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# Ubiquitin–Proteasome System in Spermatogenesis

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## 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.

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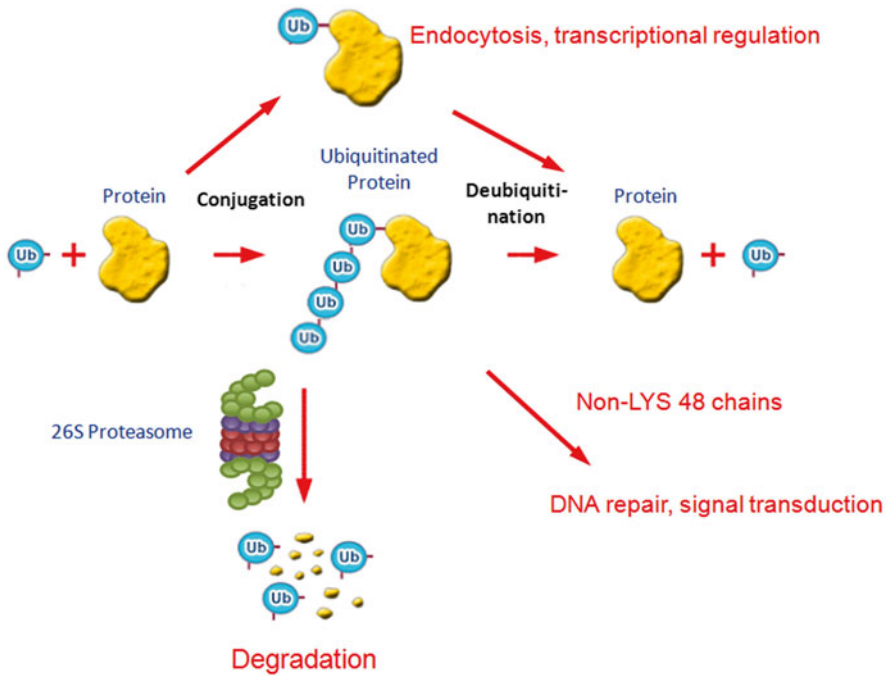
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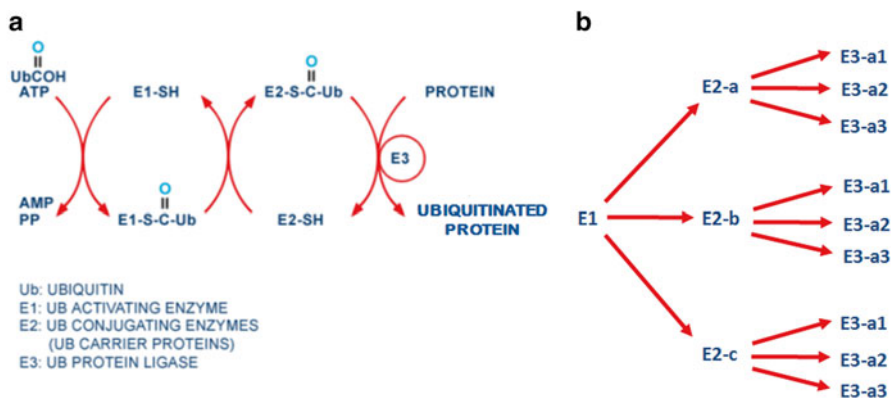
**General Features and Functions  
of the Ubiquitin–Proteasome System (UPS)**

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  $\epsilon$ -amino group of the side chain of a lysine residue on the protein substrate. Rarely, the ubiquitin is attached to the  $\alpha$ -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  $\alpha$ 1–7 and the two inner rings consist of subunits  $\beta$ 1–7. 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. PA28 $\alpha/\beta$  is a heptameric complex induced by interferon, which can bind to the ends of the core particle and is involved in adaptive immunity [10]. PA28 $\gamma$  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 I $\kappa$ B $\alpha$  [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

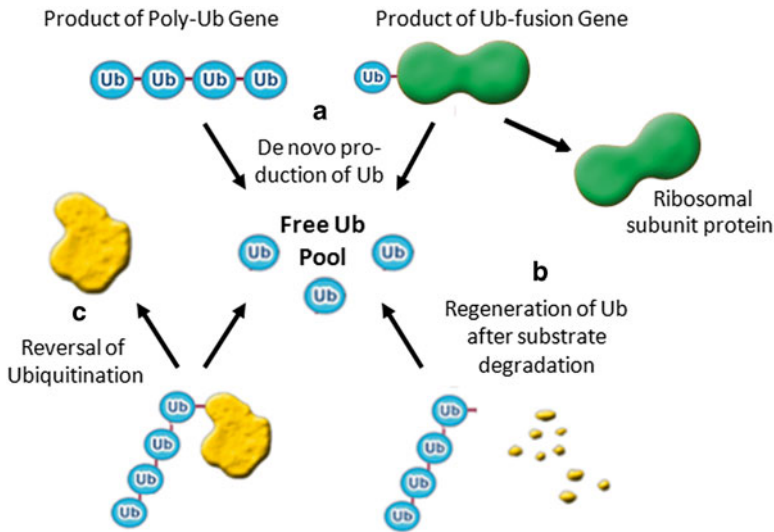


**Fig. 9.2** Mechanism of action of enzymes involved in conjugating ubiquitin (Ub) to proteins. (a) Ub is first activated by Ub activating enzyme (E1) in a reaction that involves ATP hydrolysis and results in the formation of a high energy thiolester linkage between Ub and the active site cysteine residue. 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 TNF $\alpha$  or IL-1, in which the ubiquitination of RIP1 or TRAF6 respectively results in the activation of NF $\kappa$ B (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 (*Really Interesting New Gene*) and HECT (*Homologous to E6-AP Carboxy-Terminus*—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  $17\beta$ -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  $\beta$ TrCP ubiquitin ligase is a well characterized ubiquitin ligase that plays important roles in regulating cell cycle and apoptosis. There are two isoforms of the  $\beta$ TrCP substrate recognition subunit,  $\beta$ TrCP1 and  $\beta$ TrCP2, both of which are expressed in spermatogonia. Gene inactivation of  $\beta$ TrCP1 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  $\beta$ TrCP substrate Snail1, a transcription factor that induces E-cadherin expression, as silencing Snail1 in the  $\beta$ TrCP 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.

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## 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  $\gamma$ H2AX, a histone modification normally associated with the transcriptionally silenced XY body in mid-pachytene spermatocytes. This absence of  $\gamma$ H2AX 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*<sup>-/-</sup> 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  $\gamma$ -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 multi-subunit 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  $\beta$ -TrCP ligase is also involved in meiosis. The  $\beta$ -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  $\beta$ -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 indispensable 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  $\alpha 3T$ ,  $\alpha 6T$  and Rpt3R subunits revealed that their expression is induced at different stages, but they are prominent in spermatids [110]. The functional significance of  $\alpha 6T$  and Rpt3R in spermiogenesis is evident from their inactivation, which results in a male sterile phenotype [108, 110]. Knocking out  $\alpha 6T$  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.IB* 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  $\alpha$  and  $\beta$  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<sup>l</sup>*) 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 transgenically 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- $\kappa$ B activator RIP1. Loss of CYLD leads to constitutive activation of NF- $\kappa$ B 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	<i>H. sapiens</i>	[146]
	UBA1/UBE1	Unknown	Testis-specific, abundant in spermatogonia and round spermatids	Undefined	Mammals	[147, 148]
E2	UBA6/UBE1L2/E1-L2	Unknown	High expression in the testis, gonocytes	Undefined	Mammals	[34, 36]
	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	<i>R. norvegicus</i>	[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 <i>Drosophila</i>	<i>R. norvegicus</i> <i>D. melanogaster</i>	[34, 100, 101, 154]
	HUWE1/LASU1/E3histone, ARF-BP1	Histones (?)	Gonocytes, spermatogonia, spermatocytes	Undefined	<i>R. norvegicus</i>	[38, 39]
HERC4	HERC4	Unknown	Spermatogonia, spermatocytes, spermatids	Subfertility, reduced sperm motility, angulated sperm tail, retention of cytoplasmic droplet on the sperm tail	<i>M. musculus</i>	[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	<i>M. musculus</i>	[122]

UBR2	Histones H2A and H2B	High expression in the testis	Infertility, arrest of spermatocytes at prophase I of meiosis and subsequent apoptosis	<i>M. musculus</i>	[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	<i>M. musculus</i>	[155, 156]
RFP	Unknown	High expression in male germ cells particularly in primary spermatocytes and round spermatids	Undefined	<i>M. musculus</i>	[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	<i>C. elegans</i>	[96]
CUL4A	Histones H3, H4	Gonocytes, spermatogonia, spermatocytes, low in spermatids	Infertility, increased apoptosis of primary spermatocytes, decreased sperm number, motility, defective acrosome formation	<i>M. musculus</i>	[97, 158]
SHAH1A	Unknown	High expression in the testis	Infertility, accumulation of spermatocytes at metaphase to telophase transition of meiosis I and subsequent apoptosis	<i>M. musculus</i>	[98, 159]
Parkin	Unknown	Undefined	Sterility, defects in mitochondrial morphogenesis during spermiogenesis leading to defective spermatid individualization	<i>D. melanogaster</i>	[160, 161]
E6-AP	Unknown	Undefined	Subfertility, reduced testis size and sperm count	<i>M. musculus</i>	[162]
MARCH7	Unknown	High expression in the testis, high expression in round, elongating and elongated spermatids	Undefined	<i>R. norvegicus</i>	[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	<i>R. norvegicus</i>	[129]
	MARCH11	Unknown	Developing spermatids	Undefined	<i>R. norvegicus</i>	[138]
	ZNF645	Unknown	Spermatocytes, round and elongated spermatids, Leydig cells	Undefined	<i>H. sapiens</i>	[131]
	ITCH	Ooccludin (a major component of tight junctions)	Testis, Sertoli cells	Subfertility, age dependent impairment in spermatogenesis with increased apoptosis, delayed spermatid development and organization	<i>M. musculus</i>	[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	<i>M. musculus</i>	[68]
	RNF4	MDC1 and BRCA1 (DNA repair proteins)	Undefined	Infertility, age-dependent testicular atrophy, depletion of germ cells	<i>M. musculus</i>	[67]
	RNF8	Histone H2A and H2AX	Undefined	Infertility, reduction of histone ubiquitination in the testes defects in global nucleosome removal during spermiogenesis	<i>M. musculus</i>	[76, 82, 83, 165]
	RNF19A	Unknown	Pachytene spermatocytes, round and elongating spermatids, mature sperm	Undefined	<i>R. norvegicus</i>	[137]
	RNF133	Unknown	Round and elongating spermatids	Undefined	<i>M. musculus</i>	[166]
	RNF149	Unknown	Gonocytes	Undefined	<i>R. norvegicus</i>	[34]
	RNF151	Dysbindin (?) (involved in membrane biogenesis and fusion)	Round and elongating spermatids	Undefined	<i>M. musculus</i>	[167]

RNF168	Unknown	Undefined	Age-dependent impairment of spermatogenesis leading to subfertility or infertility at 12 months age, testicular atrophy	<i>M. musculus</i>	[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	<i>M. musculus</i>	[42, 99]
APC/C	MIWI (murine homologs of PIWI)	Spermatocytes, round and late spermatids	Defects in spermatid maturation	<i>M. musculus</i>	[169]
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	<i>H. sapiens</i>	[175-178]
USP14	Unknown	Round and elongating spermatids and mature sperm	Spermatid differentiation	<i>M. musculus</i>	[128]
USP26	Unknown	Testis specific expression	Polymorphisms linked to azoospermia, oligozoospermia, asthenozoospermia	<i>H. sapiens</i>	[179-184]
USP42	Unknown	High in round spermatids, low in condensing spermatids	Undefined	<i>M. musculus</i>	[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	<i>M. musculus</i>	[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	<i>M. musculus</i>	[187, 188]
	UCH-L4	Unknown	Spermatogonia	Undefined	<i>M. musculus</i>	[188, 189]
	UCH-L5	Unknown	Spermatocytes and spermatids	Undefined	<i>M. musculus</i>	[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	<i>M. musculus</i>	[136]
Proteasome	Prosalpha3T	Unknown	Low expression in late phases of second meiotic division, prominent in post-meiotic spermatids	Undefined	<i>D. melanogaster</i>	[109]
	Prosalpha6T	DIAP (?)	Post-meiotic spermatids, mature sperm	Sterility, defects in sperm individualization and nuclear maturation	<i>D. melanogaster</i>	[108, 109]
	PROS28.1A	Unknown	Primary spermatocytes and spermatids	Undefined	<i>D. melanogaster</i>	[107]
	PROS28.1B	Unknown	Elongating spermatids	Undefined	<i>D. melanogaster</i>	[107]
	PA200/PSME4	Acetylated histones	Constitutive	Subfertility, reduced sperm concentration and motility, hypospermatocytogenesis, apoptotic spermatocytes and spermatids with defective nucleosomal removal	<i>M. musculus</i>	[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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