required for chromosome congression [138]. Septin 1 is required for spindle assembly and chromosome congression [139] and Septin 7 is required for orderly meiosis [140]. These studies have opened up an important new area for further investigations into septin functions and PTMs during fertilization and cell divisions throughout embryo development when septin may play a role in cellular polarization.

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Deubiquitinating Enzymes in Oocyte Maturation, Fertilization and Preimplantation Embryo Development

5

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Abstract

Post-translational modifications of cellular proteins by ubiquitin and ubiquitin-like protein modifiers are important regulatory events involved in diverse aspects of gamete and embryo physiology including oocyte maturation, fertilization and development of embryos to term. Deubiquitinating enzymes (DUBs) regulate proteolysis by reversing ubiquitination, which targets proteins to the 26S proteasome. The ubiquitin C-terminal hydrolases (UCHs) comprise are DUBs that play a role in the removal of multi-ubiquitin chains. We review here the roles of UCHs in oocytes maturation, fertilization and development in mouse, bovine, porcine and rhesus monkeys. Oocyte UCHs contributes to fertilization and embryogenesis by regulating the physiology of the oocyte and blastomere cortex as well as oocyte spindle. Lack of UCHs in embryos reduces fertilization, while mutant embryos fail to undergo compaction and blastocyst formation. In addition to advancing our understanding of reproductive process, research on the role of

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deubiquitinating enzymes will allow us to better understand and treat human infertility, and to optimize reproductive performance in agriculturally important livestock species.

Keywords
Oocyte • Sperm • Fertilization • Embryo • UCHL1 • UCHL3 • Ubiquitin • Proteasome

Introduction

The multicellular organism proteome is more complex than its genome. In humans, scientists estimate that the genome contains between 20,000 and 25,000 genes [1]. The total number of proteins and protein variants in the human proteome is estimated at over one million [2]. Genomic recombination, transcription initiation at alternative promoters, differential transcription termination, and alternative splicing of the transcript are mechanisms that generate different mRNA transcripts from a single gene [3]. In addition, post-translational protein modifications (PTMs) play key roles in protein function by regulating activity, localization and interactions with other proteins, nucleic acids, lipids, and cofactors. The protein dynamics and changes are usually a result of a response to stimuli [4].

The proteome is in a dynamic state of synthesis and degradation. During proteolysis, the peptide bonds that link amino acids are hydrolyzed, and free amino acids are released [5]. The protease-enzymes are responsible for protein degradation. During proteolysis, the energy invested in the synthesis of the peptide bond is released. Distinct proteolytic mechanisms serve different physiological requirements and allow the organism to accommodate to changing environmental and pathophysiological conditions.

Proteins are divided between extracellular and intracellular compartments, and the paths of degradation differ for the two. The intracellular substrate-specific degradation of a protein may be achieved in two ways—proteolysis in lysosomes, or an ubiquitin-dependent process that targets unwanted proteins to the 26S proteasome. Lysosome deals primarily with extracellular proteins (e.g. plasma proteins, that are taken into the cell, by endocytosis; cell-surface membrane proteins that are used in receptor-mediated endocytosis, and the proteins (and other macromolecules) engulfed by autophagosomes); while ubiquitin–proteasome system deal primarily with endogenous proteins; that is, proteins that were synthesized within the cell. The autophagy–lysosomal pathway is normally a non-selective process, but it may become selective upon starvation, when proteins with the peptide sequence KFERQ or similar are selectively degraded [6, 7]. The lysosome contains a large number of cysteine proteases such as cathepsins, and aspartic or serine proteinase families of hydrolytic enzymes [8–10]. During this process, the extracellular proteins are never exposed to the intracellular environment (the cytosol) and remain “extracellular” (topologically). Degradation of proteins in lysosomes is not specific, and all engulfed proteins exposed to lysosomal proteases are degraded at approximately the same rate.

A specialized branch of lysosomal proteolysis, autophagy, is responsible for the engulfment of protein aggregates and even whole organelles in the autophagophore, which subsequently fuses with a lysosome to form an autophagosome. Contrary to lysosomal degradation, autophagy may have a certain degree of substrate specificity conveyed by its interactions with the ubiquitin–proteasome system [11].

Several observations led to the prediction that degradation of intracellular proteins must be carried out by a dedicated, substrate-specific mechanism [12]. The process is highly substrate-specific, and different proteins half-lives vary from a few minutes to several days. Selective ubiquitin-mediated protein degradation fulfills these criteria. Proteins marked for degradation are covalently linked to ubiquitin (UB), a small chaperone protein capable of reversible, yet stable covalent binding to other proteins [5]. Multiple molecules of ubiquitin may be linked in tandem to a protein destined for degradation. These "polyubiquitinated" proteins are targeted to an ATP-dependent protease complex; the 26S proteasome where the ubiquitin moiety is released and reused, while the targeted protein is degraded. In this chapter, we will discuss the roles of post-translational modification of proteins in oocyte maturation and fertilization by protein ubiquitination and protein turnover by ubiquitin–proteasome system. We will also discuss situations where these processes are disrupted, resulting in abnormal oocytes that are deficient in supporting embryo development.

Protein Ubiquitination and Ubiquitin–Proteasome System

Ubiquitination is a covalent posttranslational modification of proteins that controls a wide range of physiological processes including but not limited to protein degradation, cell cycle progression and transcriptional regulation [13–15], Proteins are conjugated to ubiquitin and ubiquitin-like proteins. Ubiquitination regulates the interactions of proteins with other macromolecules protein complexes, for example binding to the proteasome or recruitment to chromatin.

Ubiquitin (UB) is a small protein (8.5-kDa polypeptide) consisting of 76 amino acids that attaches itself to the substrate proteins through a covalent bond between the glycine at the C-terminus of UB and the side chains of lysine in target proteins. Single UB molecules can be conjugated to the lysine of these proteins (monoubiquitination), or more commonly, multi-UB chains can be attached (polyubiqutination). Ubiquitin-protein conjugation requires ATP hydrolysis. Polyubiquitinated proteins are recognized by the 26S proteasome, a multi-subunit holoenzyme that catalyzes the degradation of the ubiquitinated proteins and the recycling of ubiquitin. This last process is mediated by ubiquitin recycling enzymes called the deubiquitinating enzymes (DUBs). The number and conformation of appended ubiquitin molecules determine the fate of the target protein. Ubiquitination is a reversible process, and deubiquitination plays an important role in regulating ubiquitin-dependent pathways [16].

Ubiquitin is attached to other proteins by a series of four enzymatic activities (Fig. 5.1). Ubiquitin molecules are activated by ubiquitin activating enzymes (E1 or

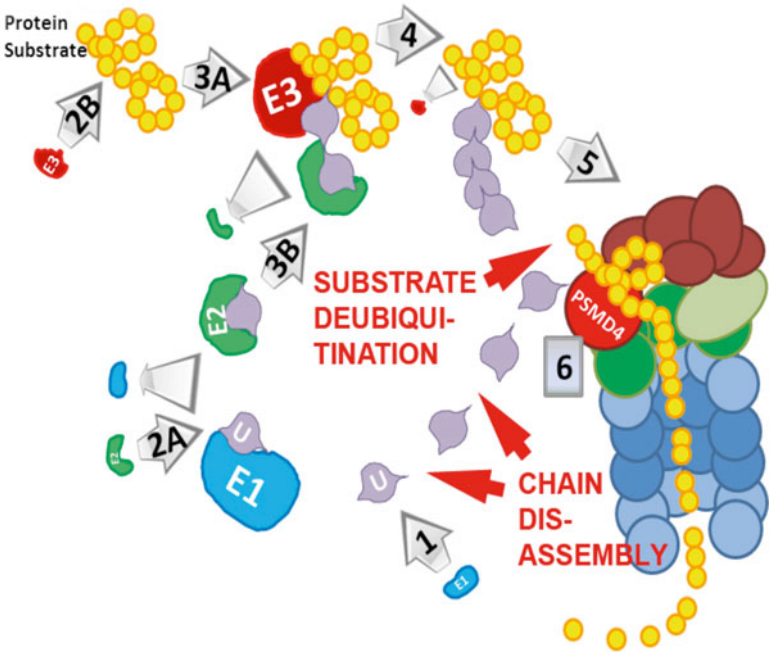

Fig. 5.1 Deubiquitination (CAP. RED) is essential for at least two different steps of UPS-dependent protein recycling, i.e. during substrate deubiquitination by deubiquitinating enzymes (DUBs) associates with proteasomal cap/regulatory complex, and during the disassembly of liberated or nascent multi-ubiquitin chains into single UBB molecules that can re-enter the protein degradation process. Steps of protein recycling by UPS: **1**: Enzyme UBE1 (E1) activates ubiquitin. **2A**: UBE1 is supplanted by ubiquitin carrier E2. **2B**: Substrate protein is flanked by an E3-type ubiquitin ligase. **3A**: E3 covalently links activated ubiquitin to substrate protein. **3B**: Second activated ubiquitin is linked to substrate bound ubiquitin. **4**: Ensuing tandem ligation of additional activated ubiquitins produces a multi-ubiquitin chain. **5**: Multi-ubiquitin chain is recognized and engaged by subunit PSMD4 of the 26S proteasome. STEP **6**: Substrate protein is deubiquitinated (liberated ubiquitin molecules re-enter the cycle) and broken down into small peptides (adapted from [25] with permission of Bioscientifica)

UBA1 enzymes) and transferred to ubiquitin conjugating enzymes (E2 or UBE2-enzymes). From there, the ubiquitin is transferred to the substrate, which is recognized by ubiquitin protein ligase enzymes (E3 or UBE3 enzymes). Depending on the type of E3 enzyme, the activated ubiquitin molecule is transferred either from the E2 to the E3 and then to the substrate, or directly from the E2 to the substrate (reviewed by Weissman [17]). At times, an additional enzyme, called an E4 (UB chain elongation enzyme), may become involved in this reaction, resulting in long polyubiquitin chains forming on substrate proteins [18]. There are at least four classes of E3 ligases: HECT-type, RING-type, PHD-type, and U-box containing type [19]. The E3 ligases are regulated during protein ubiquitination, but balance in the UB system is also achieved through a set of deubiquitinating isopeptidases that

cleave UB off the substrates [12, 20, 21]. The selectivity of UB conjugation is determined by specific degradation signals (degrons) in short-lived proteins, or the N-end rule that determines protein-half life through a specific set of amino acid residues near the N-terminus of substrate protein [22–24].

Polyubiquitination provides a signal for proteasomal degradation. The 26S proteasome is a large multiprotein complex with three proteolytic active sites sequestered inside a barrel-like core 20S complex capped on one or both ends with a 19S regulatory particle. The 19S particle recognizes and engages the multi-ubiquitin chain attached to a protein destined for proteasomal degradation [25]. The multi-ubiquitin chain is removed by the 19S subunits with deubiquitinating activity or by extrinsic deubiquitinating enzymes that can associate with the 19S complex. Then, the substrate protein is unfolded and translocated to the 20S core. As the primed protein enters the 20S core, it is broken into small peptides that are released from the 20S core (Fig. 5.1).

Deubiquitinating Enzymes

Deubiquitinating enzymes (DUBs) comprise more than 100 enzymes in humans that cleave ubiquitin from substrate proteins or from other ubiquitin molecules [26–29]. DUBs are classified into five families, four of which are thiol proteases including the ubiquitin-C-terminal hydrolases (UCHs, ubiquitin-specific proteases (USPs), ovarian-tumor (OTU) domain DUBs and Machado-Joseph domain (MJD) DUBs. The DUBs of the fifth family contain a Jab1/MPN metalloenzyme (JAMM) domain and act as zinc-dependent metalloproteases (reviewed by Wilkinson [16]). The large number of gene families, each with multiple members, suggests that selective pressure to evolve such catalysts has occurred multiple times. In addition, this diversity implies that considerable substrate specificity exists. This finding supports the assumption that the mutation, deletion or down-regulation of specific DUBs induces very limited and specific cellular responses, phenotypes or pathologies [30, 31]. For example, the mutation or deletion of the major neuronal DUB in mammals, the ubiquitin C-terminal hydrolase L1 (UCHL1), causes a localized axonal dystrophy but few other overt effects [32].

Few DUB substrates have been identified but recent studies have revealed the importance of DUBs and their regulation by transcriptional, protein–protein interactions and post-translational modifications [33]. DUB activities include:

1. Ubiquitin precursor processing: Following transcription from tandem-translated transcripts encoded by multi-repeat ubiquitin genes, ubiquitin is processed from its linear polyprotein precursor to a free mono-ubiquitin by DUBs with ubiquitin hydrolase activities [34].
2. Ubiquitin recycling: Isopeptide-linked ubiquitin is removed from substrate prior to substrate degradation by the 26S proteasome to recycle free ubiquitin. Once removed from the substrate, unanchored multi-ubiquitin chains can be further processed into free mono-ubiquitin [35, 36].

3. Regulation of substrate degradation: DUBs can antagonize E3 ubiquitin ligase function by either directly deubiquitinating a substrate or by modifying E3 activity and/or abundance [37–39].
4. Regulation of substrate activity: DUBs can regulate substrate activity by removing monoubiquitin or non-degradative polyubiquitin from substrate [40].
5. Ubiquitin chain editing: Mixed polyubiquitin chains can be edited by DUBs or DUBs can remove one chain linkage type prior to elongation of second chain [41, 42].
6. Regulation of DUB activity: DUB-binding partners can regulate DUBs by either recruiting the DUB to substrate or by modulating the catalytic properties of the substrate-bound DUB [43, 44].

Ubiquitin C Terminal Hydrolases (UCH'S)

Ubiquitin (UB) C-terminal hydrolases (UCHL's) belong to a larger group of enzymes called deubiquitinases. They catalyze the hydrolysis of the peptide or isopeptide bond through which UB is attached to other proteins or other UB moieties in monoubiquitin or polyubiquitin chains to generate the ubiquitin monomer [16, 45].

UCHL1 is a 223-amino acid, primarily neuronal DUB that is involved in several neurodegenerative diseases such as Parkinson disease, Alzheimer, Lewy body disease, and other diseases such as cystic fibrosis and cancer [46]. UCHL1, through its hydrolase activity, is responsible for generating free monomeric ubiquitin from precursor poly-ubiquitin chains [47] and associated with mono-ubiquitin to inhibit its degradation. This stabilizes and maintains ubiquitin levels [48]. UCHL1 is one of the most abundant proteins in mammalian oocytes [49, 50] and comprises 2 % of brain proteins [51, 52]. UCHL1 accumulates in the oocyte cortex. The overall structure of UCHL1 is very similar to that of UCHL3, which has a similar size and shares 51 % of sequence identity [53]. Despite the similarities between UCHL1 and UCHL3, the expression pattern of these enzymes is tissue and cell-type specific. Unlike UCHL1, UCHL3 is uniformly expressed in all tissues, including brain; in mammalian MII oocytes, UCHL1 is localized in the cortex (plasma membrane) and UCHL3 associates with the spindle [54–57]. This may suggest distinct sets of in vivo substrates. The in vitro hydrolytic activities of the two enzymes are very different: UCHL3 is >700-fold more active toward a model ubiquitin substrate than UCHL1, whereas UCHL1 has an in vitro ligase activity correlated with dimerization of the enzyme [58].

Evolutionarily conserved localization of UCHL1 in the oocyte cortex and UCHL3 in the meiotic spindle (identical in human, monkey, mouse, pig and cow ovum (Fig. 5.2) suggests the nature of their respective substrates. UCHL1 is most likely to regulate the cortical microfilament cytoskeleton responsible for cortical granule (CG) maturation and PB1 extrusion during oocyte maturation, for sperm incorporation in the oocyte cytoplasm during fertilization, and for morula compaction during preimplantation embryo development. Localized in the meiotic spindle, UCHL3 is predicted to control the function of spindle microtubules, but consideration should also be given to effects on the centromere and chromosomes.

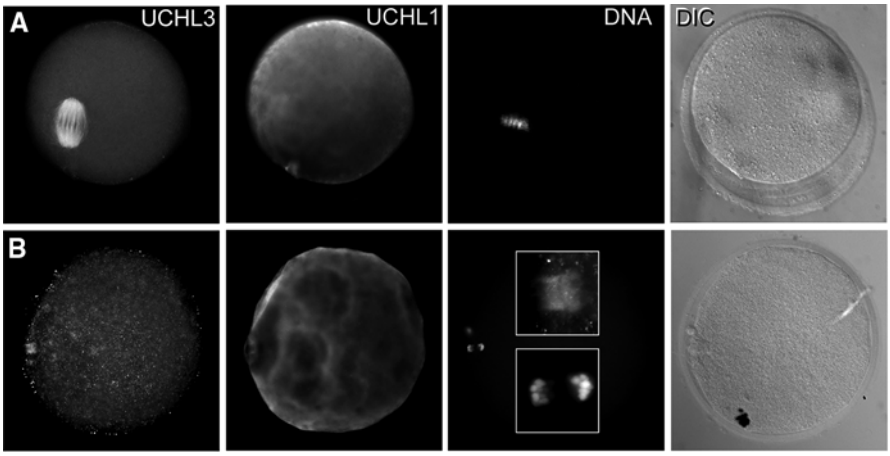

Fig. 5.2 Cortical localization of UCHL1 and meiotic spindle-association of UCHL3 proteins in the oocytes of mouse (**a**; metaphase II) and rhesus monkey (**b**; telophase I). Oocyte DNA was counterstained with DAPI and parforcal transmitted light images recorded using differential interference contrast (DIC) optics. *Insets* in DNA panel of **b** shown detail of oocyte chromosomes labeled with DAPI (*left*) and UCHL3 immunolabeling (*right*) in the same rhesus monkey spindle (previously unpublished data)

Essential Role of UCHs in Oocyte Maturation

Oocytes provide half of the nuclear genetic material-and endowing the embryo with nearly all membrane and cytoplasmic determinants that are required for development (reviewed in [59, 60]). Regulated protein degradation is believed to be essential for oocyte maturation [55, 61–64]. Metabolic cooperation occurs between the oocyte and somatic cells ensures metabolite availability to the growing oocyte (reviewed in [60]). UCHs have a complimentary distribution in porcine, bovine, and murine oocytes, with UCHL1 accumulation in the oocyte cortex, and UCHL3 association with oocyte spindle [55, 56, 63, 65]. Application of a small molecule UCHL3-inhibitor to the mouse oocyte-cumulus complexes (OCC) cultured in vitro reduces the expansion of cumulus cells compared to control (Unpublished Data). Cumulus cells support oocyte maturation and control the access of spermatozoa to the oocyte during fertilization. Reduction of expansion of cumulus cells, which can be inhibited by proteasomal inhibitors during oocyte maturation [66], may have impact on proper and complete nuclear and cytoplasmic maturation. Among other mechanisms, UPS is involved in the regulation of cumulus extracellular matrix deposition and steroidogenesis during cumulus expansion in the domestic pig [67].

Inhibiting UCH activity also leads to excessively large polar bodies (PB), indicating a disruption in spindle localization or a dysfunction of microfilament cytoskeleton in the oocyte cortex ([63], Fig. 5.3). The meiotic spindle in the maturing oocyte is asymmetrically located to enable extrusion of a polar body and maximal

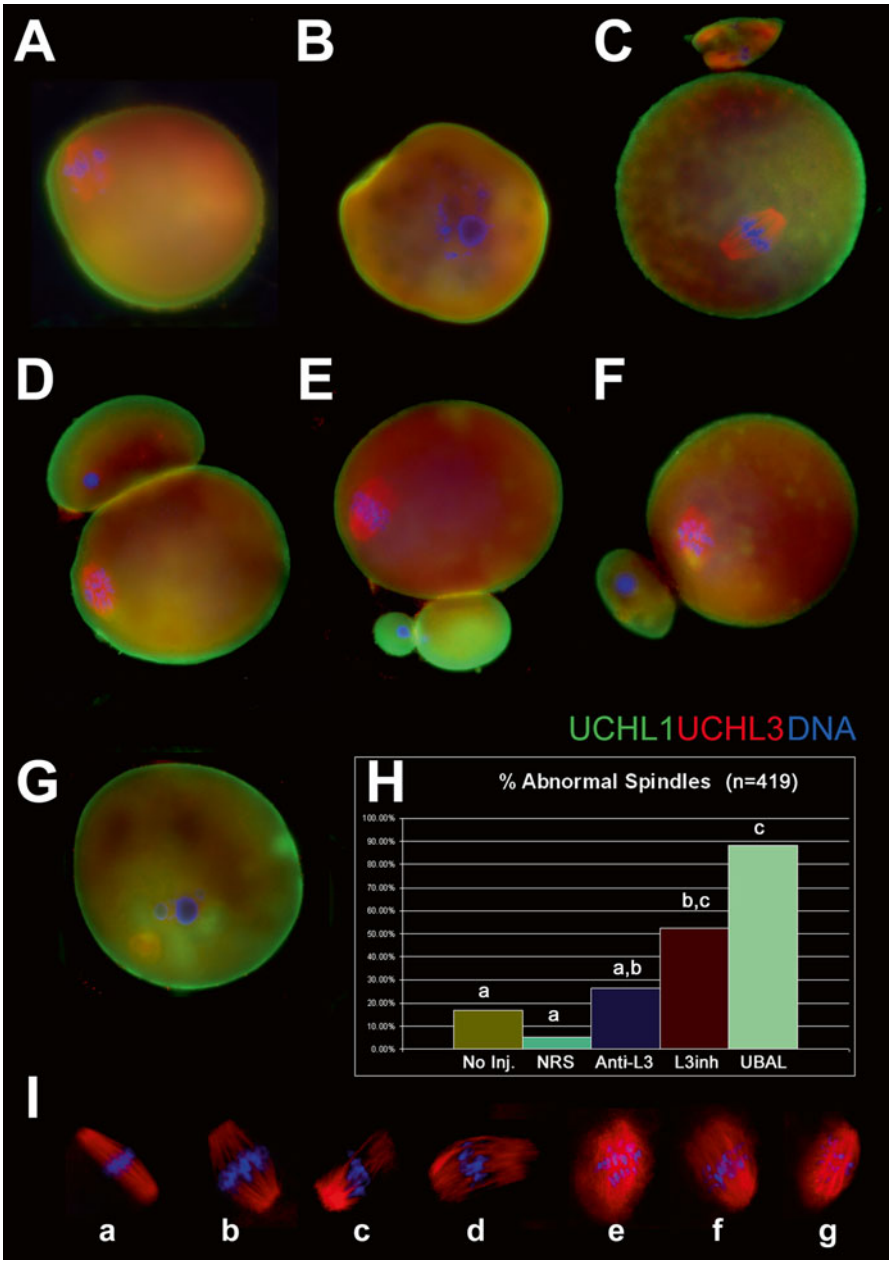

Fig. 5.3 Mouse oocyte maturation is affected by the inhibition of oocyte ubiquitin C-terminal hydrolases. Oocytes were labeled with antibodies against UCHL1 (*green*) and UCHL3 (*red*); DNA was counterstained with DAPI. **A**, **B**: Oocyte matured in the presence of UCHL3 inhibitor showed increased incidence of abnormal metaphase-II spindles (**A**; note uneven chromosome distribution and wide spindle poles) or a failure to complete GVBD (**B**; a large nucleolus precursor body typical of a GV-stage oocyte is present). **C**: Control oocyte with well-focused spindle poles and a first

retention of ooplasm to maximize oocyte developmental competence [68, 69]. Excessive allocation of cytoplasm and organelles to a polar body will negatively affect fertilization and preimplantation embryo development (Fig. 5.3). PB extrusion in the mouse is controlled by spindle microtubules and cortical actin microfilaments [56, 70–74]. Putative factors controlling F-actin assembly during oocyte meiosis include microfilament associated proteins formin-2 (FMN2) and myosins [75, 76], as well as ADP-ribosylation factor 1 (ARF1 [77]). There is no observed gross alteration of cortical microfilaments in mouse oocytes subjected to UCH-inhibition [63]. But the UCHs, and particularly oocyte-cortical UCHL1, may regulate the activity or turnover of the microfilament-regulating proteins. Examples of actomyosin turnover by UPS are well known [78] and the formin-2-related proteins such as DIAPH3 are degraded by UPP during cell division [79]. Proteasomal inhibitors increase poly-ADP ribosylation [80], which is dependent on ARF1 and other ARFs acting as stimulators of ADP-ribosyltransferase [81]. ADP ribosylation regulates many cellular processes such as DNA repair, apoptosis and is involved in cell signaling interestingly, expression of the mutated *Arf1* gene (Arf1T31N) causes mouse oocytes to cleave symmetrically instead of extruding the first PB during meiosis [77]. This resembles the effects of UCH inhibition seen in our oocyte studies [63]. At least one of the ARF proteins, ARF6 appears to be regulated by ubiquitination [82], which could be reversed by UCHs. UCHL1 and related UCHs may regulate actin and myosin containing microfilaments during polar body extrusion. Microfilament-controlled cytokinsesis, cleavage-furrow formation and cell polarity establishment in the early embryos of *Caenorhabditis elegans* are controlled by ubiquitin C-terminal hydrolase CYK3 [83]. The CYK-3 protein contains UCH domains homologous to mammalian ubiquitin-specific proteases USP11 and USP32. Completion of the first meiotic division, manifested by the extrusion of the first polar body (PB-I), depends on proteasomal degradation of cyclin B1 and securing, and the subsequent respective CDK1 inactivation and chromosome segregation [84]. Altogether, the studies of mammalian oocytes [55, 63] demonstrate the importance of UCHL1 and UCHL3 in maturation.

Inhibition of UCHs during oocyte maturation distorts the meiotic spindle, altering spindle lengths/pole-to-pole distances and disrupting other spindle attributes, such as chromosome alignment [63] (Fig. 5.4). Such defects may lead to aneuploidy in the oocyte and resulting embryo [85–88]. Aneuploidy is a major factor

Fig. 5.3 (continued) polar body (PB1). **D–G**: Oocyte preinjected with UBAL showed abnormally large PB1 (**D**) or multiple PB1s (**E**), abnormal spindles with misaligned chromosomes (**D–F**), or premature chromatin condensation following GVBD (**G**). (**H**): Diagram of abnormal spindle frequencies in various UCHL3-affecting treatments shows average percentages of abnormal spindles from two replicates in mouse metaphase-II oocyte (nU419) that were injected at the GV-stage with UBAL, affinity-purified rabbit antibody against UCHL3 (Anti-L3) or NRS, or matured without preinjection (No Inj.), or without preinjection and in the presence of a specific inhibitor of UCHL3 (L3inh). **I:** Examples of normal (*a*, *b*; control oocyte) and abnormal (*c–g*; UBAL-injected oocyte) metaphase II spindles and chromosomes from this trial (adapted from [63])

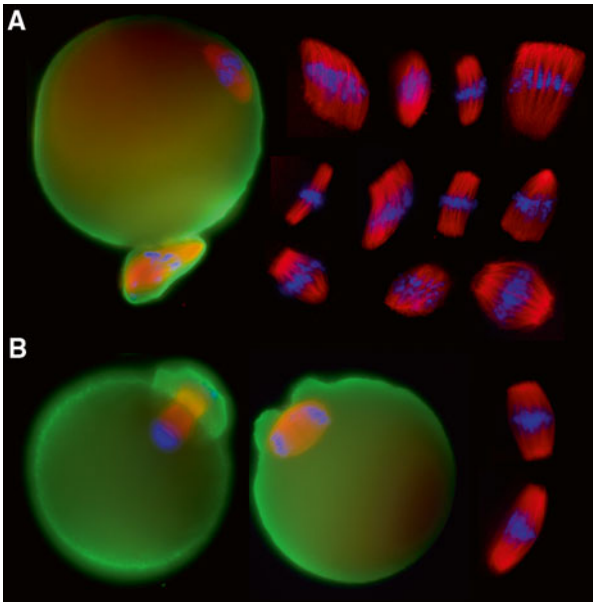

Fig. 5.4 Metaphase II spindles in the oocyte injected with anti-UCHL3-antibody or NRS at GV stage and processed 16 h later for immunofluorescence with antibodies against UCHL1 (*green*) and UCHL3 (*red*), combined with DNA stain DAPI (*blue*). **a**: Oocyte injected with a rabbit anti-UCHL3 antibody prior to IVM show various abnormalities such as lack of pole microtubule focusing, very narrow or very wide spindles and disarrayed metaphase chromosome plates. Note the presence of a second spindle pole within the polar body of an oocyte shown on the *left*. **b**: Control oocyte injected at GV-stage with NRS show focused spindle poles even in cases where they did not reach metaphase II after 16 h of IVM (adapted from [63])

contributing to infertility, fetal loss and conception resulting in birth defects, notably trisomy 21 or Down syndrome in human [89–93]. The spindle pole–pole distance remains constant from metaphase to anaphase with UCH inhibition. The pole-to-pole distance of the metaphase spindle is reasonably constant in a given cell type; in the case of vertebrate female oocytes, this steady-state length can be maintained for substantial lengths of time, during which microtubules remain highly dynamic [94]. Overexpression of Aurora-B kinase mutant in *Xenopus* oocytes resulted in short bipolar spindle or monopolar spindle [95]. Oocytes matured in suboptimal conditions are more likely to have a shorter spindle length (long axis) and smaller spindle area [96]. Though a number of molecular perturbations are known to influence spindle integrity [73, 97–99], a global understanding of the factors that determine metaphase spindle length has not been achieved. The UCHs may regulate germinal vesicle (GV) breakdown, spindle assembly, spindle pole focusing as well as ubiquitin-dependent cyclin degradation and chromosome segregation during metaphase–anaphase transition [55, 62, 64, 66, 100]. Also, we have observed meiotic spindle colocalization of UCHL3 with separase/separin [101], an enzyme that may be a substrate of UCHL3 during mitotic metaphase–anaphase transition

(PS; unpublished data). The ubiquitin-like protein NEDD8, a known substrate of UCHL3 has also been implicated in the control of spindle positioning and cell cycle progression during mitosis [102].

UCH'S in Fertilization

The UCHs regulate fertilization in different species such as ascidians [103, 104], mouse [57, 98, 105, 106], cattle [55], pigs [56], the rhesus monkey [60, 107] and humans [108]. Sperm acrosomal UCHL3 participates in sperm–ZP interactions and regulation of polyspermy in the domestic pig, while UCHL1 may also act in polyspermy defense at the oolemma due to its abundance and accumulation in the oocyte cortex [56].

Fertilization is a well-coordinated cascade of events, rather than a single, isolated reaction. Interruption of any step in the sequence can cause fertilization failure. Only a fully mature oocyte will be recognized and penetrated by a fertilizing spermatozoon to ensure rapid and synchronous male and female pronucleus formation [109]. Fertilization by more than one spermatozoon, called polyspermy, causes aberrant development and death of the embryo at an early stage of development [110–112]. An active block of polyspermic fertilization is essential, and mammalian oocytes rely on zona pellucida (ZP) hardening, plasma membrane depolarization and exocytosis of cortical granules to achieve this block. The ZP and plasma membrane changes occur immediately after sperm head fusion with the oolemma [113, 114]. Little is known about the plasma membrane block to polyspermy in mammals. Cortical granule exocytosis in the oocyte begins within 20 min and is complete by 1 h after sperm addition to oocytes [115] and is associated with ZP2 cleavage after gamete fusion in the mouse [116, 117]. The cleavage of ZP2 also occurs in other mammalian species, but may not be sufficient to prevent polyspermy [118].

Effective ways to study protein function include using a double knockout organism or use specific antibodies and inhibitors. Our study of deubiquitinating enzymes in the mouse oocyte used microinjection of antibodies specific to UCHL1 and UCHL3, variety of UCH-inhibitors (ubiquitin-aldehyde (UBAL), which is a full-length, C-terminally modified ubiquitin protein with an aldehyde group. It is a highly potent, extremely stable inhibitor of most members of ubiquitin-C-terminal hydrolases/isopeptidase family [119, 120], and UCHL3-inhibitor 4, 5, 6, 7-tetrachloroidan-1-3-dione [63, 105] and UCHL1-inhibitors LDN-57444, UBEI-41, C16 and C30 [55, 63, 105]) used during IVF as well as a genetic model, the subfertile *Uchl1*$^{gad-/-}$ mutant mouse [63, 105]. *Gad* mutant oocytes lacking functional UCHL1 [121] display an increase in polyspermy during in vitro fertilization (IVF) [57]. Porcine zygotes treated with ubiquitin aldehyde (a specific inhibitor of UCHs) also have an increase in polyspermy [56]. In bovine oocytes, UCHL1 inhibition during oocyte maturation impairs cortical granules exocytosis and increases polyspermy rates [55]. The inhibition of ooplasmic UCHs by the intracytoplasmic microinjection of UBAL, reduces fertilization rates in zona-intact oocytes and to a lesser extent in the zona-free oocytes [105]. The effect of UBAL on fertilization/

sperm incorporation is most likely mediated by UCHL1, because intracytoplasmic injection of anti-UCHL1 antibody almost completely abolishes fertilization of zona-enclosed oocytes. These results suggest an effect of UCHL1 on post-fertilization modification of the ZP, oolemma, or cortical cytoskeleton. For example, UCHL1 activity may control cortical granule release, with premature release causing premature cleavage of ZP proteins to block sperm binding and sperm–zona penetration. Other effects on the ZP are also possible. Interestingly, inhibiting UCH function at the GV stage (i.e. prior to oocyte maturation) can also prevent fertilization after the oocytes have matured, indicating additional targets of UCH action that control fertilization [63]. This could include changes in the oolemma and oocyte cortex, and changes in the release of chemoattractant molecules from oocytes and/or cumulus cells. The ubiquitin–proteasome system participates in the release of an oocyte-produced sperm chemoattractant during ascidian fertilization [122]. If UCHs only controlled the oolemma and oocyte cortex functions in fertilization, one would expect a fertilization block by intracytoplasmic injection of UBAL or anti-UCHL1 antibody to result in the accumulation of spermatozoa in the perivitelline space. But this is not seen [105]. There are no differences in cortical microfilament distribution in control and UBAL injected oocytes stained with rhodamine-phalloidin. Altered distribution or function of cortical granules, as observed in bovine oocytes matured in the presence of UCH-inhibitors [55], could also alter fertilization rate in the UCH manipulated mouse oocyte. However, such a treatment, preventing cortical granule exocytosis and post-fertilization ZP alterations, would be expected to increase fertilization/polyspermy rate, not reduce it, as we observed [105]. The inability of the UBAL-injected mouse oocytes to attract spermatozoa thus remains a distinct possibility. A recent study in ascidians indicated that valosin containing protein (VCP/p97), an OTU-class DUB involved in the extraction and proteasomal presentation of ubiquitinated proteins found in the asicidian oocyte cortex, interacts with the oocyte-produced sperm-attracting factor SAAF and may be essential for fertilization [122].

Single gene deletion of *Uchl1* or *Uchl3* genes is neither infertility causing nor it is overtly embryo lethal (although the number of progeny is reduced), presumably due to a mutual compensating ability between these closely related *Uch* genes [123]. In contrast, the inhibitors and antibodies targeting oocyte UCHs have a profound negative effect on sperm–zona penetration and sperm incorporation in the ooplasm during mouse fertilization (Table 5.1).

Targeting of UCHL1 in the oocyte cortex inhibits fertilization completely [105]. Interestingly, we found partial compensation in function between members of the UCH family. Loss of UCHL1 from the egg cortex of *Uchl1*$^{gad-/-}$ mice was accompanied by a potentially compensatory translocation of UCHL3 to the cortex (Fig. 5.5F) [105]. This suggests that UCH functions associated with the oocyte cortex and oolemma may be at least partially restored by UCHL3 function in the *Uchl1*$^{gad-/-}$ mice, if opportunity for this is presented during oogenesis. Such a compensatory translocation of UCHL3 may be a long term process occurring during oogenesis in

Table 5.1 Summarizes the published effects of various types of interference on UCHL1 and L3 function during murine, porcine and bovine gametogenesis, fertilization and pre-embryo development

Species	Effect	Induced by
Sus	High rate of sperm–ZP penetration and polyspermy	Ubiquitin-aldehyde (UBAL), a C-terminal modified full length ubiquitin derivative that blocks both UCHL1 and UCHL3
Bos	Impaired extrusion of cortical granules, high rate of polyspermy	UCHL1 inhibitor applied during oocyte maturation
Mus	Decreased oocyte meiotic progression beyond metaphase I, spindle and first polar body anomalies (large polar body), cortical granule disruption. Near complete block of fertilization	UBAL, anti-UCHL1 antibody, Uchl3 si RNA
Mus	Abnormal MII spindles (uneven chromosome distribution and wide spindle poles), failure to complete GVBD. Reduced fertilization rates and abnormal pronuclear formation	UCHL3 inhibitor, Uchl3 si RNA

the *Uchl1*$^{gad-/-}$ mice. In contrast, the inhibition of both proteins after maturation does not allow this compensation, resulting in more severe consequences of UCHL1 deficiency [63, 105]. The translocation of UCHL3 and possibly other related UCHs to the oocyte cortex may not be fast enough when inhibitors and antibodies, injected into the ooplasm, target UCHL1 that is already in the oocyte cortex. The UCHL1 and UCHL3 are very closely related, sharing high amino acid sequence homology, similar sizes, and similar abilities to deubiquitinate substrate proteins. Consequently, the UCHL1 and UCHL3 proteins may, under certain experimental conditions, provide compensatory functions [123].

High polyspermy is seen in porcine oocytes fertilized with the addition of UBAL in IVF medium (reference). In this case, the most likely target of UBAL was UCHL3 associated with boar sperm acrosome [56]. High polyspermy was also proposed to be the cause of subfertility in *Uchl1*$^{gad-/-}$ mice in vitro [57]. Neither our studies using UBAL addition in mouse IVF medium nor our findings in zygotes fertilized in vivo and recovered from *Uchl1*$^{gad-/-}$ females provide indication of increased polyspermy. However, it is possible that the *Uchl1*$^{gad-/-}$ oocyte would show polyspermy if challenged by higher number of spermatozoa during IVF, as reported previously [57]. Alternatively, polyspermy might only be an in vitro phenomenon in the *Uchl1*$^{gad-/-}$ mutants, one that may be ameliorated by the exposure of spermatozoa to seminal plasma and oviductal fluid in vivo. A deficiency in functional UCHL1 enzyme [57] could lead to different phenotypes in vitro versus in vivo. Even so, the subfertility in *Uchl1*$^{gad-/-}$ mice [57, 127] is most likely due to impaired preimplantation embryo development at the morula stage, as demonstrated by our data (Fig. 5.5J, K) [105]. While rodents and other mammals seem to use the ubiquitin–proteasome pathway during sperm–oocyte interactions, our data hint at differences between taxa in how this pathway contributes to the fertilization process. Based on our studies in

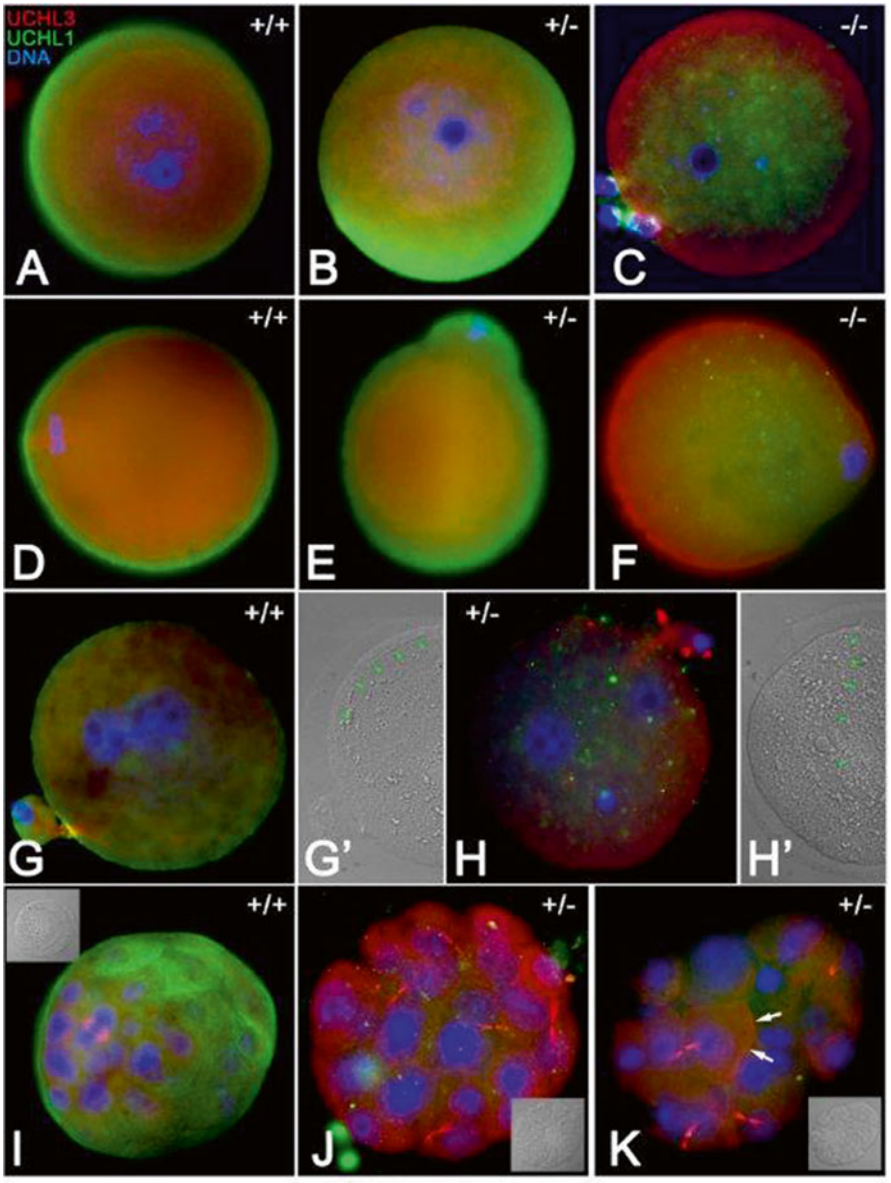

Fig. 5.5 Oocyte maturation, fertilization and embryo development in *Uchl1*^{gad} mice. **A**–**F**: Homozygous *Uchl1*$^{gad-/-}$ oocytes (–/–), heterozygous *Uchl1*$^{gad+/-}$ oocytes (+/–) and wild type *Uchl1*$^{gad+/+}$ oocytes (+/+) were obtained from the properly phenotyped daughters of *Uchl1*$^{gad+/-}$ female mice mated with *Uchl1*$^{gad+/-}$ males. *Red* labeling denotes UCHL3 while UCHL1 is shown in *green*. **A**–**C**: The GV-stage oocytes; note the replacement of cortical UCHL1 with UCHL3 in *Uchl1*$^{gad-/-}$ ovum. **D**–**F**: Metaphase-II ova; UCHL3 translocation to oocyte cortex becomes even more obvious in *Uchl1*$^{gad-/-}$ ova. **G**–**K**: Heterozygous *Uchl1*$^{gad+/-}$ zygotes and later stage embryos were obtained from *Uchl1*$^{gad-/-}$ females mated with *Uchl1*$^{gad+/+}$ males; wild type *Uchl1*$^{gad+/+}$ zygotes/

ungulate models, we anticipated that the inclusion of UBAL and antibodies against UCHL1 or UCHL3 in the IVF medium would stimulate murine fertilization and cause polyspermy. In ungulates, UCHL3 in the sperm acrosome may participate in sperm passage through the ZP by interacting with the sperm acrosome-borne proteasomes. In accord with the stimulatory effect of UBAL on proteasomal proteolysis, inhibition of sperm acrosomal UCHs increases ZP penetration rate of sperm for porcine [56] and bovine [55] oocytes. This is in line with the observation that the inhibition of 20S proteasomal core proteolytic activities, an intervention that has opposite effect to that of UCH-inhibition, blocks sperm–ZP penetration in higher mammals [124]. In these experiments, zona free porcine oocytes were readily fertilized in the presence of proteasomal inhibitors (MG132 and lactacystin) or anti-proteasome antibodies. In the mouse, however, proteasomal inhibitors such as ALLN blocked sperm–oolemma fusion and sperm incorporation in the ooplasm [106]. Our data on UCH inhibitors and antibodies affecting sperm incorporation in the ooplasm but not ZP penetration in the mouse are consistent with this observation. Thus, caution should be exercised when hypotheses on mammalian fertilization are generalized based solely on mouse data.

In spite of the aforementioned species differences, the participation of ubiquitin system in fertilization is extremely conserved, with communalities found between mammals including humans [125], ascidians [103], echinoderms [104], and even plants [126]. While the mechanism of their action may vary, the expression and localization patterns of UCHs appear to be evolutionarily conserved between rodents and primates. High expression levels of proteasomal subunits and enzymes of the ubiquitin system appear to be a common feature of mammalian oocytes and early embryos [107, 127]. Collectively, our studies confirmed a conserved localization of UCHs between rodents, ungulates and primates [55, 56, 63]. Some etiologies of human infertility and developmental failure after assisted fertilization may arise from abnormal expression/functioning of human oocyte UCHs. A strong maternal effect was also seen for preimplantation embryo development in heterozygous *Uchl1*$^{gad+/-}$ embryos produced by mating homozygous *Uchl1*$^{gad-/-}$ females to homozygous wild type males (reference). Many of these embryos arrest between the morula and blastocyst stages [105]. Such strong maternal effects could arise due to haploinsufficieny; however previous reports indicated a matrilineal origin of the fertility defect [57, 128]. This suggests that embryo lethality most likely would arise from triploidy/multiplody (e.g., from polyspermy) or from aneuploidy

Fig. 5.5 (continued) embryos were obtained from *Uchl1*$^{gad+/+}$ females mated with *Uchl1*$^{gad+/+}$ males. **G**, **H**: Neither the wild type nor the *Uchl1*$^{gad+/-}$ ova showed polyspermy or fertilization failure. Normal fertilization is revealed by the presence of two pronuclei (*blue*) and one sperm tail (*arrows* in DIC, panels **G'** and **H'**). **I**–**K**: Day 4 embryos; wild type, *Uchl1*$^{gad+/+}$ embryo has a normal blastocyst appearance with a dividing blastomere within its inner cell mass. The *Uchl1*$^{gad+/-}$ embryos failed to reach blastocyst stage and became arrested at pre-compaction morula stage. Note the accumulation of UCHL3 in blastomere cortex in *Uchl1*$^{gad+/-}$ morula (*arrows*, panel **K**). Insets show corresponding DIC images (adapted from [105])

resulting perhaps from meiotic defects in the *Uchl1*$^{gad-/-}$ oocytes. Because we find that polyspermy is not increased with the mutant oocytes, it is most likely that the increased embryo lethality is due to decreased developmental competence of *Uchl1*$^{gad-/-}$ oocytes, which could be exacerbated by altered oviductal environment. While UCHL1 does not seem to associate with the meiotic spindle, it could influence cytokinesis during oocyte maturation by directing cortical cytoskeleton changes during polar body extrusion, and also have an effect on the association of UCHL3 with the spindle by maintaining the balance mono- and poly-ubiquitin. Consequently, mis-segregation of chromosomes during meiosis could arise from defects in the polar body extrusion (as indeed observed in oocytes and zygotes treated with UCH-inhibitors), or spindle function in the oocyte. Thus, although UCHL3 may compensate for UCHL1 functions in the oocyte by translocating to the cortex, this compensation may come at the high cost of incorrect chromosome segregation. The resulting aneuploidy would yield embryos that are unable to undergo compaction and progress beyond the morula stage, as observed [105]. Some reduction in blastocyst formation and quality has also been observed in oocytes fertilized by ICSI after pre-injection with UBAL. The aberrant blastocyst phenotype was less robust than that of *Uchl1*$^{gad-/-}$ mice, possibly because the injected UBAL was metabolized by the ICSI zygotes prior to morula stage [105]. Additional to the effects on spindle function and cytokinesis, a deubiquitinating enzyme insufficiency could reduce oocyte developmental competence by diminishing the pool of unconjugated monoubiquitin available for protein modifications, necessary for cell cycle regulation and any other processes controlled by protein ubiquitination in the preimplantation embryo.

Altogether, these studies demonstrate the importance of UCH enzymes for mammalian oocyte maturation, fertilization and reveal a key maternal effect of the *Uchl1* gene [63, 105]. These results signify new, key roles for deubiquitinating enzymes in the equilibrium of posttranslational modifications during early development.

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Posttranslational Modifications of Zona Pellucida Proteins

6

Naoto Yonezawa

Abstract

The zona pellucida (ZP), which surrounds the mammalian oocyte, functions in various aspects of fertilization. The ZP consists of three or four glycoproteins, which are derived from transmembrane proteins that lack the ability to self-assemble. Following posttranslational processing at specific sites, ectodomains of ZP precursor proteins are released from the membrane and begin to form a matrix. Glycosylational modification is thought to be involved in species-selective sperm recognition by ZP proteins. However, in mice, the supramolecular structure of the zona matrix is also important in sperm recognition. One ZP protein, ZP2, is processed at a specific site upon fertilization by ovastacin, which is released from cortical granules inside the oocyte. This phenomenon is involved in the block to polyspermy. The proteolysis of ubiquitinated ZP proteins by a sperm-associated proteasome is involved in penetration of the zona matrix by sperm, at least in the pigs. Thus, the posttranslational modification of ZP proteins is closely tied to ZP formation and the regulation of sperm–oocyte interactions.

Keywords

Zona pellucida • *N*-linked chain • *O*-linked chain • Glycosylation site • Postfertilization processing • Disulfide bond

Abbreviations

CFCS	Consensus furin cleavage site
EHP	External hydrophobic patch
Fuc	Fucose

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Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GlcNAc	*N*-acetylglucosamine
HPLC	High-performance liquid chromatography
IHP	Internal hydrophobic patch
Man	Mannose
NeuAc	*N*-acetylneuraminic acid
NeuGc	*N*-glycolylneuraminic acid
PNGase F	Peptide *N*-glycosidase F
SDS	Sodium dodecyl sulfate
Sia	Sialic acid
sLe^x	Sialyl-Lewis^x
TMD	Transmembrane domain
ZP	Zona pellucida

Introduction

Mammalian oocytes are surrounded by a transparent envelope called the zona pellucida (ZP) (Fig. 6.1), which is involved in several critical aspects of fertilization, including the species-selective sperm recognition, induction of the acrosome reaction upon sperm binding, the block to polyspermy, and protection of the oocyte and embryo until implantation [1–3]. Changes in structure of the zona matrix occur via the actions of enzymes released from cortical granules upon fertilization. These structural changes, called the zona reaction, are part of the mechanism underlying the block to polyspermy.

The ZP consists of three or four proteins. About 30 years ago, it was reported that the ZP in mice consists of three glycoproteins [4, 5]. These were called ZP1, ZP2, and ZP3 in order of their apparent molecular masses (200, 120, and 83 kDa, respectively) on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, under non-reducing conditions. This nomenclature has been used in other species. ZP proteins are also named in descending order based on the sizes of the cDNAs encoding the polypeptides (e.g., ZPA, ZPB, and ZPC) [6]. This nomenclature has been used for species other than mice (e.g., pigs and cows). ZPA and ZPC correspond to ZP2 and ZP3, respectively. Mouse ZP1 is a disulfide-bonded dimer; the size of the cDNA encoding ZP1 is between that of the cDNAs encoding ZP2 and ZP3. It was unclear why ZP2 (ZPA) and ZP3 (ZPC) are highly homologous between mice and pigs while mouse ZP1 and porcine ZPB are weakly homologous, until it was discovered that human ZP consists of four proteins: ZP1, ZP2, ZP3, and ZPB [7]. At that point, ZPB was renamed ZP4. Thus, the present nomenclature is ZP1, ZP2, ZP3, and ZP4.

In many mammalian species, including hamsters, humans, monkeys, rabbits, and rats, there are four ZP proteins (Fig. 6.2) [8], whereas the mouse ZP (ZP1, ZP2, and ZP3), and porcine and bovine ZP (ZP2, ZP3, and ZP4) [9] are comprised of three proteins (Fig. 6.3). Thus, ZP2 and ZP3 are common to all mammals studied so far. In those species that lack ZP1 or ZP4, the corresponding gene is a pseudogene [8]. All ZP proteins contain a ZP domain (Fig. 6.2), which

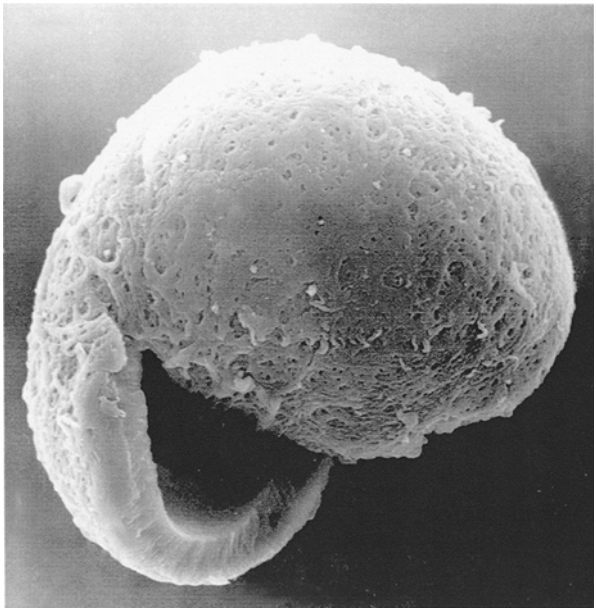

Fig. 6.1 Scanning electron micrograph of porcine zona pellucida

is comprised of ~260 amino acids and contains eight conserved Cys residues (Fig. 6.4) [10]. A ZP domain is found at the C-terminal region of other secretory and/or glycosylphosphatidyl inositol (GPI)-anchored proteins, including α- and β-tectorin, Tamm-Horsfall protein (also called uromodulin), and transforming growth factor-β receptor III (also called betaglycan) [11].

The ZP appears as a porous matrix consisting of interconnected filaments (Fig. 6.1) [12]. The diameter of the ZP in mice and pigs is about 80 and 150 μm, respectively. The porcine ZP is about 16 μm in width and contains 30–33 ng of glycoproteins, compared to 3 ng in the murine ZP. The bovine ZP is similar in size to the porcine ZP. The estimated protein molar ratio of ZP2/ZP3/ZP4 in the porcine ZP is 1:6:6 [13], compared to 1:2:1 in the bovine ZP [14]. In mice, the estimated molar ratio of ZP1/ZP2/ZP3 is 1:4:4 [15]. ZP2 and ZP3 form a filamentous equimolar complex while ZP1 crosslinks the ZP2/ZP3 complex in mice (Fig. 6.3) [16]. It appears that the architecture of the zona is similar between pigs and mice based on the similarity in their protein molar ratios; that is, in pigs ZP3 and ZP4 form a filamentous equimolar complex, which is crosslinked by ZP2 (Fig. 6.3). The architecture of the zona in cows appears to be different from that in pigs and mice because of differences in the molar ratios of ZP proteins.

In mice, the three ZP protein genes are transcribed in growing oocytes [15]. The expression of ZP protein genes is also limited to oocytes in rats and monkeys [17, 18], whereas expression is observed in cumulus cells as well as oocytes in rabbits and pigs [19–21]. The egg coat, called the vitelline membrane in non-mammals, consists of ZP protein homologs. The chick ZP1 homolog is synthesized in liver cells [22], then transported to the ovary where it is assembled with a ZP3 homolog synthesized in granulosa cells [23, 24]. Thus, the mechanisms of egg coat formation

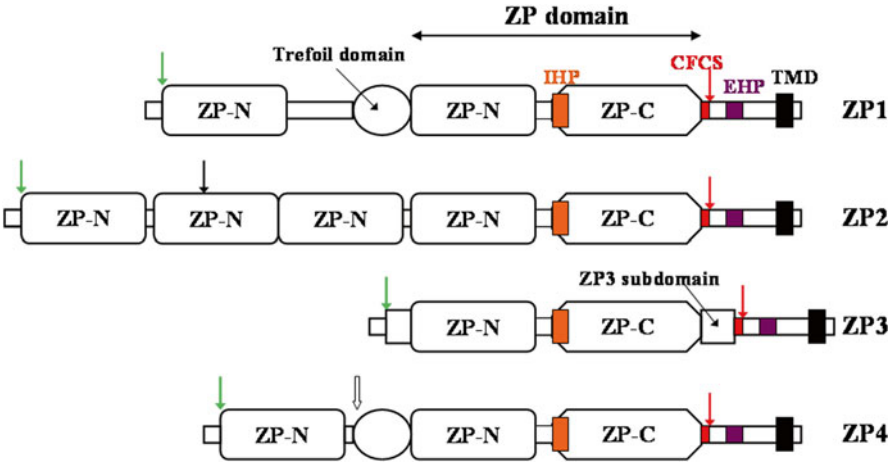

Fig. 6.2 Schematic representation of mammalian zona pellucida proteins. *Green arrows* show the sites cleaved by the signal peptidase. *Red arrows* show the positions of the consensus furin cleavage site (CFCS). It is suggested that furin or furin-like processing enzymes cleave the CFCS; the mature polypeptides in the zona pellucida lack the external hydrophobic patch (EHP), shown by the *purple box*, and the transmembrane domain (TMD) shown by the *black box*, both of which are downstream of the CFCS. The ZP domain consists of an N-terminal subdomain (ZP-N) and C-terminal subdomain (ZP-C). These subdomains are connected by a hinge region. An internal hydrophobic patch (IHP), shown by the *orange box*, exists at the beginning of ZP-C, and the interaction of the IHP with the EHP inhibits assembly of the zona pellucida precursor proteins. The regions, N-terminal to the ZP domains, contain the subdomain indicated as ZP-N, which is homologous to ZP-N in the ZP domain [114]. The *black arrow* in ZP2 shows the site cleaved by ovastacin, a cortical granule enzyme released upon fertilization. The ZP3 protein has a unique subdomain (referred to as the ZP3 subdomain in this chapter) downstream of ZP-C. Among the species studied so far, only pig ZP4 is processed on the N-terminal side of the trefoil domain, to yield the N-terminus indicated by the *white arrow*

on the surface of growing oocytes may differ between mammals and nonmammals (e.g., birds and fishes).

The posttranslational modification of ZP proteins, including glycosylation, specific cleavage by processing enzymes, and ubiquitination, is closely related to functioning of the ZP proteins during zona matrix formation, sperm recognition, the block to polyspermy, and sperm penetration into the ZP.

N-termini and C-termini of the Mature ZP Polypeptides

The cloning and sequencing of pig ZP4 cDNA revealed that ZP proteins have a putative consensus furin cleavage site (CFCS, R-X-[R/K]-R) upstream of the transmembrane domain [25, 26], suggesting that the precursors of ZP proteins are translated as transmembrane proteins, which then become secretory proteins following processing by furin or furin-like processing enzymes. Biochemical analyses, including automated Edman degradation and mass spectrometry, have been used to

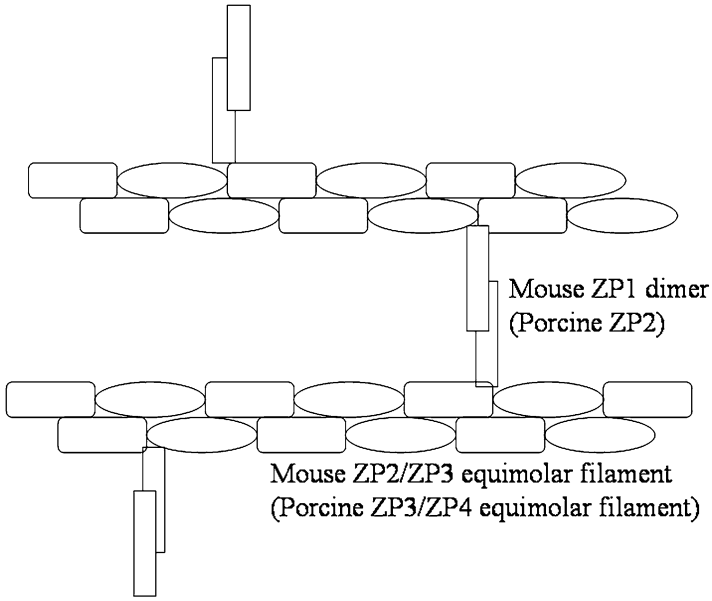

Fig. 6.3 Proposed model of the mouse zona pellucida architecture. Among the three mouse zona pellucida proteins, the major components ZP2 and ZP3 are believed to form equimolar filaments, which are cross-linked by ZP1 dimers (the minor component). Since the molar ratio of porcine ZP2, ZP3, and ZP4 is similar to that of mouse ZP1, ZP2, and ZP3, the architecture of the zona pellucida in these species may be similar. In that case, porcine ZP2, ZP3, and ZP4 correspond to mouse ZP1, ZP2, and ZP3, respectively, in the zona matrix

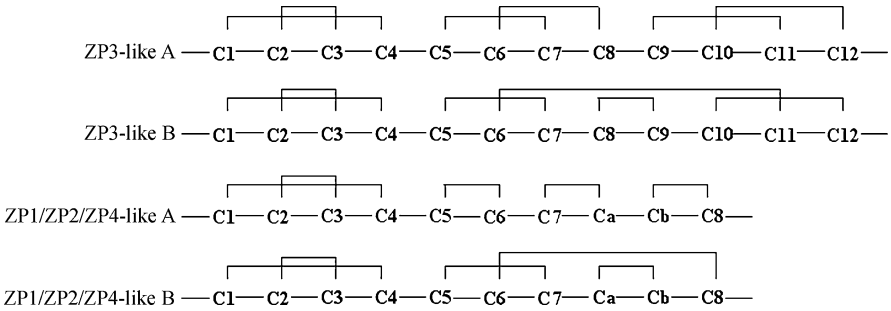

Fig. 6.4 Disulfide bond patterns in the zona pellucida domains of ZP proteins. The ZP3 has eight Cys residues (C1–C8) in the zona pellucida (ZP) domain and four Cys residues (C9–C12) in the ZP3 subdomain. There are two patterns in the region from C5 to C12 of ZP3, referred to as ZP3-like A and ZP3-like B. The Cys residues of ZP1, ZP2, and ZP4 are conserved among these proteins. The first Cys residue in the ZP domain is shown as C1 and the number of Cys residues corresponds to those in ZP3. The ZP domains of ZP1, ZP2, and ZP4 have two additional Cys residues, Ca and Cb, between C7 and C8. The ten Cys residues from C1 to C8 in the ZP domains of ZP1, ZP2, and ZP4 correspond to the Cys residues shown as C12/11/11 to C21/20/20 (ZP1/ZP2/ZP4 in Figs. 6.5, 6.6, and 6.8, respectively). There are two patterns in ZP1/ZP2/ZP4, which are referred to as ZP1/ZP2/ZP4-like A and ZP1/ZP2/ZP4-like B

characterize the N- and C-termini of ZP proteins from pigs, mice, and rats. The N-terminus of mature porcine ZP2, ZP3, and ZP4 purified from ovaries contains Ile-36, pyroGln-23, and Asp-137 (the translational initiation site [Met] is numbered as 1), respectively (Figs. 6.6, 6.7, and 6.8) [27–29]. Porcine ZP4 lacks an N-terminal region; the polypeptide begins with a trefoil domain (Figs. 6.2 and 6.8), probably due to cleavage between Leu-136 and Asp-137 by an unknown enzyme. The other ZP4 proteins analyzed so far, including bovine ZP4 and rat ZP4, retain the N-terminal region. The C-termini of pig ZP3 and ZP4 end with Ser-332 and Ala-462, respectively (Figs. 6.7 and 6.8) [30], while the C-terminal amino acid residue of pig ZP2 has not yet been determined.

The N- and C-terminal amino acids of native murine ZP proteins are pyroGln-21 and Arg-546 for ZP1, Val-35 and Ser-633 for ZP2, and pyroGln-23 and Asn-351 for ZP3, respectively (Figs. 6.5, 6.6, and 6.7) [31]. The N- and C-terminal amino acids of native rat ZP proteins are pyroGln-21 and His-544 for ZP1, pyroGln-23 and Asn-351 for ZP3, and Gln-29 and Arg-473 for ZP4, respectively (Figs. 6.5, 6.7, and 6.8) [32, 33]. For rat ZP2, only the N-terminal amino acid has been determined (Ser-25; Fig. 6.6) [32]. Thus, the N-terminus of ZP2 is not blocked, but the N-terminal Gln in ZP1 and ZP3 is pyroglutaminated in all species examined so far. It has been suggested that porcine ZP4 is first processed at the putative CFCS Arg-Arg-Arg-Arg (463–466) by furin or a furin-like enzyme, after which the basic residues are removed by a carboxypeptidase. C-Terminal processing of mouse and rat ZP proteins may also be dependent on cleavage at the CFCS followed by the trimming of dibasic residues by a carboxypeptidase. Dibasic Arg-Arg residues remain at the C-termini of mouse ZP1 (545–546) and rat ZP4 (472–473), suggesting an alternative processing procedure. The peptide bond N-terminal to these dibasic residues may be cleaved by a processing enzyme in mouse and rat ZP proteins. Mouse ZP3 mutated at the CFCS is secreted [34], and the C-terminus of pig ZP3 is upstream of the CFCS, as mentioned above. These findings suggest the involvement of processing at other sites in addition to the CFCS, in the secretion of ZP3. Thus, the essential role of CFCS in the secretion of ZP proteins is not yet clarified.

Processing at the CFCS Regulates ZP Protein Assembly

Studies of mouse ZP proteins have shown that ZP precursor proteins are transported to the plasma membrane and processed at the CFCS [35, 36]. The polymerization of ZP proteins is controlled by an external hydrophobic patch (EHP) located between the CFCS and transmembrane domain and an internal hydrophobic patch (IHP) located in the ZP domain [37]. An X-ray structural analysis of the chick ZP3 precursor homolog revealed that EHP constitutes a β strand in the C-terminal half of the ZP domain (ZP-C subdomain) and blocks premature polymerization by interacting with interfaces between the N-terminal halves of ZP domains (ZP-N subdomains) and ZP-C subdomains. Upon cleavage at the CFCS and dissociation of the EHP

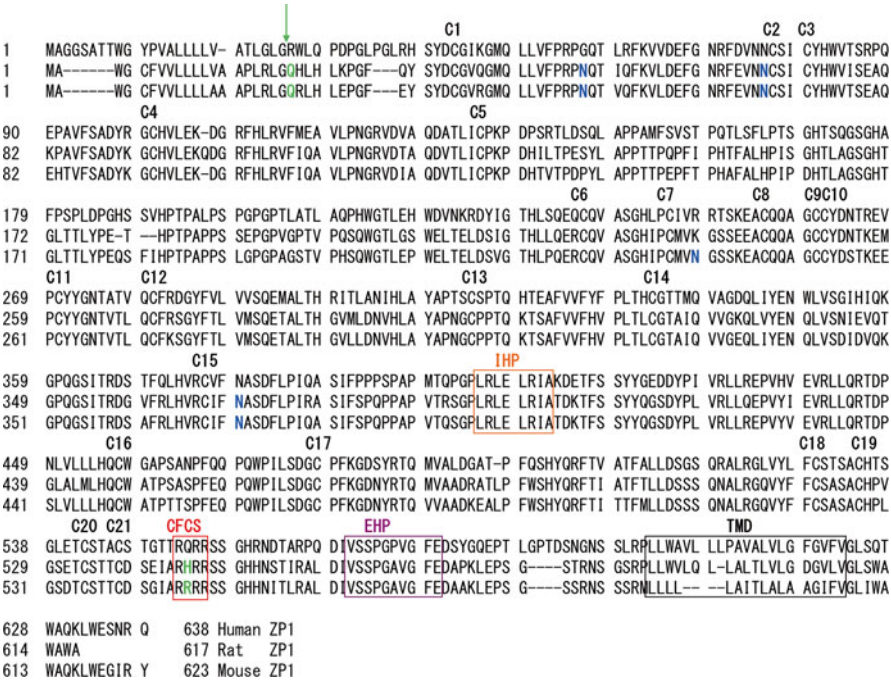

Fig. 6.5 Amino acid sequences of ZP1. The complete amino acid sequences, starting from the translation initiation site (Met; numbered as 1), are shown for the human, rat, and mouse ZP1 precursor polypeptides. The *green arrow* indicates the site cleaved by the signal peptidase. A *green Q* indicates the biochemically determined N-terminal pyroglutamate in the mature polypeptides. A *blue N* shows *N*-glycosylated Asn residues; *O*-glycosylation sites have not yet been determined. *Orange* and *purple boxes* indicate the positions of the internal hydrophobic patch (IHP) and external hydrophobic patch (EHP), respectively, which regulate assembly of the zona pellucida proteins. The *red box* shows the position of the consensus furin cleavage site (CFCS). *Green letters* in the *red box* indicate the C-termini of the mature polypeptides. The *black box* indicates the transmembrane domain (TMD). Annotations C1–C21 refer to the positions of the Cys residues that are conserved among the ZP1 polypeptides. Five Cys residues (C1–C5) are involved in two intramolecular disulfide bonds and one intermolecular disulfide bond. Six Cys residues (C6–C11) belong to the trefoil domain. Four Cys residues (C12–C15) belong to the N-terminal half of the zona pellucida domain (ZP-N subdomain). Six Cys residues (C16–C21) belong to the C-terminal half of the zona pellucida domain (ZP-C subdomain). The biochemical information shown in this figure is not yet available for human ZP1, but it can be surmised, based on sequence homology, that the conserved sites are also posttranslationally modified

from the ZP-C subdomain, a hydrophobic interaction between the surface of the ZP-N subdomain and IHP in the ZP-C subdomain triggers a conformational change in ZP3 that is followed by ZP protein assembly (see Fig. 6.2) [38]. However, the details of this conformational change are unknown. The short cytoplasmic tails of ZP proteins also have important roles in preventing intracellular interactions among ZP precursor proteins and the incorporation of processed proteins into the ZP [36].

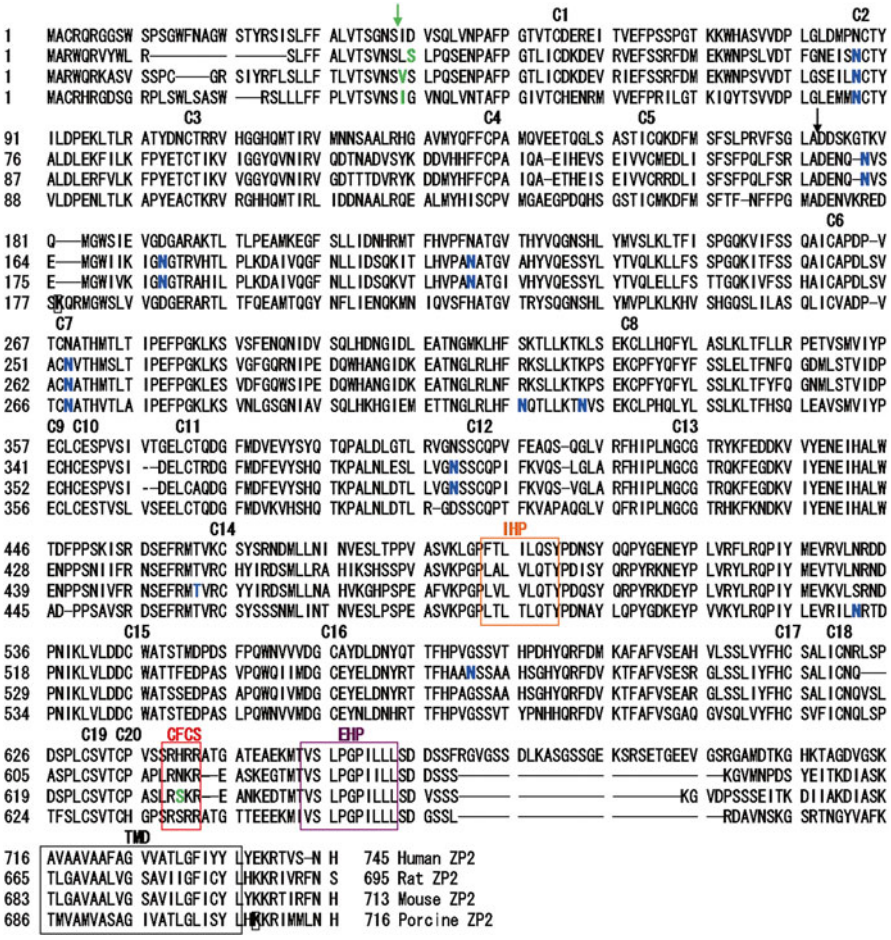

Fig. 6.6 Amino acid sequences of ZP2. The complete amino acid sequences, starting from the translation initiation site (Met; numbered as 1), of the human, rat, mouse, and porcine ZP2 precursor polypeptides are shown. The *green arrow* shows the sites cleaved by the signal peptidase in mouse and porcine ZP2. A *green S*, *V*, or *I* indicate the biochemically determined N-termini of the mature polypeptides. The N-termini are not blocked. A *blue N* indicates *N*-glycosylated Asn residues; the only *O*-glycosylation site identified so far is Thr-455 (shown by a *blue T*). *Orange*, *red*, *purple*, and *black boxes* show the positions of the internal hydrophobic patch (IHP), consensus furin cleavage site (CFCS), external hydrophobic patch (EHP), and transmembrane domain (TMD), respectively. *Green letters* in the *red box* indicate the C-terminus of the mature polypeptide. *Black arrows* indicate the site cleaved by ovastacin upon fertilization. A *boxed K* in porcine ZP2 indicates ubiquitinated Lys residues. Annotations C1–C20 refer to the positions of the Cys residues that are conserved among the ZP2 polypeptides. Four Cys residues (C11–C14) belong to the N-terminal half of the zona pellucida domain (ZP-N subdomain). Six Cys residues (C15–C20) belong to the C-terminal half of the zona pellucida domain (ZP-C subdomain). The equivalent biochemical information shown in this figure is not yet available for human ZP2, but it can be surmised, based on sequence homology, that the conserved sites are also posttranslationally modified

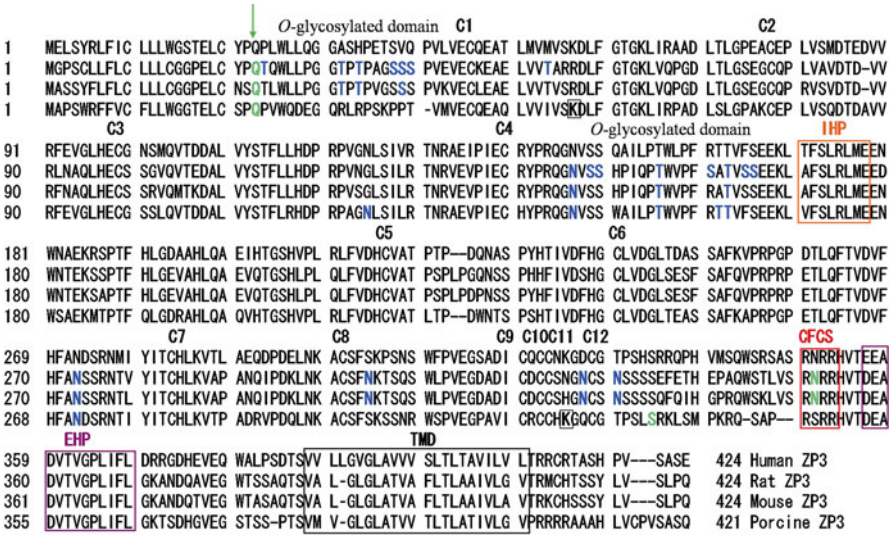

Fig. 6.7 Amino acid sequences of ZP3. The complete amino acid sequences, starting from the translation initiation site (Met; numbered as 1), of the human, rat, mouse, and porcine ZP3 precursor polypeptides are shown. The *green arrow* shows the site cleaved by signal peptidase. A *green Q* indicates the biochemically determined N-terminal pyroglutamate of the mature polypeptides. A *blue N* indicates the *N*-glycosylated sites; a *blue S* or *T* indicates *O*-glycosylated sites. There are two *O*-glycosylated domains in the N-terminal region and in the hinge region close to the internal hydrophobic patch (IHP) (shown by an *orange box*). *Red*, *purple*, and *black boxes* show the positions of the consensus furin cleavage site (CFCS), external hydrophobic patch (EHP), and transmembrane domain (TMD), respectively. *Green letters* in the *red box* and upstream of the *red box* indicate the C-termini of the mature polypeptides. A *boxed K* indicates ubiquitinated Lys residues. Annotations C1–C12 show the positions of the Cys residues that are conserved among the ZP3 polypeptides. Four Cys residues (C1–C4) belong to the N-terminal half of the zona pellucida domain (ZP-N subdomain). Four Cys residues (C5–C8) belong to the C-terminal half of the zona pellucida domain (ZP-C subdomain). Four Cys residues (C9–C12) belong to the region unique to ZP3 (ZP3 subdomain). The biochemical information shown in this figure is not yet available for human ZP3, but it can be surmised, based on sequence homology, that the conserved sites are also posttranslationally modified

Disulfide Linkages in ZP Proteins

The Cys residues in the ZP domain are well conserved among homologs (Figs. 6.5, 6.6, 6.7, and 6.8). These Cys residues are well conserved not only in ZP proteins and their non-mammalian homologs but also in ZP domain proteins other than egg coat proteins [11].

Disulfide linkages between four Cys residues (C1–C4 in Fig. 6.4) in the ZP-N subdomains have been identified in mammalian ZP proteins and their nonmammalian homologs (C1–C4 and C2–C3) [31–33, 39–41]. The ZP-N subdomain of mouse

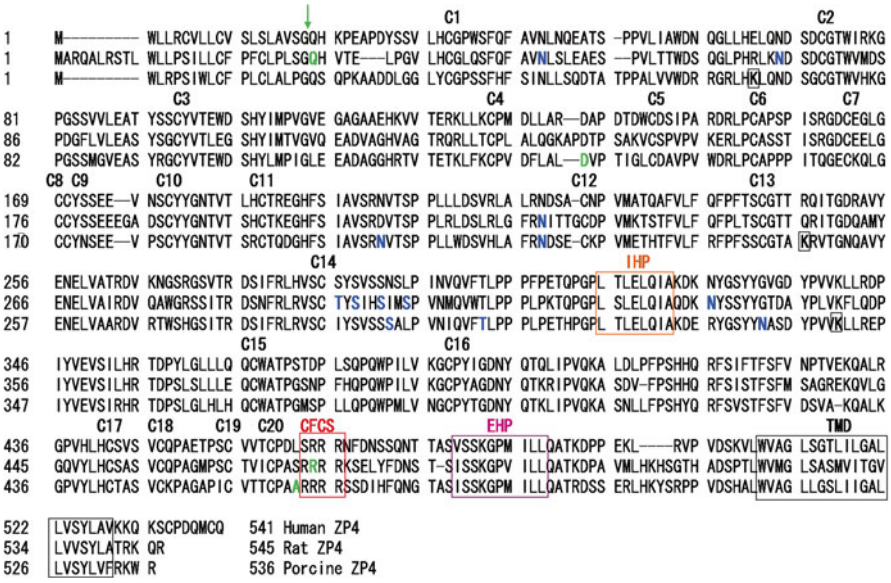

Fig. 6.8 Amino acid sequences of ZP4. The complete amino acid sequences, starting from the translation initiation site (Met; numbered as 1) of the human, rat, and porcine ZP4 precursor polypeptides are shown. The *green arrow* shows the site cleaved by the signal peptidase. A *green Q* or *D* (Asp-137 of porcine ZP4) indicates the biochemically determined N-termini of the mature polypeptides. A *blue N* indicates an *N*-glycosylated site; a *blue S* or *T* indicates *O*-glycosylated sites. There is one *O*-glycosylated domain in the hinge region near internal hydrophobic patch (IHP) (shown by an *orange box*). *Red*, *purple*, and *black boxes* show the positions of the consensus furin cleavage site (CFCS), external hydrophobic patch (EHP), and transmembrane domain (TMD), respectively. *Green letters* in a *red box* and next to the *red box* indicate C-termini of the mature polypeptides. A *boxed K* indicates ubiquitinated Lys residues. C1–C20 show the positions of the Cys residues that are conserved among the ZP4 polypeptides. Six Cys residues (C5–C10) belong to the trefoil domain. Four Cys residues (C11–C14) belong to the N-terminal half of the zona pellucida domain (ZP-N subdomain). Six Cys residues (C15–C20) belong to the C-terminal half of the zona pellucida domain (ZP-C subdomain). The biochemical information shown in this figure is not yet available for human ZP4, but it can be surmised, based on sequence homology, that the conserved sites are also posttranslationally modified

ZP3, including C1–C4, can form filaments following expression in bacteria [42]; therefore, the ZP-N subdomain has a function by itself. These findings suggest that the ZP-N subdomains of ZP3 from other mammalian species can also form filaments by themselves.

However, the disulfide linkages between six Cys residues (C5–C8) in the ZP-C subdomain of ZP1/ZP2/ZP4 and eight Cys residues in the C-terminal region of ZP3 (four Cys residues [C5–C8] in the ZP-C subdomain and four Cys residues [C9–C12] in the subsequent region unique to ZP3, referred to as the ZP3 subdomain in this chapter) are different from each other and are classified as ZP1/ZP2/ZP4- and ZP3-like patterns, respectively (Fig. 6.4). Furthermore, two subpatterns have been found for each of these patterns. In mice, rats, and rainbow trout, the disulfide bond

patterns are C5–C6, C7–Ca, and Cb–C8 (referred to as a ZP1/ZP2/ZP4-like A pattern in this chapter), while in porcine ZP4, the disulfide linkages are C5–C7, C6–C8, and Ca–Cb (referred to as a ZP1/ZP2/ZP4-like B pattern in this chapter) [41]. In mouse, rat, and human ZP3 and the rainbow trout ZP3 homolog, the disulfide linkages are C5–C7, C6–C8, C9–C11, and C10–C12 (ZP3-like A pattern), while in pigs the ZP3 disulfide linkages are C5–C7, C6–C11, C8–C9, and C10–C12 (ZP3-like B pattern) [41].

X-ray crystallographic studies indicate that the chick homolog of the mammalian ZP3 precursor protein has a ZP3-like B pattern [38] while rat beta-glycan has a ZP1/ZP2/ZP4-like B pattern [43]. According to X-ray crystallographic analyses of the chick ZP3 homolog, the two ZP3 disulfide bond patterns make only a subtle structural difference. The C-terminal region of the ZP domain in the chick and quail ZP1 homologs is involved in the interaction with ZP3 [44, 45]. This suggests that the ZP-C subdomain interacts with other ZP proteins, although it is not yet clear whether the ZP-C subdomain in mammalian ZP proteins has the same function. The existence of the two disulfide bond patterns (A and B) creates subtle structural differences that may influence the specificity of the interaction between ZP1/ZP2/ZP4 and ZP3. Current data indicate that when the disulfide bond pattern in ZP3 is type A, the pattern in ZP1/ZP2/ZP4 within the same species is also A, and vice versa. Disulfide bond exchanges may be induced by the conformational change that occurs during ZP assembly following processing at the CFCS.

ZP2 Cleavage During Fertilization

ZP2 is cleaved at a specific site upon fertilization by a cortical granule protease. The cleavage site is well conserved among mammals (Fig. 6.6); the consensus sequence is Leu/Met-Ala-Asp-Asp/Glu, except that the Ala is replaced by Ile in rabbit ZP2. The peptide bond between Ala and Asp is cleaved by a protease. In a ZP protein mixture isolated from pig and cow ovaries, a portion of ZP2 was found to have already been cleaved, whereas ZP2 was completely cleaved when ZP was isolated from in vitro-fertilized oocytes. Ovastacin is an astacin family metalloprotease that was first identified as a candidate hatching enzyme in the ZP [46] but was recently revealed to be a ZP2-processing protease [47, 48]. When ZP2 cleavage is inhibited by mutations within the consensus sequence, spermatozoa remain bound to the ZP of fertilized oocytes, as revealed using transgenic mice, while spermatozoa do not bind to the ZP of fertilized oocytes from wild-type mice [49]. Thus, one reason for ZP2 cleavage is to inhibit sperm binding to the ZP. Based on a model of mouse sperm–ZP binding, ZP2 cleavage creates a sperm binding-inactive supramolecular structure. Recent work revealed that human spermatozoa bind to the N-terminal region of ZP2 [50], which suggests that the sperm-binding site on ZP2 is cleaved by ovastacin upon fertilization. It is known that ZP hardening occurs upon fertilization, and a bovine disulfide bond exchange from intramolecular linkages to intermolecular linkages has been suggested [51]. Thus, ZP2 cleavage is thought to trigger the changes in ZP structure that occur upon fertilization.

Ubiquitination of Porcine ZP Proteins

Proteasome subunits have been detected in the acrosome of porcine sperm [52, 53]. Proteasomal inhibitors and anti-proteasome antibodies block in vitro fertilization at the step of sperm penetration into the ZP. The substrates of the sperm proteasome are ZP proteins and sperm proteins [54]. The sperm proteasome can degrade ZP3 and intact ZP in vitro. ZP proteins isolated from ovaries are already ubiquitinated. Ubiquitinated Lys residues were identified in all three porcine ZP proteins by mass spectrometry (Figs. 6.6, 6.7, and 6.8); however, it appears that ZP3 is the major ubiquitinated component among ZP proteins. Ubiquitinated ZP proteins have been detected only on the outer face of the porcine ZP [52]. The reason for this localization is not yet known. Ubiquitinated ZP proteins may be secreted from the cumulus cells surrounding ZP-encased oocytes during the late stage of porcine oocyte growth. It is also possible that the ZP proteins secreted from cumulus cells are ubiquitinated in ovarian follicles.

Carbohydrate Chains of ZP Proteins

The ZP proteins are *N*- and *O*-glycosylated; their carbohydrate contents are estimated at 15–54 % (w/w). ZP proteins give smeared bands on SDS-polyacrylamide gel electrophoresis due to heterogeneity in their carbohydrate chains.

In mice, an essential role has been proposed for the carbohydrate moiety of ZP3 in sperm recognition based on studies showing that the *O*-linked chains of ZP3 are essential for sperm binding (glycan model) [2, 55–61]. For example, the nonreducing terminal *N*-acetylglucosamine (GlcNAc) residue of ZP3 has been proposed to be a sperm ligand that specifically binds to β1,4-galactosyltransferase on the sperm surface [62]. *N*-Acetylglucosaminidase released from cortical granules at fertilization removes the sperm ligand GlcNAc; this may account for the block to polyspermy [63]. Sperm ligands, including GlcNAc, were proposed without direct information on the carbohydrate chain structures of native mouse ZP. These proposed ligands should be reconsidered based on new structural information. Moreover, many proposed sperm ligands have not been supported by studies using transgenic mice [2, 61, 64–66]. Alternatively, a supramolecular complex model was proposed in which the supramolecular structure of the ZP, and not the carbohydrate moiety of the ZP, is necessary for sperm binding in mice [67]. However, the conditional knockout of the gene encoding *N*-acetylglucosaminyltransferase I in murine oocytes was found to reduce sperm binding, as compared to wild-type oocytes, and to reduce fertility, suggesting the involvement of complex and/or hybrid *N*-linked chains in sperm–ZP recognition [33, 68]. The current model of sperm recognition by the ZP is that both the protein and carbohydrate moieties of ZP glycoproteins constitute sperm-binding sites (domain-specific model) [68]. Thus, the sperm–ZP recognition mechanism in mice, especially the essential role of the carbohydrate moiety in the ZP, is still controversial.

Using pig, cow, human, and mouse cells, spermatozoa have been shown to bind the carbohydrate moiety of ZP glycoproteins in vitro. Thus, structural characterization of the carbohydrate moieties of ZP glycoproteins is important for understanding the detailed mechanism of sperm–ZP recognition.

Carbohydrate Chains of Porcine ZP Proteins

Since porcine ZP can be isolated in sufficient quantity for biochemical analyses, the structural characterization of the carbohydrate chains in porcine ZP has been the first one reported in mammals. The structures of the *N*-linked carbohydrate chains from a porcine ZP glycoprotein mixture [69] and from a porcine ZP3/ZP4 mixture (Fig. 6.9) [13, 70, 71] have been determined. The *N*-linked chains are composed of neutral and acidic chains at a molar ratio of about 1:3. The *N*-Linked chains released from a ZP3/ZP4 mixture by hydrazinolysis were fluorescently labeled with 2-aminopyridine and then separated into neutral and acidic fractions by anion exchange column chromatography. The neutral chains were examined by two-dimensional sugar mapping using reverse-phase and size fractionation high-performance liquid chromatography (HPLC), stepwise digestion with a variety of exoglycosidases, and proton nuclear magnetic resonance. The neutral chains include di-, tri-, and tetra-antennary complex-type chains with a fucosyl (Fuc) residue at the innermost GlcNAc (Fig. 6.9; 1–6). Di-antennary chains occupy 75 % of the neutral chains. Structural analyses of the acidic chains have been done using a ZP3/ZP4 mixture. The acidic chains are highly heterogeneous due to a variety of sulfated *N*-acetyllactosamine (Galβ1-4[HSO_3-6]GlcNAc) repeats and sialylation (Sia). Two types of repeats, Siaα2-3Galβ1-4(HSO_3-6)GlcNAcβ1-3Gal and HSO_3-6GlcNAcβ1-3Gal, were obtained from the acidic chains by digestion with endo-β-galactosidase. The acidic chains also include di-, tri-, and tetra-antennary complex-type chains with a Fuc residue at the innermost GlcNAc. The molar ratio in the di-, tri-, and tetra-antennary acidic chains is 2:3:3. It is believed that sulfated *N*-acetyllactosamine repeats are derived from a core structure and that part of the nonreducing terminal galactosyl (Gal) residues are sialylated. Di-antennary acidic chains were separated from the tri- and tetra-antennary acidic chains using concanavalin A, which binds specifically to high-mannose chains and di-antennary complex-type chains. The structures of the di-antennary acidic chains were then analyzed in detail (Fig. 6.9) [70, 71]. The chains were classified into four groups. The first group represents sialylated chains without sulfated *N*-acetyllactosamine repeats (Fig. 6.9; 7), whereas the other three groups have chains of various lengths that vary in the number of monosulfated *N*-acetyllactosamine units at one or both of the two branches (Fig. 6.9; 8–10). Not only C-6 of GlcNAc in *N*-acetyllactosamine but also C-3 in the reducing terminal GlcNAc was found to be sulfated. Fucosylated *N*-acetyllactosamine also exists in the nonreducing portion of acidic chains [72]. The acidic chains have keratan sulfate-type repeats, but *N*-acetyllactosamine disulfated at both GlcNAc and Gal is not found in porcine ZP, while disulfated *N*-acetyllactosamine is found in keratan sulfate. Sulfated *N*-acetyllactosamine elongation is found mainly at the

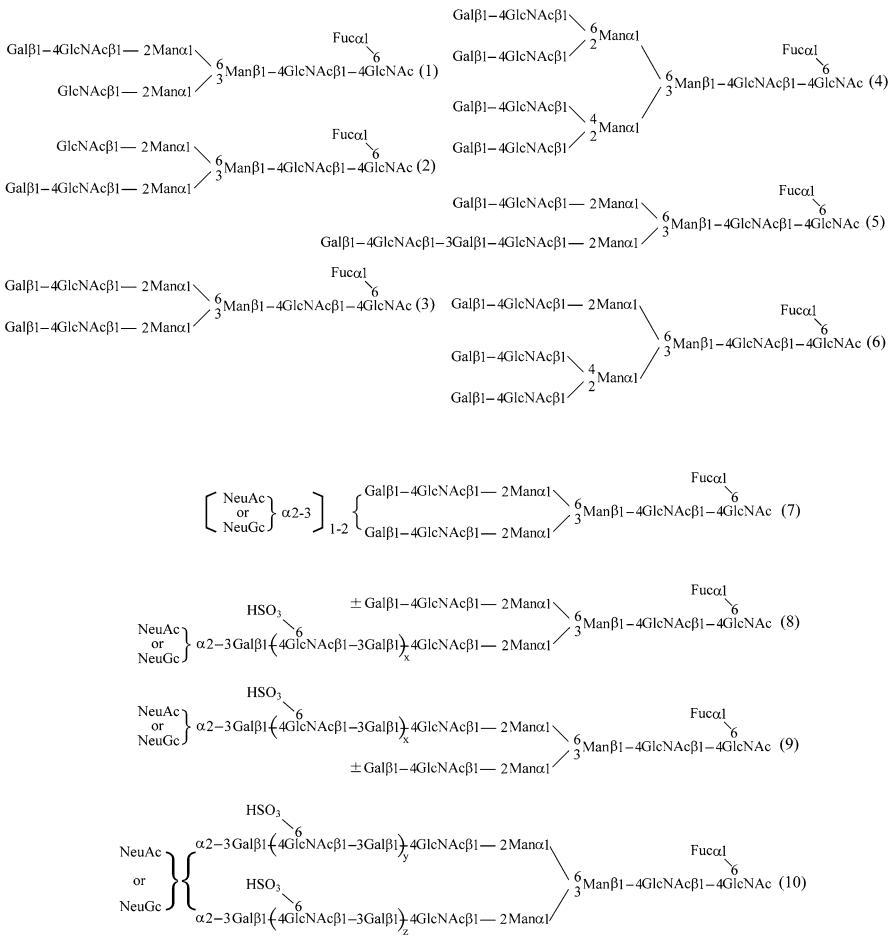

Fig. 6.9 Structures of the neutral *N*-linked chains and acidic di-antennary *N*-linked chains from a porcine ZP3/ZP4 mixture. The structures of the major neutral *N*-linked chains are shown as (1)–(6). The acidic *N*-linked chains are di-, tri-, and tetra-antennary complex-type chains, but only di-antennary chains are shown as (7)–(10). x = 1–6. y + z = 1–6

α1-6mannosyl (Man) branch of di-antennary chains in keratan sulfate, but in porcine ZP, such elongation is found at both α1-6Man and α1-3Man branches (Fig. 6.9). Thus, the structures of the acidic chains in porcine ZP are different from the carbohydrate structure of keratan sulfate. It was proposed that maturation of the carbohydrate chains of ZP glycoproteins also occurs during in vitro oocyte maturation [3]. During oocyte maturation, ZP glycoproteins show a small acidic shift. Sulfation of the *N*-acetyllactosamine repeats in *N*-linked chains during maturation is responsible for at least part of this acidification [73].

The *O*-linked chains were released from porcine ZP by alkali-borohydride treatment, labeled with tritium at the reducing end, and subjected to structural analyses [74–76]. A total of 9 neutral chains and 26 acidic chains were identified (Fig. 6.10). The major *O*-linked chains were unbranched core 1 type chains; the structures of

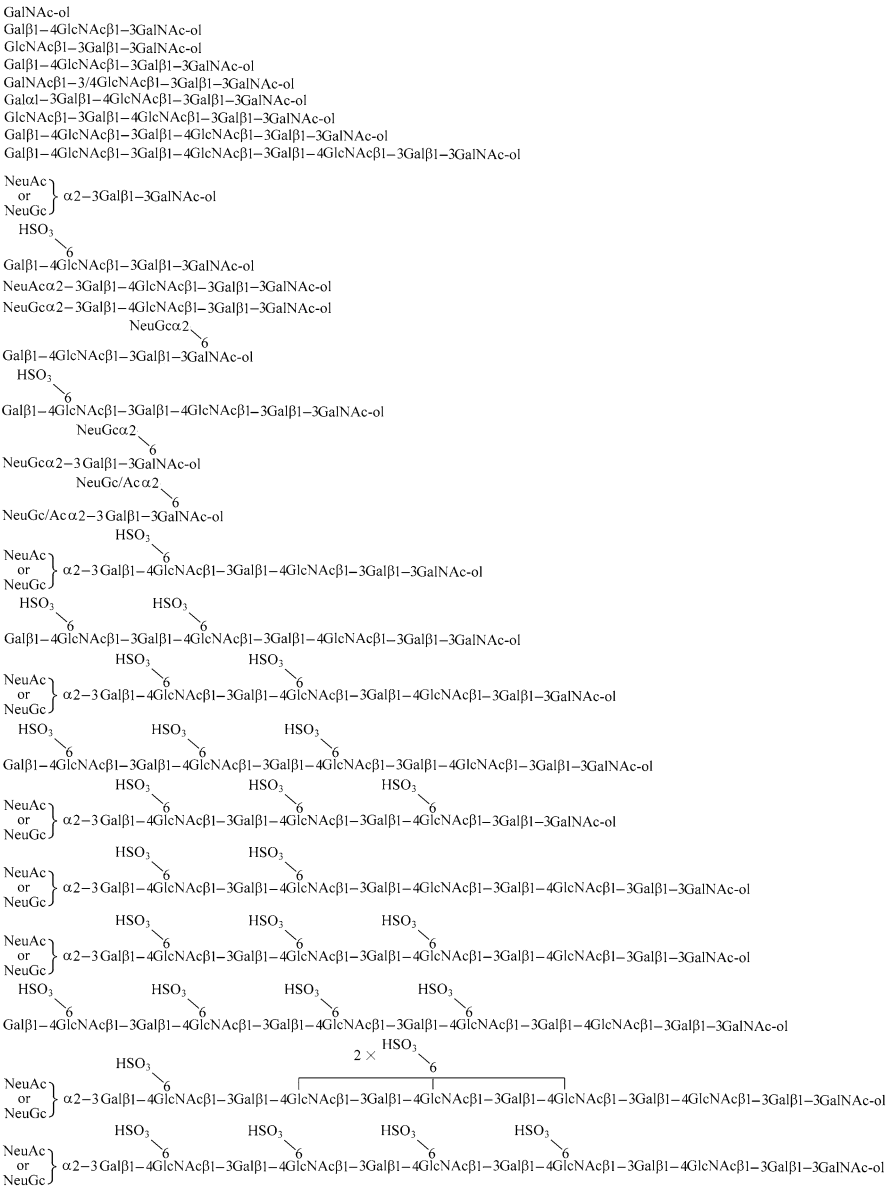

Fig. 6.10 Structures of the *O*-linked chains in porcine zona pellucida

these chains are described as $Sia_{0\text{-}1}[Gal\beta1\text{-}4(HSO_3\text{-}6)GlcNAcb1\text{-}3]_{0\text{->}6}Gal\beta1\text{-}4GlcNAc\beta1\text{-}3Gal\beta1\text{-}3GalNAc\text{-}ol$ (Fig. 6.10). Sulfation occurs at C-6 of GlcNAc in *N*-acetyllactosamine in the *O*- and *N*-linked chains. Gal and GalNAc in the core structure are sialylated in the short chains. As a minor neutral chain, the structure Galβ1-4GlcNAcβ1-3GalNAc-ol was found (Fig. 6.10). The α-Gal and β-N-acetylgalactosamine (GalNAc) were found at the nonreducing terminus.

Neutral *N*-linked chains released from a ZP3/ZP4 mixture retain sperm-binding activity [70, 71], while tri- and tetra-antennary complex-type chains show stronger activity than di-antennary complex-type chains [77]. Removal of the non-reducing terminal β-Gal residues from either tri- or tetra-antennary chains or ZP proteins significantly reduces their inhibition of sperm–egg binding, indicating that the β-Gal residues at the non-reducing ends are involved in porcine sperm–oocyte binding [78–80]. Conversely, another study reported that *O*-linked chains, and not *N*-linked chains, released specifically from a ZP3/ZP4 mixture, inhibited sperm–oocyte binding [81]. Therefore, both *N*- and *O*-linked chains are thought to act as ligands for sperm binding. The sperm-binding activities of carbohydrate chains released from the polypeptides are much lower than the activity of the ZP3/ZP4 mixture. Thus, a protein scaffold is necessary for expression of the sperm-binding activity of carbohydrate chains.

Spermadhesins such as AWN and AQN-3 are lectin-like porcine sperm surface proteins with affinity for Galβ1-3GalNAc and Galβ1-4GlcNAc in *O*- and *N*-linked chains, respectively [82, 83]. An ADAM5/ADAM20- like protein heterodimer with affinity for Galβ1-4GlcNAc has also been proposed as a candidate ZP receptor on the pig sperm surface [84].

N-Linked Carbohydrate Chains of Bovine ZP Glycoproteins

In bovine ZP, the structures of the *N*-linked chains, but not of the *O*-linked carbohydrate chains, have been reported [85]. Sulfation is dominant over sialylation in both *O*- and *N*-linked acidic chains. Most acidic *N*-linked chains become neutral through the removal of sialic acid residues, indicating that the sulfated *N*-linked chain content is small in bovine ZP, while sulfation is dominant in the acidic *N*-linked chains of porcine ZP. The molar ratio of neutral *N*-linked chains to acidic *N*-linked chains is about 1:3. The major neutral *N*-linked chain consists of only one structure, a high-mannose-type chain, $Man_5GlcNAc_2$ (Fig. 6.11). On this point, the structures of the *N*-linked chains in bovine ZP are quite different from those in porcine and human ZP, but similar to those in murine ZP. Four major core structures of acidic chains were identified after removing the non-reducing terminal *N*-acetyllactosamine repeats by digestion with endo-β-galactosidase and then removing β-Gal by digestion with β-galactosidase. The core structures of the acidic chains are di-, tri-, and tetra-antennary, fucosylated complex-type chains (Fig. 6.11). A core structure possessing α-Gal at the putative non-reducing end has also been found. Lectin staining of bovine ZP suggested that glycans in the outer region of the ZP are more highly sialylated than those in the inner region [86]. Thus, there may be a heterogeneous carbohydrate composition in the thickness of bovine ZP, as observed in other species. In cows, a relatively large number of fertilized oocytes can be obtained by in vitro fertilization. The *N*-linked chains from the ZP of fertilized bovine oocytes have far fewer acidic chains than those from unfertilized oocytes. Acidic chains are desialylated and become neutral in the ZP of fertilized oocytes [85]. The high-mannose-type chain ($Man_5GlcNAc_2$) is unchanged on fertilization.

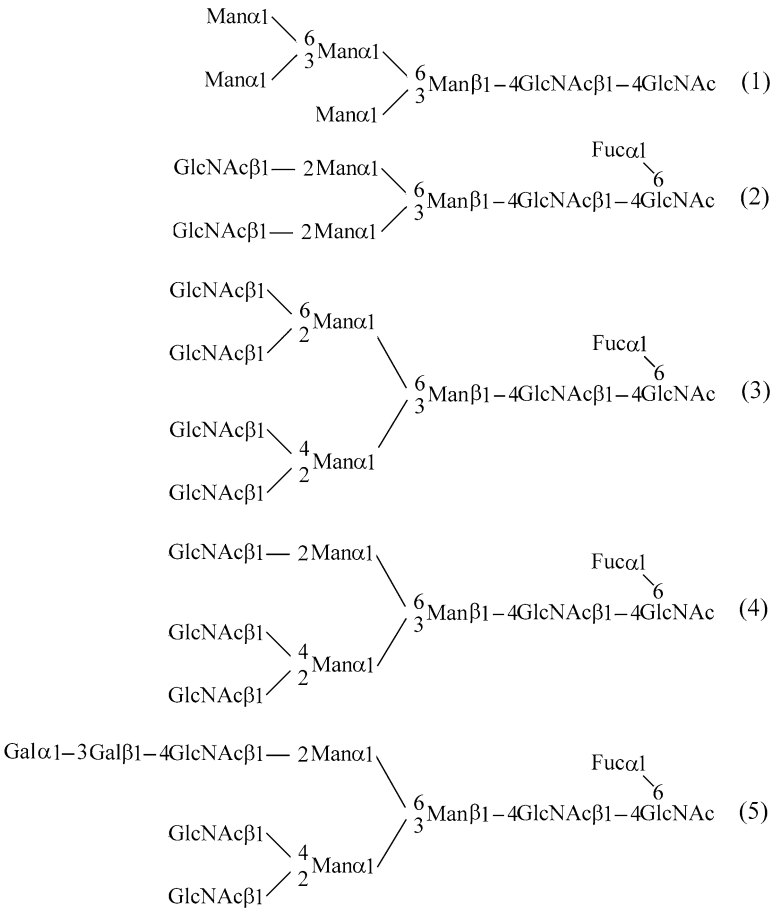

Fig. 6.11 Structures of the *N*-linked chains in bovine zona pellucida. The neutral *N*-linked chain consists of only one structure, a high-mannose-type chain containing five mannose residues, indicated as (1). The acidic *N*-linked chains were analyzed after digestion with endo-β-galactosidase followed by β-galactosidase. The structures shown are of the core region without the acidic groups (2–5)

The non-reducing terminal α-Man in the high-mannose type *N*-linked chains of ZP glycoproteins are bovine sperm ligands [80, 87, 88]. Sialic acid molecules at the non-reducing ends of acidic *N*-linked and/or *O*-linked chains of bovine ZP glycoproteins are also involved in sperm binding [89]. A reduction in the sialic acid content of the ZP from fertilized oocytes has been implicated in the block to polyspermy. Neither α-Man-binding proteins nor sialic acid-binding proteins on bovine sperm have been identified.

Carbohydrate Chains of Murine ZP Proteins

A structural analysis of carbohydrate chains from mouse ZP was conducted after similar studies of porcine and bovine ZP because the amount of protein in a single mouse ZP is about one-tenth of that in porcine or bovine ZP; therefore, biochemical analyses are more difficult to conduct for mouse ZP than for porcine or bovine ZP. The first detailed study was performed on *N*-linked chains fluorescently labeled with 2-aminopyridine by two-dimensional mapping [90]. Structural studies using lectins and radiolabeled chains have also been conducted [91, 92]. The proposed structures from these studies are partly consistent with the structures determined by high-sensitivity mass spectrometry [93]. Native mouse ZP was isolated from ovaries and digested with trypsin. The *N*-linked chains were released from the digests by peptide *N*-glycosidase F (PNGase F) digestion and then permethylated. The *O*-linked chains were released by reductive elimination from the PNGase F-treated glycopeptides and then permethylated. These glycans were then subjected to mass spectrometric analysis. The most abundant *N*-linked chain was a high-mannose-type chain consisting of five Man residues. Less abundant high-mannose-type chains consisting of six to nine Man residues were also detected. Di-antennary chains were dominant among the complex-type chains detected. Tri-antennary chains were also detected, but tetra-antennary chains were rare. These complex-type chains were identified with or without a Fuc at the innermost GlcNAc. The acidic chains contained *N*-acetylneuraminic acid (NeuAc), *N*-glycolylneuraminic acid (NeuGc), or both. The existence of heterogeneous complex-type chains possessing *N*-acetyllactosamine repeats as a minor component was suggested. Galα1-3Gal was found in the nonreducing region of the complex-type chains. A notable feature identified by mass spectrometry is an Sd^a antigen (NeuAcα2-3 or NeuGcα2-3[GalNAcβ1-4]Galβ1-4GlcNAc) in the non-reducing region of the *N*-linked chains and core-2 type *O*-linked chains. Thus, the non-reducing terminal structures of *N*-linked chains are *N*-acetyllactosamine with or without non-reducing terminal sialic acid, α-Gal, Sd^a, α-Man, and GlcNAc. The *O*-linked chains were predominantly core-2 type chains (Galβ1-4GlcNAcβ1-6[Galβ1-3]GalNAc) [93, 94]. The non-reducing terminal structures of *O*-linked chains are the same as those of *N*-linked chains, except for Man and GlcNAc.

The sulfation of murine ZP carbohydrate chains has been also reported [95], but sialylation is predominant in mice. Cytochemical staining of mouse ZP suggested that the inner and outer regions contain different nonreducing terminal carbohydrate residues to which lectins can bind [96, 97]. For example, α-Gal was detected in the inner, but not the outer, region of the ZP [98].

Carbohydrate Chains of Human ZP Proteins

Ultrasensitive mass spectrometric analyses revealed that the major *N*-linked chains are acidic di-, tri-, and tetra-antennary complex-type chains with a Fuc at the innermost GlcNAc residue [99]. High-mannose-type chains were not detected. The *O*-linked chains are core-1 and -2 type chains. A notable structural feature is

that a large part of the antennae of *N*-linked chains are terminated with the sialyl-Lewis^x (sLe^x) sequence [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc] or sLe^x-Le^x [NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNAc] sequence. Sulfated *N*-linked chains were also found as minor chains. Core-2 type *O*-linked chains, but not core-1 type chains, have sLe^x. Thus, sLe^x, well known as a selectin ligand, is abundant in human ZP. The sLe^x binds to the human sperm head and inhibits sperm–ZP binding. Sialylation is essential for the sperm-binding activity of sLe^x. Thus, sLe^x is a good candidate human sperm ligand. Cytochemical staining of human ZP using anti-sLe^x antibodies detected a denser signal in the outer region of the ZP than in the inner region, which may account for the different sperm-binding activity between the outer and inner regions of human ZP [100].

Glycosylation Sites in ZP Proteins

The glycosylation sites in native ZP proteins have been determined for porcine ZP2, ZP3, and ZP4; bovine ZP2; mouse ZP1, ZP2, and ZP3; and rat ZP1, ZP2, ZP3, and ZP4.

Glycosylation Sites in Mouse ZP Proteins

The glycosylation sites of native mouse ZP proteins were determined by mass spectrometric analyses [31]. The *N*-Glycosylated Asn is converted to Asp upon the release of *N*-linked chains by digestion with PNGase F; therefore, the PNGase F digestion of *N*-glycosylated peptides causes an increase in mass (0.98 Da). The *N*-glycosylation can occur at the Asn in the consensus sequence Asn-X-(Ser or Thr) (X cannot be Pro). Proteins ZP1, ZP2, and ZP3 have four, six, and six predicted *N*-linked glycosylation sites in the mature polypeptides, respectively. In ZP1, all four sites (Asn-94, -68, -240, and -371) are *N*-glycosylated (Fig. 6.5). In ZP2, all six sites (Asn-83, -172, -184, -217, -264, and -393) are *N*-glycosylated (Fig. 6.6). In ZP3, five out of six sites (Asn-146, -273, -304, -327, and -330) are *N*-glycosylated; Asn-227 is not glycosylated (Fig. 6.7). The *O*-glycosylation site in ZP1 has not been determined due to the small amount of protein, but the successive removal of *N*-linked chains followed by *O*-linked chains using specific enzymes showed that ZP1 contains more *O*-linked chains than *N*-linked chains. The ZP2 protein probably has only one *O*-glycosylation site, at Thr-455 (Fig. 6.6); ZP3 has two *O*-glycosylated domains. Taking the mass spectrometric data and prediction algorithm data together, two *O*-glycosylation clusters exist at Thr-32, Thr-34, and Ser-39 in the N-terminal region and at Thr-155 and Thr-162 within the flexible hinge region between the ZP-N and ZP-C subdomains (Fig. 6.7). Sperm-binding sites on mouse ZP3 were reported on *O*-linked chains at Ser-332 and Ser-334 [101], but mass spectrometric analyses showed that neither Ser was *O*-glycosylated. The carbohydrate structures at each *N*-glycosylation site have not been determined. Mass spectrometric analyses of the *O*-linked chains at Thr-155 and Thr-162 revealed that core-2 type chains were predominant at both sites [102].

Glycosylation Sites in Rat ZP Proteins

The glycosylation sites in rat ZP proteins were analyzed by mass spectrometry [32, 33]. In ZP1, all three predicted sites (Asn-49, -68, and -369) are *N*-glycosylated (Fig. 6.5). In ZP2, at least six sites (except for Asn-173) among the seven predicted sites (Asn-72, -161, -173, -206, -253, -382, and -563) are known to be *N*-glycosylated, though Asn-173 may also be *N*-glycosylated (Fig. 6.6). In ZP3, there are six predicted sites (Asn-146, -227, -273, -304, -327, and -330). Four sites (Asn-146, -273, -304, and -330) are *N*-glycosylated (Fig. 6.7). Glycosylation at Asn-327 was not ascertained because of a lack of sequence coverage, but Asn-327 may be *N*-glycosylated based on the degree of similarity between mouse and rat ZP3. In ZP4, all four of the predicted *N*-glycosylation sites (Asn-50, -74, -228, and -336) are glycosylated (Fig. 6.8). The *O*-glycosylated sites in ZP1 and ZP2 could not be determined. The ZP3 protein has two *O*-glycosylated domains. The N-terminal domain, which is heterogeneously *O*-glycosylated, includes seven potential sites (Thr-24, Thr-32, Thr-34, Ser-38, Ser-39, Ser-40, and Thr-54), all of which are maximally glycosylated. The second domain resides within the flexible hinge region between the ZP-N and ZP-C subdomains and contains seven potential sites (Ser-148, Ser-149, Thr-155, Thr-160, Thr-162, Thr-164, and Thr-165). This domain is also heterogeneously *O*-glycosylated, and all seven sites are maximally glycosylated. The ZP4 protein has one *O*-linked chain. The *O*-glycosylation site was identified as one of five potential sites (Thr-296, Ser-298, Ser-301, Ser-304, and Ser-312).

Glycosylation Sites in Porcine ZP Proteins and the N-linked Chains at Each Site

The *N*-glycosylation sites in ZP2 were determined by lectin-affinity chromatography using tryptic fragments of ZP2, and identification of the fragments using Edman degradation and mass spectrometry. Porcine ZP2 has six potential *N*-glycosylation sites. Of these, Asn-268, Asn-316, Asn-323, and Asn-530 are glycosylated, while the proteolytic peptide containing Asn-84 and Asn-93 was found to have only one *N*-linked chain [103]. Asn-93, but not Asn-84, was detected by Edman degradation [104], suggesting that Asn-84 is glycosylated while Asn-93 is not (Fig. 6.6). A mass spectrometric analysis succeeded in determining the structures of the *N*-linked chains linked to each site in ZP2 [103]. Similar to the *N*-linked chains in the ZP3/ZP4 mixture described above, di-antennary complex-type chains with Fuc are predominant, while tri- and tetra-antennary chains are the minor chains, in ZP2. The ZP domain of ZP2 has only one *N*-glycosylation site at Asn-530, and di-antennary complex-type chains are predominant at this site. Tri- and tetra-antennary chains may be linked to Asn residues outside of the ZP domain (e.g., Asn-84, Asn-268, and Asn-316), although the localization of the tri- and tetra-antennary chains has not been determined. A remarkable difference in the structures of the *N*-linked chains between ZP2 and ZP3/ZP4 is that ZP2 has a high-mannose-type chain containing five Man residues, probably located at Asn-268 [103]. High-mannose-type chains were not detected in ZP3/ZP4 mixtures. The *O*-glycosylation site in ZP2 is unknown.

The glycosylation sites in ZP3 and ZP4 were determined by the lectin affinity purification of *N*- or *O*-glycosylated proteolytic fragments and amino acid sequence analyses of the isolated fragments. Porcine ZP3 has three *N*-glycosylation sites, at Asn-124, Asn-146, and Asn-271 (Fig. 6.7) [105]. The structures of the *N*-linked chains at each site were analyzed by two-dimensional mapping. Since ZP3 was purified after digestion with endo-β-galactosidase, the *N*-linked chains were not necessarily intact. At Asn-124, a large part of the *N*-linked chains are di-antennary chains (Fig. 6.9; 1–3), while only a small part contains tri-antennary chains (Fig. 6.9, panel 6; 3 % of the chains at Asn-124). Only di-antennary chains (Fig. 6.9; 2 and 3) are found at Asn-146, while di-, tri-, and tetra-antennary chains (Fig. 6.9; 1–4 and 6) are found at Asn-271. The di-, tri-, and tetra-antennary chains possessing Gal residues at all nonreducing termini (Fig. 6.9; 3, 4 and 6) were not digested with endo-β-galactosidase and, therefore, are intact neutral chains.

ZP4 has three *N*-glycosylation sites, at Asn-203, -220, and -333 (Fig. 6.8) [77]. A solubilized porcine ZP protein mixture was digested with lysylendopeptidase. Two *N*-glycosylated fragments of ZP4, Asp-137 to Lys-324 containing Asn-203 and Asn-220, and Asp-325 to Lys-341 containing Asn-333, were isolated from the digests. Thus, the *N*-linked chains have intact structures. Acidic *N*-linked chains were dominant over neutral chains (molar ratio of neutral chains:acidic chains = 1:4) in the fragment containing Asn-203 and Asn-220, while neutral *N*-linked chains were dominant over acidic chains (73:27) at Asn-333. The neutral chains at Asn-333 are di-antennary complex-type chains (Fig. 6.9;1–3). Fragments containing Asn-203 and Asn-220 were isolated by reverse-phase HPLC from the Asp-137 to Lys-324 fragment, and digested with endo-β-galactosidase and chymotrypsin. Tri- and tetra-antennary chains (Fig. 6.9; 4 and 6) are localized at Asn-220, while di-antennary chains (Fig. 6.9; 1–3) are also present at Asn-220. Only one structure, a di-antennary chain (Fig. 6.9; 1), was detected at Asn-203. Since these *N*-linked chains (at Asn-203 and Asn-220) were prepared from the endo-β-galactosidase-digested ZP4 fragment, the *N*-linked chains are not necessarily intact. Since endo-β-galactosidase does not cleave nonreducing terminal Gal residues, the di-antennary chains possessing nonreducing terminal Gal residues at both branches and the tri- and tetra-antennary chains at Asn-220 are intact structures.

In ZP3, Thr-155, Thr-161, and Thr-162 are *O*-glycosylated. In ZP4, Ser-293 and Thr-303 are *O*-glycosylated [106]. However, the structures of the *O*-linked chains at each site have not been clarified.

Glycosylation Sites in Bovine ZP2

The *N*-glycosylation sites in bovine ZP2 have been determined; however, the *O*-glycosylation sites in bovine ZP proteins have not been determined.

The *N*-glycosylation sites in bovine ZP2 are at Asn-83, Asn-191, and Asn-527 [107]. A neutral chain, $Man_5GlcNAc_2$, exists at Asn-83 and Asn-191, but there is very little of the high-mannose-type chain at Asn-527 in the ZP domain of ZP2.

Sperm-Binding Domain Involving Glycosylated Sites on the ZP

A series of biochemical studies of porcine ZP proteins have been performed to identify the sperm-binding sites on the ZP. As mentioned above, among the neutral *N*-linked chains released from the ZP3/ZP4 mixture, the tri- and tetra-antennary complex-type chains showed greater activity than the di-antennary complex-type chains [77]. The β-Gal residues at the nonreducing ends are involved in porcine sperm–ZP binding [78, 80].

It has been proposed that ZP4 has a sperm-binding site in porcine ZP because ZP4 purified from porcine ovaries shows sperm-binding activity, while ZP3 does not [25, 26, 108, 109]. An N-terminal fragment (from Asp-137 to Lys-324) of ZP4 containing two *N*-glycosylation sites at Asn-203 and Asn-220 was found to have sperm-binding activity, and *N*-linked chains located at this fragment are involved in porcine sperm–egg binding [110]. The porcine ZP4 used in these studies was actually contaminated with a trace amount of ZP3, and ZP3/ZP4 heterocomplex formation is essential for the sperm-binding activity of the glycoproteins [111]. Neither pure ZP3 nor pure ZP4 shows sperm-binding activity.

The carbohydrate structures of recombinant porcine ZP proteins expressed using a baculovirus-Sf9 cell system are quite different from those of native porcine ZP proteins. The major structures of the *N*-linked chains of recombinant ZP proteins were estimated to be pauci- and high-mannose-type chains with or without a Fuc at the innermost GlcNAc by mass spectrometry; these results were confirmed using several lectins [79]. The *N*-linked chains possess a nonreducing terminal α-Man and, at this point, they are similar to the neutral *N*-linked chain of bovine ZP proteins, $Man_5GlcNAc_2$, while the neutral *N*-linked chains of porcine ZP have β-Gal and β-GlcNAc at their nonreducing ends. Porcine ZP3 or ZP4 expressed alone in Sf9 cells does not show sperm-binding activity, while co-expressed ZP3 and ZP4 show binding activity toward bovine spermatozoa but not toward pig spermatozoa. Porcine spermatozoa recognize the *N*-linked chains of ZP4 on the ZP3/ZP4 complex [78, 79, 112]. The carbohydrate moieties of ZP proteins are suggested to be essential for the species-selective recognition of bovine and porcine spermatozoa. Heterocomplex formation between ZP3 and ZP4 is necessary for formation of the sperm-binding domain, including the carbohydrate chains. Two posttranslational modifications, glycosylation of the ZP proteins and removal of the C-terminal propeptide, are related to formation of the sperm-binding domain in porcine ZP.

Site-directed mutagenesis at each one of three *N*-glycosylation sites, Asn-203, Asn-222, and Asn-333, to Asp in the polypeptide sequence of porcine ZP4 eliminates the *N*-linked glycosylation at specific sites on ZP4 [79]. The mutation of Asn-333 does not reduce the sperm-binding activity of the ZP3/ZP4 complex, while the mutation of Asn-220 dramatically reduces the activity of the ZP3/ZP4 complex; the mutation of Asn-203 also reduces ZP3/ZP4 complex activity. The localization of the neutral tri- and tetra-antennary chains on ZP4 is related to the sperm-binding site of ZP4 [113]. As mentioned above, the N-terminal fragment containing two *N*-linked chains at Asn-203 and Asn-220 exhibits sperm-binding activity [110], and the tri- and tetra-antennary chains are localized at Asn-220 of porcine ZP4 [77]. Taken

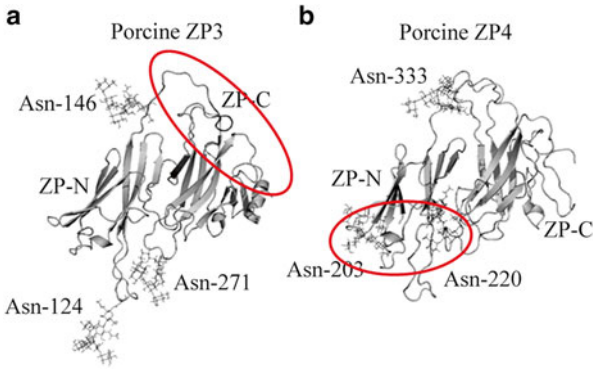

Fig. 6.12 Models showing the three-dimensional structure of porcine ZP3 and ZP4. Three-dimensional structure models of porcine ZP3 (**a**) and porcine ZP4 (**b**) were produced using I-TASSER ONLINE (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The region of ZP3 surrounded by a *red oval* corresponds to the sperm-binding domain of mouse ZP3 [38, 68]. The *N*-glycosylated region of ZP4 indicated by a *red oval* shows the sperm-binding domain of porcine ZP4 [110], but the domain on ZP4 only becomes active when it is part of the ZP3/ZP4 complex

together, these findings suggest that Asn-220 is the most important of the three *N*-glycosylation sites of ZP4 for the sperm-binding activity of ZP3/ZP4. Asn-203 and Asn-220 are located close to each other in the primary structure and in the three-dimensional model of porcine ZP4; thus, the *N*-glycosylated region may form part of the sperm-binding domain (Fig. 6.12). Conversely, another study reported that the *O*-linked carbohydrate chains, and not the *N*-linked chains, which are released specifically from the ZP3/ZP4 mixture, inhibit sperm–ZP binding [81].

In bovine ZP, neither ZP3 nor ZP4 shows sperm-binding activity by itself, but ZP3/ZP4 complex co-expressed in Sf9 cells shows sperm-binding activity [88]. Similar to porcine ZP, the carbohydrate moieties of the ZP may be essential for sperm binding; however, protein scaffold formation caused by the interaction of ZP3 with ZP4 is necessary for the activity of the carbohydrate moieties. Although it was revealed that the region of ZP4 N-terminal to the ZP domain is dispensable for sperm-binding activity, a detailed assessment of the sperm-binding domain on ZP3/ZP4 has not been done.

In contrast to the ZP in pigs and cows, murine ZP3 has sperm-binding activity on its own. The sperm-binding site on ZP3 was determined to be the *O*-linked chains. Furthermore, it was revealed that the sperm-ligand chains are linked to Ser-332 and Ser-334 [101]. This model was challenged by mass spectrometric analyses of native mouse ZP3, as mentioned above. This model was also excluded by an in vivo study using transgenic mice [49]. An alternative model was proposed based on the three-dimensional structure of the chick homolog of the ZP3 precursor. An *O*-linked sugar residue at Thr-168 in the chick ZP3 homolog precursor is involved in sperm-binding activity [38]. Mouse ZP3 has *O*-linked chains at Thr-155 and Thr-162 in the hinge region. The *O*-glycosylation sites are close to the C-terminal region containing Ser-332 and Ser-334 in the three-dimensional structure (Fig. 6.12).

An alternative model suggests that the *O*-linked chains in the hinge and C-terminal regions form the sperm-binding domain (Fig. 6.12) [38, 68].

In porcine ZP3, the *O*-glycosylation sites are also conserved in the hinge region; thus, it is possible that the *O*-glycosylated region of porcine ZP3 is involved in formation of the sperm-binding domain together with the *N*-glycosylated sperm-binding domain on porcine ZP4 (Fig. 6.12).

On the other hand, a recent study revealed that the N-terminal region of ZP2 has sperm-binding activity in humans [50]. The involvement of carbohydrate chains in this binding is not known. Thus, it is not yet known whether sperm-binding domains are conserved in mammals.

Conclusions

Posttranslational processing at specific sites and the ubiquitination of ZP proteins play important roles in regulating zona assembly, the block to polyspermy, and sperm penetration. Conformational changes in ZP proteins, which occur during processing, remain to be clarified. The carbohydrate structures in these proteins are unique to each species analyzed so far. Since carbohydrate chains are at least part of the sperm ligand in vitro, glycosylation is related to species-specific or species-selective sperm recognition by ZP proteins. Additional detailed studies on the interaction of spermatozoa with sperm-binding domains consisting of carbohydrate chains as well as polypeptides should be pursued in order to clarify the mechanism of sperm–ZP binding.

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Role of Aberrant Protein Modification, Assembly, and Localization in Cloned Embryo Phenotypes

7

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Abstract

Aberrant post-translational modifications of proteins contribute markedly to the abnormal characteristics of cloned embryos. This review summarizes aberrant aspects of protein modifications and protein interactions, taking an inside–outside view to the cell. These aberrant aspects affect a range of processes including the control of chromatin structure, expression of pluripotency genes, propagation of epigenetic inheritance, protein trafficking, localization and signaling, cytoskeletal structure, mitosis, and correct localization of membrane proteins. By observing these aberrant features of cloned embryos, how they arise, and their impacts on development, it is possible to gain insight into normal development and identify novel strategies for enhancing cloning outcomes.

Keywords

Somatic cell nuclear transfer • Cloning • Embryo • Protein modification • Protein localization

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Introduction

Cloning by nuclear transfer provides a remarkable applied technology, useful for propagating individuals with valuable phenotypes, genetically modified organisms, and endangered species, with broader potential application in generating pluripotent stem cells for therapeutic use. It also provides a remarkable and unique means of studying normal embryogenesis. With nuclear transfer, it is possible to recapitulate key early events within a different temporal context, create novel nuclear–cytoplasmic dialogs, and characterize the consequences of aberrant gene regulation and defects in other processes. A great deal of effort has been invested in recent years to characterize differences between cloned and fertilized embryos. There are many such differences, and their analysis has provided new insight onto normal gametogenesis and embryogenesis, as well as yielding novel strategies for enhancing the efficiency of cloning. This chapter will examine some of these differences, taking an inside-outside approach. We begin by evaluating aberrant post-translational modifications and localization of nuclear proteins, including chromatin proteins, transcription factors, and other proteins associated with the nucleus. We then examine differences in the cytoplasm, primarily focusing on the cytoskeleton. Last, we address alterations at the cell surface. By examining the differences between cloned and fertilized embryos within these different cellular domains, a new appreciation is emerging for the integral connections between these compartments during early embryogenesis.

Nuclear Proteins and Chromatin Regulation

Many differences in nuclear proteins have been described in cloned embryos. These encompass proteins involved in chromatin structure, DNA methylation, and gene transcription. Chromatin structure is highly regulated by post-translational protein modifications. Modifications arise after nuclear transfer, during developmental progression, or may fail to occur on their normal schedule in cloned embryos.

Histone Modifications

One of the most significant aberrations recognized in cloned embryos relates to abnormal patterns of histone post-translational modifications. Such modifications play key roles in the control of DNA transcription, replication and repair by controlling accessibility of the DNA template. Donor cell nuclei are endowed with a somatic profile of histone post-translational modifications appropriate to their gene expression programs. These profiles differ from normal embryonic patterns. Conversion of a somatic cell genome to an embryonic state would therefore necessitate removal of many histone “marks” and the establishment of others. Experimentally, treating cells with “epigenetic drugs” that modify histone marks

can facilitate this process. The drugs can be applied either to the donor cells to initiate the reprogramming process before nuclear transfer, or to the cloned constructs for different durations after nuclear transfer. Overall, the results from descriptive comparisons of cloned and normal embryos reveal that many somatic marks persist after nuclear transfer, many are removed and new ones established. Epigenetic drugs can enhance cloning outcomes, particularly when applied to cloned constructs, though some treatments can have negative effects, and effects on one type of epigenetic modification can influence other types of modifications. This section reviews some of the key results supporting these observations.

The fates of donor cell-derived histone marks and the patterns of genesis of new ones in the cloned embryo are complex and dynamic. Table 7.1 summarizes many of the observations for different histone marks from different species. In general, repressive histone marks may persist in cloned embryos. Conversely, marks related to gene activation may be more dynamic in clones, and more variable amongst cells. In most situations, increased presence of these marks in donor cells favors cloning outcomes, and treatments that enhance these marks after nuclear transfer do as well. Interestingly, some of the more predictive histone marks are those that represent specialized domains within the nucleus, such as histone 3 serine 10 and 28 phosphorylation (H3S10P, H3S28P), and combined histone H3 lysine 9 methylation and phosphorylation (H3K9me/H3S10P). These marks appear to reflect the reprogramming state of specific chromatin domains, and may prove highly predictive of cloning potential. The histone H3 lysine 4 dimethylation mark (H3K4me2) is also intriguing for its potential role in propagating memory of gene transcription state.

Studies applying epigenetic drugs have yielded a similarly complex range of results. Different drugs can exert different effects [1], different results are obtained depending on time of administration, and different effects have been described between species. However, some common themes emerge. The most efficacious drugs to date have been the histone deacetylase inhibitors (HDACi). Short-term treatments of cloned constructs can yield improvements in early development to blastocyst, placenta formation, term development rates, and phenotypes of cloned offspring. Trichostatin A (TSA) and Scriptaid have allowed cloning to succeed with donor cell types otherwise refractory to the process [2–4], as well as enhancing early development, and in some cases term development, with more tractable donor or recipient cell types [5–10]. Valproic acid also yields positive effects [11], as do oxamflatin [12] and m-carboxycinnamic acid bishydroxamide [13]. In bovine species, HDACi treatment can enhance early development but this is not always followed by increased term development [14–19].

Collectively, these observations reveal that many of the histone marks are modified after nuclear transfer. Subsequently, some marks follow a normal pattern during early embryogenesis, but other marks either persist or fail to be acquired appropriately. Epigenetic drugs applied before nuclear transfer may accelerate some changes, but may have unexpected effects as well. Epigenetic drugs applied to cloned embryos have been beneficial, though not universally so. The beneficial effects of these drugs may be limited by effects on other proteins (see below).

Table 7.1 Summary of changes in histone modifications reported for clones of different species

Mark	Effect on transcription	Mouse	Rabbit	Bovine species	Porcine species	Monkey	Interspecies	References
H3K27me3	Repression	Aberrant X Chr. Inactivation						[30, 31]
H3K9me3	Repression	Persistence signifies limited reprogramming		Increased				[80, 81]
H3K9me2	Repression			Increased				[82]
H3K4me2	Transcription memory; supp. remodeling	Persists; weaker in clones than IVF 2-cell embryos						[83]
H3K4me3	Activation			Increased	Increased			[82, 84]
H3K9me	Activation	Reprogrammed						[85]
H3K9ac	Activation	Reprogrammed; Hyper- & Hypo- acetylation different genes, variable state intergenic regions		Correlates with greater cloning outcome; enhanced in donor cells treated with 5-aza-C		Higher in clones made with cell lines yielding better outcomes	(A); enhanced early development felids	[16, 18, 82, 85–91]
H4K8ac	Activation	Persists			(A); TSA reduces DNA methylation; may have limited effect on term development			[17, 82, 85, 92, 93]
H4K5ac	Activation			(A); differences between blastomeres	Increased		Reprogrammed monkey to bovine SCNT	[82, 94–96]

H4K12ac	Activation	Persists				[85]
H4K14ac	Activation	Reprogrammed; (A) and VPA		(A) and VPA		[8, 85, 97]
H4K16ac	Activation	Reprogrammed				[85]
H3K18ac	Activation			Increased; variable in swamp buffalo		[82, 98]
H3K14ac	Activation			(B)		[17]
H3K9 K14ac2	Activation		High in donor yields better outcome	(A)		[17, 99]
H3S10P	Activation and chromatin condensation	Marks reprogrammed chromatin			Increases after SCNT	[80, 100]
H3K9me 3S10P	Reprogramming marker	Marks reprogramming chromatin				[80]
H3S28P	Reprogramming marker			(A)		[84]
MacroH2A		Removed rapidly				[21]
Histone H1 somatic		Removed rapidly				[20]

Annotation details: *me*, *me2*, *me3*, mono- di- and tri methylation; *ac*, *ac2*, acetylation and deacetylation, *P*, phosphorylation; (*A*), increases with TSA treatment beneficial to cloning; (*B*) increases with TSA with no increase term development

Histone Exchange

Histone turnover and exchange are likely key for nuclear reprogramming. The histone H1 exists in multiple forms including an oocyte form (H1FOO) and a somatic form. The somatic form is stripped from the donor nucleus within an hour after nuclear transfer, whilst the H1FOO form is rapidly acquired starting within five minutes of nuclear transfer [20]. MacroH2A is also removed after nuclear transfer [21]. Histone ubiquitination and degradation may participate in reprogramming in clones by promoting histone replacements [22–26]. Application of protease inhibitors plus HDACis has not been effective in improving cloning in pigs [27]. In the mouse, the proteasomal inhibitor MG132 inhibited the H1 transition [28]. Though protease inhibitors were suggested as a means of enhancing cloning outcome, they could work contrary to the reprogramming process by inhibiting histone degradation.

X Chromosome Inactivation and Histone Modification

X chromosome regulation and inactivation (XCI) is aberrant in cloned embryos. Clones can reactivate, count, and inactivate X chromosomes, but do so inconsistently [29]. Cloned embryos display aberrant expression of *Xist* RNA, both from the active X chromosome in female clones, and in clones made with male Sertoli cell nuclei [30], and selective deficits in essential X-linked gene expression [31], even though both X chromosomes can be initially expressed [32]. Clones made with cumulus cells display two foci of H3K27 trimethylation (H3K27me3), a marker of XCI [31], indicating aberrant inactivation [31]. The inactive X chromosome in normal embryos is marked as a bright immunofluorescent signal with antibody to H3K27me3. The same mark is prominent in cloned male embryos, along with X chromosome expression deficiencies and contrary to X chromosome counting expectations, and female clones show supernumerary foci [31]. Inhibiting *Xist* RNA expression genetically or with siRNA improves cloned embryo development, particularly when done in combination with the histone deacetylase inhibitor trichostatin A (TSA) [30, 31]. Indeed, the treatment of clones with *Xist* siRNA and TSA or the knockout of *Xist* in donor cells led to some of the largest increases in cloning efficiency documented, with increased X-linked gene expression in cloned morulae and blastocysts. These observations suggest that aberrant *Xist* RNA expression may promote aberrant XCI and that this is a major barrier to cloning. Treatment of donor cells with S-adenosylhomocysteine reduces X chromosome methylation and improves clone development [33], indicating that less DNA methylation on the X chromosome is beneficial to preimplantation stage clones. The normal relationships between DNA methylation, *Xist* expression, and H3K27me3 are disrupted in clones.

DNA Methyltransferases

Along with the alterations in histone modifications, other abnormalities in nuclear proteins likely contribute to aberrant gene regulation in cloned embryos. One of the

earliest documented changes in cloned embryos related to altered localization of DNA methyltransferase 1 (DNMT1) variants to nuclei during cleavage stages [34]. There are two forms of DNMT1—the somatic form (DNMT1s) and the oocyte form (DNMT1o). Antibodies that differentially detect the two proteins initially indicated that in fertilized embryos DNMT1o, but not DNMT1s, is enriched in the nuclei of 8-cell stage blastomeres [35, 36]. Its nuclear localization is developmentally regulated [37–39], and this may contribute to passive DNA methylation loss. The DNMT1o form is also preferentially stabilized at early stages [40], offering a possible explanation for its use as a maternally expressed variant in the embryo. Later studies using a novel antibody found that DNMT1s is expressed in oocytes, albeit about 2,000-fold less abundant than DNMT1o. It is also expressed throughout preimplantation development [41]. DNMT1s localizes to nuclei in the early embryo, but shows preferential localization to the maternal pronucleus initially, with delayed appearance within the paternal pronucleus [41]. It is proposed to contribute to maintenance DNA methylation during early embryogenesis [41].

Cloned embryos display DNMT1s nuclear localization at the same stages when DNMT1o is normally enriched in the nucleus. This is observed even using the earlier antibodies, indicating enhanced nuclear localization during this period [34]. Staining with a pan-specific antibody also indicated that some nuclei were deficient in either variant, so that DNA methylation maintenance could be compromised in a subset of cells. It is possible that a low level of DNMT1s undetected by that antibody localizes in these early clones, however the deficiency of DNMT1 staining suggests that overall DNA methylation maintenance could be compromised. This might affect a wide range of genes including imprinted genes, non-imprinted genes displaying allele-specific non-CpG methylation [42], and other genes regulated by DNA methylation. Imprinting defects have been reported for early cloned embryos of several species [43–46]. Given the finding that DNMT1 contributes to de novo DNA methylation [47], deficiencies in its nuclear localization may lead to broader deficiencies in developmental gene regulation in cloned embryos, beyond deficiencies in maintenance of imprints and other sites of DNA methylation.

Taken together, these observations indicate that the mechanisms that regulate partitioning of DNMT1 variants between the nuclear and cytoplasmic compartments are disrupted in cloned embryos. Studies revealed that DNMT1o is relatively non-mobile in the cytoplasm but more mobile in the nucleus [39], pointing to a robust mechanism controlling its localization in the oocyte. A specific domain in the amino terminus mediates this localization [38]. About 70 % of DNMT1 in the oocyte is stably retained in the ooplasm after membrane permeabilization [37]. Interestingly, entry of DNMT1 into the nuclear compartment is not temporally dependent upon DNA replication or gene transcription, but instead is linked to the elapsed time post-fertilization [37]. Interestingly, partitioning of DNMT1 to the cytoplasm also occurs in somatic cells [48] indicating that both DNMT1o and DNMT1s may be subject to this regulation in the early embryo. Moreover, DNMT1 is partitioned to the cytoplasm in neural cells by α-synuclein [49], and its translocation to the nucleus may be mediated in neural cells by the Ras-related nuclear protein RAN [50]. These observations suggest that an active DNMT1 localization mechanism may be mis-regulated in clone embryos, possibly at the level of aberrant

post-translational modification of the partitioning molecules similar to those identified in neural cells.

Additionally, post-translational control of DNMT1 stability by ubiquitination, which is in turn controlled by deubiquitinating enzyme USP7 and ubiquitin ligase UHRF1, has been reported [51]. Mosaic and deficient nuclear localization could also arise due to differences in the control of ubiquitinating/deubiquinating proteins.

Pluripotency Factors

Cloned embryos also display changes in the localization of key proteins regulating cell fate and developmental potential. Early studies revealed mosaicism and deficient expression of POU5F1 (a.k.a., OCT4) [52]. Further studies to understand the contributions of deficient localization or protein stability in clones would be interesting. More recent studies implicated other transcription factors that modulate cell fate in cloning outcome. For example, the expression of *Chd7* and *Runx1t1* mRNAs was negatively correlated with cloning outcome [53]. CHD7 is a chromodomain helicase that regulates pluripotency genes and also colocalizes with POU5F1, NANOG, SOX2 and P300 in ES cells [54], and the RUNX1T1 is a histone deacetylase that may repress target genes [55]. The differential expression of such proteins in either donor or recipient according to genotype could affect pluripotency establishment via post-translational regulation such as histone modifications.

Cytoplasmic Proteins

One area of interest to cloning biology is the potential for non-nuclear effects of HDAC inhibitors (HDACi). As described above, HDACi are widely used to enhance the success of cloning. But HDACs normally act upon many cytoplasm proteins as well as nuclear proteins, and modulate a wide range of cellular activities (e.g. [56–60]). HDAC localization is regulated post-translationally [61]. The potential effects of HDACi on these processes in cloned embryos have not been explored.

The beneficial effects of glucose in clones are accompanied by favorable changes in mitochondrial ultrastructure [62]. The regulation of mitochondrial composition may thus be altered in clones, and clones may possess a signaling mechanism coupling increased glucose availability to increased expression of genes for oxidative phosphorylation. The presence of this signaling mechanism in clones but not normal embryos could rely on differences in post-translational modifications of intermediate signaling transducers.

As alluded to above, changes in protein ubiquitination/deubiquitination and protein turnover in cloned embryos may contribute to observed changes in embryo characteristics. Cloned mouse embryos at the 2-cell stage display enhanced expression of mRNAs related to proteasomal proteolysis and protein ubiquitination [63]. This could alter the overall flow of transition from maternal to embryonic control of development, as well as embryonic protein composition as cleavage proceeds. Additionally, from a technical standpoint, a prolonged period of incubation of the

donor nucleus within the recipient ooplasm before oocyte activation could be beneficial for reprogramming. But, such a delay in activation diminishes developmental success [28], indicating a possible ongoing decay of maternal proteins and other macromolecules. One report recommended application of the protease inhibitor MG132 to prevent this decay and allow prolonged reprogramming [64], following improved outcomes in cloning the rat [65]. In mice, MG132 inhibited the loss of somatic histone H1, improved development with delayed activation, and yielded a small enhancement of blastocyst formation with timely activation [28]. No beneficial effect on term development was seen. These observations may reflect a beneficial effect of proteasomal inhibitor with respect to stabilizing essential maternal proteins, but may also signify that this treatment either has no consequence for reprogramming, or could be mildly inhibitory by retarding protein turnover within the nucleus.

The Saga of the Spindle in Cloned Embryos

A striking report was published in 2003, demonstrating an apparent reduction of abundance of key spindle proteins on the mitotic spindles of cloned monkey constructs [66]. It was suggested that removal of the spindle–chromosome complex (SCC) at the first step of cloning might irreversibly deplete these proteins, and lead to mitotic errors in the early embryo. Subsequent studies in the mouse using Western blotting revealed that SCC removal could diminish the total content of particular spindle-associated proteins, but that their abundances were partly or wholly restored during 1–3 h after nuclear transfer [67]. For some proteins, recovery required the nuclear transfer, indicating that formation of a new SCC after nuclear transfer (denoted the pseudomeiotic or pmSCC) was required. Incorporation of proteins into the spindle, therefore, may stabilize associated proteins and facilitate their recovery in total abundance. Immunofluorescence analysis revealed that a majority of proteins were reacquired onto the pmSCC, but one protein examined, calmodulin, did not. This protein remained deficient, and this deficiency was recapitulated at each of the first two mitotic divisions in the embryo [67].

Because the calmodulin mRNA was abundantly expressed in oocytes, it appeared likely that calmodulin deficiency on the pmSCC and later mitotic spindles was not the result of persistent total calmodulin protein deficit, but rather a failure of calmodulin still present in the oocyte to be recruited to the spindle appropriately. Support for this conclusions came from examination of tetraploid constructs prepared by nuclear transfer without prior SCC removal [67]. Calmodulin was present on the SCC, but again deficient on the pmSCC despite the fact that no material had been removed [67]. A more convincing argument for a failure to recruit calmodulin came from examinations of cloned constructs prepared with 8-cell stage blastomere nuclei or embryonic stem cell nuclei. In these cases, the pmSCC stained normally for calmodulin [67]. Thus, the nature of the donor genome controls the pmSCC protein composition by influencing protein recruitment to the pmSCC.

In an effort to understand the basis for the failure of pmSCCs formed after somatic cell nuclear transfer to acquire the appropriate array of proteins, a proteomics analysis was undertaken to compare isolated SCCs from normal metaphase II stage

oocytes, isolated pmSCC obtained 2 h post-somatic cell nuclear transfer, and SCC-depleted ooplasts, using a mass spectroscopy (MS) based approach [68], combined with a survey of candidate proteins identified in prior proteome studies of somatic cell mitotic spindles. The proteomic analysis identified 64 proteins enriched on the SCC compared to the ooplasts, 25 deficient on the pmSCC, and 6 with increased abundance on the pmSCC [68]. Combined with the candidate protein analysis, the study revealed deficiencies in four additional proteins, clathrin heavy chain (CLTC), aurora B (AURKB), dynactin 4 (DCTN4) and casein kinase 1 alpha (CSKN1A1).

CLTC displayed the most robust signal on the MS analysis and a prolonged deficiency on Western blots. CLTC was chosen for more in-depth study. One of the hallmarks of cloned constructs is that there is substantial scattering of the donor cell condensed chromosomes and an apparent delay in chromosome congression [67]. Deficiency on the pmSCC might have no functional consequence, because polar body extrusion is prevented during activation. Deficiency during mitosis, however, could lead to mitotic errors and aneuploidy. The potential for generating aneuploidy was thus a significant concern. Earlier studies examining aneuploidy in cloned blastocysts indicated a small but increased fraction of aneuploid cells [29]. More recent studies indicated that aneuploidy can be more pervasive [69]. To learn whether manipulating CLTC expression affected chromosome congression in the oocyte, its expression was reduced prior to maturation using siRNA microinjection [68]. This led to CLTC-deficient SCC, and chromosome congression defects. Conversely, microinjecting CLTC mRNA to enhance CLTC expression after cloned construct activation enhanced chromosome congression efficiency at first mitosis [68]. Despite this dramatic rescue of an aberrant cloned embryo phenotype, which constituted the first functional test of the original hypothesis that SCC defects contribute to limited cloning success, term development was not elevated, confirming that other barriers to success must exist.

Interestingly, CLTC is most widely known for its role in endocytosis. Other endocytosis-related proteins were detected in the MS analysis of SCC [68]. A role for CLTC in mitosis had been reported earlier [70, 71]. CLTC plays a vital role in bundling microtubules together [71] and is required for recruiting proteins to the spindle [70]. It was relevant, therefore, that the pmSCC also appears to stain less intensely for tubulin [68]. Thus CLTC may play a key role in bundling microtubules together to form the basic framework of the spindles, and then recruit other proteins to the spindle as cargo, mediated by additional endocytosis-related proteins. It is interesting to speculate that the general deficiency of multiple proteins on the pmSCC obtained with somatic cell nuclear transfer, but not embryonic cell nuclear transfer, may result from a difference in the initial association of these key endocytosis-related proteins with the nucleus. In any case, it appears that spindle protein composition is altered in clones through a combination of protein deficiency and altered post-translational mechanisms that affect the ability of proteins to be recruited to the newly formed pmSCC and the mitotic spindles. Understanding how protein assembly at the spindle is altered in clones may provide new insight into spindle regulation in normal oocytes, embryos, and somatic cells.

A Role for Cytoskeleton Scaffold Proteins in Clones

Another story has unfolded recently indicating the apparent importance of cytoskeletal proteins (including spindle proteins) in the cloning process. To identify ooplasmic factors that contribute to successful early cloned embryogenesis, a systems genetics approach was taken to map genome regions that correlate with early developmental progression [53]. This study was based on the initial observation that the oocytes of two inbred mouse strains (C57BL/6 and DBA/2) differ markedly in their abilities to support early clone development [72]. The efficiency of preimplantation cloned embryo development was evaluated for 28 of the C57BL/6 X DBA/2 (BXD) recombinant inbred mouse lines. The trait data were then analyzed to yield a primary locus on chromosome 17 and two additional loci on chromosomes 1 and 4 as being significantly associated with early cloned embryo development. These three regions were flanked by additional regions having suggestive associations with the traits. A total of 195 candidate genes for the three significant and six suggestive intervals were evaluated by a variety of parameters, eventually narrowing the list of candidates to a set of 26 genes. The two strongest candidates, lying within the chromosome 17 significant interval, were EPB4.1L3 and DLGAP1, which displayed negative mRNA expression correlations with cloning outcome. Both proteins encode cytoskeleton scaffold proteins that orchestrate numerous protein interactions within the cortical cytoplasm. Additional candidate genes encoded spindle-associated proteins, and signaling proteins known to associate with the cytoskeleton, or to regulate cytoskeletal structure.

That some candidates encode spindle proteins and that these may contribute to cloning outcome is not surprising. However, the potential involvement of cytoskeletal scaffold proteins that control protein interactions within the cortical cytoplasm raises novel possibilities for contemplating the molecular basis for limited cloning success. These proteins likely play critical roles in mediating diverse cellular functions, ion and molecular transport, protein trafficking, and signal transduction. Because clones express many somatic cell-like characteristics and molecules, with precocious or delayed activation of some processes, the robustness with which protein interactions at the cytoskeleton occur within the cortical cytoplasm, or throughout the cytoplasm, could have significant effects on the overall physiology and viability of cells in cloned embryos. Further studies exploring the mechanism by which the cytoskeletal scaffolding proteins modulate cloned embryo development should prove interesting. One mechanism might be by modulating the cortical cytoplasmic lattices or subcortical maternal complex [73–75], which provide vital, though as yet incompletely characterized functions in the early embryo. Another possible role could lie in controlling cellular potency. Earlier studies revealed differential association of acetylated tubulin with inner versus outer cells in the early embryo, a difference that may affect allocation to the inner cell mass versus trophectoderm lineage [76]. Wherever the answer is discovered, understanding the role of the cytoskeleton in cloned embryo and normal embryo development should prove very interesting.

Membrane Proteins–Molecular Transporters

An example of post-translational effects on this category of proteins is found in the glucose transporters. Cloned mouse embryos require glucose in the culture medium during early cleavage stages, whereas normal embryos do not [77]. Clones also take up more glucose [78], have increased glucose metabolism and increased glycogen stores, but lower ATP levels [62]. Increased uptake may be mediated by changes in glucose transporter regulation, as SLC2A1 (GLUT1) first localizes to the cell membrane precociously (2-cell stage) in clones [78]. The somatic cell form SLC2A4 (GLUT4) is aberrantly expressed in myoblast-cloned embryos [78]. In some cancer cells, GLUT1 localization to the plasma membrane is promoted by IKKB and NFkappaB via separate points in the AKT signaling pathway [79]. This raises the interesting possibility that this signaling pathway may be precociously active in clones, and could exert widespread changes in post-translational modification and activities of many target proteins.

Conclusions and Perspectives

Cloned embryos manifest a wide spectrum of aberrant characteristics, as indicated in the foregoing inside-to-outside summary of what has been observed in the cells of cloned constructs. Many of these deficiencies are attributable to slow or incomplete nuclear reprogramming. Consequently, a great deal of effort has been invested in the examination of post-translational modifications and localization of nuclear proteins in cloned embryos, and several exciting and encouraging advances have been realized in improving the technology by manipulating these features, most notably histone acetylation and expression of the *Xist* RNA. Other studies, however, point to a significant role for aberrant expression or regulation of cytoplasmic and membrane proteins in limiting cloned embryo developmental capacity. In particular, genetic, immunolocalization, and proteomic studies indicate a key role for cytoskeletal proteins in normal development, and disruptions in these functions in clone development. Some technical refinements to improve cloning have related to using less disruptive treatments of the cytoskeleton during the process. Collectively, these more recent studies indicate that the cloned embryo still has much to teach us about normal development, and that cloning may be further improved by a deeper understanding of the post-translational regulation and function of the cytoskeleton and associated molecules. Accordingly, as our knowledge of the cloned embryo evolves along this same inside-to-outside trajectory, so too should evolve our understanding of the early embryo. This continued growth in our understanding should provide new opportunities for advancing the role for reproductive biology in the clinical and applied arenas as well.

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Role of Posttranslational Protein Modifications in Epididymal Sperm Maturation and Extracellular Quality Control

8

Gail A. Cornwall

Abstract

The epididymal lumen is a complex microenvironment in which spermatozoa acquire motility and fertility. Spermatozoa are synthetically inactive and therefore the maturation process requires their interaction with proteins that are synthesized and secreted in a highly regionalized manner by the epididymal epithelium. In addition to the integration of epididymal secretory proteins, posttranslational modifications of existing sperm proteins are important for sperm maturation and acquisition of fertilizing potential. Phosphorylation, glycosylation, and processing are several of the posttranslational modifications that sperm proteins undergo during epididymal transit resulting in changes in protein function and localization ultimately leading to mature spermatozoa. In addition to these well-characterized modifications, protein aggregation and cross-linking also occur within the epididymal lumen and may represent unique mechanisms for controlling protein function including that for maturation as well as for extracellular quality control.

Keywords

Epididymis • Luminal fluid • Spermatozoa • Aggregation • Amyloid • Transglutaminase • Phosphorylation • Glycosylation • Ubiquitination

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Introduction

The most recent estimate suggests there are 20–25,000 genes in the human genome [1]. The use of alternative promoters and splicing events estimates approximately 100,000 transcripts are derived from these genes. However, by the use of posttranslational modifications (PTM), the number of proteins that results from these 100,000 transcripts is amplified considerably and is predicted to be greater than 1,000,000 [2]. Because PTM affect protein structure, resulting changes in protein localization, interactions, activity and stability can occur. Thus PTM of proteins are a powerful means by which an organism can develop complex mechanisms for regulation of function and allow new and rapid adaptations. PTM are currently numbered at 200 different modifications and involves the modification of amino acids and the attachment of functional groups such as GPI anchors, acetyl, alkyl, phosphate, or glycosyl groups, addition of peptides including SUMO or ubiquitin, and structural changes by formation of disulfide bonds or proteolytic cleavage [3]. Phosphorylation and N-linked glycosylation have been shown to be the most prevalent PTM reported experimentally in the Swiss-Protein Knowledgebase [4]. Most PTM are reversible allowing the fine-tuning of protein function by the addition or removal of specific modifying groups.

The epididymis is a long tubule through which spermatozoa must pass after they migrate out of the testis. As they move from the proximal (caput) to the distal (cauda) end of the epididymis, spermatozoa gradually mature and acquire the capacity for progressive motility and fertility. Because spermatozoa are synthetically inactive, the maturation process involves the interaction of spermatozoa with proteins that are synthesized and secreted into the luminal environment by the epididymal epithelium. The highly regionalized synthesis and secretion of proteins from the epithelium result in spermatozoa being exposed to a constantly changing luminal environment that ultimately causes maturation. Thus during epididymal transit spermatozoa are exposed to an assembly line that includes sequential interactions with a variety of proteins such as those that become integrated into spermatozoa as well as those that induce PTM. Indeed, PTM of spermatozoa appear to be the primary mechanism by which sperm maturation occurs. These sperm protein modifications, as we will discuss in greater detail below, include those commonly found in other biological systems including protein phosphorylation, glycosylation, and processing. Proteins present within the epididymal lumen and not directly associated with spermatozoa also undergo similar types of PTM. However, within the epididymal lumen protein modifications less well-described in other organ systems, including changes in protein structure by altered protein folding resulting in protein aggregates or by protein cross-linking also occur and are integral to epididymal function. Why these particular protein modifications occur within the epididymal lumen has yet to be established. However, it may reflect the unique functions of the epididymis itself and the critical role it plays not only in the maturation but also in the protection of spermatozoa. Considering that perpetuation of the species requires functionally intact spermatozoa, it is likely that quality control mechanisms are in place within the epididymal lumen to deal with the extremely active synthetic and

secretory properties of the epithelium including any possible protein misfolding that could occur within the lumen. As will be discussed, the formation of protein aggregates may be a means for proteins to acquire new functions as well as prevent function. This review will briefly discuss the different types of PTM that have been described in the epididymis and associated with sperm maturation as well as how different PTM may mediate extracellular protein quality control within the epididymal lumen.

Phosphorylation

Phosphorylation is the addition of a phosphate group to serine, threonine, tyrosine (O-linked) or histidine (N-linked) amino acids. This involves the transfer of phosphate groups from ATP or GTP to the protein by the action of protein kinases while dephosphorylation utilizes protein phosphatases. In general, changes in protein phosphorylation are associated with the activation or inactivation of protein function during cell signaling cascades. Both protein kinases and phosphatases are present in epididymal spermatozoa. Furthermore, changes in the phosphorylation status of proteins have been observed in spermatozoa during epididymal transit with resulting alterations in protein localization and/or functions suggesting that this PTM plays an important role during sperm maturation.

Although activation of a cAMP/PKA pathway with an upregulation of protein tyrosine phosphorylation is a hallmark of the sperm capacitation process and necessary for the acrosome reaction, less is known about specific phosphorylation events that occur in spermatozoa during epididymal transit and how these events affect maturation. Early studies in both rat and boar suggested maturational changes in sperm protein tyrosine phosphorylation with caput spermatozoa showing a more dispersed localization of phosphotyrosine labeling over the acrosome while cauda spermatozoa exhibited a pattern of protein phosphorylation confined to the posterior aspect of the acrosome, suggesting that dephosphorylation of some sperm proteins was associated with epididymal sperm maturation [5, 6]. Increased tyrosine phosphorylation labeling was then observed over the whole boar sperm head after capacitation suggesting a reversal of some of the maturation-associated modifications or activation of new populations of proteins [6]. The marked decrease in tyrosine phosphorylation in rat and boar spermatozoa during epididymal transit is in marked contrast to that observed in the mouse where overall protein tyrosine phosphorylation, as determined by Western blot analysis, was relatively low in both caput and cauda spermatozoa [7]. Individual sperm proteins that have been shown to be tyrosine phosphorylated in the epididymis include the ATP-binding cassette membrane transporter G2 (ABCG2). ABCG2 is present in the plasma membrane overlying the sperm acrosome and is proposed to play a role in the translocation of cholesterol across the plasma membrane in epididymal spermatozoa [8]. The tyrosine phosphorylation of ABCG2 has been shown to be necessary for its function within epididymal spermatozoa while dephosphorylation is believed to result in inactivation of ABCG2 function.

In addition to tyrosine phosphorylation, phosphorylation of sperm proteins at serine and threonine residues is also associated with epididymal maturation. The beta subunit of F1 ATPase in spermatozoa was shown to be serine phosphorylated during epididymal transit perhaps as a means to stabilize the protein [9]. This enzyme has been shown in a lymphoma cell line to undergo cAMP-dependent phosphorylation suggesting that the F1 ATPase may be a target of a PKA signaling pathway (cAMP-dependent kinase activity) that becomes active in spermatozoa as they mature in the epididymis [10–12]. Functionally, phosphorylation of the mitochondrial F1 ATPase may then increase ATP production, facilitating sperm motility maturation.

The use of mass spectroscopy to identify phosphoproteins in spermatozoa has revealed the breadth this particular modification plays in sperm maturation and has identified a number of sperm associated proteins that are differentially phosphorylated depending on the epididymal region from which they are isolated [13]. Using titanium dioxide to enrich for phosphopeptides, Baker et al. [14] identified several proteins including the fertilization molecule IZUMO, testis lipid binding protein, A-kinase anchoring protein 4, outer dense fiber I, sodium bicarbonate cotransporter, dynein intermediate chain I, and several others that showed changes in phosphorylation between epididymal regions. In this study, the majority of phosphopeptide changes occurred in spermatozoa between the corpus and cauda regions where significant maturational changes in motility are observed. Furthermore, of the proteins that showed maturational changes in phosphorylation status, many of these had previously described roles in sperm capacitation. It may be that in the more distal epididymal regions, important signaling complexes are becoming activated in spermatozoa in preparation for downstream fertilization events. Together, these studies show that sperm proteins with proposed functions not only in cell signaling but in other cellular processes as well are modified by phosphorylation during epididymal transit as an integral part of the maturation process.

The phosphorylation of proteins is mediated by the activity of kinases that are present in spermatozoa including those of the MAP kinase pathway [15–17], CaMKII alpha [18], FYN kinase [19], JAK/STAT [20] and many others. Most of these kinases have been studied from the standpoint of their roles in sperm capacitation and fertilization, and will not be addressed here. However, several other sperm-associated kinases have been shown to undergo modifications themselves during epididymal transit and others have been implicated in specific maturation events. For example, the testis specific serine kinase I undergoes phosphorylation during epididymal transit, perhaps as an activation step to ultimately allow additional downstream phosphorylation of target proteins [14].

Other sperm kinases include glycogen synthase kinase-3 alpha (GSK3-apha), a signaling kinase that becomes serine phosphorylated in spermatozoa during epididymal transit. GSK-3 was shown to be more active in caput than in cauda spermatozoa, suggesting that phosphorylation of GSK-3 is associated with a loss of its function. Stimulation of sperm motility by the phosphodiesterase inhibitor isobutylmethyl-xanthine also caused an increase in GSK-3 serine phosphorylation, suggesting a role for this kinase in the regulation of sperm motility [21]. Upstream signaling

molecules protein kinase B and phosphoinositide 3-kinase, that are involved in GSK-3 phosphorylation, are also present in spermatozoa [21].

The tyrosine kinase cSrc has been proposed as the kinase responsible for protein tyrosine phosphorylation during sperm capacitation since this kinase is activated during capacitation and specific inhibitors blocked sperm capacitation and its associated protein tyrosine phosphorylation [22, 23]. However, despite exhibiting reduced motility and fertility in vitro, protein tyrosine phosphorylation still increased during capacitation of spermatozoa from cSrc null mice suggesting that cSrc does not play a direct role in this process [24]. Recently, it was shown that cSrc is secreted by the epididymis and is acquired by spermatozoa during epididymal transit [24]. Together these studies suggest an important role for cSrc in epididymal sperm maturation possibly as a mediator of critical signaling events that contribute to the acquisition of sperm motility.

Protein kinase A RI, RIα, and RIIα subunits are active in regulating tyrosine phosphorylation in both caput and cauda epididymal spermatozoa yet caput sperm exhibit lower and different patterns of tyrosine phosphorylation when exposed to dbcAMP and PTX, suggesting other maturational changes are also involved [5, 7, 25]. RIIα itself also appears to undergo region-dependent serine phosphorylation [14].

The removal of phosphate groups by the action of phosphatases has also been implicated in epididymal sperm maturation. The serine–threonine protein phosphatase I gamma 2 (PP1γ2) plays an important role in the development and regulation of sperm motility. Specifically, high PP1 activity is present in immotile caput spermatozoa with lower activity in motile cauda spermatozoa. Furthermore, inhibition of PP1 activity by okadaic acid and calyculin A stimulated motility in the normally quiescent caput spermatozoa, suggesting that the potential for motility is already present in caput epididymal spermatozoa and that the high PP1 activity keeps this motility in check [26]. PP1 has also been proposed to regulate sperm motility by the suppression of the full activation of PKA [27]. A population of phosphorylated PP1γ2 was shown to localize to the sperm head, suggesting additional roles for this phosphatase in fertilization [28].

Glycosylation

Glycosylation refers to the enzymatic process that attaches glycans/sugars to proteins, lipids, or other organic molecules and is distinct from glycation which is the nonenzymatic attachment of sugars. N-linked glycosylation involves the transfer of sugars to a nitrogen of asparagine or arginine residues and occurs in the lumen of the endoplasmic reticulum. O-linked glycosylation is the transfer of sugars to the hydroxy oxygen of serine, threonine, tyrosine, hydroxylysine, or hydroxyproline residues and occurs in the Golgi. Cellular processes associated with glycosylation include modifications of the cell surface and host defense. The glycan modifying enzymes glycosyltransferases, which add sugars to proteins, and glycosidases, which cleave sugar residues from glycoproteins, are present in the epididymal fluid. Both N-linked and O-linked glycosylation has been observed in proteins present in

the epididymal fluid and associated with spermatozoa; thus glycosylation appears to be a significant PTM that modifies epididymal sperm surface proteins affecting functions important for sperm maturation. Indeed, using a variety of different approaches including treatment with glycosidases or by lectin binding, it appears that the sperm surface, like other cells, is dominated by the presence of carbohydrates. These carbohydrates linked to protein, lipids or other molecules create what is referred to as the glycocalyx on the sperm surface and which plays an integral role in fertilization [29].

Sperm plasma membrane glycoproteins that likely contribute to the glycocalyx are extensively modified during epididymal transit as shown by the profound changes in the binding of the lectin PNA to spermatozoa from different epididymal regions. Rat cauda epididymal spermatozoa showed a loss of PNA binding compared to that in caput spermatozoa which was thought to reflect either the masking of galactosyl residues by the addition of other sugars or the cleavage of the galactosyl residues by β-D-galactosidase [30]. In the rhesus monkey, regional modifications of epididymal glycoproteins (O- and N-linked) including those associated with spermatozoa was observed using biotinylated lectins [31, 32]. An analysis of the boar sperm glycocalyx was recently performed by lectin binding and increases in sperm galactose, glucose/mannose, and N-acetylglucosamine were observed in spermatozoa from distal epididymal regions [33]. Similar studies have also been performed in the cat [34]. Examples of specific proteins that undergo changes in glycosylation during epididymal transit include SPAM 1 (PH-20). The hyaluronidase activity of SPAM1 in caput spermatozoa is much lower than in cauda epididymal spermatozoa. Furthermore, the increase in enzymatic activity in spermatozoa during epididymal transit was correlated with a reduction in the molecular weight of SPAM1. It was proposed that the deglycosylation of the N-linked glycosylated SPAM1 may be important for the activation of its activity [35]. The transmembrane glycoprotein basigin belongs to the immunoglobin superfamily and is localized to the sperm tail. During epididymal transit, basigin localization changes from the principal piece to the midpiece, with a concurrent reduction in molecular mass that is thought to represent deglycosylation [36]. In most species, there is a general increase in negative surface charge during epididymal sperm maturation [37, 38]. This is likely due to the acquisition of sialic acid in spermatozoa during epididymal transit since increased binding of the lectin WGA, specific for sialic acid and N-acetylglucosamine, is observed [39]. Two sialoproteins, proteins D and E, are secreted by the epididymal epithelium and associated with spermatozoa [40]. Other glycoproteins that become associated with the sperm surface during epididymal transit that may contribute to the sperm glycocalyx include CD59, fertilin, HE2, HE4, and HE5 (reviewed in [29]).

Of the several glycosyltransferase and glycosidase activities that have been examined in the rat epididymis, most are in the luminal fluid fraction with only a small percentage of enzyme activities associated with spermatozoa [41]. Namely, galactosyltransferase, glucosaminyltransferase, fucosyltransferase, and sialyltransferases were primarily detected in the supernatant after low and high speed centrifugation to pellet spermatozoa and insoluble components in the luminal fluid.

Similarly, the glycosidases β-D-galactosidase, β-D-glucuronidase, α-D-mannosidase, and β-*N*-acetylglucosaminidase were present in the supernatant fractions [41]. While glycosyltransferases exhibit pH optima at neutral pH, compatible with the epididymal luminal pH of 6.6–6.8, most glycosidases are optimally active at acidic pH (pH 3–5). However, examination of the luminal fluid β-D-galactosidase showed optimal activity at pH 6.8, suggesting that the epididymal enzymes may have unique properties allowing them to function within the epididymal lumen [42]. While galactosyltransferase and glucosaminyltransferase exhibited similar activities in spermatozoa isolated from the different epididymal regions, both fucosyltransferase and sialyltransferase activities showed regional differences with highest activity present in caput spermatozoa and very low activity in cauda spermatozoa [43]. These results are consistent with the idea that addition of sialic acid to spermatozoa occurs during epididymal transit as well as that fucosylation appears to also contribute to region-dependent changes in glycosylation [43]. Although the functional significance for the addition of sialic acid to the sperm surface has not been established, studies in several species including the mouse and bull suggest that fucose residues on sperm surface glycoproteins play a role during sperm–oocyte fusion [44, 45]. Addition of fucose during sperm–oocyte fusion assays or exposure of capacitated spermatozoa to fucosidase, which removes fucose, inhibited sperm–oocyte fusion during fertilization [46].

Using a proteomic approach, several other enzymes involved in glycoprotein metabolism have been identified in the epididymal fluid from the bull including β-mannosidase, β-hexosaminidase, and sialidase [47].

Protein Processing

In addition to changes in phosphorylation and glycosylation, a critical component of the sperm maturation process is the remodeling of the sperm surface by proteolytic processing. Proteolytic processing involves the breaking of peptide bonds between amino acids in proteins which is carried out by peptidases and proteases. These proteolytic processing events are associated with changes in protein localization in spermatozoa and/or activation of protein function and thus protein processing is thought to contribute to the formation of sperm cell surface domains [48].

Several ADAM (A Disintegrin and Metalloprotease) family members with proposed roles in fertilization are present in the epididymal lumen and/or associated with epididymal spermatozoa, and the majority of these have been shown to undergo protein processing in the epididymis [49, 50]. Specifically, ADAMs are transmembrane domain proteins composed of a signal sequence, pro- and metalloprotease, and disintegrin domains, a cysteine-rich region, transmembrane domain, and cytoplasmic tail. Both ADAM1B (fertilin α) and ADAM2 (fertilin β) have their pro- and metalloprotease domains removed with the disintegrin domain becoming the N-terminus on mature spermatozoa. The disintegrin domain has been shown to be important for sperm–oocyte membrane interactions during fertilization [48]. In the mouse, ADAM2 undergoes additional modifications in its C-terminus [51].

Following protein processing, ADAM2 is redistributed on the sperm surface and becomes localized to the posterior sperm head [52]. ADAM1B–ADAM2 and ADAM2–ADAM3 (cyritestin), which is also processed during epididymal transit, have been shown to form complexes in spermatozoa [53, 54]. ADAM3 is thought to promote sperm migration in the female reproductive tract [55]. Trimeric complexes between ADAM2–ADAM3–ADAM4, ADAM2–ADAM3–ADAM5, and ADAM2–ADAM3–ADAM6 have also been described [56]. Interestingly, ADAM3 and ADAM6 are lost from spermatozoa in mice lacking tyrosylprotein sulfotransferase-2 activity, which catalyzes the post-translational modification of tyrosine O-sulfation, suggesting additional PTM of ADAM proteins contribute to their functionality or quality control [57]. Tyrosine O-sulfation has been shown in other cell systems to play a role in protein complex formation [58].

Although ADAM15 and ADAM24 (testase1) are also proteolytically processed during epididymal transit, they only lose their prodomains with the active metalloprotease domain remaining. ADAM15 later undergoes additional protein processing during the acrosome reaction, ultimately exposing the disintegrin domain [59, 60]. While the function of ADAM15 is not clear, roles in sperm–egg interactions have been proposed. ADAM24 has been proposed to function as a protease during epididymal sperm maturation [61]. ADAM24 has a cytoplasmic PKC phosphorylation site and can be phosphorylated in vitro by PKC [61]. It was proposed that protein processing in the epididymis may be the initial activation step while later phosphorylation by PKC during capacitation/acrosome reaction is the final step for activation of ADAM24 protease function during fertilization [61].

Other sperm proteins that undergo protein processing during epididymal transit include the proteins D/E [62], guanylyl cyclase-G [63], the adaptor protein CASK [64], CE9 [65], and α-D-mannosidase [66]. For several of these proteins, protein processing is associated with changes in cellular localization. CE9 is redistributed from the posterior to the anterior tail of the rat spermatozoa following proteolytic cleavage [65]. In addition to proposed changes in glycosylation status, protein processing has also been implicated in the changes in molecular mass of SPAM1 (PH-20) and its localization on spermatozoa. The processing of SPAM1 from a 74 kDa form to a 67 kDa form correlates with its change in localization from a broad distribution on the head of caput spermatozoa to a more defined region in cauda spermatozoa [67]. This change in localization was proposed to be due to a trypsin like activity in the luminal fluid since treatment of caput spermatozoa with trypsin in vitro changed the SPAM1 localization to that characteristic of cauda spermatozoa [48].

For many of the processing events described above, the proteases involved have yet to be described. However, angiotensin-converting enzyme (ACE), a key regulator of blood pressure by its ability to cleave small peptides resulting in a change in their biological activities, has also been shown to exhibit GPIase activity by its ability to release GPI anchored proteins from the cell surface including PH-20 and TESP5 from spermatozoa [68]. ACE has also been shown to regulate the movement of ADAM3 in the sperm membrane [55]. ACE itself is shed from the sperm surface by the action of an unknown serine protease activity that appears to be in the epididymal fluid [69].

The protease(s) responsible for ADAM protein processing was proposed to be a serine protease [70], in particular, a proprotein convertase, since predicted cleavage sites for this family of serine proteases are present in ADAM1 [71]. Furthermore, proprotein convertases have been implicated in the processing of several other ADAMs [72]. ADAM15 also possesses a proprotein convertase cleavage site [60]. Several proprotein convertases are present in the epididymal fluid including furin [73] [Cornwall, unpublished observations]. Furthermore, mice lacking proprotein convertase 4, a testis-specific convertase, exhibit fertility defects including reduced zona pellucid binding suggesting a role for PCSK4 in fertilization [74]. Spermatozoa from PSCK4 null mice showed increased ADAM2 processing possibly reflecting the upregulation of PCSK7 activity in response to the loss of PCSK4, supporting a role for proprotein convertases in ADAM protein processing [75]. Furthermore, exposure of spermatozoa to a peptide inhibitor of PCSK4 resulted in decreased ADAM2 processing [76]. Finally, mice lacking PCSK4 showed reduced levels of sperm ACRBP protein processing in the epididymis, suggesting that several sperm associated proteins may be PCSK4 substrates [77]. Recent evidence suggests that in addition to PCSK4 present in spermatozoa, a population of PCSK4 is present in the epididymal fluid and may also contribute to sperm protein processing [78] [Cornwall, unpublished observations].

The serine protease PRSS21 (testin) is involved in epididymal sperm maturation since PRSS21 deficient mice show decreased motility, angulated and curled tails, and an increased susceptibility to decapitation [79]. Proteomics has also revealed a large number of proteases that are present within the epididymal luminal fluid. These include cathepsins A, D, H, L and S, dipeptidyl peptidase III, matrix metalloproteinase 2, and serine carboxypeptidase I [47]. The specific roles these proteases play in epididymal function have yet to be established.

In parallel with the number of proteases that have been identified in the epididymis that contribute to sperm protein processing and activation, many protease inhibitors have also been identified which likely control these proteolytic events. Several members of the Kazal-type serine protease inhibitor (SPINK) family such as SPINK1, SPINK2, SPINK8, SPINK10, and SPINK12 are highly expressed in the mouse epididymis [80]. These studies also discovered a new WAP 4 disulfide core domain protease inhibitor WFDC10. All of the protease inhibitors showed region-specific localizations and their expression was controlled by testis-specific factors suggesting a role for the inhibitors in the regulation of sperm maturation. Recently, SPINK13 was also identified in the epididymis [81]. SPINK13 was secreted into the epididymal lumen where it associated with the acrosomal region of maturing spermatozoa. RNAi to knockdown SPINK13 resulted in spermatozoa with an accelerated acrosome reaction and fertility defects in vitro and in vivo. These studies suggest that the protease inhibitory activity of SPINK13 may be necessary for preventing premature acrosome reaction [81]. In addition to the SPINK family, other families of protease inhibitors have been found to show epididymis-specific expression. In particular, on human chromosome 20 and mouse chromosome 2 is a cluster of genes encoding proteins with both Kunitz-type and whey acidic protein four disulfide core (WFDC) domains as well as those that possess only WFDC or only

Kunitz-type domains (SPINT3, SPINT4, SPINT5) [82, 83]. While most of the protease inhibitors are predominantly expressed in the epididymis, their functional roles have yet to be established. However, roles in innate immunity have been proposed since the Kunitz and WFDC domains have been shown to inhibit pro-inflammatory proteases such as elastin [84]. Human epididymal protein 4 (HE4), is one of the WFDC proteins that comprise the protease inhibitor locus and though its function in the epididymis is unknown, it has become a marker for the diagnosis of ovarian cancer [85].

In addition to the Kunitz–WFDC protease inhibitor locus, a second cluster of unrelated protease inhibitors is also localized to human chromosome 20 and mouse chromosome 2. The cystatin family 2 of the cystatin superfamily of cysteine protease inhibitors forms a cluster of which only cystatin C is a prototypical cystatin with potent inhibitory activities against papain-like cysteine proteases. The other cystatin family members present at this locus compose the CRES (cystatin-related epididymal spermatogenic) subgroup of reproductive specific cystatins [86]. Although structurally resembling cystatins, the eight characterized CRES subgroup members lack consensus sites for cysteine protease inhibition, suggesting distinctive functions. Indeed CRES, the defining member of the CRES subgroup, did not inhibit cysteine proteases in vitro but rather inhibited the serine proteases prohormone convertase 2 (PCSK2) and prohormone convertase 4 (PCSK4), suggesting CRES was a cross-class inhibitor with a role in the regulation of protein processing [87, 78]. As described above, PCSK4 has been implicated in the processing of several sperm associated proteins including ADAM2 and ACRBP and therefore, CRES may function within the epididymal lumen to regulate this proteolytic activity. In addition to CRES, CRES2, CRES3 and cystatin E2 are also expressed in the epididymis and are present in the epididymal lumen. However, whether they also function as cross class inhibitors or have completely lost inhibitor activity has yet to be determined.

Ubiquitination

Ubiquitination involves the modification of target proteins through the covalent attachment of ubiquitin via an isopeptide bond with target lysine residues of a substrate protein. This process requires the presence and activity of a set of ubiquitin activating and conjugating enzymes. In general, ubiquitination of proteins marks them for degradation by the 26S proteasome. While typically ubiquitination occurs intracellularly, studies have now established that the ubiquitin–proteasome system is also functional extracellularly [88, 89]. Specifically, defective spermatozoa have been shown to become ubiquitinated within the epididymal lumen, suggesting a mechanism for quality control [88]. The ubiquitin–proteasome pathway may also play an important role in zona pellucid penetration by acrosome reacted spermatozoa [90]. Further discussion on the role of ubiquitination in reproduction is presented in other chapters.

Methylation

Methylation is the addition of a methyl group to proteins usually at a lysine or arginine residue. Typically, methylation is associated with the cellular process of development and differentiation. A functional methylation system includes a methylating enzyme such as protein carboxyl methylase (PCM), a demethylating enzyme protein such as methylesterase (PME), and methyl acceptor proteins (MAPs). This system reversibly modifies by methylation the carboxyl groups of proteins affecting their charge, structure, and function. PCM and MAPs are present in mature spermatozoa and the MAP/PCM ratio increases by 20-fold as rat germ cells differentiate into mature and motile cauda epididymal spermatozoa. PME has also been found in spermatozoa with the majority of activity associated with the detergent insoluble component of the sperm flagella which is consistent with a role for protein carboxyl methylation in sperm motility maturation [91, 92]. Subsequent to these early studies, little work has been done to examine the role of protein methylation in epididymal sperm maturation.

Disulfide Bond Formation

Disulfide bonds are formed between the sulfur atoms of pairs of cysteine residues within or across proteins and can affect protein structure and function. Spermatozoa from the caput epididymal region are rich in sulfhydryls and lack disulfides while cauda epididymal spermatozoa are rich in disulfides, suggesting that sperm disulfide bond formation is part of the sperm maturation process in the epididymis [93, 94]. Within spermatozoa, disulfide bond formation occurs in both the head, particularly in nuclear protamines as part of sperm chromatin condensation, as well as within the sperm tail [95]. The sulfhydryl rich organelles in the sperm tail that undergo oxidation to disulfides include the outer dense fibers, including the protein outer dense fiber 1, connecting piece, outer mitochondrial membranes, and the fibrous sheath [96]. Disulfide bond formation is thought to be important for sperm motility maturation since incubation of spermatozoa with a sulfhydryl-specific membrane impermeant dye inhibited the motility of goat spermatozoa [97]. Furthermore, induction of motility in hamster caput epididymal spermatozoa resulted in angulated spermatozoa that were prevented by the addition of the sulfhydryl oxidant diamide [98]. Together, these studies suggest that sulfhydryl oxidation is important for sperm tail structure stabilization and contributes to normal wave patterns of sperm motility. Sperm disulfides are also important for fertility since treatment of guinea pig spermatozoa with dithiothreitol (DTT) to reduce sperm disulfides to sulfhydryls resulted in inhibition of sperm capacitation, acrosome reaction and sperm–oocyte interactions [99]. The thiol status of spermatozoa is regulated by androgens since cauda epididymal spermatozoa isolated from castrated or anti-androgen treated rats showed increased levels of sulfhydryls, including those in

protamines that correlated with reduced sperm fertilizing ability [100]. While the enzymes involved in sperm sulfhydryl oxidation are not well characterized, a sulfhydryl oxidase activity is present in epididymal luminal fluid which prevented flagellar angulation in hamster caput epididymal spermatozoa induced to acquire motility [98, 101]. Protein disulfide isomerase which catalyzes disulfide bond formation is also present in the epididymal fluid and may participate in sperm disulfide bond formation [102]. Finally, sperm sulfhydryl oxidation has been shown to facilitate protein tyrosine phosphorylation. Spermatozoa exposed to diamide to form disulfides exhibited enhanced tail protein phosphorylation while the reduction of disulfides by treatment with DTT decreased phosphorylation [103]. Further studies also showed a correlation of protein phosphotyrosine phosphatase activity with sperm thiol status [103].

Protein Aggregation

After translation, proteins fold into their native and functional three-dimensional conformations. Proteins that do not fold correctly or that unfold after adopting their native state can aggregate if the cell does not degrade the misfolded protein or assist in protein refolding. Unfolded proteins with exposed hydrophobic sites will attempt to stabilize by interacting with the exposed hydrophobic regions of other unfolded proteins leading to protein aggregation. Cells have the ability to sort misfolded proteins into two different compartments including the JUNQ (juxta-nuclear quality control compartment) for soluble or ubiquitinated misfolded proteins and the IPOD (insoluble protein deposit) for non-ubiquitinated insoluble proteins [104]. This differential sequestration of misfolded proteins into two quality control compartments is conserved from yeast to mammals.

Proteins that self-aggregate and form higher ordered structures with a cross β sheet fibrillar structure are known as amyloids. Although typically associated with neurodegenerative diseases including Alzheimer's and Parkinson's disease, amyloid structures have also been shown to be nonpathological and carry out biological functions. Functional amyloids have long been known to exist in bacteria and yeast. However, it has been only recently that functional amyloids were described in mammals. The PMEL protein forms amyloid structures that contributes to the formation of a stable scaffold within melanosomes and is involved in melanin synthesis [105]. Also, in the anterior pituitary gland several protein hormones are stored as amyloids in secretory granules [106].

Within the epididymis, the cystatin CRES contributes to the formation of a functional amyloid structure present within the epididymal lumen [107, 108]. Specifically, CRES localized to a film-like amyloid-containing material that was present in the lumen throughout the epididymis. Gel filtration and Western blotting experiments showed that monomeric forms of CRES were prevalent in the proximal caput epididymal lumen and then disappeared by the distal caput region. The loss of monomeric forms of CRES correlated with the appearance of CRES in SDS-resistant high molecular mass complexes, presumably reflecting a transition of

CRES to an aggregated amyloid state [107]. Because the CRES structures were part of the epididymal mileu from normal mice with no pathologies or fertility defects, a functional role for the CRES amyloid structures in epididymal function was implied. The role of CRES amyloid in the epididymis has yet to be determined. One possibility is that the aggregation of CRES is a means to regulate CRES protease-inhibitory function. Surprisingly, in vitro the CRES dimer was a more potent inhibitor of the proprotein convertase 4 (PCSK4) than the monomer [78]. However, the related protein cystatin C loses its cysteine protease-inhibitory function following dimerization [109]. Alternatively, CRES amyloid may function as a scaffold structure within the epididymal lumen perhaps as an organizational center for proteins that may then be transferred to discrete domains of the spermatozoa during epididymal maturation.

The formation of aggregate structures seems to be a common theme within the epididymal lumen. In addition to the CRES amyloid, several other particulate structures have been described. These include epididymosomes which are small membrane bound structures released from the epididymal epithelium by apocrine secretion and which are thought to be a means to transfer hydrophobic proteins to the maturing spermatozoa [110]. The prion protein was found in both the epididymosomes and in a soluble high molecular mass lipophilic complex with the chaperone clusterin [111]. Other structures in the lumen include dense bodies, which appear larger than epididymosomes, and which contain the heat shock proteins HSPD1/HSP60 and tumor rejection antigen 1 (TRA1), a member of the heat shock protein 90 family [112]. Proposed functions for TRA1 include the folding of denatured proteins and multimer assembly. While the epididymosomes are thought to be released from multivesicular bodies/apical blebs that bud off from the epididymal epithelium indicating an intracellular packaging of the proteins into these structures, the presence of several nonmembrane bound proteinaceous structures in the lumen suggests that protein aggregation of several other proteins, like CRES, may form following their secretion into the lumen. In support, like prion protein, SPAM1 (PH20) has been found in both epididymosomes as well as in monomeric and oligomeric complexes in the epididymal lumen [113].

The presence of several distinct types of aggregate structures in the epididymal lumen raises the possibility that some of these may be extracellular equivalents of the JUNQ and IPOD inclusions that are present intracellularly and which are used by the cell to differentially sort aggregated proteins depending on their solubility and ubiquitination state. The fact that the ubiquitin–proteasome pathway is present in the epididymal lumen would suggest that, in addition to ubiquitination of spermatozoa, ubiquitination of luminal proteins also occurs and these proteins may be targeted to the luminal inclusions for downstream removal by endocytosis. Another possibility is that these inclusions represent preassembled protein complexes that are then delivered to the spermatozoa during maturation. In support of this idea, several proteins with proposed roles in fertilization have been found in large molecular mass complexes with chaperone proteins, perhaps as a means to maintain appropriate folding/stability of the complex [112, 114–116]. Also, several proteins, including prion protein and SPAM1, that become associated with spermatozoa

during epididymal transit, are present in the luminal fluid with GPI-anchors. It is possible that these proteins associate with lipoproteins/chaperones such as clusterin or with lipid raft structures in luminal inclusions as a means to keep the proteins stable and soluble prior to their interactions with spermatozoa [113].

Transglutaminase Cross-Linking

Transglutaminases (TGase) are a family of calcium-dependent enzymes that catalyze the formation of a covalent bond either through protein cross-linking via e-(g-glutamyl) lysine bonds or through incorporation of primary amines at selected peptide-bound glutamine residues. These bonds can be intra- or inter-molecular, and result in extremely stable cross-linked products that are resistant to proteolysis [117]. TGase activities have previously been documented in the male reproductive tract including testis, epididymis, prostate and spermatozoa, and have been suggested to play a role in stabilization of FSH-receptor complexes [118], formation of the seminal coagulum [119], sperm motility [120], and suppressing sperm antigenicity [121, 122]. Little is known regarding the expression or function of TGase in the epididymis other than that spermatozoa and fluid from the head of the epididymis exhibited higher levels of activity than the tail [107]. Studies by von Horsten et al. [107] showed that the TGase activity present in the epididymal luminal fluid was a tissue-type TGase, as evidenced by inhibition of its activity in the presence of a tissue type TGase-specific peptide inhibitor. In addition, although in vitro studies indicated that tissue-type TGase activity is optimal at basic pH, ^{14}C-putrescine incorporation studies to assess TGase activity in the epididymis revealed TGase activity at pH 6.8, which closely approximates luminal pH in the caput epididymis [107]. Thus, these studies indicated that a functional TGase activity is present within the caput epididymal lumen. Although TGase activity has also been detected in spermatozoa, including the activity associated with the sperm head and the cytoplasmic droplet, its roles in sperm maturation are unclear [123]. However, TGase was shown to mediate the binding of spermidine as well as a seminal vesicle secreted protein to the rat sperm surface [124].

PTM and Extracellular Quality Control

The epididymal lumen is a complex microenvironment that is continually being modified by the addition and removal of proteins along the tubule. The proximal or caput region is the most metabolically active region secreting approximately 80 % of the total overall protein secretion in the lumen. Within this same region, more than 99 % of the fluid accompanying the testicular spermatozoa is removed resulting in an extreme concentration of spermatozoa and luminal contents. This concentration of luminal components appears to be necessary for normal sperm maturation events. However, the loss of water content can result in macromolecular crowding which leads to protein misfolding and aggregation [125]. Since an important role of

the epididymis is to protect the maturing spermatozoa, it is likely that extracellular quality control mechanisms are in place. Several lines of evidence suggest that this indeed is the case. First, as mentioned above, components of the ubiquitin–proteasome pathway are present in the epididymal lumen suggesting that this well-characterized intracellular quality control system also is functional extracellularly [90]. These observations in the epididymis were one of the first to describe a mechanism for extracellular quality control in any organ system. A number of chaperones are also present in the epididymal lumen where they may bind to misfolded proteins and either prevent their aggregation or assist in refolding. It is intriguing that the chaperone clusterin contributes approximately 41 % of the total luminal protein content in the rat caput epididymis, possibly functioning as a mediator of extracellular quality control [126]. The observation that many of the particulate structures found in the epididymal lumen contain chaperones suggests an active role for these proteins in quality control systems. Studies have also suggested that the PTM by transglutaminase crosslinking may contribute to quality control in the epididymis. The cystatin CRES is a substrate for transglutaminase cross-linking [107]. Furthermore, exposure of CRES to TGase resulted in the formation of an aggregate structure that was distinct from the highly organized structures of amyloid that CRES forms in the absence of TGase. These data suggest that TGase cross-linking may be a PTM that shifts potentially cytotoxic amyloidogenic structures into stable cross-linked amorphous structures that are not inherently cytotoxic and which may then be taken up by endocytosis [107].

It is likely that within the epididymal lumen, there is a delicate balance between monomeric and oligomeric forms of proteins with aggregate structures carrying out active roles in sperm maturation as well as in the removal or sequestration of luminal proteins [127]. Although extracellular quality control mechanisms are not well described in any organ system, the presence of the ubiquitin–proteasome system, significant levels of TGase activity, and extremely highly levels of chaperones suggest that the epididymal luminal environment has adopted extreme and perhaps unusual measures to ensure viability of the maturing spermatozoa and thus provides an interesting model system for dissecting out the roles these components play in quality control.

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Ubiquitin–Proteasome System in Spermatogenesis

9

Rohini Bose, Gurpreet Manku, Martine Culty, and Simon S. Wing

Abstract

Spermatogenesis represents a complex succession of cell division and differentiation events resulting in the continuous formation of spermatozoa. Such a complex program requires precise expression of enzymes and structural proteins which is effected not only by regulation of gene transcription and translation, but also by targeted protein degradation. In this chapter, we review current knowledge about the role of the ubiquitin–proteasome system in spermatogenesis, describing both proteolytic and non-proteolytic functions of ubiquitination. Ubiquitination plays essential roles in the establishment of both spermatogonial stem cells and differentiating spermatogonia from gonocytes. It also plays critical roles in several key processes during meiosis such as genetic recombination and sex chromosome silencing. Finally, in spermiogenesis, we summarize current knowledge of the role of the ubiquitin–proteasome system in nucleosome removal and establishment of key structures in the mature spermatid. Many

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mechanisms remain to be precisely defined, but present knowledge indicates that research in this area has significant potential to translate into benefits that will address problems in both human and animal reproduction.

Keywords
Ubiquitin • Proteasome • Deubiquitinating enzymes • Gonocytes • Spermatogonia • Histones • Ubiquitinated histones • Meiotic sex chromosome inactivation • Recombination • Nucleosome • Acrosome

General Features and Functions of the Ubiquitin–Proteasome System (UPS)

Ubiquitination is the process by which ubiquitin, an 8.5 kDa peptide highly conserved throughout evolution, becomes covalently linked to its target proteins (rev. in [1]). This linkage usually occurs between the carboxy-terminal end of the ubiquitin peptide and the ε-amino group of the side chain of a lysine residue on the protein substrate. Rarely, the ubiquitin is attached to the α-amino group at the N-terminus of the protein [2, 3] or the thiol group of a cysteine residue [4]. Often a chain of ubiquitin moieties is attached to the substrate protein in which each distal moiety of the chain is linked to one of the seven lysine residues found in the more proximal ubiquitin moiety. The ubiquitin chains, particularly when the number of ubiquitin moieties is greater than four and linked via the lysine 48 residue, are recognized by the 26S proteasome complex, resulting in the degradation of the protein substrate [5]. The 26S proteasome consists of a 20S catalytic core particle bound at one or both ends by a 19S cap particle [6]. The 20S core has a cylindrical structure formed by the stacking of four rings, each composed of seven distinct subunits [7]. The two outer rings consist of subunits α1–7 and the two inner rings consist of subunits β1–7. These latter subunits contain the active sites of the protease. The 19S caps cover the ends of the 20S cylindrical core particle, thereby acting as gates to prevent uncontrolled proteolysis. The 19S particles contain subunits that recognize and bind ubiquitin chains or adaptor proteins which in turn bind ubiquitinated proteins, targeting them to the proteasome (rev. in [8]). The 19S particles also contain deubiquitinating enzymes that remove the ubiquitin from the targeted protein to recycle the ubiquitin for new conjugation (rev. in [9]). Subunits with ATPase activity are present and likely involved in unfolding the protein, opening of the gate of the 20S core particle, and subsequent translocation of the substrate into the channel for hydrolysis. The 20S core particle can also interact with three other regulators. PA28α/β is a heptameric complex induced by interferon, which can bind to the ends of the core particle and is involved in adaptive immunity [10]. PA28γ is a structurally related protein whose function remains unclear. PA200 is a monomeric protein (rev. in [11]) that can also bind to the 20S core and has been suggested to play roles in DNA repair, mitochondrial inheritance and, as will be reviewed here, in spermatogenesis.

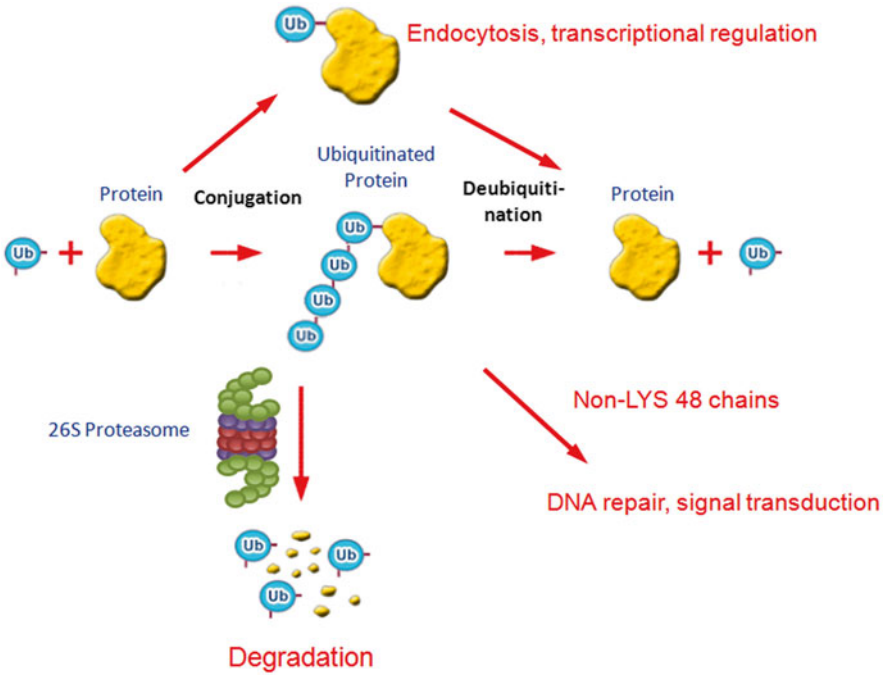

Fig. 9.1 Outline of the ubiquitin–proteasome system. Ubiquitination is the process by which the peptide ubiquitin (Ub) is conjugated at its C-terminal end to the side chain of a lysine residue in the target protein. Proteins can be either monoubiquitinated or conjugated to polyubiquitin chains formed by the linkage of the more distal Ub moiety to a lysine residue in the more proximal Ub moiety. Ub contains seven lysine residues allowing different types of chains to be formed depending on which lysine is used to form the chain. Classically, chains formed using lysine 48 on Ub target the attached protein for recognition and degradation by the 26S proteasome. Proteins conjugated with chains formed using other lysines can mediate non-proteolytic functions of ubiquitination such as DNA repair or signal transduction. Monoubiquitination of proteins generally does not lead to degradation, but is involved in processes such as trafficking of plasma membrane proteins through the endocytic pathway or regulation of gene transcription (through monoubiquitination of histones). Recent studies indicate, though, that these distinctions in functions depending on type of ubiquitination are not absolute. Ubiquitination can also be reversed through the action of deubiquitinating enzymes

The UPS has numerous substrates—perhaps as many as half of all cellular proteins [12]. Many of these proteins are regulatory proteins and include transcription factors such as Myc [13], cell cycle proteins such as p27 [14], cyclins [15], and signaling pathway molecules such as IκBα [16, 17]. By regulating the cellular levels of proteins, the UPS is able to modulate a variety of physiological processes in the cell (Fig. 9.1). In addition, ubiquitination can serve nonproteolytic functions, particularly when the target proteins are monoubiquitinated or ubiquitinated by chains formed by non-Lys 48 linkages (rev. in [18]). In these situations, the ubiquitination can alter the structure and function of the target protein or serve to recruit other proteins. For example, ubiquitination of plasma membrane proteins can target them for internalization and trafficking through the endocytic pathway to multivesicular

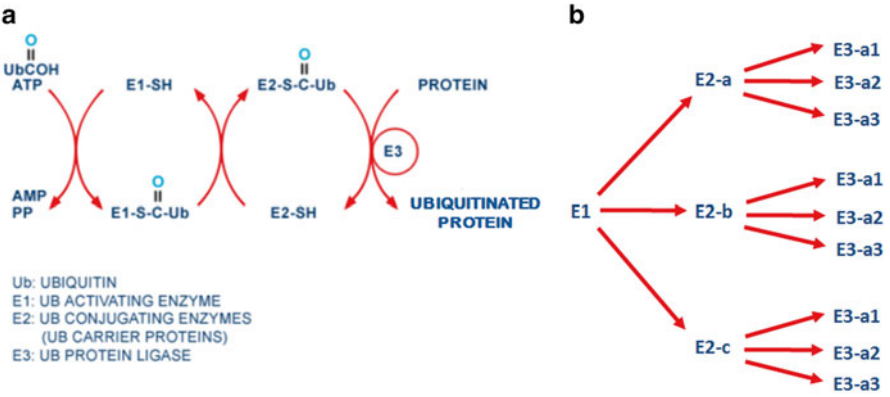

Fig. 9.2 Mechanism of action of enzymes involved in conjugating ubiquitin (Ub) to proteins. (**a**) Ub is first activated by Ub activating enzyme (E1) in a reaction that involves ATP hydrolysis and results in the formation of a high energy thiolester linkage between Ub and the active site cysteine residue. The E1 then transfers the activated Ub onto the active site cysteine residue of one of a family of Ub conjugating enzymes (E2s or UBCs). The E2 then conjugates Ub to protein substrates in concert with a third protein, Ub protein ligase (E3) that plays the critical role of binding the substrate. (**b**) Ub activating enzyme (E1, 2 genes in mammals) supplies activated Ub to a family of Ub conjugating enzymes (E2s, ~30 genes in mammals). Each E2 can interact with a subset of Ub ligases (E3s, ~800 in mammals) to mediate conjugation of Ub to specific protein substrates

bodies, for subsequent degradation in lysosomes (rev. in [19]). Ubiquitination can also result in the activation of signaling pathways, as seen downstream of the inflammatory cytokine receptors for TNFα or IL-1, in which the ubiquitination of RIP1 or TRAF6 respectively results in the activation of NFκB (rev. in [20]). As detailed later in this chapter, monoubiquitination of histones can result in recruitment of other proteins/enzymes that mediate other chromatin modifications.

Conjugation of ubiquitin is mediated by a sophisticated three-step enzyme cascade (Fig. 9.2) [21, 22]. In the first step, the E1 ubiquitin activating enzyme activates ubiquitin in an ATP-dependent manner and charges it onto the cysteine residue of the active site of an E2 ubiquitin conjugating enzyme. The E2 then conjugates the ubiquitin to protein substrates in concert with a third protein, the E3 ubiquitin ligase that is essential for binding the substrate. There are two major classes of E3s, namely the RING (*R*eally *I*nteresting *N*ew *G*ene) and HECT (*H*omologous to *E*6-AP *C*arboxy-*T*erminus—named after E6AP, the first E3 described in this class) that can be recognized by specific conserved protein domains/motifs. In addition, there are U-box containing E3s presenting a distinct sequence motif, but whose tertiary structure is similar to a RING domain. There are two E1s in mammalian cells—Uba1, Uba6—with the former being the dominant E1 activity in most cell types. Approximately 30 genes encode E2s, each of which can interact with a subset of the approximately 800 E3s in the mammalian genome.

Protein ubiquitination may be reversed by deubiquitinating enzymes. These enzymes number approximately 100 and can be divided into five distinct classes,

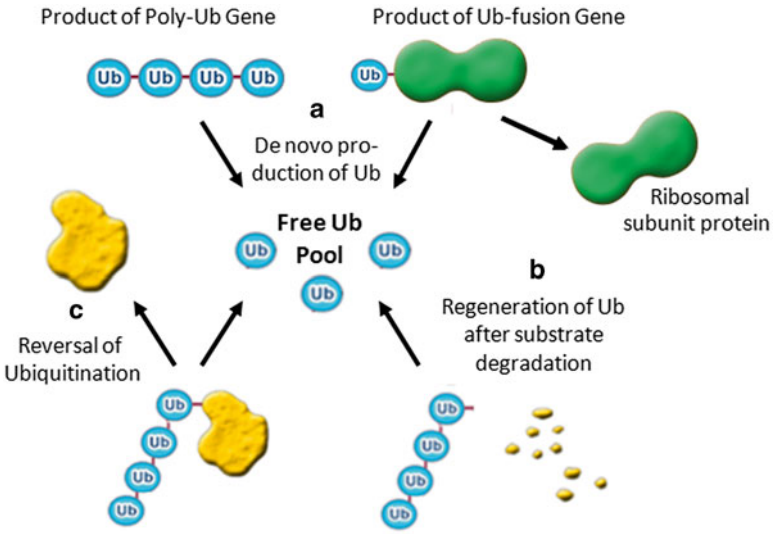

Fig. 9.3 Functions of deubiquitinating enzymes. Deubiquitinating enzymes function to maintain a free Ub pool by processing products of polyUb genes or Ub-fusion genes (**a**), or by processing polyubiquitin chains after substrate degradation (**b**), or through the reversal of ubiquitination of target proteins (**c**)

based on sequence conservation [23]. Four of these classes—USP (Ubiquitin Specific Proteases, largest class with ~60 genes), Otubains, UCH (Ubiquitin C-terminal Hydrolases), and MJD (Machado Joseph Disease) enzymes - are cysteine proteases. The fifth class contains the JAMM (JAB1/MPN/Mov34) domain containing proteins which are metalloproteinases. In addition to reversing the effects of ubiquitination, these enzymes also function to recycle ubiquitin after their protein targets have been committed to degradation by the proteasome or within the lysosome (Fig. 9.3). There are four genes that encode ubiquitin [24]. Two are polyubiquitin genes which encode linear polymers of multiple copies of ubiquitin and the other two encode a single copy of ubiquitin fused at its carboxy terminal end to a ribosomal subunit. Deubiquitinating enzymes are responsible for processing the products of these four genes into the monomeric proteins and this appears to occur co-translationally.

The large families of enzymes involved in ubiquitination allow for highly selective and precisely regulated ubiquitination of target proteins. The number of potential cellular functions regulated by ubiquitination is extensive. In this chapter, we will review the current knowledge about some of the functions of the UPS in spermatogenesis, highlighting particular examples that have been well defined, as well as identifying important gaps in our current knowledge.

UPS: Essential for Gonocyte and Spermatogonial Development

Spermatogenesis represents a complex succession of cell division and differentiation events resulting in the continuous formation of spermatozoa. This process relies on a reservoir of spermatogonial stem cells (SSCs) that can undergo self-renewal or differentiation [25, 26]. The SSCs arise from precursor cells known as gonocytes (also called pre- or pro-spermatogonia), 1 week after birth in rodents, and a few weeks after birth in humans [27]. Neonatal gonocyte development involves three main processes: proliferation, migration, and differentiation [27]. Proliferation is regulated by platelet-derived growth factor (PDGF) and 17β-estradiol [28, 29], while differentiation is induced by retinoic acid [30]. During these processes, a major reorganization of cellular structure takes place [27].

The role of the UPS in cell remodeling and protein turnover in gonocytes was unknown until recently. Isolated rat gonocytes can be induced, upon exposure to retinoic acid, to express the differentiation marker Stra8 (stimulated by retinoic acid gene 8) [30] which is expressed in differentiating spermatogonia and pre-meiotic germ cells [31–33]. This retinoic acid dependent induction can be blocked by the presence of the proteasome inhibitors lactacystin or bortezomib, indicating that proteasome activity is necessary for gonocyte differentiation [34].

Gene expression analysis using microarrays identified 91 UPS genes that are significantly expressed in post natal day (PND) 3 rat gonocytes. Of these, at least five genes have a higher expression level in PND3 gonocytes compared to PND8 spermatogonia, suggesting that down-regulation of these genes may be involved in this developmental transition. These genes include ubiquitin activating enzymes Uba1 and Uba6, and ubiquitin ligases Huwe1, Trim47, and Rnf149. Uba1 has previously been shown to be expressed in adult germ cells and is needed for both capacitation and fertilization [35]. Uba6 was suggested to play a role in the transition between mitosis to meiosis [36, 37]. Huwe1 is highly expressed in gonocytes, spermatogonia and spermatocytes [38, 39]. Interestingly, it translocates from the cytoplasm to the nucleus during the gonocyte to spermatogonia transition [34]. We have recently inactivated the gene specifically in germ cells and our preliminary results demonstrate a defect in SSC renewal and differentiation confirming an important role for Huwe1 in this early stage of spermatogenesis. No literature on the function of Trim47 is available, while a recent study showed that Rnf149 is the ubiquitin ligase for the serine/threonine kinase BRAF [40]. When these five genes were analyzed in gonocytes treated with or without retinoic acid, only Rnf149 was significantly decreased upon retinoic acid treatment. This indicates that Rnf149 is actively down-regulated during both in vitro and in vivo differentiation.

There are other genes highly expressed in gonocytes that may also be involved in differentiation based on their functions in other systems. Among these is the conjugating enzyme Ube2e3 which is essential for epithelial cell proliferation and is down-regulated during differentiation [41]. Other UPS genes abundant in gonocytes and spermatogonia include the ubiquitin ligases Mdm2, Huwe1 and Ubr5, reported to interact with p53 in other cell types [34]. These genes could potentially regulate cell cycle progression in proliferating gonocytes or spermatogonia.

The SCF βTrCP ubiquitin ligase is a well characterized ubiquitin ligase that plays important roles in regulating cell cycle and apoptosis. There are two isoforms of the βTrCP substrate recognition subunit, βTrCP1 and βTrCP2, both of which are expressed in spermatogonia. Gene inactivation of βTrCP1 has no effect on spermatogonial development but insufficiency of both isoforms results in disrupted organization of these cells due to loss of adherens junctions and E-cadherin. This effect appears to be due to stabilization of a single βTrCP substrate Snail1, a transcription factor that induces E-cadherin expression, as silencing Snail1 in the βTrCP deficient testis was able to reverse the defect in spermatogenesis [42].

The deubiquitinating enzyme Uchl1 (alt. name PGP9.5) is highly expressed in spermatogonia. It has been proposed that spermatogonia undergo both symmetrical and asymmetrical division to maintain the stem cell pool and differentiation of progeny [43]. Interestingly, Uchl1 protein segregates asymmetrically to the two daughter cells in asymmetric division. The cells that inherit a high level of Uchl1 express the undifferentiated spermatogonia marker Plzf, whereas those with a low level of Uchl1 express the differentiated spermatogonia marker c-Kit. This suggests that Uchl1 may be an intrinsic determinant for spermatogonial self-renewal or differentiation [44]. P97/VCP, a chaperonin that can bind ubiquitinated proteins and target them to the UPS, may through this function act as a regulator of signaling pathways such as the BMP pathway in gonocytes and spermatogonia in neonatal testes [45, 46]. Further analysis of these and other relevant genes using loss of function approaches in the testis is required to confirm the roles of these proteins. Specific functions of the UPS in this early mitotic stage of spermatogenesis will likely be found in view of the known importance of the system in proliferation of other cell types.

UPS: An Important Regulator of Meiosis

Since the UPS has a critical role in mitotic division (rev. in [47, 48]), it was anticipated that it would also be involved in the meiotic division leading to the development of haploid round spermatids during spermatogenesis. Indeed, over the past decade, considerable work has been done to elucidate the role of the UPS in meiosis, which is marked by highly regulated events such as meiotic entry, genetic recombination of homologous chromosomes, meiotic sex chromosome inactivation (MSCI), and meiotic exit. Defects in these important events often lead to developmental arrest in germ cells and consequent infertility. In this section, we will describe a few general observations regarding the importance of ubiquitin and the proteasome, and then highlight examples that demonstrate the highly efficient and selective ability of the UPS to tightly regulate some of these processes, thereby promoting adequate production of normal spermatozoa.

The importance of the UPS during meiosis is illustrated by defects observed upon depletion of ubiquitin or impairment of the proteasome. For example, disruption of the polyubiquitin gene Ubb in mice leads to infertility with a germ cell arrest at the prophase stage of meiosis I [49]. Ubb is one of the two polyubiquitin genes

and is therefore important for de novo synthesis of ubiquitin. Inactivation of Ubb results in a significant decrease in free ubiquitin in the testis, which, associated with the meiotic defect, implies that insufficient levels of ubiquitin in the mutant testis disrupt the ubiquitination required during meiosis. The spermatocytes in the *ubb* null mice are marked by the absence of discrete foci of γH2AX, a histone modification normally associated with the transcriptionally silenced XY body in mid-pachytene spermatocytes. This absence of γH2AX foci could be due to either a delay in the progression of the pachynema in mutant spermatocytes or to a delay in the formation of the XY body. Despite decreased ubiquitin levels in mutant testes, histone H2A was still ubiquitinated (uH2A) and the uH2A remained associated with the XY body, but the levels of uH2A were about 50 % lower than those found in normal testes [49, 50]. The uH2A has been suggested to be essential for maintaining silencing of the unsynapsed chromosomes. Microarray analysis of the testicular transcriptome did indeed reveal many more upregulated genes than downregulated in the mutant testes. There was abnormal expression of many testis-specific/meiosis-specific genes which may explain in part the meiotic defect seen in these mice [50].

The requirement for ubiquitin in meiosis appears conserved in eukaryotic evolution. Mutants of the polyubiquitin gene Ubi4(+) in *S. pombe* abort meiosis at the first or second division and are characterized by short, condensed non-separated chromosomes, abnormal spindle and disintegrated spindle pole bodies. Ubi4(+) mRNA is normally strongly induced prior to meiosis, with a slight increase in protein levels. However, in these mutants, there is a significant decrease in ubiquitin levels when the cells enter meiosis, suggesting that this impaired availability of ubiquitin is the cause of the aborted meiosis [51].

With respect to the proteasome, silencing of two subunits of the catalytic core of the proteasome PBS-4 and PAS-5, in *C. elegans*, results in impaired entry into meiosis, suggesting that the UPS regulates the switch from proliferation to meiosis by degrading proteins necessary for proliferation [52]. In mice, inactivation of a proteasome activator, PA200, which is highly expressed in the testis, results in subfertility and significant histological defects in spermatocytes, including a high level of apoptosis. Given that both the proteasome and PA200 have previously been suggested to regulate repair of DNA double strand breaks (DSB) [53, 54], this study hinted at a role for the proteasome in the repair of DSBs during meiosis, possibly by regulating the metabolism of repair proteins or histones [55].

UPS and Meiotic Recombination

Meiotic recombination, one of the key steps in the prophase of the first meiotic division, ensures efficient exchange of genetic material supporting the production of germ cells with genetic variability, whilst maintaining genomic integrity. During this time, double strand breaks (DSBs), which are a prerequisite for homologous recombination, are introduced in a controlled manner. A role of the UPS in this process has been best elucidated in the yeast *S. cerevisiae* in which mutations of the ubiquitin conjugating enzyme Rad6 (Ubc2) result in defective sporulation and DNA

repair [56–59]. Mutation of the Rad6 gene leads to meiotic prophase arrest, with only 10-20 % of the rad6 mutant cells entering meiosis I. These mutants are characterized by an overall decrease in the frequency of DSBs in the cell, which could be explained by a delay in their entry into the pre-meiotic S-phase. Rad6 interacts with the Bre1 ubiquitin ligase and together, they mediate ubiquitination of histone H2B. Disruption of Bre1 or substitution of the lysine residue of H2B that is ubiquitinated, also leads to a reduction in DSB formation during meiosis, indicating that these effects are due to Rad6/Bre1 mediated ubiquitination of H2B. The uH2B may be required for recruitment of the DSB-forming machinery to DSB hotspots [59].

Roles for these enzymes in meiotic recombination appear to be conserved in the mammalian testis. The ubiquitin conjugating enzymes Hr6a and Hr6b are mammalian orthologs of Rad6/Ubc2. Hr6b knockout mice showed increased apoptosis of primary spermatocytes in the first wave of spermatogenesis, with longer synaptonemal complexes in the pachytene spermatocyte nuclei, depletion of synaptonemal complex proteins from near telomeric regions and increased foci containing the mismatch DNA repair protein MLH1, indicating a high crossing-over frequency [60, 61]. The knockout mice, however, have no defect in overall ubiquitinated H2A levels, indicating the existence of other ubiquitin-conjugating enzymes that can perform this function.

The Hr6a/Hr6b ubiquitin conjugating enzymes interact with a number of E3s, which may be involved in their functions in meiotic recombination. In particular, they interact with the Ubr family of ligases which function in the N-end rule pathway (rev. in [62]). This pathway was initially described as a set of enzymes that are involved in recognizing ubiquitination substrates by their N-terminal amino acid. Loss of the Ubr2 ligase leads to infertility in males with degeneration of the postnatal testis beyond 3 weeks. This is due to an arrest at the leptotene/zygotene and pachytene stages of spermatocytes, which are conspicuous by their lack of intact synaptonemal complexes and eventually die through apoptosis [63]. This phenotype could be explained by a role of Ubr2 in the maintenance of genomic integrity and homologous recombination repair of double strand breaks, as was observed in mouse fibroblast cells derived from $Ubr2^{-/-}$ embryos [64]. The exact substrates of Ubr2 that mediate these effects are unknown. Ubr2 has been shown to interact with TEX19.1, a germ cell-specific protein. However, TEX19.1 does not appear to be a substrate of Ubr2 as it is unexpectedly destabilized in *Ubr2* knockout mice and the loss of TEX19.1 phenocopies loss of Ubr2. Thus, the role of Ubr2 may be to stabilize TEX19.1 by preventing the ability of other ubiquitin ligases to interact and ubiquitinate this protein [65]. Clinically, single nucleotide polymorphisms in the *Ubr2* gene have been associated with azoospermia in men who presented meiotic stage arrest in spermatogenesis, suggesting a molecular basis for the infertility in this type of non-obstructive azoospermia [66].

Another ubiquitin ligase that has an essential role during homologous recombination is Rnf4. Rnf4 ubiquitinates specifically the SUMO-modified proteins. Inactivation of Rnf4 results in the persistence of DNA damage signaling following an ionizing radiation insult. Mechanistically, Rnf4 ubiquitinates SUMO-modified MDC1 (Mediator of DNA damage checkpoint protein 1) and SUMO-modified BRCA1 (Breast cancer type I susceptibility protein, involved in DNA damage

response), both events being required for the loading of RAD51, a component of the homologous recombination repair machinery [67]. Mice lacking Rnf4 have defective spermatogenesis characterized by increased apoptosis and depletion of spermatocytes [67]. Mouse mutants of a putative ubiquitin ligase Mei4, a mouse ortholog of Human Enhancer of Invasion 10 (Hei10), show defective assembly of chromosomes at the metaphase plate during meiosis I, leading to arrest and apoptosis [68]. Late meiotic prophase in these germ cells is marked by the absence of cyclin-dependent kinase 2 and mismatch repair proteins at the chromosome cores, correlating with immature chromosome separation due to lack of chiasmata formation.

UPS and Meiotic Sex Chromosome Inactivation

During the pachytene stage of prophase I, the X and Y chromosomes are partially synapsed at their pseudoautosomal regions, and undergo transcriptional silencing, a process called meiotic sex chromosome inactivation (MSCI), which is vital for the progression of meiosis and production of spermatozoa. The sex body containing the heterochromatic X and Y chromosomes is enriched in ubiquitinated H2A and levels normally peak in pachytene spermatocytes [69]. Ubiquitination of H2A has been linked to gene repression in both *Drosophila* and mammals [70, 71] and therefore may be essential for MSCI. Supporting this idea, mice deficient in the previously mentioned ubiquitin ligase Ubr2 are also characterized by a failure in H2A ubiquitination and impaired chromosome-wide silencing of genes linked to unsynapsed X and Y chromosomes. Ubr2 interacts with the ubiquitin conjugating enzyme Hr6b, which can conjugate ubiquitin to histone H2A in vitro, suggesting that these enzymes may be responsible for generating the uH2A in the pachytene spermatocytes. The lack of MSCI probably activates a pachytene checkpoint system and results in a consequent arrest at meiotic prophase I [72, 73]. MSCI is also marked by decreased H3K4 dimethylation which results in persistent gene silencing in round spermatids. Mediators of this process appear to be the Hr6b conjugating enzyme along with another of its interacting ubiquitin ligases, Rad18. Loss of function of either Hr6b or Rad18 leads to increased level of H3K4 dimethylation on the XY body and the whole nucleus at the diplotene stage, an indication of derepression of silenced genes [74]. Rad18 has previously been shown to be an orchestrator of homologous recombination repair after DNA damage by binding to the recombinase RAD51C [75]. Silencing Rad18 in mice leads to subfertility, and reduced body weight and testis size. The mice show increased asynapsis of the X and Y-chromosomes in the pachytene spermatocytes, as well as increased dimethylation of H3K4 and corresponding derepression of X-linked genes that are normally silenced [74].

The importance of uH2A for MSCI has been questioned by findings in mice deficient in the ubiquitin ligase Rnf8. This ubiquitin ligase has multiple roles including involvement in DSB repair. Rnf8 recognizes ATM–mediated phosphorylated MDC1 bound to γ-H2AX, which allows histone ubiquitination and the ubiquitin-dependent recruitment of 53BP1 and Brca1, helping in the repair of DSB [76–79]. Another ubiquitin ligase, Rnf168 may act in concert with Rnf8 to propagate the

ubiquitination signal at the DSBs [80]. Mice lacking Rnf8 are sterile and present defective ubiquitination of the XY body in pachytene spermatocytes [81–83]. However, MSCI occurred normally indicating that uH2A on the XY body is not essential for this silencing [83]. More recently, it has been shown that Rnf8 dependent ubiquitination of H2A is instead essential for the establishment of H3K4 dimethylation which is required for escape gene activation in post meiotic stages of spermatogenesis [81, 84].

Other components of the UPS have been reported to associate with the sex chromosomes during this phase of meiosis, but their functions are still unclear. The ubiquitin ligase Ret finger protein, a transcriptional repressor that is able to interact with nuclear matrix associated proteins and double stranded DNA, plays a possible role in the positioning and attachment of the XY body to the nuclear lamina next to the nuclear membrane in pachytene spermatocytes [85]. This seclusion of the XY body from the rest of the chromosomes is critical to handle the asynchronous synapsis of the sex chromosomes post homologous recombination during meiosis (rev. in [86, 87]).

UPS and Meiotic Progression

There are a number of UPS genes that are required at various time-points of meiotic progression such as during the transition from mitosis to meiosis, the preparatory stage for homologous recombination, transitions from metaphase to anaphase during meiosis I, chromosome segregation, transitions during meiosis II and the exit from meiosis. The anaphase promoting complex or cyclosome (APC/C) is a multisubunit ubiquitin ligase that targets various proteins for degradation, notably securin, to facilitate chromosome segregation during mitosis (rev. in [88, 89]). The APC/C has also been demonstrated to play a role in meiosis and this function is conserved amongst several organisms. Null mutations in the APC5 subunit in yeast [90–92] and loss of function of APC/C subunits in *C. elegans* embryos cause an arrest at metaphase of meiosis I [93–95]. However, the role of the APC/C in male meiosis of mammals is yet to be explored. Moreover, in *C. elegans*, a temperature sensitive mutation of the ubiquitin ligase Cul2 results in inhibition of the first steps of meiotic prophase I progression because of the accumulation of HTP-3, a member of the HORMA (Hop1–Rev1–Mad2) domain-containing proteins, required for preparing chromosomes for meiosis. The protein is needed for loading of cohesion REC8 and components of the synaptonemal complex, mainly SYP-1 and HIM-3. In the absence of Cul2, there is premature binding of the synaptonemal complex members, probably leading to the activation of a DNA replication checkpoint [96].

Furthermore, there are a few mutation studies in higher organisms that indicate the importance of the UPS in other aspects of meiotic progression. Among these, gene targeting of the ubiquitin ligase Cullin4A in mice leads to increased accumulation of DNA licensing protein CDT1, phospho-p53 and MLH1 with disrupted meiosis II and increased cell death in pachytene–diplotene cells [97]. Inactivation of another murine RING ubiquitin ligase, Siah1a was found to cause abnormal meiotic division in spermatocytes, with an increased accumulation and then consequent

degeneration of the metaphase and anaphase cells. There were no post-meiotic round spermatids and the cells that progressed beyond metaphase were binucleated/multinucleated, indicating defective chromosome segregation. The accumulation and death of the spermatocytes from the metaphase I to telophase I transition may be due to the accumulation of KID, an anaphase I inhibitor protein and a substrate for Siah1A [98]. Besides its role in spermatogonial development, the β-TrCP ligase is also involved in meiosis. The β-TrCP1 mRNA is upregulated in spermatocytes and indeed, mice lacking this isoform are subfertile with marked decreased production of spermatids and increased number of metaphase I spermatocytes consistent with a defect in meiosis. The mutant testis also showed increased levels of the cell cycle regulators and β-TrCP substrates, Emi1 and cyclin A, providing a possible molecular mechanism for this defect [99]. Rat100/EDD/HYD/UBR5, a UBC-4 dependent HECT ubiquitin ligase, is induced during postnatal development, peaking around PND 25 in rats. It is highly expressed in spermatocytes with low expression in the round and elongating spermatids [100]. It is a homolog of the *Drosophila* hyperplastic discs gene, point mutants of which cause male infertility due to a lack of progression of the germ cells past the primary spermatocyte stage [101].

Ubiquitin C-terminal hydrolases or UCHs are believed to be essential for maintaining ubiquitination activity by releasing ubiquitin from its substrates. Overexpressing Uchl1 in the testis of male mice results in sterility due to blockage of spermatogenesis at the pachytene stage of spermatocytes and increased apoptosis in these cells. PCNA (proliferating cell nuclear antigen) was strongly expressed in the primary spermatocytes with little expression in spermatogonia and Sertoli cells [102]. Knocking out Uchl1 led to an increased number of premeiotic germ cells between PND 7 and 14, concomitant with increased levels of apoptotic proteins TRP53, Bax, and caspase-3 [103]. A study looking at the profiles of genes exhibiting expression patterns similar to that of retinoic acid target gene Stra8, an initiator of meiosis known to peak at PND 10, identified ubiquitin-activating enzyme Uba6 [37]. Uba6 transcript levels are highest in the human and mouse testis compared to other tissues [36] with the protein expression pattern coinciding with its mRNA expression. Besides being expressed in neonatal gonocytes [34, 37], it is highly expressed in the cytoplasm of spermatogonia and preleptotene spermatocytes at PND 10, with localization shifting to the nuclei of preleptotene, leptotene and zygotene spermatocytes in PND 20 mice. These observations suggest a possible role of Uba6 in meiotic initiation [37].

It is evident from the aforementioned studies that the UPS is indispensible for the progression of major meiotic events during spermatogenesis. Collectively, several loss-of function studies clearly associated the absence of UPS genes with profound defects in homologous recombination, meiotic sex chromosome inactivation and metaphase I-anaphase I transitions. Ubiquitination of histones appears to be particularly important in mediating some of the key events in homologous recombination and regulation of gene expression from the sex chromosomes. As an important regulator of these events, the UPS ensures accurate exchange of genetic information between homologous chromosomes, chromosome assembly/segregation and expression/silencing of genes thus setting the foundation for the germ cells to undergo successful transition through the last stage of spermatogenesis.

UPS: A Modulator of Spermiogenesis

The haploid round spermatids derived at the end of meiosis II undergo extensive nuclear and morphological remodeling, leading to the formation of elongated, compact, transcriptionally quiescent and highly specialized spermatozoa [104]. This remodeling is mediated by a wide array of highly regulated molecular events. The UPS appears to play important roles during spermiogenesis by removing proteins that are no longer needed in the more differentiated spermatids, as well as in modulating the regulatory mechanisms that control these processes.

A role for the UPS in spermiogenesis was suggested early on by observations that levels of ubiquitinated proteins are markedly increased during the first wave of spermatogenesis in the rat at times when haploid spermatids first appear and are becoming the prominent germ cell in the testis. This is likely due to an activation of ubiquitin conjugating activity, which is dependent mostly on the Ubc4 family of ubiquitin conjugating enzymes whose levels are dramatically increased in spermatids [105]. Proteasome activity is also high in elongating spermatids as well as in mature spermatozoa [105, 106].

Studies in *Drosophila* support a role for the UPS during spermiogenesis. In *D. melanogaster*, 12 of the 33 subunits of the 26S proteasome are represented by multiple paralogous genes [107–109], and in each case, one of the paralogs is testis-specific [110]. Analyses of the testis specific α3T, α6T and Rpt3R subunits revealed that their expression is induced at different stages, but they are prominent in spermatids [110]. The functional significance of α6T and Rpt3R in spermiogenesis is evident from their inactivation, which results in a male sterile phenotype [108, 110]. Knocking out α6T results in defective actin cone movement during sperm individualization (terminal differentiation), and abnormal nuclear morphology and maturation, the first defect being either due to improper degradation of important components of the actin cone movement machinery or due to the defective regulation of the caspase activation which is essential for sperm individualization [108]. In addition, two testis-specific subunits of the 20S core proteasome, *Pros28.IA* and *Pros28.1B* are expressed during spermatid differentiation [107] suggestive of proteasomal functions that are unique to spermiogenesis.

These results demonstrate a general implication of ubiquitination and proteasome mediated degradation during spermiogenesis, and the conservation of this function across species. In the following sections, we will highlight examples that illustrate how specific components of the UPS play essential roles in spermiogenesis in a carefully regulated and selective manner.

UPS Genes and Nucleosome Remodeling

The round spermatid nuclei initially house a transcriptionally active genome consisting of DNA and histones. The nucleosome, the basic subunit of chromatin, is formed by a 147 bp segment of DNA wrapped around a histone octamer core consisting of two copies of histones H2A, H2B, H3 and H4 [111]. During spermiogenesis, these histones are replaced initially by transition proteins 1 and 2, and then

finally by protamines 1 and 2 that give rise to a more compact and hence transcriptionally silent genome [82, 112, 113]. Although the exact reason behind this replacement is not clear yet, data suggests that this is necessary for DNA condensation and packaging into the sperm head nucleus and to protect the DNA cargo from damage. Indeed, aberrations in the process are associated with male infertility [113–117]. Ubiquitinated H2A as well as [69] polyubiquitinated forms of H3, TH3 (testis-specific H3) and H2B have been observed in the elongating spermatids, suggesting that this post-translational modification might be essential for loosening up the chromatin for accurate nucleosome removal, or may simply target these histones to the proteasome [118]. In *Drosophila*, histones are lost prior to individualization and this is preceded by H4 hyperacetylation and H2A monoubiquitination. The spermatid nuclei are also marked by high levels of ubiquitin, UbcD6 (drosophila homolog of yeast Rad6 and mammalian Hr6a and Hr6b), SUMO and DNA strand breaks [119].

Studies using mice deficient in the RING ubiquitin ligase Rnf8 [82] and the PA200 activator of the proteasome [120] have provided insights into the roles of uH2A and H4 acetylation in nucleosome removal. Both of these mutant mice show defective removal of nucleosomes in spermatids. As described in the previous section, Rnf8 mutant testes are deficient in sex body associated uH2A in meiosis. Rnf8 dependent ubiquitination of H2A was shown to recruit MOF, an acetyltransferase required for H4K16 acetylation [82]. The PA200 activator of the proteasome can recognize and bind this acetylated histone, leading to its degradation in the 20S core particle [120]. Together, these studies provide a model for nucleosome removal. However, it should be pointed out that other investigators did not find any changes in H4K16 acetylation levels or in histone–protamine exchange in Rnf8 inactivated mice [84]. Instead, they observed that Rnf8-dependent H2A ubiquitination was shown to be important for the establishment of active epigenetic modifications on the sex chromosomes during meiosis such as dimethylation of H3K4. This in turn, led to the establishment of other active epigenetic marks on the XY body in post-meiotic spermatids such as trimethylation of H3K4, histone lysine crotonylation, and incorporation of the histone variant H2AFZ. These modifications regulate escape gene activation and hence epigenetic programming in the sperm.

UPS and Acrosome Biogenesis

One of the critical structures that are formed during spermiogenesis is the acrosome, which is believed to arise from the fusion of Golgi-derived vesicles. Ubiquitinated proteins have been detected in every step of acrosome formation during rat spermiogenesis [121]. Using electron microscopy, ubiquitin signals were shown to exist in the matrix of transport vesicles between the acrosome and the Golgi apparatus, with strong expression in different regions of the developing acrosome after vesicle enlargement and fusion with the nucleus. Additionally, there are a number of UPS enzymes that have been functionally linked to sperm morphogenesis. TMF/ARA160, a Golgi associated protein that was shown to have ubiquitin ligase activity, is detected in the Golgi of spermatocytes and spermatids but disappears in the

mature spermatozoa. Homozygous null male mice are sterile, showing absence of homing of Golgi-derived proacrosomal vesicles to the perinuclear surface in the spermatids. Other defects in these mice include improper removal of the cytoplasm, misshapen sperm head, coiled tail and lack of motility indicating a broader role of TMF/ARA160 in the regulation of sperm differentiation [122].

A number of other UPS components are localized in compartments suggestive of a role in acrosome biogenesis. Usp8 (mUBPy) is a deubiquitinating enzyme highly expressed in the mouse testis [123]. While the localization in round spermatids is diffuse and scattered within the perinuclear zone, a region that holds both the Golgi and the centrosome, the protein marks every step of the developing acrosomal vesicle in the differentiating spermatids, and then relocates to the cytoplasmic surface of the acrosome and to the centrosomal region in the mature sperm. This expression pattern is very similar to that of the molecular chaperone MSJ1 and subunits of the 20S proteasome [123], suggesting that Usp8 may have a functional association with MSJ1 and the proteasome, acting together to balance protein folding and degradation during the time of acrosome biogenesis. The role of Usp8 in acrosome biogenesis was further studied by protein–protein interaction assays and immunolabeling experiments where it was found to interact with components of the early endosomal machinery [124]. Antibodies to Usp8 or ESCRT-0 (endosomal-sorting complex required for transport-0) co-labeled vesicles contributing to acrosome formation. Usp8, through its MIT (microtubule interacting and trafficking/transport) domain, links the labeled vesicles with microtubules, which participate in acrosome formation by promoting the migration of proacrosomic vesicles [124, 125].

UPS and Sperm Tail Biogenesis

Sperm tail structuring is vital to the process of spermiogenesis as it ensures proper mitochondrial arrangement and microtubule assembly essential for generation of motile spermatozoa. Different UPS enzymes have been implicated in the structuring of the flagellum/tail of the developing spermatozoa. Male mice homozygous null for the ubiquitin ligase Herc4 sired litters that were 50 % smaller and associated with a 50 % reduction in sperm motility. Many of the mature spermatozoa had angulated tails with cytoplasmic droplets being retained at the region of angulation, indicating the essential role of Herc4 in removal of cytoplasmic droplets and efficient post-testicular sperm maturation [126]. It would be apt to mention here that the sperm cytoplasmic droplet contains several components of the UPS such as ubiquitin, ubiquitin conjugating enzyme E2, the ubiquitin C-terminal hydrolase Uchl1/PGP9.5 and various proteasome core subunits types α and β MECL-1/b2i [127] that may regulate the processing of the cytoplasmic droplet contents.

Usp14 is a proteasome associated ubiquitin specific protease whose expression is reduced in homozygous ataxia (ax^J) mice leading to neurological defects and death within 2 months of age. These mice are sterile. To study non-neuronal functions of Usp14, the enzyme was expressed trangenically only in the brain of the mutant mice. Deficiency of Usp14 in the testis resulted in reduced testis size,

decreased sperm production, morphologically abnormal spermatids and infertility. In general, Usp14 is diffusely distributed in the cytoplasm of the round and elongating spermatids and becomes associated with the post-acrosomal segment of the spermatid nucleus in steps 14–16. In step 16, Usp14 is restricted to the redundant nuclear envelopes (a region at the base of the elongating spermatid nucleus arising due to removal of nuclear pore complexes) and the cytoplasmic droplets in elongating spermatids and differentiated spermatozoa, which appear to be sites of ubiquitin dependent proteolysis. Usp14 deficiency may lead to low availability of free monoubiquitin necessary for the degradation of substrates or may lead to decreased deubiquitinating activity of the proteasome. The mutant phenotype was also characterized by the up-regulation of other deubiquitinating enzymes, namely Uchl1, Uchl3, Uchl5 and Usp5, possibly a cellular response to cope with decreased levels of free circulating monoubiquitin [128].

Other UPS enzymes that may be involved in sperm tail remodeling based on their localization include the MARCH10 ubiquitin ligases. These enzymes are expressed in elongating and elongated spermatids, and specifically localize to the cytoplasmic lobes, the principal piece and the annulus of the flagella. Overexpression of MARCH10a in COS7 cells showed that it is associated with microtubules, while in sperm MARCH10b was distributed in the cytoplasm [129]. In COS7 cells, MARCH 10a was found to have auto-ubiquitinating activity that was abolished upon microtubule disassembly, suggesting a possible similar function in sperm flagellum formation. MARCH7 is highly expressed in spermiogenic cells from round spermatids to elongated spermatids and spermatozoa. The localization of the protein on the neck, midpiece, cytoplasmic lobes and acroplaxome of the elongated spermatid suggests that this ligase participates in spermiogenesis by regulating the structural and functional integrity of these different parts of the developing spermatozoon [130]. ZNF645, a human RING finger protein, is localized to the post-acrosomal perinuclear theca and the entire tail of the mature sperm, suggesting a possible role of the UPS in development of these structures [131].

UPS and Overall Spermatid Maturation

Some components of the UPS appear important for multiple facets of spermatid development. Deficiency of the ubiquitin ligase Itch is associated with an increase in germ cell apoptotic index during the peri-pubertal stage at PND 28, as well as in adults at PND 56. The mutant mice are characterized by a developmental delay in spermatogenesis at PND28 and spermatid head defects and disorganization of the spermatids at PND56 [132]. Apart from the already mentioned defects in meiosis, the Hr6b/Ubc2 null mutants are also characterized by an increased flagellar diameter, abnormal periaxonemal structures and decreased motility [133]. The spermatids and spermatozoa also show head shape defects such as an increased space between the nucleus and acrosome cap, association of flat membranous saccule-like

structures with the manchette, manchette associated with nucleus with an irregular outline, and nuclear evagination/invagination [134, 135]. Inactivation of the deubiquitinating enzyme CYLD in mice leads to sterility with a drastic testicular atrophy at PND 28. Round and elongating spermatids are scarce in these animals, with complete absence of spermatozoa in the epididymis. The mutant elongating spermatids have an incorrectly formed acrosome. Other defects include a lack of radial testicular organization and a significant increase in apoptosis. CYLD was shown to interact and negatively regulate ubiquitin-dependent NF-κB activator RIP1. Loss of CYLD leads to constitutive activation of NF-κB signaling with aberrant expression of anti-apoptotic genes [136].

Some of the structures that are established during spermiogenesis include the acrosome–acroplaxome complex and the head–tail coupling apparatus (HTCA) in the sperm head. The microtubular manchette formed on the caudal end of the acrosome–acroplaxome complex is a transitory structure suggested to be required for the transport of cargo between the nucleus and cytoplasm and towards the HTCA and tail. The RING finger ligase Rnf19A was shown to interact with Psmc3, a component of the 19S regulatory cap of the 26S proteasome; both co-localized on the Golgi-derived proacrosomal vesicles, and subsequently on the cytosolic side of the acrosome and acroplaxome and at the acroplaxome ring–manchette perinuclear ring region, thus suggesting a role for the UPS in acrosome biogenesis, sperm head shaping and sperm tail development [137]. The transmembrane ubiquitin ligase MARCH-XI, expressed predominantly in developing spermatids of rats, is localized on the trans-Golgi network (TGN) and multivesicular bodies (MVB). It forms complexes with the adaptor protein complex I and ubiquitinated/non ubiquitinated forms of fucose-containing glycoproteins, which are transported to the MVB from the TGN during acrosome biogenesis. This association suggests that the ubiquitin ligase has a ubiquitin-mediated role in the sorting of cargo in the TGN-MVB body transport pathway involved in spermiogenesis [138].

There are also studies hinting at the role of the UPS in centrosome reduction, a hallmark of spermatid elongation [139, 140]. After generating the sperm axoneme, the centrosome is either completely removed or reduced to a single inactive centriole in mammals [140]. Ubiquitin immune-reactivity was detected in the centrosomal part of the human and rhesus sperm tail, and proteasome subunits were also detected inside or near the sperm centriole [141, 142]. Deubiquitinating enzyme Usp8 also associates with gamma tubulin, a centrosomal protein marker suggesting a role in centrosome function [143].

These studies have shown that the UPS is a vital mediator of spermiogenesis, regulating events such as chromatin remodeling, acrosome biogenesis, sperm head shaping and structuring of the flagellum with consequences on fertility. While some functions are speculative based on localization and association studies, especially the ones looking at the role of UPS in acrosome biogenesis and flagellum formation, a significant number of functions are well established based on gene inactivation models.

Table 9.1 UPS genes implicated in spermatogenesis

Class of enzyme	Name	Substrates involved in spermatogenesis	Regulation of expression	Testis phenotype of mutants	Species	References
E1	UBA7/UBE1L (Putative E1)	Unknown	Abundant in adult testis	Undefined	*H. sapiens*	[146]
	UBA1/UBE1	Unknown	Testis-specific, abundant in spermatogonia and round spermatids	Undefined	Mammals	[147, 148]
	UBA6/UBE1L2/ E1–L2	Unknown	High expression in the testis, gonocytes	Undefined	Mammals	[34, 36]
E2	HR6A/UBE2A	Histones	Testis, spermatocytes, low in spermatids	Normal progression of spermatogenesis	Mammals	[60, 69, 149]
	HR6B/UBE2B	Histones	Testis, prominent in spermatocytes and spermatids	Infertility, apoptosis of primary spermatocytes in the first wave, sperm head shape anomalies, flagellar anomalies such as abnormal distribution of periaxonemal structures	Mammals	[60, 133, 134, 149]
	UBC4 (UBC4-testis, UBC4-1, UBC4-2)	Histones	Constitutively expressed with high expression in spermatids	UBC4-testis mutants show a slight delay in postnatal testis development	*R. norvegicus*	[34, 105, 150–153]
	Rat100/EDD/ UBR5/HYD	Unknown	High in gonocytes, spermatocytes, low in round and elongated spermatids	Undefined in vertebrates Infertility, primary spermatocyte stage arrest defects, defects in spermatid elongation seen in the *Drosophila*	*R. norvegicus* *D. melanogaster*	[34, 100, 101, 154]
	HUWE1/LASU1/ E3histone, ARF-BP1	Histones (?)	Gonocytes, spermatogonia, spermatocytes	Undefined	*R. norvegicus*	[38, 39]
	HERC4	Unknown	Spermatogonia, spermatocytes, spermatids	Subfertility, reduced sperm motility, angulated sperm tail, retention of cytoplasmic droplet on the sperm tail	*M. musculus*	[126]
	TMF/ARA160	Unknown	Spermatocytes, spermatids	Infertility, lack of motility, developmental defects include lack of acrosome in the spermatozoa, presence of cytoplasmic remnant around the condensed sperm nuclei, misshapen heads and tail coiling in sperm	*M. musculus*	[122]

UBR2	Histones H2A and H2B	High expression in the testis	Infertility, arrest of spermatocytes at prophase I of meiosis and subsequent apoptosis	*M. musculus*	[63–65, 72, 73]
RAD18/RAD18Sc	Unknown	High expression in the testis, highest expression in pachytene and diplotene spermatocytes	Subfertility, defective DSB repair during meiosis, defective MSCI	*M. musculus*	[155, 156]
RFP	Unknown	High expression in male germ cells particularly in primary spermatocytes and round spermatids	Undefined	*M. musculus*	[85, 157]
CUL2	HTP3 (Belongs to the family of HORMA domain-containing proteins, required for preparing chromosomes for meiosis)	Germ line, high levels in meiotic cells	Infertility, cell cycle arrest, premature meiotic entry	*C. elegans*	[96]
CUL4A	Histones H3, H4	Gonocytes, spermatogonia, spermatocytes, low in spermatids	Infertility, increased apoptosis of primary spermatocytes, decreased sperm number, motility, defective acrosome formation	*M. musculus*	[97, 158]
SIAH1A	Unknown	High expression in the testis	Infertility, accumulation of spermatocytes at metaphase to telophase transition of meiosis I and subsequent apoptosis	*M. musculus*	[98, 159]
Parkin	Unknown	Undefined	Sterility, defects in mitochondrial morphogenesis during spermiogenesis leading to defective spermatid individualization	*D. melanogaster*	[160, 161]
E6-AP	Unknown	Undefined	Subfertility, reduced testis size and sperm count	*M. musculus*	[162]
MARCH7	Unknown	High expression in the testis, high expression in round, elongating and elongated spermatids	Undefined	*R. norvegicus*	[130]

(continued)

Table 9.1 (continued)

Class of enzyme	Name	Substrates involved in spermatogenesis	Regulation of expression	Testis phenotype of mutants	Species	References
	MARCH10	Unknown	Elongating and elongated spermatids	Undefined	*R. norvegicus*	[129]
	MARCH11	Unknown	Developing spermatids	Undefined	*R. norvegicus*	[138]
	ZNF645	Unknown	Spermatocytes, round and elongated spermatids, Leydig cells	Undefined	*H. sapiens*	[131]
	ITCH	Occludin (a major component of tight junctions)	Testis, Sertoli cells	Subfertility, age dependent impairment in spermatogenesis with increased apoptosis, delayed spermatid development and organization	*M. musculus*	[132, 163, 164]
	Mei4 (mouse homolog of HEI10)	CCNB3 (?) (a meiotic B-type cyclin expressed maximally during leptotene to zygotene transition)	Undefined	Infertility, inability of mutant spermatocytes to maintain interhomolog associations due to lack of crossing over, failure of chromosome aggregation on the metaphase plate leading to arrest in the spermatocyte stage and subsequent apoptosis	*M. musculus*	[68]
	RNF4	MDC1 and BRCA1 (DNA repair proteins)	Undefined	Infertility, age-dependent testicular atrophy, depletion of germ cells	*M. musculus*	[67]
	RNF8	Histone H2A and H2AX	Undefined	Infertility, reduction of histone ubiquitination in the testes defects in global nucleosome removal during spermiogenesis	*M. musculus*	[76, 82, 83, 165]
	RNF19A	Unknown	Pachytene spermatocytes, round and elongating spermatids, mature sperm	Undefined	*R. norvegicus*	[137]
	RNF133	Unknown	Round and elongating spermatids	Undefined	*M. musculus*	[166]
	RNF149	Unknown	Gonocytes	Undefined	*R. norvegicus*	[34]
	RNF151	Dysbindin (?) (involved in membrane biogenesis and fusion)	Round and elongating spermatids	Undefined	*M. musculus*	[167]

	RNF168	Unknown	Undefined	Age-dependent impairment of spermatogenesis leading to subfertility or infertility at 12 months age, testicular atrophy	*M. musculus*	[168]
	SCF(beta)-TrCP	Snail1 (a transcription factor inducing E-cadherin expression) Emi1 (?) (a cell cycle regulator)	Spermatogonia, spermatocytes	Failure of spermatogonial stem cell adhesion in the seminiferous tubules, accumulation of metaphase I spermatocytes, decrease in number of spermatids and sperm	*M. musculus*	[42, 99]
	APC/C	MIWI (murine homologs of PIWI)	Spermatocytes, round and late spermatids	Defects in spermatid maturation	*M. musculus*	[169]
DUB	USP2/UBP-testis	Unknown	Constitutively expressed with high expression in the elongating spermatids	Subfertility, abnormal aggregation of elongated spermatids, presence of multinucleated cells in some tubules, decreased motility in nutrient-deprived conditions	Mammals	[34, 170, 171]
	USP8/mUBPy	MSJ-1 (?) (sperm-specific DnaJ chaperone protein)	High expression in the testis, round, elongating and mature sperm	Undefined	Vertebrates	[123, 124, 143, 172–174]
	USP9Y	Unknown	Undefined	Single nucleotide polymorphisms associated with azoospermia/oligospermia	*H. sapiens*	[175–178]
	USP14	Unknown	Round and elongating spermatids and mature sperm	Spermatid differentiation	*M. musculus*	[128]
	USP26	Unknown	Testis specific expression	Polymorphisms linked to azoospermia, oligozoospermia, asthenozoospermia	*H. sapiens*	[179–184]
	USP42	Unknown	High in round spermatids, low in condensing spermatids	Undefined	*M. musculus*	[185]
	UCH-L1	Unknown	Spermatogonia, Sertoli cells	Impaired spermatogenesis, decreased spermatogonial stem cell proliferation and increased number of pre-meiotic germ cells, resistance to early wave of apoptosis in immature testis, decreased sperm concentration and motility	*M. musculus*	[44, 103, 186, 187]

(continued)

Table 9.1 (continued)

Class of enzyme	Name	Substrates involved in spermatogenesis	Regulation of expression	Testis phenotype of mutants	Species	References
	UCH-L3	Unknown	Pachytene spermatocytes and post-meiotic spermatids	Increased germ cell loss following cryptorchid injury of the testis	*M. musculus*	[187, 188]
	UCH-L4	Unknown	Spermatogonia	Undefined	*M. musculus*	[188, 189]
	UCH-L5	Unknown	Spermatocytes and spermatids	Undefined	*M. musculus*	[188]
	CYLD	Receptor-interacting protein 1 (RIP1)	Undefined	Infertility, attenuation of early wave of germ cell apoptosis, drastic testicular atrophy by post-natal day 28, spermatid deficiency	*M. musculus*	[136]
Proteasome	Prosalpha3T	Unknown	Low expression in late phases of second meiotic division, prominent in post-meiotic spermatids	Undefined	*D. melanogaster*	[109]
	Prosalpha6T	DIAP (?)	Post-meiotic spermatids, mature sperm	Sterility, defects in sperm individualization and nuclear maturation	*D. melanogaster*	[108, 109]
	PROS28.1A	Unknown	Primary spermatocytes and spermatids	Undefined	*D. melanogaster*	[107]
	PROS28.1B	Unknown	Elongating spermatids	Undefined	*D. melanogaster*	[107]
	PA200/PSME4	Acetylated histones	Constitutive	Subfertility, reduced sperm concentration and motility, hypospermatocytogenesis, apoptotic spermatocytes and spermatids with defective nucleosomal removal	*M. musculus*	[55, 120]

Summary and Future Perspectives

From the studies cited in this review, it is clear that the UPS plays important roles at every step of spermatogenesis starting from gonocytes to differentiated spermatids, ready to be released into the epididymis. To date, there is a large volume of evidence for the regulation of various enzymes of the UPS at different stages of spermatogenesis. This is particularly true for regulation of levels of expression of the enzymes or their subcellular localization. Though less abundant, there are still plentiful examples of loss-of-function studies, especially gene inactivation models, that identify clear physiological functions of the UPS in spermatogenesis (Table 9.1). Often, these are critical functions as the inactivation of these genes results in abnormal progression or the developmental arrest of germ cells leading to deficits in infertility. Although the number of physiological functions of the UPS identified in the testis is increasing quickly, in most cases, there is still limited information on the molecular mechanisms by which the system exerts these effects. Specifically, the exact substrates of the enzymes involved and therefore the molecular pathways affected are often unknown. Such information is important to confirm that the genes implicated from loss of function studies play a direct role in the physiological process. In developmental processes that require multiple sequential steps, such as occurs in spermatogenesis, loss of function at an upstream step may impact indirectly a downstream step.

The extent of our knowledge on the roles of the UPS in spermatogenesis is also variable depending on the stage considered. The functions of this system in gonocytes and spermatogonial stem cell renewal and differentiation remain largely to be evaluated. A more detailed understanding of the roles of the UPS in regulating germ cell proliferation and differentiation will likely be useful in identifying new approaches to enhance the preservation of gonocytes and spermatogonial stem cells for translational purposes such as fertility preservation in prepubertal boys subjected to sterilizing chemotherapy, transgenesis and improved animal husbandry.

The most detailed understanding of the functions of the UPS in spermatogenesis is in meiosis. As detailed above, many loss of function studies have demonstrated impaired meiotic progression. Indeed, it is in early meiosis that spermatogenesis is arrested when the supply of free ubiquitin is limited [49, 50], suggesting that a critical increase in ubiquitinating activity occurs at this stage. There are examples in which molecular mechanisms have been delineated at a reasonably high resolution. This is particularly true for the roles of ubiquitination of histones in the repair of double strand breaks in DNA. Ubiquitinated H2A also appears important in meiotic sex chromosome inactivation [49, 50], but controversy as to whether it is essential remains to be resolved [81, 84].

A number of functions of the UPS in spermiogenesis have been proposed, but most remain to be confirmed through loss of function approaches. The relative paucity of such data may be related to technical limitations. In gene knockout studies, effects in spermiogenesis may be masked if the inactivated gene also plays critical roles earlier in spermatogenesis and causes arrest of development in spermatogonia or spermatocytes. Histones in spermatids are known to be ubiquitinated, but whether

this ubiquitination plays an essential role in their replacement by protamines, in the epigenetic regulation of gene expression in these cells or in the resulting embryo remain intriguing and important questions at this time. Much additional work is required to establish firmly the role of ubiquitination in acrosome biogenesis and tail formation. In other cellular systems, ubiquitination has been shown to be important in mitochondrial remodeling and turnover [144]. Its potential role in the critical mitochondrial rearrangement in the mid-piece during spermiogenesis is largely unexplored.

Although emphasis is often placed on the proteolytic functions of ubiquitination, it is now well established that this post-translational modification serves many non-proteolytic functions. This is particularly true in the case of chromatin proteins such as histones. It is clear that the monoubiquitination of histone H2B or H2A recruits other proteins involved in DNA repair and studies cited in this review suggest that ubiquitination of histones plays important roles in regulating gene expression. Such modifications in the spermatozoa may have important epigenetic functions if they alter gene expression in the resulting embryo. Since spermatozoa with disordered nuclear condensation are often used in intracytoplasmic sperm injection in assisted reproduction, abnormalities in chromatin ubiquitination may be important in the developmental defects that have been observed at increased frequency in the offspring born from these procedures (rev in [145]).

As the number of loss of function studies with clear defects in spermatogenesis continues to increase (Table 9.1), it provides an opportunity to test whether abnormalities in these genes may be responsible for similar phenotypes in infertile men. This will enhance the possibilities for precise molecular diagnoses which are lacking in this clinical syndrome and may one day lead to specific therapies. Perhaps of more obvious translational potential is that this growing catalog of genes whose loss results in infertility are potential targets for the development of male contraceptives. Since they are mostly enzymes, they are in theory amenable to high throughput assays for screening of candidate drug inhibitors. Some are also germ cell specific and so inhibition may be less likely to result in toxicity in other cell types of the body.

The vast numbers of UPS genes that are expressed in the testis but have not been studied in this context indicate that we are still in the early phases of discovery in this area. Nonetheless, current insights confirm that the UPS plays important physiological roles in spermatogenesis and holds translational potential that will surely be revealed in the years to come.

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Role of Posttranslational Modifications in *C. elegans* and *Ascaris* Spermatogenesis and Sperm Function

10

Long Miao and Steven W. L'Hernault

Abstract

Generally, spermatogenesis and sperm function involve widespread posttranslational modification of regulatory proteins in many different species. Nematode spermatogenesis has been studied in detail, mostly by genetic/molecular genetic techniques in the free-living *Caenorhabditis elegans* and by biochemistry/cell biology in the pig parasite *Ascaris suum*. Like other nematodes, both of these species produce sperm that use a form of amoeboid motility termed crawling, and many aspects of spermatogenesis are likely to be similar in both species. Consequently, work in these two nematode species has been largely complementary. Work in *C. elegans* has identified a number of spermatogenesis-defective genes and, so far, 12 encode enzymes that are implicated as catalysts of posttranslational protein modification. Crawling motility involves extension of a single pseudopod and this process is powered by a unique cytoskeleton composed of Major Sperm Protein (MSP) and accessory proteins, instead of the more widely observed actin. In *Ascaris*, pseudopod extension and crawling motility can be reconstituted in vitro, and biochemical studies have begun to reveal how posttranslational protein modifications, including phosphorylation, dephosphorylation and proteolysis, participate in these processes.

Keywords

C. elegans • *Ascaris suum* • Spermatogenesis • Major sperm protein • *spe* mutant • Phosphorylation • Dephosphorylation • Proteolysis • Ubiquitination • Acyl transferase

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Introduction

Nematodes, including the free-living species *Caenorhaditis elegans* and the parasitic species *Ascaris suum*, undergo spermatogenesis to produce spermatozoa that form a single pseudopod (Fig. 10.1a) and move by a crawling, amoeboid-like motility mechanism [1, 2]. In several ways, studies in these two species complement each other. While detailed cytological and genetic studies of spermatogenesis are feasible in *C. elegans* [3], such studies have not been done in *Ascaris*, due to its lengthy and complex life cycle [4]. In contrast, the ready availability of abundant numbers of spermatids from dissected male *Ascaris* (which are up to 30 cm in length) [5] have allowed biochemical studies of spermiogenesis, the conversion of sessile spermatids into crawling spermatozoa. These biochemical studies have included identification and analyses of posttranslational modifications, with the principal focus on those related to sperm motility [2]. *C. elegans* males are small (~1 mm in length), making it is difficult to recover the large numbers of spermatids required for biochemical analyses of posttranslational modifications. However, the genes affected in *C. elegans spe*rmatogenesis-defective (*spe*) mutants include those that encode putative kinases, phosphatases, proteases, palmitoyl transferases, and enzymes in the protein ubiquitination pathway [3]. Consequently, posttranslational modifications are likely an important part of *C. elegans* spermatogenesis. Despite the fact that *Caenorhabditis* and *Ascaris* last shared a common ancestor ~540 MY ago [6], there is strong conservation in both the phenomenological and molecular genetic aspects of spermatogenesis [2, 7–10]. This indicates that the complementary approaches to posttranslational protein modification possible in these two species will eventually allow a unified picture of these processes. Below we summarize the data available for each nematode.

C. elegans Reproductive Biology

C. elegans exists as two sexes, a male and a protandrous hermaphrodite [11]. The male engages in spermatogenesis upon reaching sexual maturity, and this continues until he dies [12]. The hermaphrodite germline first matures as a testis during larval life, when several hundred sperm are proliferated and then the germline sex determination mechanism (Fig. 10.2a) causes a switch in sexual identity so that it becomes an ovary [11, 13]. Generally, hermaphrodites use the spermatozoa generated during the first phase of germline maturation to self-fertilize the oocytes produced during the second phase of germline maturation. This is an efficient process and virtually 100 % of ovulated oocytes are self-fertilized [14]. This peculiar self-fertility aspect of *C. elegans* reproductive biology has been exploited to identify and recover mutants that are defective in spermatogenesis (*spe* mutants) [15].

The *spe* mutants are initially identified as hermaphrodites that are self-sterile and lay unfertilized oocytes onto the agar growth plate [16, 17]. Such self-sterile hermaphrodites can be mated to wild type males and, if progeny form, this means that wild type spermatozoa are both necessary and sufficient to correct the self-sterility

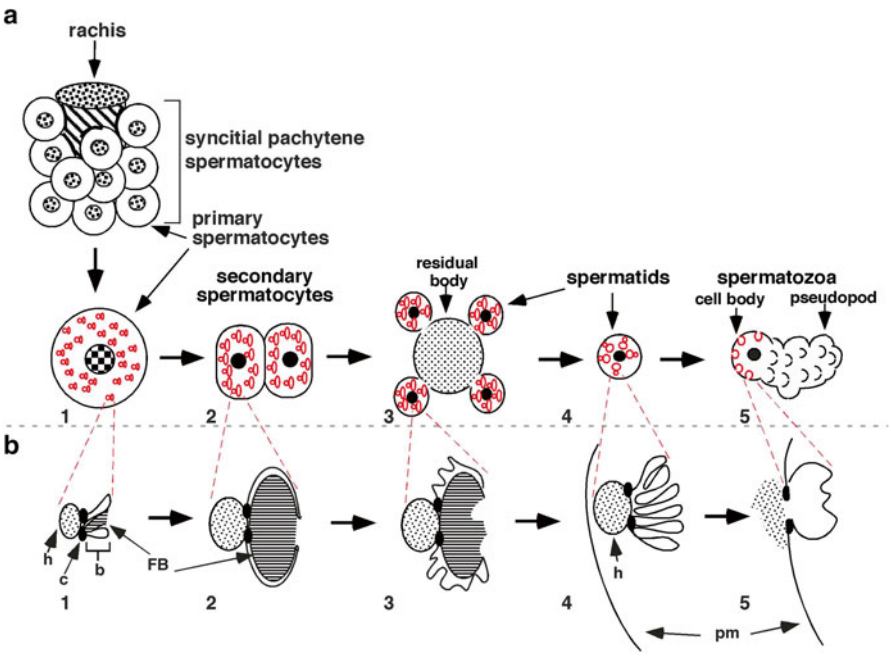

Fig. 10.1 (**a**) A summary of the cellular stages of wild type spermatogenesis and its relationship to FB–MO morphogenesis. *1*, Syncytial pachytene spermatocytes with many FB–MOs bud from the rachis and divide to form; *2*, secondary spermatocytes (FB–MOs are shown in *red*); *3*, the residual body forms and spermatids begin to bud from it; *4*, budded spermatids selectively retain FB–MOs; *5*, MOs fuse with the spermatid plasma membrane as a pseudopod extends from the cell body during spermiogenesis. Nuclei are the *circles* in the center of each cell. Nuclei are speckled during stages when chromatin is in condensed meiotic chromosomes or completely filled (in *black*) after chromatin forms a single highly condensed sphere. (**b**) Summary of wild type FB–MO morphogenesis. At each stage, *red*, *dashed lines* connect the detailed FB–MO drawing in panel **b** to one example in the relevant cellular stage in panel **a**. *1*—The fibrous body (FB) develops in close association with, and is surrounded by, the membranous organelle (MO) within the primary spermatocyte. The MO is separated by a collar region (*c*) into a head (*h*; speckled region at *left*) and body (*b*; region to the *right* of the collar). *2*—The FB–MO complex reaches its maximum size within primary/secondary spermatocytes. The double layered MO-derived membrane surrounds the FB, which is composed of the major sperm protein filaments. *3*—The MO-derived membranes surrounding the FB retract and fold up while FB filaments depolymerize and disperse as spermatids bud from the residual body. *4*—The head of each MO (*arrow*) moves to a position just below the plasma membrane (pm) of the spermatid after the FBs have depolymerized and disappeared. The irregular shapes within the FB represent retracted membrane that had covered the exterior of the FB. *5*—The head of the MO fuses at the collar to the plasma membrane and exocytoses its contents (*dots at arrow*) onto the cell surface. A permanent fusion pore remains at the point of each MO fusion (each cell has many MOs). This figure and legends are modified from [109].

of the mutant hermaphrodite. Subsequent cytological analyses frequently reveal that *spe* mutant worms (male or hermaphrodite) usually produce spermatozoa that have abnormal cytology. Less frequently, a *spe* mutant produces spermatozoa that have a cytology that is indistinguishable from wild type when viewed by light microscopy.

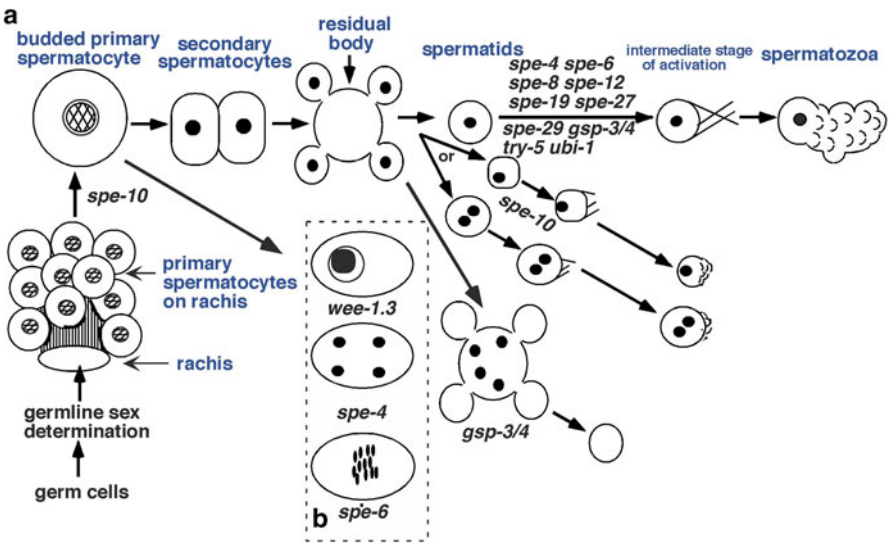

Fig. 10.2 (**a**) Stages of wild type spermatogenesis are shown as an ordered pathway of morphogenesis with the stages labeled in *blue*. Of the >44 known genes known to cause a spermatogenesis-defective (*spe*) phenotype, only those that have been implicated in posttranslational protein modification are placed on the pathway at the point(s) where ultrastructural or light microscopic defects are evident. For several mutants, a point at which morphogenesis arrest varies from cell to cell which is indicated by placing the gene name at more than one place in the figure. (**b**) Most common terminal stages of mutants that arrest morphogenesis without forming spermatids. Please note that individual drawings are not to scale; for reference, wild type spermatids are 5–6 μm in diameter. This figure and legend are modified from [109]. Copyright: © 2006 S.W. L'Hernault, distributed under the terms of the Creative Commons Attribution

In such cases, more sophisticated tests reveal that these *spe* mutant spermatozoa lack one or more traits that are present in wild type spermatozoa [3, 18].

Overview of *C. elegans* Spermatogenesis

Within broad outlines, spermatogenesis is similar within the two sexes [12–14, 19, 20], except as noted below. The initial stages of germline mitotic proliferation involve nuclear division in a syncitium. This is governed by germ cell proximity to the distal tip cell, which is a somatic gonadal cell that supplies a paracrine signal to maintain mitotic proliferation [21–23]. Once germ cell nuclei move some distance from the distal tip cell, mitotic proliferation ceases and the syncitial nuclei begin to cellularize. The initial stage looks like "kernels on a corn cob", with the kernels representing the developing spermatogonial cells and the "cob" being a central nuclear-free cytoplasmic zone known as the rachis (Fig. 10.1a). During this stage, spermatogonial cells initiate meiosis and bud from the rachis to become fully

cellularized primary spermatocytes (Figs. 10.1a and 10.2a) [19, 20]. Interestingly, unlike during mammalian spermatogenesis, budded *C. elegans* primary spermatocytes can complete both meiotic divisions and form spermatids in vitro without any additional paracrine or endocrine input [24, 25].

In *C. elegans*, the completion of meiosis occurs just prior to formation of spermatids (Fig. 10.1a) [19, 20]. While this process is analogous to spermatid formation in mammals (Fig. 10.1a), there are some important differences. *C. elegans* spermatids bud from the residual body as they complete meiosis II, while mammalian meiosis is completed well before spermatids bud from the residual body. As in mammals, the *C. elegans* residual body is the repository for all cellular components that are no longer needed to complete formation of mature spermatozoa; components that are placed into the residual body include all of the ribosomes [26]. This means that, unlike mammals, the transition from spermatid to spermatozoon occurs without any post meiotic proteins synthesis in *C. elegans* [27]. This absence of post meiotic protein synthesis means that spermatids must activate into spermatozoa while utilizing existing components. This, in turn, means that there must be a way of ensuring that required proteins and other components segregate into spermatids during their budding from the residual body. One prominent way in which spermatids receive required components is through unusual fibrous body–membranous organelles (FB–MOs).

MOs are first observed in association with the Golgi during pachytene of meiosis I (Fig. 10.1b1) [19, 20]. During subsequent spermatocyte development, they polymerize fibers composed of major sperm protein (MSP); MSP is a highly conserved protein in nematodes that later plays a role is spermatozoon pseudopodial structure and function [28, 29], which has been extensively studied in *Ascaris* (see below). These fibers are hexagonally packed and encased by a double-layered membrane envelope derived from the MO; at this stage, it is termed the fibrous body–membranous organelle (FB–MO) complex (Fig. 10.1b2). FB–MOs are vectorially transported into the spermatid as it buds and separates from the residual body. The double-layered membrane envelope surrounding the FB retracts and remains a part of the MO as the FB fibers depolymerize, causing soluble MSP dimers to accumulate in the cytoplasm (Fig. 10.1b3) [30]. The FB–MOs store and transport both membrane and cytoplasmic proteins used subsequently by spermatids to complete maturation and become functional spermatozoa [31]. After the FB's disassemble, the MOs localize near the plasma membrane (Fig. 10.1b4). During spermatid activation (see below), the MOs fuse with the plasma membrane, place integral membrane proteins into the plasma membrane and release their contents, which include lectin-positive glycoproteins of unknown identity, to the extracellular space (Fig. 10.1b5) [32].

Spermatid activation occurs when spermatids transition to become spermatozoa (Figs. 10.1a and 10.2a). This occurs in both males and hermaphrodites, but by sex-specific mechanisms. As discussed above, the *C. elegans* hermaphrodite gonad is bi-potential and differentiates first into a testis that proliferates several hundred spermatids before switching to become an ovary [13]. After this sexual switch,

spermatids are pushed from the proximal germline into the spermatheca by the first ovulated oocyte, in a piston-like fashion. Movement of spermatids into the spermatheca is associated with their rapid activation into spermatozoa. Unlike hermaphrodites, males accumulate spermatids in a seminal vesicle and they remain arrested at this stage until the male mates with a hermaphrodite [12]. During mating, ejaculated spermatids are mixed with seminal fluid and this causes them to activate into spermatozoa [14]. Both male and hermaphrodite-derived spermatozoa move by crawling [1, 24], which is essential for them to fertilize an ovulated oocyte. As an oocyte is ovulated into the spermatheca, only one spermatozoon will fertilize it and many of the remaining sperm will be physically pushed out of the spermatheca into the uterus as that fertilized egg exits the spermatheca and enters the uterus. Crawling motility allows these pushed out sperm to leave the uterus and re-enter the spermatheca for another try at fertilization; this cycle typically repeats hundreds of times until all spermatozoa have successfully fertilized an oocyte [14].

Spermatid activation can also be elicited in vitro by treating either male or hermaphrodite-derived spermatids with any of a wide variety of substances that include proteases (Pronase or trypsin), weak unprotonated bases (such as triethanolamine) [32] or ionophores that raise intracellular pH (such as monensin) [24], chloride channel inhibitors [25] or Zn^{2+} ions [33]. These in vitro studies have shown that activation is characterized by an initial, transient stage where fine spikes are extended ("intermediate stage of activation", in Fig. 10.2a). This is followed by coalescence of spikes into a single, motile pseudopod that is used for crawling motility (Fig. 10.2a) [24]. Spermatids can be continually treated with very high concentrations (200 μg/ml) of broad-spectrum proteases, such as Pronase, and they will activate into spermatozoa that remain viable and motile for hours. This suggests that proteolytic digestion of one or more surface proteins can elicit spermatid activation. Currently, the identity of the affected protein(s) is unknown. However, unlike in vivo or TEA activated spermatids, Pronase-treated spermatids become spermatozoa that are not competent to fertilize oocytes after artificial insemination [34]. This suggests that broad-spectrum protease (like Pronase) treatment of live spermatozoa affects one or more surface proteins required for motility and/or fertilization.

Mutants and Posttranslational Modifications During *C. elegans* Spermatogenesis

As mentioned above, there has been little biochemical work to directly identify and analyze posttranslational modifications during *C. elegans* spermatogenesis. Rather, most work starts from recovering *spe* mutants, cloning the genes and inferring the function of the encoded protein [3]. In a number of cases, the conserved function of identified proteins reveals that they likely participate in posttranslational processes, as we discuss below.

Putative Kinases that Function During *C. elegans* Spermatogenesis

WEE-1.3

Six dominant mutants that arrest spermatocyte meiosis have been identified and they all affect the *wee-1.3* gene [35]. The *wee-1.3* gene encodes a one-pass integral membrane protein that is a regulatory kinase orthologous to Myt1, which is found throughout the metazoa [36]. Normally, WEE-1.3/Myt1 phosphorylation of its CDC2 target is balanced by the opposing CDC25 encoded phosphatase [37]. When CDC25 predominates, the phosphate added by the Myt1/WEE-1.3 kinase is removed from the CDC2 substrate, activating the CDC2 kinase so that it can phosphorylate its targets and allow completion of either mitosis or meiosis. Various genetic techniques have shown that *wee-1.3* loss of function (recessive) mutants widely affect *C. elegans* cells, with the earliest observed defect being embryonic lethality. Surprisingly, the six above-mentioned dominant *wee-1.3* mutants only affect spermatocyte meiosis, most likely arresting at the G2/M boundary of meiosis I (Fig. 10.2b). These dominant mutations all cluster within a 4 amino acid region that is near the C terminal end of WEE-1.3. The phenotype associated with these dominant mutants indicates that WEE-1.3 activity during spermatogenesis is regulated by a tissue-specific mechanism. The most parsimonious hypothesis is that dominant *wee-1.3* mutants produce a WEE-1.3 that inappropriately continues phosphorylation of CDC2, and that this cannot be overcome by CDC25 phosphatase activity. This suggests that part of the balancing of WEE-1.3 by CDC25 during *C. elegans* spermatogenesis involves negative regulation of WEE-1.3 kinase activity. The amino acids in the WEE-1.3 region affected in the dominant mutants mediate this negative regulation, perhaps because interaction with a regulatory protein is disturbed. This speculation, while plausible, is solely based on developmental genetics and will need to be confirmed biochemically.

SPE-6

SPE-6 is a casein I type serine/threonine kinase that participates in multiple aspects of spermatogenesis [38]. Null *spe-6* mutations are unable to form spermatids and they arrest as aberrant spermatocytes. These aberrant spermatocytes can form condensed chromosomes and initiate meiosis, but they arrest at diakinesis of meiosis I (Fig. 10.2b). Somehow, the chromosomes disengage from the meiotic spindle and can replicate. This results in arrested spermatocytes containing four half meiotic spindles and condensed chromosomes that do not complete meiosis. Additionally, these aberrant spermatocytes are unable to polymerize MSP dimers into the FBs, so FB–MO morphogenesis is severely disrupted. While the substrate(s) for the SPE-6 kinase have not yet been identified, it has been suggested that MSP fibers are a phosphorylated substrate [39, 40].

Eighteen non-null mutants of *spe-6* have been recovered based on their suppression of the *spe-8* pathway ([38]; also, see below). These mutants were identified based on their ability to restore self-fertility to *spe-27* loss of function mutants. These non-null *spe-6* mutations are capable of suppressing mutants in at least several of the genes (*spe-8*, *spe-12* and *spe-29*) in the *spe-8* pathway (Fig. 10.2a). The simplest interpretation of these data is that, in the presence of a non-null *spe-6* suppressor mutation, the rest of the *spe-8* pathway is not required for spermatid activation to occur in hermaphrodites.

SPE-8

The SPE-8 is a soluble, non-receptor tyrosine kinase with one Src homology 2 domain (P. J. Muhlrad and S. Ward, personal communication). The *spe-8* kinase is one of five genes, *spe-8*, *spe-12*, *spe-19*, *spe-27* and *spe-29* that collectively are known as the "*spe-8* class" of genes/mutants (Fig. 10.2a). SPE-12 (41), SPE-19 [42] and SPE-29 [43] are transmembrane proteins while SPE-27 [44] is a small soluble protein; SPE-8 is the only protein in which the sequence suggests an enzymatic function. Loss of function mutations in any of the *spe-8* class genes causes hermaphrodites to accumulate spermatids that do not activate into spermatozoa, so these mutants are self-sterile. In contrast, male mutants of the *spe-8* class produce spermatids that activate into normal spermatozoa in vivo, and these spermatozoa can successfully fertilize oocytes [15].

All *spe-8* class mutants have in vitro spermatid activation that has distinctive phenotypic characteristics, and the below-discussed properties apply to spermatids derived from either hermaphrodites or males. When treated with triethanolamine, spermatids of the *spe-8* mutant class activate into spermatozoa that are cytologically indistinguishable from wild type. In contrast, Pronase (a mix of proteases with different substrate specificities) treatment of spermatids from *spe-8* class mutants causes them to extend fine, spiky surface projections [45]. In wild type, these projections would be a transient stage that precedes formation of a pseudopod (like "intermediate stage of activation" in Fig. 10.2a; [24]; also, see above), but spermatids from *spe-8* class mutants arrest at this intermediate stage. Additionally, wild type spermatids activate into spermatozoa after treatment with micromolar Zn^{2+} but this treatment is without effect on *spe-8* class mutant spermatids. This suggests that Zn^{2+} works through a pathway that employs proteins encoded by the *spe-8* class genes to mediate spermatid activation into spermatozoa [33].

Putative Phosphatases that Function During *C. elegans* Spermatogenesis

GSP-3 and GSP-4

There are two yeast Glc Seven-like phosphatase (GSP)/Protein Phosphatase 1-γ like genes in *C. elegans* that play a role during spermatogenesis, *gsp-3* and *gsp-4*, and

these genes share ~95 % identity over much of their coding sequence [46]. Consequently, RNAi knockdown of either *gsp-3* or *gsp-4* gene expression affects both genes, causing a ~40 % reduction in self-fertility [46–48]. Loss of function mutations in each of these genes were subsequently recovered and either *gsp-3* or *gsp-4* single mutants showed a very modest (~10 %) reduction in hermaphrodite self-fertility. However, *gsp-3* and *gsp-4* are functionally redundant because the double mutant hermaphrodite is completely self-sterile, and this self-sterile hermaphrodite is cross-fertile after mating to a wild type male, consistent with defects in spermatogenesis [40]. The *gsp-3*/*gsp-4* double mutant has a spectrum of defects during spermatogenesis (Fig. 10.2a). During meiosis, chromosome segregation is defective in a significant number of spermatocytes so that anucleate spermatids are observed. Among the spermatocytes that manage to divide and form spermatids with nuclei, these activate to become defective spermatozoa where the pseudopods do not move properly. The movement defects shown by pseudopods are associated with defects in the MSP-based cytoskeleton, suggesting that *gsp-3*/*gsp-4* play roles in modulating MSP assembly/disassembly, perhaps analogous to what occurs in *Ascaris* spermatozoa, which employs a PP2A homolog for this purpose [49]. Currently, the only known kinase that affects the MSP cytoskeleton is SPE-6, suggesting that it might be responsible for some or, perhaps, all of the protein phosphorylation that is removed by GSP-3/GSP-4 [40].

Putative Proteases that Function During *C. elegans* Spermatogenesis

SPE-4

The *spe-4* gene encodes what is likely an eight-pass integral membrane protein [50] that is homologous to the presenilins (PS1 and PS2), which are implicated in early-onset Alzheimer's disease (AD) [51, 52]. The presenilins are the catalytic subunit of an aspartyl protease known as γ secretase [53]. This unusual protease catalyzes the intramembranous cleavage of certain type I transmembrane (TM) proteins, including the β amyloid precursor protein (APP) and Notch/LIN-12. This proteolytic activity of γ secretase generates fragments of its substrate TM proteins that have biological activity and cause specific defects when absent or altered. For instance, loss of PS1 function in mice results in severe developmental defects, including defects in the nervous system where Notch signaling is required to generate a normal distribution of neurons; neonatal death is the result [54]. A large number of missense mutations in human PS1 or a smaller number of PS2 mutations, which all appear to be partial loss of function [55], alter its ability to cleave APP and the form produced has greatly enhanced ability to accumulate in neuronal plaques, which are a hallmark of AD [56].

SPE-4 is the most distant homolog of the presenilins encoded by *C. elegans*, with the other two homologs, SEL-12 [57] and HOP-1 [58], performing all somatic presenilin functions, including regulation of the Notch ortholog LIN-12.

The *spe-4* gene is expressed only during spermatogenesis and loss of function *spe-4* mutant hermaphrodites show complete penetrance for a self-sterile phenotype due to spermatogenesis failure [50]. The *spe-4* null mutants are not able to make spermatids but, instead, accumulate terminal spermatocytes where one cell includes all four haploid nuclei (Fig. 10.2b). These defective terminal spermatocytes also contain disrupted FB–MOs. Localization studies with specific antibodies indicated that SPE-4 resides in FB–MOs, suggesting that SPE-4 functions by interacting with one or more FB–MO resident TM proteins [59].

Overall, the homology between SPE-4 and PS1 is low, but a number of critical residues, including the aspartate residues implicated in proteolysis, are conserved [60]. Much insight into both wild type and mutant PS1 has been gained by studies using expression constructs in cultured mammalian cells. One of these studies included creation of a chimeric construct that had the region surrounding and including the protease catalytic site from SPE-4 embedded within the amino acid sequence of PS1 protein [61]. This and wild type PS1 were expressed in cultured cells and tested for their ability to digest known PS1 TM substrates. While wild type PS1 is capable of specific, intramembranous cleavage of both APP and Notch, the PS1/SPE-4 chimeric molecule was only able to cleave APP. While this result is intriguing, the in vivo relevance of SPE-4 is unknown because its substrates have not been identified.

While initially implicated in MO morphogenesis, SPE-4 has also been shown to participate in spermatid activation (Fig. 10.2a). Mutant hermaphrodites that are homozygous for *spe-4*(*hc196*) have a temperature sensitive phenotype, exhibiting a self-brood size of ~85 when grown at 15° but only ~17 progeny when grown at 25°; for reference, the wild type controls showed, respectively, self-broods of ~304 and ~191 at these two growth temperatures [62]. So, this mutation profoundly affects self-fertility, but it is a partial loss of function because, under equivalent conditions, any of several different null *spe-4* mutants exhibit zero self-fertility [15, 50]. The *spe-4*(*hc196*) mutant was identified based on its ability to restore modest self-fertility to *spe-27* loss of function mutants; non suppressed *spe-27* mutants are self-sterile and *spe-27 spe-4*(*hc196*) double mutants produce ~9 self progeny [62]. The *spe-4*(*hc196*) mutation can also suppress the self-sterile phenotype of *spe-8* class mutants (*spe-8*, *spe-19* and *spe-29* have all been examined). The *spe-4*(*hc196*) missense mutation affects a membrane spanning region in SPE-4 and, while it is unclear if this reduces or alters SPE-4 aspartate protease activity, this seems to be the most plausible explanation. If so, this suggests that the wild type role of SPE-4 is to inhibit sperm maturation and reducing/altering SPE-4 activity permits some spermatozoa to complete maturation independent of the *spe-8* pathway.

TRY-5

The route to showing how proteases are involved in spermatid activation was indirect. As described above, mutants of the *spe-8* class can produce spermatids in both hermaphrodites and males [15, 41–45], but they have different sex-dependent fates in vivo: those produced by hermaphrodites fail to become spermatozoa but those

produced by males become spermatozoa when ejaculated during mating. This indicates that there must be a distinct pathway for spermatid activation that is restricted to males. The first insight into how male-derived spermatids activate into spermatozoa came from recovering the *swm-1* mutant [63]. Unlike wild type males that accumulate spermatids in their seminal vesicle, seminal vesicles in *swm-1* mutants are full of spermatozoa. One consequence of having a seminal vesicle full of spermatozoa is that these sticky cells clog the male ductwork so they cannot be properly ejaculated, and *swm-1* males cannot sire progeny. The *swm-1* gene encodes a protein with high similarity to serine protease inhibitors, which suggests that a protease capable of activating spermatids must be inhibited by SWM-1.

The cross sterility of *swm-1* mutant males offered a way to discover the protease that interacted with SWM-1 through suppressor genetics [64]. The *swm-1* mutants were mutagenized and screened for males that could sire cross progeny. These *swm-1* suppressed strains had, in addition to the original mutation in *swm-1*, a mutation in a second gene that prevents the premature activation of spermatids into spermatozoa. Subsequent analysis revealed that the suppressed line accumulated spermatids, not spermatozoa, because it lacked TRY-5 protease-induced spermatid activation. The *swm-1 try-5* double mutant allowed males to successfully mate because the male ductwork was not clogged with sticky spermatozoa, so spermatids could be ejaculated. Once these male-derived *swm-1 try-5* double mutant spermatids were within the hermaphrodite uterus, they were exposed to the hermaphrodite-derived spermatid activator. Consequently, they activated into spermatozoa because, as far as is known, the hermaphrodite-specific and male-specific spermatid activator pathways are distinct and do not share components.

The TRY-5 protease likely acts by the proteolytic digestion of spermatid surface proteins. Although it is a serine protease like one of the proteases found in Pronase (which is a mixture of proteases), TRY-5 affects its substrate(s) on the spermatid surface in a manner that results in fertilization-competent spermatozoa (Fig. 10.2a) [64], whereas Pronase activity triggers formation of abnormal spermatozoa. Consistent with this interpretation, spermatozoa that form after Pronase activation of spermatids are not able to fertilize oocytes in artificial insemination assays [34]. It will be very interesting to define the critical surface proteins found on spermatids that, when proteolytically digested by TRY-5, promote the transition of spermatids into fertilization-competent spermatozoa.

A Role for the Ubiquitin System During *C. elegans* Spermatogenesis

UBI-1

UBI-1, which is part of the ubiquitin-dependent pathway for posttranslational protein modification, has been shown to affect *C. elegans* sperm function. Ubiquitin conjugation occurs widely throughout most cell types [65], including during mammalian spermatogenesis [66], and is essential for viability. Ubiquitin is a small protein that is catalytically attached to a target protein by a peptide bond, posttranslationally.

A series of enzymes participates in ubiquitin-substrate conjugation, usually onto the ε amino side chain of lysine residues of a target protein, and a frequent fate of such a protein is degradation via the 26S proteasome. Alternatively, ubiquitin attachment can change the physiological role played by the modified protein, so ubiquitin conjugation can be a regulatory step. The enzymology has been characterized in great detail and is highly conserved. Briefly, the E1 enzyme is conjugated to ubiquitin, it catalytically transfers its ubiquitin to an E2, then the E2 transfers ubiquitin to an E3 enzyme responsible for substrate specificity of ubiquitination and, finally, the E3 transfers ubiquitin to the protein that is to be regulated or degraded [65].

Like most organisms, the *C. elegans* genome encodes a single E1 (named *ubi-1*) that is essential for viability, and the *ubi-1*(*ok1374*) null mutation causes recessive embryonic or larval lethality [67]. However, *ubi-1*(*it129*), initially known as *spe-32*, is a temperature sensitive mutation that can be conditionally inactivated by manipulating the growth temperature of worms [67]. If *ubi-1*(*it129*) worms are grown at 15° and shifted to 25° during the third larval stage, the resulting hermaphrodites are self-sterile. In wild type, the numerous spermatozoa located in the spermatheca, where fertilization occurs, compete with each other to fertilize oocytes that enter one at a time, as described above. Many spermatozoa that lost this competition are pushed out of the spermatheca and into the uterus, but they crawl back into the spermatheca where they try again to fertilize an oocyte. In *ubi-1*(*it129*), self-sterility occurs because mutant spermatozoa are unable to maintain their position in the spermatheca so that large numbers are observed in the uterus. When examined by light microscopy, this effect is subtle because spermatozoa appear to have cytology and motility that is superficially like wild type. Overall, it is known that *ubi-1*(*it129*), which has a mutation that is located near the probable enzyme active site, significantly reduces the amount of ubiquitin-conjugated proteins found in whole worms. Consequently, the sperm defect seen in *ubi-1*(*it129*) mutants most likely involves reduced or absent ubiquitin transfer to one or more sperm proteins required for proper sperm function (Fig. 10.2a). The *ubi-1*(*ok1374*) is a likely null mutant, but it causes recessive lethality so spermatogenesis cannot be directly studied in this mutant. However, the spermatogenesis phenotype can be studied in the *ubi-1*(*it129*)/(*ok1374*) *trans* heterozygotes and it is enhanced because effects on sperm proliferation are evident, in addition to the defects observed in spermatozoa. At this time, the critical sperm protein(s) that shows altered ubiquitin conjugation in *ubi-1*(*it129*) mutants have not yet been identified.

Palmitoyl Transferase Activity During *C. elegans* Spermatogenesis

SPE-10

The *spe-10* gene encodes a DHHC cysteine rich domain (CRD) protein [68]. Earlier work in the yeast *Saccharomyces cerevisiae* showed that the DHHC–CRD domain in *ERF2* is the catalytic site of a protein acyl transferase that catalyzes the reversible S-palmitoylation of RAS [69]. This *ERF2*-catalyzed protein lipidation regulates

RAS association with the plasma membrane. Later, it became clear that DHHC–CRD proteins are widespread, and eukaryotic genomes encode multiple proteins with this domain, ranging from five in *Schizosaccharomyces pombe* to 23 in humans [70]. *C. elegans* has 15 genes that encode DHHC–CRD proteins, including SPE-10 (see www.wormbase.org).

C. elegans spe-10 mutants have severe abnormalities in FB–MO morphogenesis that first become evident in primary spermatocytes (Fig. 10.2a) [71]. In wild type, all FBs are intimately associated with MOs in spermatocytes with a double-layered membrane from the MO extending around and mostly enveloping the FB (as in Fig. 10.1b1). This enveloping membrane is retracted from around the FB as spermatids are formed, and loss of this association is correlated with disassembly of FB MSP fiber polymers into individual, soluble MSP monomers. In contrast, *spe-10* mutant spermatocytes contain many intact FBs that are not enveloped by membranes derived from the MO. Instead, they are distinct structures in the spermatocyte cytoplasm. In some cases, *spe-10* spermatocytes manage to form a residual body from which spermatids bud. Unlike wild type, many of the MOs that partition into spermatids do not have associated FBs, which end up in the residual body. If the FBs in the residual body (RB) get close to the plasma membrane, they will be enveloped by it and form cytoplasts released from the RB. The MOs found in *spe-10* spermatids are scattered in the cytoplasm instead of localizing just below the plasma membrane, as is the case in wild type spermatids (see Fig. 10.1a4). Furthermore, *spe-10* mutant spermatids are more than 25 % smaller than wild type spermatids (Fig. 10.2a). During spermiogenesis, many *spe-10* mutant spermatids fail to become spermatozoa and the ones that do extend abnormally short pseudopods. Unlike wild type, many MOs fail to fuse with the plasma membrane during *spe-10* spermiogenesis.

DHHC–CRD proteins are a large and diverse class that can have 0–11 transmembrane (TM) domains [70]. The SPE-10 protein has four TM domains, placing it within a sub family that has many members throughout the eukaryotes. Currently, there are seven mutant alleles of *spe-10* that include the *spe-10*(*ok1149*) deletion mutation. The *spe-10*(*ok1149*) must be a null mutant because it deletes the promoter and the majority of the SPE-10 coding sequence, including the entire DHHC–CRD region. Surprisingly, *spe-10*(*ok1149*) mutant hermaphrodites can produce a few self-progeny when propagated at 16° growth temperature but are completely self-sterile when grown at 25°. This suggests that a few fertilization-competent spermatozoa must be produced when this mutant is grown at 16°. The *spe-10*(*eb64*) missense mutant encodes a tyrosine in place of the underlined, bold histidine (**H**) in the consensus DHHC_CRD, C-x_2-Cx_9-**H**-C-x_2-C-x_2-C-x_4-D-H-H-C-x_5-C; this H is invariant in over 300 DHHC–CRD proteins. The *spe-10*(*eb64*) mutant hermaphrodite self-fertility and mutant sperm cytology are not significantly different from *spe-10*(*ok1149*) mutants. These data indicate that loss of this invariant H produces a protein that completely lacks SPE-10 function [68].

The SPE-10 protein has been localized by immunofluorescence; it resides in FB–MOs and is completely missing from *spe-10*(*ok1149*) mutants, as expected. The DHHC–CRD domain is predicted to face the FB–MO interior (see Fig. 10.1b), indicating that SPE-10 catalyzes palmitoylation of one or more proteins that reside in this membrane-bound compartment [68]. Currently, it is not possible to take an

informatics-based approach to identify candidate substrates for SPE-10 because there is no defined consensus site for the S-palmitoylation catalyzed by DHHC–CRD proteins [70].

Introduction to *Ascaris*

Unlike *C. elegans*, *Ascaris suum* is an obligate dioecious species with true males and females. While much of *Ascaris* spermatogenesis prior to spermatid formation has not been examined in detail, it seems probable that it is similar to *C. elegans*. Unlike *C. elegans*, the large size of *Ascaris* allows for straightforward isolation of many spermatids and reproductive tract fluids for biochemical and biophysical studies [5, 72, 73]. Usually, spermatids are studied in vitro because, since *Ascaris* is a parasite, mating experiments are not feasible. Unlike *C. elegans*, *Ascaris* spermatozoa contain randomly scattered electron-dense vacuoles that fuse into a single refringent granule during spermiogenesis [74–76]. The function of these granules is not clear and some speculate that they might be an energy reservoir used by spermatozoa during their migration in the female reproductive tract [76]. During ejaculation, *Ascaris* spermatids pass through the vas deferens where they are mixed with a secreted, trypsin-like serine protease TRY-5 that mediates spermatid activation (spermiogenesis) [77], as is the case for *C. elegans* (see above and [64]). As for *C. elegans* spermatozoa (see above), MOs in *Ascaris* spermatozoa fuse with the plasma membrane during spermiogenesis and exocytose their contents into the surrounding extracellular space, mediating both spermatid activation and sperm competition. Once spermatids have been activated after ejaculation, their motility is necessary if they are to be successful at fertilizing an oocyte.

Generally, eukaryotic cells employ actin to form the cytoskeletal structures utilized for amoeboid motility [78, 79]. In contrast, lamellipodial extension and amoeboid motility in nematode spermatozoa occurs in the absence of actin filaments and, instead, is powered by the dynamics of a cytoskeleton composed of Major Sperm Protein (MSP) [2]. Like actin-based cell migration, MSP-based cell crawling by nematode spermatozoa involves extension of the leading edge, adhesion to the substratum, and retraction of the cell body. MSP polymerization has been reconstituted in vitro [80], and this has provided a simplified model for the study of amoeboid cell motility. Several of the accessory components that modulate the MSP-based cell motility by protein phosphorylation and dephosphorylation have been identified and characterized.

Major Sperm Protein (MSP): The Actin Substitute in the Nematode Spermatozoa

Ascaris spermatozoa, either activated in vitro or dissected from the female reproductive tract, display a pseudopod that is, essentially, a flattened lamellipodium (a broad, sheet-like structure) with a trailing spherical cell body. The lamellipodium is packed with MSP filaments that assemble at the leading edge and remain stationary with respect to the substrate as the spermatozoon advances.

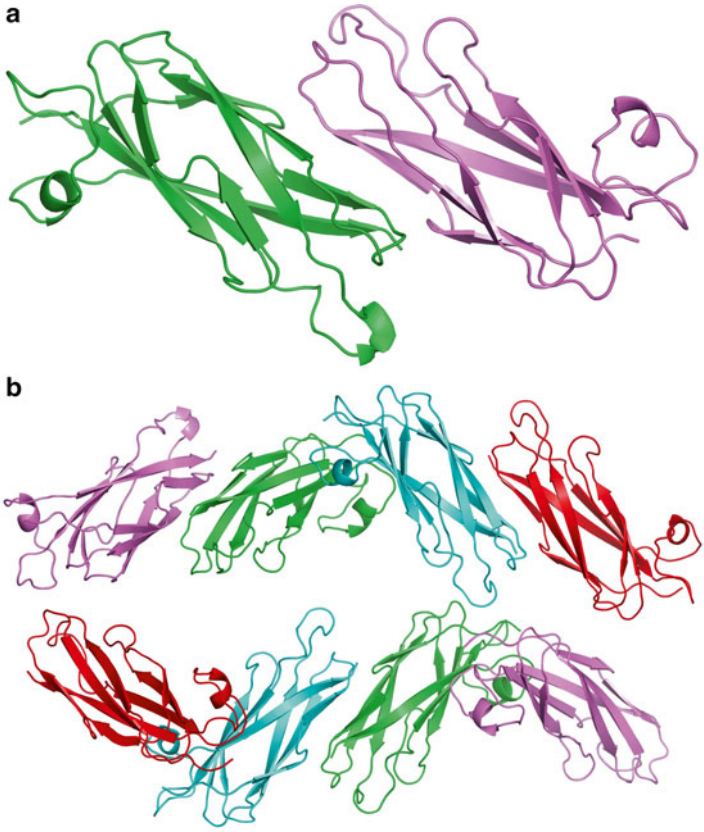

Fig. 10.3 Structure of MSP dimer, subfilament and filament. (**a**) MSP is a symmetrical dimer in which each polypeptide chain (shown as *green* and *violet*) has an immunoglobulin-like fold based on a seven-stranded β sandwich. Two strands contain *cis*-proline residues that impart distinctive kinks [82]. (**b**) The MSP dimers polymerize into helical subfilaments that wind together in pairs to form higher-order filaments. The twofold rotation axis of each dimer is oriented perpendicular to the helix axis [83, 110], resulting in the filaments having no overall polarity. Images are generated using PyMOL (DeLano, W.L. The PyMOL Molecular Graphics System, http://www.pymol.org) from PDB accession numbers 1MSP and 2MSP, respectively

The MSP is a nematode specific small protein that is composed of 126 amino acids and has a molecular weight of 14 kDa [2, 81]. It is the most abundant protein present in nematode spermatozoa, comprising ~15 % of the total protein and more than 40 % of the soluble protein. MSP structure and interactions have been determined by X-ray crystallography, electron microscopy [82, 83], NMR spectroscopy in solution [84], the yeast two-hybrid system and biochemical analyses of bacterially expressed wild type and mutant proteins [85]. Taken together, these data suggest that MSP structure is highly similar between *C. elegans* and *Ascaris*, consistent with the fact that they share 83 % sequence identity. The overall structure of monomeric MSP (Fig. 10.3a) resembles an immunoglobulin fold, which is completely different from the structure of actin [82, 86]. The subunit for MSP polymerization

is a structurally symmetric dimer, rather than the monomeric G actin that polymerizes into actin microfilaments. The constructed subfilaments and filaments also lack obvious structural polarity [86] (Fig. 10.3b). Therefore, it is unlikely that molecular motor proteins, which depend on the polarity of actin microfilaments or microtubules for function, are involved in the MSP-based cell motility [87].

Spermatid Activation

Spermatids in the seminal vesicles of *Ascaris*, like those in *C. elegans*, are round, sessile cells that lack both a flagellum or an acrosome [88]. The role of the glandular vas deferens, through which spermatids must pass during copulation, in spermatid activation was first demonstrated by injecting crude gland homogenates directly into the seminal vesicle [89]. Adding the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) to a vas deferens extract inhibited its ability to cause spermatid activation. Spermatid activation can also be triggered by trypsin treatment in vitro, implying that a serine protease secreted from the glandular vas deferens is essential for this process [90, 91].

As in *C. elegans* (see above), the protease that causes in vivo spermatid activation was identified by an indirect approach. The monoclonal antibody 1CB4 was first defined as a marker that localized to MOs in *C. elegans* sperm [59, 92], and was subsequently shown to also recognize MOs in *Ascaris* spermatozoa by immunostaining. Biochemical analysis showed that 1CB4 recognizes a serine protease inhibitor (serpin) in *Ascaris* [77]. During spermatid activation, serpin was exocytosed into the extracellular medium to modulate the activity of a serine protease from the glandular vas deferens. This modulation occurs by a well-defined suicide substrate mechanism, whereby serpin is cleaved by the serine protease at the reactive site loop and the cleaved serpin forms a covalently linked complex with the protease. Analysis of the purified complex allowed identifying the protease-derived protein fragment as the trypsin-like serine protease TRY-5 [77]. While the substrate for TRY-5 is currently unknown, it seems plausible that it might cleave a channel protein such as the epithelial sodium channel (ENaC) [93] or a Protease-Activated Receptor (PAR) [94] to signal the downstream cascade required for sperm activation. From analogous work in *C. elegans* (see above), precocious sperm activation in the *Ascaris* seminal vesicle would likely causes male fertility defects because activated spermatozoa are sticky and not easily transferred to the female reproductive track during copulation [63, 64]. Thus, there is a negative feedback loop between a protease secreted by vas deferens and the protease inhibitor released from spermatids during activation [95].

Sperm Activation: From Filopodium to Lamellipodium

Activating spermatids extend several filopodia-like spikes from the cell surface, like "molecular antennae" exploring their local environment. Spikes are highly dynamic and transient structures. Some spikes retract quickly back to the cell surface while

others thicken into blebs. Blebs can also be formed directly from the cell surface. Like spikes, blebs are highly dynamic and can either protrude, retract or move over the cell surface. Several dynamic blebs at one side of the cell will coalesce and mark the position of lamellipodial extension. Once a lamellipod extends, all spikes and blebs remaining on the surface are retracted and the lamellipod will dominate cell surface protrusion, reaching its full length within a minute [96]. This contrasts with what happens during actin-mediated cell motility, where filopodia extend from the leading edge of the lamellipod of migrating cells [97]. The coordination of leading edge protrusion with cell body retraction, coupled with modulation of adhesion to the substrate, generate *Ascaris* sperm cell locomotion.

The MSP-based protrusion and retraction was reconstituted in cell-free extracts of *Ascaris* spermatozoa [80, 98]. The addition of ATP to the sperm extract triggers the assembly of columnar MSP fibers. Each fiber is a meshwork of MSP filaments behind a vesicle, which is derived from plasma membrane that was at the leading edge of the advancing cell. The fiber elongates by assembling filaments at the vesicle that then pushes the vesicle forward; this resembles how MSP cytoskeletal dynamics drives lamellipod leading edge protrusion in intact spermatozoa. This cell-free reconstitution system has been utilized in biochemical experiments to identify several key components of the MSP-based amoeboid locomotion [98, 99].

Protein Phosphorylation/Dephosphorylation Modulates MSP-Based Cell Motility

As mentioned above, fibers of the MSP motile apparatus require ATP or they will not elongate in vitro. Since molecular motors were not thought to be likely participants in MSP based motility, this suggested that the prerequisite ATP was likely used for posttranslational protein modification by phosphorylation [80]. The MSP fibers interact with and are dynamically controlled by a number of interacting proteins (Fig. 10.4). Fractionation studies indicated that fiber elongation required a membrane-associated factor and a soluble, cytoplasmic factor that was not MSP itself [100]. The only membrane protein required to nucleate MSP assembly, the MSP Polymerization Organizing Protein (MPOP), is a tyrosine-phosphorylated 48 kDa integral membrane protein localized at the leading edge of the sperm lamellipod [101]. Phosphorylation of MPOP is pH-sensitive and seems to be catalyzed by a yet to be identified cytosolic tyrosine kinase; the protein sequence of MPOP is currently unknown. Immuno-staining of membrane-intact spermatozoa with an anti-phosphotyrosine (pY) antibody showed that tyrosine phosphorylation of a sperm surface protein occurs during spermatid activation, especially at the protrusion site of the leading edge [77]. This extracellular tyrosine phosphorylation might be induced by cytoplasmic phosphorylation that, perhaps, is autophosphorylation, similar to what occurs on receptor tyrosine kinases [102]. Phospho-MPOP recruits a soluble, 34 kDa serine/threonine kinase, which shares sequence homology with the casein kinase 1 family SPE-6 from *C. elegans* (see above), to the cytoplasmic surface of the plasma membrane [103]. This kinase has been named MSP

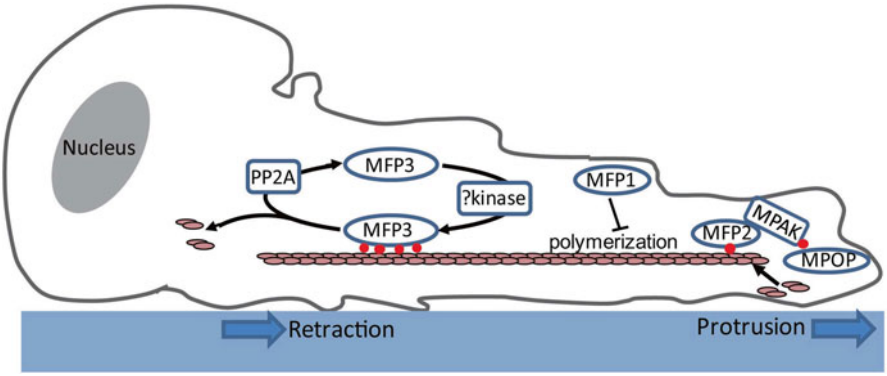

Fig. 10.4 Nematode sperm migration involves differential regulation of protein phosphorylation/dephosphorylation at the leading edge during protrusion and at the cell body during retraction. The MSP-based sperm motility is tightly coupled to these processes. At the leading edge, tyrosine phosphorylated MPOP recruits MPAK, which in turn phosphorylates MFP2. Phospho-MFP2 promotes MSP assembly, generating protrusive force. The MFP1 inhibits MSP polymerization. The cytosolic MFP3 is phosphorylated by an as yet unknown kinase, and then binds to and stabilizes MSP filaments. At the cell body-pseudopod junction, PP2A becomes activated by a phosphatase and dephosphorylates phospho-MFP3, resulting in the release of MFP3 from MSP fibers. Ultimately, MSP fibers disassemble, generating a retraction force to pull the cell body forward. Reproduced from Protein Cell. 2012;3:755–61. doi: 10.1007/s13238-012-2936-2 by copyright permission of Higher Education Press

Polymerization-Activating Kinase (MPAK) because it catalyzes phosphorylation of MSP Fiber Protein 2 (MFP2). This phosphorylation regulates MSP polymerization because, when it occurs, the rate of fiber growth is accelerated [104, 105]. The MPAK, MFP2 and MPOP are all also involved in the extension of filopodia or blebs during spermatid activation [96, 99]. In contrast, MFP1, another component that is enriched in fiber complexes, is not required for membrane-associated MSP polymerization and, instead, is a negative regulator of fiber assembly. The MFP1 might be an MSP fiber capping protein; its role would be analogous to that of an actin capping protein that blocks polymerization by binding to the end of the growing F-actin filament [104].

The motile machinery of amoeboid nematode spermatozoa is confined to the pseudopod, which is sharply separated from the cell body (Fig. 10.4). This means that there must be a mechanism to retract MSP fibers at the cell body/pseudopod border, as fibers do not extend into the cell body. This has been examined in vitro and retraction of fibers occurs when they are treated with sperm extract to which *Yersinia enterocolytica* tyrosine phosphatase (YOP) has been added. Treating this preparation with sodium orthovanadate, which is a potent and broad-spectrum tyrosine phosphatase inhibitor, allows fiber assembly to resume. However, YOP activity on MSP fibers requires crude sperm extract, which indicates that the protein tyrosine phosphorylation/dephosphorylation of sperm extract components functions as an on/off switch for fiber retraction [98, 99]. The YOP plays its regulatory role by dephosphorylating the Ser/Thr phosphatase PP2A, which is necessary and sufficient

for fiber retraction in the absence of any other sperm extract components. In turn, PP2A acts on MSP fiber protein 3 (MFP3), which is a 43 kDa cytosolic threonine-phosphorylated protein [49]. The MFP3 contains 19 tandem repeats of a 15-residue motif (flanked by ~90-amino acid N and C termini), and each repeat has at least one threonine residue that might be phosphorylated. The phosphorylated form of MFP3 binds to and seems to stabilize MSP filaments. Dephosphorylation of MFP3 by PP2A results in its release from the filament, causing depolymerization of MSP fibers [49] (Fig. 10.4).

Pharmacological experiments in *C. elegans* suggested that Ca^{2+} homeostasis played a role in sperm pseudopod dynamics and that this might be a significant second messenger, both in initiating and regulating these dynamics. This relationship was first revealed when the calmodulin (CaM) inhibitors trifluoperazine (TFP), chlorpromazine (CPZ) and W7 were shown to stimulate pseudopod extension in *C. elegans* [45]. More recently, TFP and CPZ were shown to also initiate spermatid activation in *Ascaris*, resulting in pseudopod extension. A CaM inhibitor might induce pseudopod extension via decreasing the phosphatase activity of calcineurin, a Ca^{2+}/CaM-dependent serine/threonine phosphatase [106]. Indeed, adding Ca^{2+} at physiological concentrations to the in vitro reconstitution system (described above) inhibits MSP fiber assembly significantly. Perfusing the assembled fibers with a cell-free sperm extract that contains Ca^{2+} promotes MSP fiber disassembly and retraction, mimicking what occurs when fibers are perfused with sperm extract to which YOP was added; this provides a link between the second messenger Ca^{2+} and MSP dynamics [106].

Much remains to be done in order to develop a fully integrated view of how MSP fibers and their associated phosphorylations are dynamically reorganized during sperm crawling. Some of the proteins implicated in *Ascaris* sperm motility do not yet have clear homologs encoded by the *C. elegans* genome and two phosphatase paralogs involved in *C. elegans* sperm motility have not yet been found to be involved in *Ascaris* sperm motility [40]. Perhaps this reflects the differing experimental approaches used in these systems and the rapid evolution of orthologous components. While it does not seem feasible to develop *Ascaris* as a system for developmental genetics, the in vitro techniques developed for *Ascaris* MSP filament assembly and motility offers a guide to create a similar system for *C. elegans*. The *Ascaris* in vitro system continues to offer a robust way to define additional components required for sperm motility. For instance, simultaneous protrusion and retraction in a single fiber can be reconstituted by merely lowering the ratio of added ATP to sperm extract [107]. This suggests that localized regulation of ATP concentration may contribute to MSP fiber dynamics. It will be interesting to determine how ATP is transported into and regulated within the pseudopod, where there are no mitochondria or other identified membrane bound organelles. Additionally, crawling motility is not a random phenomenon as *C. elegans* spermatozoa are strongly attracted by F-series prostaglandins synthesized and released from oocytes [108]. It seems probable that an analogous process occurs in *Ascaris*, which means that the posttranslational regulatory cascade within the pseudopod we describe above is somehow harnessed to allow sperm chemotaxis towards the oocyte in vivo.

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