Published in final edited form as:

Semin Cell Dev Biol. 2014 June ; 0: 27–35. doi:10.1016/j.semcdb.2014.03.001.

THE ROLE OF E3 LIGASES IN THE UBIQUITIN-DEPENDENT REGULATION OF SPERMATOGENESIS*

John H. Richburg^{1,3}, Jessica L. Myers¹, and Shawn B. Bratton²

¹Center for Molecular and Cellular Toxicology, College of Pharmacy, The University of Texas at Austin, Austin, Texas

²The University of Texas MD Anderson Cancer Center, Department of Molecular Carcinogenesis, Smithville, Texas

Abstract

The ubiquitination of proteins is a post-translational modification that was first described as a means to target misfolded or unwanted proteins for degradation by the proteasome. It is now appreciated that the ubiquitination of proteins also serves as a mechanism to modify protein function and cellular functions such as protein trafficking, cell signaling, DNA repair, chromatin modifications, cell-cycle progression and cell death. The ubiquitination of proteins occurs through the hierarchal transfer of ubiquitin from an E1 ubiquitin-activating enzyme to an E2 ubiquitin-conjugating enzyme and finally to an E3 ubiquitin ligase that transfers the ubiquitin to its target protein. It is the final E3 ubiquitin ligase that confers the substrate specificity for ubiquitination and is the focus of this review. Spermatogenesis is a complex and highly regulated process by which spermatogonial stem cells undergo mitotic proliferation and expansion of the diploid spermatogonial population, differentiate into spermatocytes and progress through two meiotic divisions to produce haploid spermatids that proceed through a final morphogenesis to generate mature spermatozoa. The ubiquitination of proteins in the cells of the testis occurs in many of the processes required for the progression of mature spermatozoa. Since it is the E3 ubiquitin ligase that recognizes the target protein and provides the specificity and selectivity for ubiquitination, this review highlights known examples of E3 ligases in the testis and the differing roles that they play in maintaining functional spermatogenesis.

Keywords

Ubiquitin; E3 ligase; spermatogenesis; ubiquitination

*Supported, in part, by grants from the National Institutes of Health (ES016591 & ES007784 to J.H.R.; CA129521 & GM096101 to S.B.B) and The University of Texas at Austin's Center for Molecular and Cellular Toxicology.

© 2014 Elsevier Ltd. All rights reserved.

³To whom correspondence should be addressed at The University of Texas at Austin, College of Pharmacy, 107 W Dean Keeton, C0875, Austin, TX 78712 Telephone: (512) 471-4736 Fax: (512) 471-5002. john.richburg@austin.utexas.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. INTRODUCTION

1.1 Ubiquitin Overview

Ubiquitination is a posttranslational modification of proteins that involves the attachment of ubiquitin, a small (8.5 kDa) regulatory protein, to a target protein. Ubiquitin was discovered by Goldstein in 1975 where it was described to be “ubiquitously” expressed in all mammalian cells analyzed as well as in yeast, bacteria and higher plants; hence the designation of ubiquitin for this protein [1]. It wasn’t until 1982 that Ciechanover *et al.* described in detail a role for protein ubiquitination in targeting proteins for degradation in nucleated cells [2]; a process that has become known as the ubiquitin proteasome system (UPS). It is now appreciated that the ubiquitination of proteins can also mediate processes in cells that occur independent of protein degradation. Like other posttranslational modifications, ubiquitination can influence various cellular signaling processes. It is worth noting that whole body tissue analysis of the rat revealed that the testis has the highest rate of ubiquitination, approximately four-fold greater, among all of the organs examined [3]. This finding implies that functional spermatogenesis is particularly dependent on ubiquitin-regulated protein function and stability. Spermatogenesis is the complex process by which spermatogonial stem cells lead to the production of mature sperm in the testis. This process, like many other biological processes, relies heavily on the remodeling of protein complexes and the rapid turnover of proteins in both the developing germ cells and in the supportive Sertoli cells of the seminiferous epithelium. In this review, the role that the ubiquitination of proteins plays in the cellular events responsible for spermatogenesis is described. Since it is the E3 ubiquitin ligase that provides the specificity and selectivity for target protein ubiquitination, this review highlights known examples of E3 ligases in the testis and the differing roles that they play in maintaining functional spermatogenesis.

1.2. The Process of Ubiquitination

The ubiquitin molecule is covalently attached to its target protein substrate through a three-step enzymatic process (reviewed in [4]; diagrammed in Fig. 1A). First, an E1 ubiquitin-activating enzyme hydrolyzes ATP to ADP to activate the ubiquitin molecule, allowing for the ubiquitin molecule to covalently attach to the E1 itself. Second, an E2 conjugating enzyme facilitates the transfer of the ubiquitin molecule from the E1 enzyme to itself. Finally, an E3 ubiquitin ligase covalently links the ubiquitin to its targets the substrate protein by transferring the ubiquitin molecule from the E2. The protein substrate can then either be released for its specific purpose or continue through this process multiple times, a process called polyubiquitination, to add on a series of ubiquitin molecules linked to the first ubiquitin. For proteins that are polyubiquitinated, it is thought that the initial ubiquitin molecule is the signal to add on the additional ubiquitin chain.

The specific E3 ubiquitin ligase involved provides the specificity for the protein substrate to be ubiquitinated. While only a limited number of E1 and E2 ligases have been described, there are hundreds of known E3 ligases, and likely many more yet to be discovered. There are two major classes of E3 ligases that differ in their sequence and the mechanism by which they transfer the ubiquitin molecule: *homologous to E6-AP carboxy terminus* (HECT) [5] and *really interesting new gene* (RING) [6] family members. RING E3 ligases act as

scaffold proteins, bringing the E2 conjugating enzyme and target protein in close proximity and transferring the ubiquitin from one to the other. On the other hand, HECT E3 ligases display catalytic activity and act as intermediates, first transferring the ubiquitin molecule from the E2 to itself, and then transferring it onto the target protein. This allows for the detection of an E3-ligase ubiquitin intermediate that is not observed in RING E3 ligases.

The number of ubiquitin molecules, and the specific amino acid residues of ubiquitin that are linked to the target protein, ultimately determine the influence that ubiquitination has on the target protein (Fig. 1B). For example, ubiquitination can mark the protein for trafficking to different cellular locations, such as the plasma membrane or lysosomes, or target them for degradation by the 26S proteasome. There are seven different lysine residues in ubiquitin that can be used to bind to the target protein and to form ubiquitin chains. The most common and well-studied linkages utilize lysine 48 (K48) and lysine 63 (K63) of ubiquitin, although linkages to the other five lysines of ubiquitin can occur but are much less common [7]. Target proteins can be mono-ubiquitinated or poly-ubiquitinated. For example, a mono- or poly-ubiquitinated chain using K63 usually signals a target protein for transport to a specific cellular localization, while a poly-ubiquitinated chain using K48 is typically used as means of protein expression control and directs the protein to the 26S proteasome for degradation.

The ubiquitination processes described above are also subject to regulation by various extracellular stimuli that induce other posttranscriptional modifications, such as protein phosphorylation and the attachment of *small ubiquitin-like modifiers* (SUMOs), a process referred to as SUMOylation. SUMO is part of a family of ubiquitin-like proteins (UBLs) that are structurally similar to ubiquitin and can interfere with the ubiquitination process. In this way the expression of UBLs can modify the capacity of certain proteins to be ubiquitinated. It is interesting to note that one UBL, SUMO-1, is highly expressed in the testis and appears to be involved in multiple processes necessary for regulating spermatogenesis [8, 9]. Paradoxically, the components of the ubiquitinating process itself are regulated by ubiquitination. These modifications can influence the activity of the E3 ligase itself as well as the ability for the target protein to be receptive to ubiquitination. The reader is referred to recent comprehensive reviews describing these regulatory processes [10, 11]. Finally, a family of enzymes termed DUBs are able to deubiquitinate proteins and alter/fine tune the balance between the ubiquitinated and deubiquitinated state of a protein and its overall influence on the cell. The reader is referred to a recent review describing DUBs and their post-translational regulation [12].

1.2.1. The ubiquitin proteasomal system (UPS)—Prior to 1977, the degradation of cellular proteins was largely thought to occur in a nonspecific manner by proteases within the cellular lysosomes. This thinking began to change with a report by Joseph Etlinger and Alfred Goldberg in 1977 that showed a rapid degradation of an abnormal globin protein in cell-free extracts of reticulocytes, a preparation that lacks lysosomes and other membrane-bound organelles [13]. Furthermore, this proteolysis was shown to be ATP-dependent, an important finding as prior to this study it was thought that ATP was only needed for the transport of the protein substrate into the lysosome and/or for maintaining an acidic environment within the lysosome [14]. Etlinger and Goldberg concluded that a soluble, non-lysosomal proteolytic system was responsible for the energy-dependent degradation of

abnormal proteins in this cell type [13]. The components of this proteolytic system were initially described using the reticulocyte cell model system by Aaron Ciechanover, a graduate student at the Technion-Israel Institute under the direction of Avram Herskho who was performing a year-long sabbatical in the laboratory of Irwin Rose at the Fox Chase Center in Philadelphia. Their initial study revealed that the formation of a heat-stable polypeptide was an important early event for proteolysis of the abnormal globin protein [15]. It wasn't until 1982 that this group described for the first time the role of the ubiquitin proteasome system (UPS) in nucleated cells [2]. This early work on the characterization of the UPS by Ciechanover, Herskho and Rose, and the elegant work that followed defining the components of the UPS, led to their award of the Noble Prize in Chemistry in 2004[†].

The ubiquitin-proteasome pathway (UPS) refers to both the cascade of enzymatic reactions leading to the ubiquitination of proteins (as described in detail in section 1.2.1 above) and the consequent proteolytic degradation of the ubiquitinated protein by the proteasome. Numerous reviews on the UPS have been published since the first comprehensive review in 2000 by Ciechanover [4]. In brief, the mammalian 26S proteasome is a cylinder-shaped protein complex composed of one 20S protein subunit and two 19S regulatory cap subunit. The 20S proteasome of eukaryotes is composed of seven different α -type subunits and seven different β -type subunits [16] (Fig. 1B), that together form the four-stacked ring cylindrical architecture that is conserved across living species. The stacked rings of proteins form a central pore with the active protease sites inside the pore structure on the face of the β subunits that make up the two inner rings. However, only three of the seven β -type subunits are catalytically active. Since the protease sites face the inner surface of the cylinder, the protein has to enter into this central pore to be degraded. The outer two α subunits serve to regulate the entry of proteins at either end of the cylinder by binding to one of the two regulatory caps (also know as regulatory particles) that recognize specific polyubiquitin chains and promote the entry of the protein into the cylinder and its subsequent degradation by the proteases within. Alterations in UPS-associated genes have been correlated with male infertility, signifying a critical role for the UPS in functional adult spermatogenesis [17]. Furthermore, a tissue specific proteasome subtype has been identified in the testis and is described in detail below.

1.2.1.1 The spermatoproteasome: Currently, three tissue-specific versions of the eukaryotic 20S protein subunit of the proteasome have been described: the immunoproteasome, the thymoproteasome, and the spermatoproteasome (reviewed in [18]). These tissue specific versions of the proteasome are thought to enable them to functional more efficiently in the degradation of protein substrates that are involved in their unique these tissues where the constitutive proteasome may only act sub-optimally act [18]. The 20S proteasome of the mammalian testis is composed of an alternative α 4-type subunit. This α subunit, termed α 4s/PSMA8, has been to been shown to be restricted to round and elongating spermatids in the testis of the Rhesus macaque [19] and cows [20]. Though the α 4-type subunits don't possess proteolytic activity, they do interact with regulatory particles that provide substrate specificity. One example in the mammalian testis is the specialized

[†]The Nobel Prize in Chemistry 2004". Nobelprize.org. Nobel Media AB 2013. Web. 20 Feb 2014. <http://www.nobelprize.org/nobel_prizes/chemistry/laureates/2004/>

process requiring the degradation of core histones during the process of spermiogenesis. Interestingly, it has been reported that the PA200 regulatory particle is highly expressed in the testis [20, 21]. It has been proposed that the $\alpha 4$ s/PSMA8 subunit may support the docking of the PA200 regulatory particle more than the conventional $\alpha 4$ -type subunit and this may play a role in promoting the acetylation-dependent, but not polyubiquitin-dependent, degradation of core histones that occur as they are replaced by transition protamines during elongate sperm formation [18, 20]. Another example is the involvement of the sperm proteasome in the process of sperm penetration of the egg and the capacitation reaction required for fertilization (reviewed in [22, 23]).

1.2.2 Non-proteolytic functions of ubiquitination—It is now well appreciated that the ubiquitination of proteins does not solely target them for degradation but can also serve to modulate the function of proteins independent of proteolysis. This includes cellular processes such as cell division, differentiation, signal transduction, protein trafficking, DNA repair, chromatin modifications, and cell-cycle progression and cell death (reviewed in [11, 24–26]). It is interesting that this appreciation for the non-proteolytic function of ubiquitin is fairly recent when the original paper by Goldstein first describing ubiquitin found that it induced the differentiation of T and B cells *in vitro* [1]. As with other well-known protein posttranslational modifications, the linkage of ubiquitin to proteins can alter their function by changing their affinity to interact with other proteins. Their linkage to lysine 63, and the length of the ubiquitin chain that is attached, largely determine the non-proteolytic functions that result. Therefore, a concise understanding of which lysine residue of ubiquitin is attached to the target protein, and the length of the ubiquitin chain, is key to understanding its influence on the proteins cellular function.

2. KNOWN TESTICULAR E3 LIGASES AND THEIR INFLUENCE ON SPERMATOGENESIS

2.1 Overview

It is clear that spermatogenesis, like numerous other biological processes, is dependent on both the proteolytic and non-proteolytic actions of protein ubiquitination for its regulation. Although ubiquitination is dependent on the hierarchal action of the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme and the E3 ubiquitin ligase, it is the E3 ubiquitin ligase that recognizes the target protein and provides the specificity for substrate ubiquitination and will therefore provide the focus of this section. Nevertheless, it should be appreciated that differences in the expression of various E1 activating and E2 conjugating enzymes, as well as a number of deubiquitinating enzymes, may also be important in modulating ubiquitination-mediated cellular processes. For example, the specific E2 conjugating enzyme UBC4 has been shown to be necessary for functional spermatogenesis in mice [27] and the importance of the deubiquitinating enzyme, UCH-L1, that regenerates mono-ubiquitin from ubiquitin-protein complexes, is highly expressed in spermatogonia [28–33]. A comprehensive analysis of genes of the UPS in neonatal rat gonocytes and spermatogonia has identified a number of novel ubiquitin-related genes specific to these cells [17]. The intent of this section is to provide examples that illustrate the participation of specific E3 ubiquitin ligases in the regulation of spermatogenesis. This section is organized

according to four key processes of spermatogenesis in which E3 ligases have been shown to play a role: spermiogenesis and the condensation of the sperm nucleus; the regulation of junctional complexes between cells of the seminiferous epithelium; the process of meiosis in spermatocytes; and the regulation of germ cell apoptosis. Readers interested in a comprehensive listing of purported testicular E3 ligase genes discovered by mining microarray data from mouse testis samples are directed to another recent review [34].

2.2 Spermiogenesis and Sperm DNA

Spermiogenesis is the final phase of spermatogenesis where the round spermatid undergoes a series of morphological and molecular alterations to produce the elongate spermatid. This process is divided into a number of defined steps first described by Leblond and Clermont in the rat [35] and defined in detail for the mouse, rat and dog by Russell et al. [36]. In short, this process is characterized by the condensation of the spermatid DNA, reduction of the cytoplasm, creation of the acrosome and formation of the sperm tail. During the final phase of this process, mature elongate spermatozoa are released from their attachment with Sertoli cells (a process defined as spermiation) into the lumen of the seminiferous tubules and the excess spermatid cytoplasm, in the form of cytoplasmic vesicles called residual bodies, is phagocytosed by Sertoli cells and degraded. A number of different E3 ligases have been implicated in each of the processes of spermiation, particularly in the removal and degradation of the histones that allows for the condensation of the sperm DNA as well as in the final phase of spermiation and the degradation of the residual bodies. Many of the testicular protein targets of the ubiquitin-proteasome pathway have been identified in germ cells likely as a result of the extensive cellular remodeling that occurs during their differentiation as well as the removal and degradation of the histones that occurs to allow for the packaging of DNA in the sperm head.

In elongating spermatids, chromatin undergoes significant changes to allow for the condensation of the DNA. This process involves the replacement of nearly all of the histones from the DNA by first transiently substituting them with transition proteins, followed by their replacement with protamines [37]; a process first suggested by Mills in 1977 [38]. In 1988 Chen *et al.* revealed that the ubiquitination of histones in post-meiotic germ cells is important for their degradation [39]. A high level of ubiquitination occurs within the testis during the germ cell stages when histones are degraded and is associated with the activity of a testis specific isoform of the ubiquitin-conjugating enzyme (E2) UBC4 (UBC4-testis) [3]. A screen for UBC4-dependent ubiquitin E3 ligases capable of ubiquitinating histones identified a HECT-containing E3 ligase, termed E3^{Histone}, that is capable of ubiquitinating all of the core histones and able to form poly-ubiquitin chains [40], suggesting that it may participate in the degradation of spermatid histones. Mass spectrometry and gel filtration analysis indicated that E3^{Histone} is a monomer of the HECT-containing E3 ligase previously termed LASU1 [40]. A subsequent evaluation of the cell-specific expression of the E3^{Histone} revealed that its mRNA expression in the testis is highest in rats during postnatal days 10 and 20 and that it decreases with age whereas its protein was found to be highly expressed in the nuclei of spermatogonia through mid-pachytene spermatocytes but was not detected in spermatid subtypes when histones are ubiquitinated and degraded [41]. Therefore, the

definitive participation of this E3 ligase in the removal of spermatid histones remains unresolved.

2.2.1. Spermatid Individualization—Investigations using classical genetic approaches in *Drosophila* have provided unique insights into a role for the inhibitor of apoptosis (IAP) proteins in the final phase of spermiogenesis where cytoplasmic bridges between elongate sperm are cleaved to produce separate individual sperm. The IAPs are a class of RING E3 ligases that have been characteristically shown to regulate the activity of the caspase family of proteases that are involved in the initiation and execution phases of apoptosis [42]. In flies, each primary diploid spermatogonium undergoes four mitotic and two meiotic divisions to give rise to a germline syncytium, or cyst, which contains 64 haploid spermatid nuclei interconnected by a network of cytoplasmic bridges. Syncytial spermatids differentiate into single motile sperm through a process known as “spermatid individualization”, wherein an actin-based “individualization complex” assembles around each spermatid nucleus and progresses down the length of the cyst, forcing cytoplasm and organelles into a “cystic bulge” that eventually reaches the base of the cyst and pinches off as a “waste bag” [43]. During this process protamines are incorporated into chromatin, resulting in nuclear condensation, and individual spermatids are encapsulated by an independent plasma membrane derived from the original cyst [43]. A similar process occurs during mammalian spermatogenesis [44], and human infertility can result from a disruption in this process [45].

Recent studies in *Drosophila* indicate that the caspase-9 ortholog DRONC, its adapter protein DARK/dApaf-1, and the caspase-3 ortholog DrICE are expressed in the cyst, and a testis-specific form of cytochrome c (Cyt-c-d) appears to stimulate DARK-dependent activation of DRONC [46–48]. Moreover, loss-of-function mutants for Cyt-c-d, DARK, or DRONC are male sterile, as are transgenic flies expressing the caspase inhibitors, p35 or *Drosophila* IAP1 (DIAP1) [46–48]. DIAP1 contains a C-terminal RING domain and distinct baculovirus IAP repeat (BIR) domains, through which it specifically binds to certain caspases and regulates their activities through ubiquitination [49, 50]. That being said, the IAP Bruce appears to play a more prominent role in spermatid individualization, since loss of function mutants exhibit features of apoptosis including widespread caspase activation along the cyst and hypercondensation of spermatid nuclei [46]. Interestingly, Bruce is itself the target of a Cullin-3-based E3 ubiquitin ligase complex, composed of a testis-specific isoform of Cullin-3, the RING protein Roc1b, and the BTB-Kelch protein Khl10. Soti, an inhibitor of this complex, competes with Bruce for binding to Khl10 [51, 52], and since Soti is expressed in a subcellular gradient, it creates inverse gradients for dBruce and active caspases along the length of the cyst. Thus, as the cystic bulge moves down the cyst, the Cul3_{testis}-Roc1b-Khl10 complex ubiquitinates Bruce near the bulge, triggering its relocalization to the distal end of the cyst. In the absence of Bruce, DARK-DRONC complexes are allowed to activate DrICE near the bulge, allowing DrICE to target the necessary substrates for spermatid individualization without initiating full-blown apoptosis of the spermatid [51, 52]. Finally, though not as well described, an SCF complex composed of Cullin-1, SkpA, and the F-box protein Nutcracker has been shown to promote caspase

activation in the cyst through its interaction with Bruce and/or by directly promoting proteasome activity [53].

Whether removal of cytoplasm and organelles from developing sperm in mammals requires the same pathways as described in *Drosophila* remains to be determined, but several IAPs are widely-expressed in the testis, including Bruce, X-linked IAP (XIAP), Livin/ML-IAP, and the testis-specific IAP, ILP-2 [54–57]. The specific roles for Bruce, Apaf-1, and caspase-9 are likewise unknown, primarily because in most cases the corresponding knockout mice are embryonic lethal [58–60]. However, in the approximately 5% of Apaf-1-deficient mice that survive to adulthood, the males exhibit a severe defect in spermatogenesis [61]. Thus, the generation of tissue restricted knockouts should prove invaluable in the future for determining the roles of caspases and the E3 ligases that regulate them during spermatogenesis.

2.3 Regulation of Junctional Complexes in the Seminiferous Epithelium

There are a number of specialized junctions between cells of the seminiferous epithelium and these junctions are dynamic and changing. Tight junctions between adjacent Sertoli cells are responsible, in part, for creating the “blood-testis” barrier (BTB) that segregates meiotic germ cells from the systemic circulation and prevents a possible immune response to these cells that are generated after immune tolerance has been established. In the testis, the diploid spermatogonial stem cells and differentiating spermatogonia reside below the BTB near the basal membrane of the Sertoli cells whereas the meiotic spermatocytes and round and elongating spermatids reside above the BTB. Therefore, a sophisticated and dynamic process for restructuring the BTB allows the preleptotene and leptotene spermatocytes to cross through the BTB into the adluminal compartment. This restructuring process is regulated by several mechanisms including transcriptional regulation of junction proteins as well as posttranslational modifications of junctional proteins, such as ubiquitination, that target these proteins for degradation. In addition, adherens junctions are dynamic structures between Sertoli cells and germ cells that are involved in the movement of differentiating germ cells from the basal compartment below the Sertoli-Sertoli cell tight junctions to the adluminal compartment above [62]. Functional spermatogenesis is dependent on the continuous restructuring of these junctions.

2.3.1 Tight junctions (TJ)—Itch has been implicated in rat testis as the E3 ligase responsible for targeting the ubiquitination and proteolytic destruction of the TJ protein occludin, an important mediator in the BTB [63]. The regulation of the *Itch* gene in the TM4 Sertoli cell line indicates that both the E2F and GATA-a motifs in the promoter are involved in *Itch* gene transcription, though they have differing roles [64]. Using a rat primary Sertoli cell culture model at a time when inter-Sertoli TJs were assembled, an increase in the levels of occludin were observed to coincide with a reduction in the level of Itch [63]. Itch belongs to the HECT family of E3 ubiquitin ligases and is widely expressed in tissues and organs throughout development (reviewed in [65]). Itch consists of a N-terminal lipid binding C2 domain, four internal WW protein-binding domains, and a C-terminal enzymatically-active HECT domain. The WW protein interacting domains are so called for the two conserved tryptophan residues that bind proline-rich sequences in target proteins, specifically PPLP or

PPXY sequences (reviewed in [66]). However, in *Itch* gene-deficient mice, the protein levels of occludin were not found to be significantly altered compared to those seen in wild-type mice [67], suggesting that either occludin isn't a significant target for Itch in mouse testis or that the *Itch* gene deficient mice have developed a compensatory mechanism to maintain the rate of occludin protein turnover.

2.3.2 Adherens junctions (AJ)—Functional spermatogenesis is dependent on the continuous restructuring of AJs between Sertoli cells and germ cells. The SCF (Skp1/Cullin/F-box protein) comprises a large subfamily of modular E3 ligases that are widely expressed and known to target many regulatory proteins for proteasomal degradation [68–70] by ubiquitinating their substrates in a phosphorylation-dependent manner [71, 72]. The E3 ligase β -TrCP is the substrate recognition subunit of SCF and appears to be uniquely required for functional spermatogenesis. Mammals express two distinct paralogs of β -TrCP; β -TrCP1 and β -TrCP2, with apparently similar biochemical properties [73, 74]. Male β -TrCP1 gene-deficient mice are reported to have a moderate disruption of spermatogenesis and fertility; though no other signs of illness or gross tissue abnormalities are evident [75]. The authors speculate that β -TrCP2 expression likely compensates for the loss of β -TrCP1. In a study by Kanarek, using β -TrCP1 gene-deficient mice crossed with tetracycline inducible β -TrCP2 hypomorphic mice, it was shown that these two β -TrCP paralogs in fact have non-redundant roles in spermatogenesis [76]. Through an elegant series of experiments using these mice, it was demonstrated that the β -TrCP2 hypomorphic mice show a significant disruption of spermatogenesis with the complete loss of spermatocytes, spermatids and spermatogonia that are displaced from their basal location towards the lumen. These observations pointed to an alteration in AJs between germ cells to the Sertoli cells. The disruption of spermatogenesis in these mice was reversible as near normal spermatogenesis was seen 4 weeks after ending tetracycline treatment. In addition, the authors discovered that the spermatogenesis defects following the decrease in β -TrCP1 and β -TrCP2 are due to the stabilization of the transcriptional regulator snail, a known substrate of β -TrCP that is targeted for proteasomal degradation upon its ubiquitination [77]. The mechanism for the alteration in cell adhesion was revealed when it was shown that the increased level in snail in the β -TrCP2 hypomorphic mice instigates a reduction in the expression of E-cadherin resulting in an impaired structure of AJs. Further, if the levels of snail were decreased in the testis by using a shRNA lentivirus to inhibit its expression, the protein levels of E-cadherin were restored and functional spermatogenesis returned. This work reveals the importance of the β -TrCP E3 ligases for the maintenance of AJs through its ubiquitination and modulation of snail and its negative influence on E-cadherin expression.

2.4 Spermatocyte Meiosis

During spermatogenesis the most mature differentiating spermatogonial subtype, type B spermatogonia, divide to form primary spermatocytes. Primary spermatocytes are characterized both biochemically and morphologically based upon the state of meiotic prophase in which they reside, as exquisitely detailed in the definitive work of Russell *et al.*, [36]. Preleptotene spermatocytes undergo meiotic S phase in which the chromosomal homologs are duplicated. Leptotene primary spermatocytes begin the long meiotic prophase characteristic of meiosis I in spermatocytes. In the zygotene spermatocytes, homologous

chromosomes become paired. In the pachytene spermatocytes, the process of homologous recombination and the exchange of genetic material occur between homologous chromosomes. Interestingly, the process of meiotic recombination is initiated through a programmed induction of DNA double strand breaks that is initiated by the topoisomerase-like protein SPO11 and utilizes similar cellular machinery that transpires with DNA double-strand break response (reviewed in [78]). Finally, the end of the long meiotic prophase occurs with the diplotene phase of meiosis and the swift progression through metaphase, anaphase and telophase to complete the first meiotic division and the production of secondary spermatocytes. The second meiotic division rapidly follows to produce haploid spermatids. Not surprisingly, a number of E3 ligases have been shown to be important regulators for the progression of spermatocytes through meiosis I and II to produce haploid spermatids.

Siah1a is a highly conserved mammalian protein containing a RING domain. The Siah1a protein is a component of an E3 ligase that controls the degradation of β -catenin, N-CoR and DCC [79]. The participation of Siah1a in the progression of germ cell meiosis was demonstrated by disrupting the *Siah1a* gene in mice *via* gene targeting. The resultant mutant mice were found to be infertile due to the failure of the germ cells to progress through meiosis I, resulting in the loss of these cells by apoptosis [79]. Similar observations were also made in *Itch* gene-deficient mice that showed a failure and/or delay in meiotic progression. However, the phenotype in *Itch* gene-deficient mice was not as robust as that seen in the Siah1a mutant mice as the *Itch* gene-deficient mice were still able to produce mature functional sperm, albeit at significantly lower numbers [67].

CUL4A is an E3 ligase that belongs to the Cullin family of ubiquitin ligases, the largest ligase family found in mammals [80]. It has been shown to play an important role during DNA replication, chromatin condensation, cell cycling, and DNA damage repair, suggesting that it may also play an essential role in the testis, particularly during meiotic germ cell division. One group generated *Cul4A* gene deficient mice (*Cul4A*^{-/-}) by deleting exons 4–8, and found that the male mice are infertile as they failed to develop spermatids [81]. The male *Cul4A*^{-/-} mice had significantly decreased testis weights, lower amounts of spermatid heads in both their epididymides and testes, and the spermatids that were present were severely deformed with very low motility. The testes of these mice had increases in abnormal multinucleated and apoptotic germ cells. The primary spermatocytes in these mice failed in the progression through late prophase I and the pachytene spermatocytes underwent persistent double strand breaks, likely due to failed homologous recombination.

Interestingly, work into the functional role of CUL4A during germ cell meiosis led to its discovery as a key protein in the double stranded break repair pathway, which plays an essential role during homologous recombination in meiotic spermatocytes [82]. A separate research team developed their own set of *Cul4A* gene-deficient mice, deleting exons 17–19, and although the gross reproductive phenotype still remained, the apparent molecular function of CUL4A appeared to be different [82]. These mice also had decreased testis weights and spermatid heads, and increases in abnormal multinucleated and apoptotic germ cells. Taken together, these two reports point to a role for CUL4A in maintaining spermatogenesis by facilitating the meiotic differentiation of germ cells.

The ubiquitination of histones occurs during meiosis in response to the double strand breaks that occur during this process (reviewed in [83]). A number of E3 ligases have been implicated in this process including the RING E3 ligase RNF8 [84–87]. This ligase works in conjunction with the E2 conjugating enzyme, UBC13, to ubiquitinate histones H2A and H2AX during this process. Li et al generated a line of *Rnf8* gene-deficient mice and showed that the loss of RNF8 did not affect the female ovary but did cause a spectrum of histopathological alterations in the testis of male mice [88]. The observed disruption of spermatogenesis within the testis of *Rnf8* gene-deficient testes varied from seminiferous tubules with a build up of pre-meiotic germ cells suggesting a meiotic arrest, to tubules with a severe disorganization of the seminiferous epithelium, to tubules that only showed a moderate disruption of spermatogenesis. Surprisingly, despite the apparent meiotic alterations, the *Rnf8* gene-deficient mice were able to produce viable litters, though with significantly reduced numbers of pups. The ubiquitination of histones by RNF8 has also been suggested to play a role in nucleosome removal in post-meiotic germ cells [89] in order for histones to be replaced by protamines as discussed in *section 2.2*.

2.5 Germ cell apoptosis

Apoptosis of testicular germ cells occurs spontaneously in the testis and serves as an important physiological mechanism for matching the numbers of germ cells to the supportive capacity of the seminiferous epithelium (reviewed in [90]). An unexpected role for *Itch* in the regulation of testicular germ cell apoptosis was revealed in the evaluation of *Itch* gene-deficient mice. The *Itch* mediated ubiquitination and subsequent proteasomal degradation of the protein c-FLIP, an inhibitor of death receptor-mediated cell apoptosis, was first shown in liver cells [91]. Since, the Fas/FasL death receptor signaling pathway has been demonstrated to be a mechanism by which testicular germ cell apoptosis is controlled [92–94], *Itch* mediated decreases in c-FLIP could regulate death receptor mediated germ cell apoptosis similar to that seen in hepatocytes. However, the c-FLIP protein does not appear to be highly expressed in the testis and levels of c-FLIP were not different between *Itch* gene-deficient or wild-type C57Bl6/J mice [67]. On the other hand, the loss of *Itch* in the testis of these mice did result in an increased basal rate of apoptosis during both a critical peripubertal period as well as in adult mice [67]. A corresponding increase in caspase-9, the prototypical initiator caspase family member of the intrinsic apoptotic signaling pathway, implies that *Itch* may influence the cleavage of caspase-9 in an age-dependent manner [67].

MEX (MEKK1-related protein X) is an E3 ligase with its mRNA expression, as indicated by dot-blot and northern blot analysis, to be uniquely located in the testis [95]. Unfortunately, the cell-specific localization of MEX in the cells of the seminiferous epithelium was not evaluated, though was presumed to be in germ cells. MEX has two RING fingers and biochemical analysis indicated that MEX is self-ubiquitinated and targeted for degradation by the UPS. MEX can also act as an E3 ligase through its interaction with the E2 ubiquitin conjugating enzymes UbcH5a, UbcH5c or UbcH6. The authors revealed that MEX promotes cell apoptosis that is initiated through the Fas, DR3 or DR4 signaling pathways. Since numerous investigators have revealed that testicular germ cells can undergo Fas-mediated germ cell apoptosis [92, 94, 96–98], it would suggest that MEX most likely is expressed in

the germ cells and acts to modify their sensitivity to death-receptor induced apoptosis; though definitive evidence is still lacking.

3. CONCLUSION AND PERSPECTIVES

Spermatogenesis is an elaborate process of germ cell proliferation and differentiation that is dependent on dynamic and complex interactions between Sertoli cells and germ cells of the seminiferous epithelium as well as with cells residing outside of the seminiferous epithelium such as Leydig cells, peritubular myoid cells and resident leukocytes. It is now widely appreciated that the ubiquitination of proteins is an important posttranslational modification for regulating protein stability and function. The fact that the rat testis has the highest rate of ubiquitination among all the organs examined [3] further underscores the extensive role that this posttranslational modification plays in the synchronized cellular differentiation that is required for spermatogenesis. The specificity for protein ubiquitination is determined by the specific E3 ubiquitin ligase that recognizes it. Therefore, the further identification of the E3 ligases involved in modulating specific proteins in the testis will provide important insights into the cellular mechanisms regulating spermatogenesis. Although many E3 ligases have now been described in the testis, the next steps in understanding these enzymes is to reveal how E3 ligases themselves are regulated. Ironically, the ubiquitination or deubiquitination of the E3 ligase itself is proving to be an key mechanism to regulate their activity [10]. Finally, although the non-proteolytic function of protein ubiquitination has been characterized in other systems, however few examples are known in the testis. This area of research would certainly be a “fertile” area of research in the years to come.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge the skillful participation of Belinda Gonzalez-Lehmkuhle in the production of the illustration for figure 1.

REFERENCES CITED

1. Goldstein G, Scheid M, Hammerling U, Schlesinger DH, Niall HD, Boyse EA. Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proc Natl Acad Sci U S A*. 1975; 72:11–5. [PubMed: 1078892]
2. Hershko A, Eytan E, Ciechanover A, Haas AL. Immunochemical analysis of the turnover of ubiquitin-protein conjugates in intact cells. Relationship to the breakdown of abnormal proteins. *J Biol Chem*. 1982; 257:13964–70. [PubMed: 6292216]
3. Rajapurohitam V, Bedard N, Wing SS. Control of ubiquitination of proteins in rat tissues by ubiquitin conjugating enzymes and isopeptidases. *Am J Physiol Endocrinol Metab*. 2002; 282:E739–45. [PubMed: 11882492]
4. Ciechanover A, Orian A, Schwartz AL. Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays*. 2000; 22:442–51. [PubMed: 10797484]
5. Huijbregtse JM, Scheffner M, Beaudenon S, Howley PM. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc Natl Acad Sci U S A*. 1995; 92:2563–7. [PubMed: 7708685]

6. Jackson PK, Eldridge AG, Freed E, Furstenthal L, Hsu JY, Kaiser BK, et al. The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. *Trends Cell Biol.* 2000; 10:429–39. [PubMed: 10998601]
7. Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, et al. A proteomics approach to understanding protein ubiquitination. *Nature biotechnology.* 2003; 21:921–6.
8. Vigodner M, Ishikawa T, Schlegel PN, Morris PL. SUMO-1, human male germ cell development, and the androgen receptor in the testis of men with normal and abnormal spermatogenesis. *Am J Physiol Endocrinol Metab.* 2006; 290:E1022–33. [PubMed: 16352666]
9. Vigodner M, Morris PL. Testicular expression of small ubiquitin-related modifier-1 (SUMO-1) supports multiple roles in spermatogenesis: silencing of sex chromosomes in spermatocytes, spermatid microtubule nucleation, and nuclear reshaping. *Dev Biol.* 2005; 282:480–92. [PubMed: 15950612]
10. Gao M, Karin M. Regulating the regulators: control of protein ubiquitination and ubiquitin-like modifications by extracellular stimuli. *Mol Cell.* 2005; 19:581–93. [PubMed: 16137616]
11. Weissman AM, Shabek N, Ciechanover A. The predator becomes the prey: regulating the ubiquitin system by ubiquitylation and degradation. *Nature reviews Molecular cell biology.* 2011; 12:605–20.
12. Kessler BM, Edelman MJ. PTMs in conversation: activity and function of deubiquitinating enzymes regulated via post-translational modifications. *Cell biochemistry and biophysics.* 2011; 60:21–38. [PubMed: 21480003]
13. Etlinger JD, Goldberg AL. A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. *Proc Natl Acad Sci U S A.* 1977; 74:54–8. [PubMed: 264694]
14. Goldberg AL, St John AC. Intracellular protein degradation in mammalian and bacterial cells: Part 2. *Annu Rev Biochem.* 1976; 45:747–803. [PubMed: 786161]
15. Ciechanover A, Hod Y, Hershko A. A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. *Biochem Biophys Res Commun.* 1978; 81:1100–5. [PubMed: 666810]
16. Groll M, Ditzel L, Lowe J, Stock D, Bochtler M, Bartunik HD, et al. Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature.* 1997; 386:463–71. [PubMed: 9087403]
17. Manku G, Wing SS, Culty M. Expression of the ubiquitin proteasome system in neonatal rat gonocytes and spermatogonia: role in gonocyte differentiation. *Biol Reprod.* 2012; 87:44. [PubMed: 22592496]
18. Kniepert A, Groettrup M. The unique functions of tissue-specific proteasomes. *Trends Biochem Sci.* 2013
19. Skerget S, Rosenow M, Polpitiya A, Petritis K, Dorus S, Karr TL. The Rhesus Macaque (*Macaca mulatta*) Sperm Proteome. *Molecular & cellular proteomics : MCP.* 2013; 12:3052–67. [PubMed: 23816990]
20. Qian MX, Pang Y, Liu CH, Haratake K, Du BY, Ji DY, et al. Acetylation-mediated proteasomal degradation of core histones during DNA repair and spermatogenesis. *Cell.* 2013; 153:1012–24. [PubMed: 23706739]
21. Ustrell V, Hoffman L, Pratt G, Rechsteiner M. PA200, a nuclear proteasome activator involved in DNA repair. *EMBO J.* 2002; 21:3516–25. [PubMed: 12093752]
22. Sutovsky P. Ubiquitin-dependent proteolysis in mammalian spermatogenesis, fertilization, and sperm quality control: killing three birds with one stone. *Microsc Res Tech.* 2003; 61:88–102. [PubMed: 12672125]
23. Sutovsky P. Sperm proteasome and fertilization. *Reproduction.* 2011; 142:1–14. [PubMed: 21606061]
24. Mukhopadhyay D, Riezman H. Proteasome-independent functions of ubiquitin in endocytosis and signaling. *Science.* 2007; 315:201–5. [PubMed: 17218518]
25. Miranda M, Sorkin A. Regulation of receptors and transporters by ubiquitination: new insights into surprisingly similar mechanisms. *Molecular interventions.* 2007; 7:157–67. [PubMed: 17609522]
26. Schnell JD, Hicke L. Non-traditional functions of ubiquitin and ubiquitin-binding proteins. *J Biol Chem.* 2003; 278:35857–60. [PubMed: 12860974]

27. Bedard N, Hingamp P, Pang Z, Karaplis A, Morales C, Trasler J, et al. Mice lacking the UBC4-testis gene have a delay in postnatal testis development but normal spermatogenesis and fertility. *Mol Cell Biol.* 2005; 25:6346–54. [PubMed: 16024774]
28. Kwon J. The new function of two ubiquitin C-terminal hydrolase isozymes as reciprocal modulators of germ cell apoptosis. *Experimental animals / Japanese Association for Laboratory Animal Science.* 2007; 56:71–7. [PubMed: 17460351]
29. Kwon J, Kikuchi T, Setsue R, Ishii Y, Kyuwa S, Yoshikawa Y. Characterization of the testis in congenitally ubiquitin carboxy-terminal hydrolase-1 (Uch-L1) defective (gad) mice. *Experimental animals / Japanese Association for Laboratory Animal Science.* 2003; 52:1–9. [PubMed: 12638230]
30. Kwon J, Mochida K, Wang YL, Sekiguchi S, Sankai T, Aoki S, et al. Ubiquitin C-terminal hydrolase L-1 is essential for the early apoptotic wave of germinal cells and for sperm quality control during spermatogenesis. *Biol Reprod.* 2005; 73:29–35. [PubMed: 15744022]
31. Kwon J, Wang YL, Setsue R, Sekiguchi S, Sakurai M, Sato Y, et al. Developmental regulation of ubiquitin C-terminal hydrolase isozyme expression during spermatogenesis in mice. *Biol Reprod.* 2004; 71:515–21. [PubMed: 15084487]
32. Kwon J, Wang YL, Setsue R, Sekiguchi S, Sato Y, Sakurai M, et al. Two closely related ubiquitin C-terminal hydrolase isozymes function as reciprocal modulators of germ cell apoptosis in cryptorchid testis. *Am J Pathol.* 2004; 165:1367–74. [PubMed: 15466400]
33. Wang YL, Liu W, Sun YJ, Kwon J, Setsue R, Osaka H, et al. Overexpression of ubiquitin carboxyl-terminal hydrolase L1 arrests spermatogenesis in transgenic mice. *Mol Reprod Dev.* 2006; 73:40–9. [PubMed: 16177983]
34. Hou X, Zhang W, Xiao Z, Gan H, Lin X, Liao S, et al. Mining and characterization of ubiquitin E3 ligases expressed in the mouse testis. *BMC Genomics.* 2012; 13:495. [PubMed: 22992278]
35. Leblond CP, Clermont Y. Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann N Y Acad Sci.* 1952; 55:548–73. [PubMed: 13139144]
36. Russell, LD.; Ettlin, RA.; Sinha Hikim, AP.; Clegg, ED. *Histological and Histopathological Evaluation of the Testis.* 1. Clearwater, FL: Cache River Press; 1990.
37. Wykes SM, Krawetz SA. The structural organization of sperm chromatin. *J Biol Chem.* 2003; 278:29471–7. [PubMed: 12775710]
38. Mills NC, Van NT, Means AR. Histones of rat testis chromatin during early postnatal development and their interactions with DNA. *Biol Reprod.* 1977; 17:760–8. [PubMed: 597543]
39. Chen HY, Sun JM, Zhang Y, Davie JR, Meistrich ML. Ubiquitination of histone H3 in elongating spermatids of rat testes. *J Biol Chem.* 1998; 273:13165–9. [PubMed: 9582357]
40. Liu Z, Oughtred R, Wing SS. Characterization of E3Histone, a novel testis ubiquitin protein ligase which ubiquitinates histones. *Mol Cell Biol.* 2005; 25:2819–31. [PubMed: 15767685]
41. Liu Z, Miao D, Xia Q, Hermo L, Wing SS. Regulated expression of the ubiquitin protein ligase, E3(Histone)/LASU1/Mule/ARF-BP1/HUWE1, during spermatogenesis. *Dev Dyn.* 2007; 236:2889–98. [PubMed: 17823942]
42. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. *Nature reviews Molecular cell biology.* 2002; 3:401–10.
43. Lindsley, DI.; Tokuyasu, KT. *Spermatogenesis.* In: Ashburner, M.; Wright, TR., editors. *Genetics and biology of Drosophila.* New York: Academic Press; 1980. p. 225-94.
44. de Krester, DM.; Kerr, JB. *The cytology of testis.* In: Knobil, E.; Neill, J., editors. *The physiology of reproduction.* New York: Raven Press; 1994. p. 1177-290.
45. Keating J, Grundy CE, Fivey PS, Elliott M, Robinson J. Investigation of the association between the presence of cytoplasmic residues on the human sperm midpiece and defective sperm function. *J Reprod Fertil.* 1997; 110:71–7. [PubMed: 9227359]
46. Arama E, Agapite J, Steller H. Caspase activity and a specific cytochrome C are required for sperm differentiation in *Drosophila*. *Dev Cell.* 2003; 4:687–97. [PubMed: 12737804]
47. Arama E, Bader M, Srivastava M, Bergmann A, Steller H. The two *Drosophila* cytochrome C proteins can function in both respiration and caspase activation. *EMBO J.* 2006; 25:232–43. [PubMed: 16362035]

48. Huh JR, Vernoooy SY, Yu H, Yan N, Shi Y, Guo M, et al. Multiple apoptotic caspase cascades are required in nonapoptotic roles for *Drosophila* spermatid individualization. *PLoS Biol.* 2004; 2:43–53.
49. Ditzel M, Broemer M, Tenev T, Bolduc C, Lee TV, Rigbolt KT, et al. Inactivation of effector caspases through nondegradative polyubiquitylation. *Mol Cell.* 2008; 32:540–53. [PubMed: 19026784]
50. Lee TV, Fan Y, Wang S, Srivastava M, Broemer M, Meier P, et al. *Drosophila* IAP1-mediated ubiquitylation controls activation of the initiator caspase DRONC independent of protein degradation. *PLoS Genet.* 2011; 7:e1002261. [PubMed: 21909282]
51. Arama E, Bader M, Rieckhof GE, Steller H. A ubiquitin ligase complex regulates caspase activation during sperm differentiation in *Drosophila*. *PLoS Biol.* 2007; 5:2270–87.
52. Kaplan Y, Gibbs-Bar L, Kalifa Y, Feinstein-Rotkopf Y, Arama E. Gradients of a ubiquitin E3 ligase inhibitor and a caspase inhibitor determine differentiation or death in spermatids. *Dev Cell.* 2010; 19:160–73. [PubMed: 20643358]
53. Bader M, Arama E, Steller H. A novel F-box protein is required for caspase activation during cellular remodeling in *Drosophila*. *Development.* 2010; 137:1679–88. [PubMed: 20392747]
54. Hauser HP, Bardroff M, Pyrowolakis G, Jentsch S. A giant ubiquitin-conjugating enzyme related to IAP apoptosis inhibitors. *J Cell Biol.* 1998; 141:1415–22. [PubMed: 9628897]
55. Lagace M, Xuan JY, Young SS, McRoberts C, Maier J, Rajcan-Separovic E, et al. Genomic organization of the X-linked inhibitor of apoptosis and identification of a novel testis-specific transcript. *Genomics.* 2001; 77:181–8. [PubMed: 11597143]
56. Richter BW, Mir SS, Eiben LJ, Lewis J, Reffey SB, Frattini A, et al. Molecular cloning of ILP-2, a novel member of the inhibitor of apoptosis protein family. *Mol Cell Biol.* 2001; 21:4292–301. [PubMed: 11390657]
57. Vucic D, Stennicke HR, Pisabarro MT, Salvesen GS, Dixit VM. ML-IAP, a novel inhibitor of apoptosis that is preferentially expressed in human melanomas. *Curr Biol.* 2000; 10:1359–66. [PubMed: 11084335]
58. Hao Y, Sekine K, Kawabata A, Nakamura H, Ishioka T, Ohata H, et al. Apollon ubiquitinates SMAC and caspase-9, and has an essential cytoprotection function. *Nat Cell Biol.* 2004; 6:849–60. [PubMed: 15300255]
59. Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R, et al. Apaf-1 is required for mitochondrial pathways of apoptosis and brain development. *Cell.* 1998; 94:739–50. [PubMed: 9753321]
60. Hakem R, Hakem A, Duncan GS, Henderson JT, Woo M, Soengas MS, et al. Differential requirement for caspase 9 in apoptotic pathways in vivo. *Cell.* 1998; 94:339–52. [PubMed: 9708736]
61. Honarpour N, Du C, Richardson JA, Hammer RE, Wang X, Herz J. Adult Apaf-1-deficient mice exhibit male infertility. *Dev Biol.* 2000; 218:248–58. [PubMed: 10656767]
62. Mok KW, Mruk DD, Cheng CY. Regulation of blood-testis barrier (BTB) dynamics during spermatogenesis via the “Yin” and “Yang” effects of mammalian target of rapamycin complex 1 (mTORC1) and mTORC2. *International review of cell and molecular biology.* 2013; 301:291–358. [PubMed: 23317821]
63. Lui WY, Lee WM. cAMP perturbs inter-Sertoli tight junction permeability barrier in vitro via its effect on proteasome-sensitive ubiquitination of occludin. *J Cell Physiol.* 2005; 203:564–72. [PubMed: 15605377]
64. Li MW, Lee WM, Lui WY. Expression of Itch in Sertoli cells is controlled via the interaction of E2F1/DP1 complex with E2F and GATA motifs. *Spermatogenesis.* 2011; 1:152–8. [PubMed: 22319664]
65. Melino G, Gallagher E, Aqeilan RI, Knight R, Peschiaroli A, Rossi M, et al. Itch: a HECT-type E3 ligase regulating immunity, skin and cancer. *Cell Death Differ.* 2008; 15:1103–12. [PubMed: 18552861]
66. Matesic LE, Copeland NG, Jenkins NA. Itchy mice: the identification of a new pathway for the development of autoimmunity. *Current topics in microbiology and immunology.* 2008; 321:185–200. [PubMed: 18727493]

67. Dwyer JL, Richburg JH. Age-dependent alterations in spermatogenesis in itchy mice. *Spermatogenesis*. 2012; 2:104–16. [PubMed: 22670220]
68. Frescas D, Pagano M. Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. *Nat Rev Cancer*. 2008; 8:438–49. [PubMed: 18500245]
69. Fuchs SY, Spiegelman VS, Kumar KG. The many faces of beta-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer. *Oncogene*. 2004; 23:2028–36. [PubMed: 15021890]
70. Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer*. 2006; 6:369–81. [PubMed: 16633365]
71. Deshaies RJ, Joazeiro CA. RING domain E3 ubiquitin ligases. *Annu Rev Biochem*. 2009; 78:399–434. [PubMed: 19489725]
72. Willems AR, Schwab M, Tyers M. A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. *Biochim Biophys Acta*. 2004; 1695:133–70. [PubMed: 15571813]
73. Suzuki H, Chiba T, Kobayashi M, Takeuchi M, Suzuki T, Ichiyama A, et al. IkappaBalpha ubiquitination is catalyzed by an SCF-like complex containing Skp1, cullin-1, and two F-box/WD40-repeat proteins, betaTrCP1 and betaTrCP2. *Biochem Biophys Res Commun*. 1999; 256:127–32. [PubMed: 10066435]
74. Tan P, Fuchs SY, Chen A, Wu K, Gomez C, Ronai Z, et al. Recruitment of a ROC1-CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of I kappa B alpha. *Mol Cell*. 1999; 3:527–33. [PubMed: 10230406]
75. Guardavaccaro D, Kudo Y, Boulaire J, Barchi M, Busino L, Donzelli M, et al. Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo. *Dev Cell*. 2003; 4:799–812. [PubMed: 12791266]
76. Kanarek N, Horwitz E, Mayan I, Leshets M, Cojocaru G, Davis M, et al. Spermatogenesis rescue in a mouse deficient for the ubiquitin ligase SCF{beta}-TrCP by single substrate depletion. *Genes Dev*. 2010; 24:470–7. [PubMed: 20194439]
77. Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, et al. Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol*. 2004; 6:931–40. [PubMed: 15448698]
78. Baudat F, Imai Y, de Massy B. Meiotic recombination in mammals: localization and regulation. *Nature reviews Genetics*. 2013; 14:794–806.
79. Dickins RA, Frew IJ, House CM, O'Bryan MK, Holloway AJ, Haviv I, et al. The ubiquitin ligase component Siah1a is required for completion of meiosis I in male mice. *Mol Cell Biol*. 2002; 22:2294–303. [PubMed: 11884614]
80. Sarikas A, Hartmann T, Pan ZQ. The cullin protein family. *Genome Biol*. 2011; 12:220. [PubMed: 21554755]
81. Kopanja D, Roy N, Stoyanova T, Hess RA, Bagchi S, Raychaudhuri P. Cul4A is essential for spermatogenesis and male fertility. *Dev Biol*. 2011; 352:278–87. [PubMed: 21291880]
82. Yin Y, Lin C, Kim ST, Roig I, Chen H, Liu L, et al. The E3 ubiquitin ligase Cullin 4A regulates meiotic progression in mouse spermatogenesis. *Dev Biol*. 2011; 356:51–62. [PubMed: 21624359]
83. Ma T, Keller JA, Yu X. RNF8-dependent histone ubiquitination during DNA damage response and spermatogenesis. *Acta biochimica et biophysica Sinica*. 2011; 43:339–45. [PubMed: 21444325]
84. Huen MS, Grant R, Manke I, Minn K, Yu X, Yaffe MB, et al. RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly. *Cell*. 2007; 131:901–14. [PubMed: 18001825]
85. Kolas NK, Chapman JR, Nakada S, Ylanko J, Chahwan R, Sweeney FD, et al. Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase. *Science*. 2007; 318:1637–40. [PubMed: 18006705]
86. Mailand N, Bekker-Jensen S, Fastrup H, Melander F, Bartek J, Lukas C, et al. RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. *Cell*. 2007; 131:887–900. [PubMed: 18001824]
87. Wang B, Elledge SJ. Ubc13/Rnf8 ubiquitin ligases control foci formation of the Rap80/Abraxas/Brca1/Brcc36 complex in response to DNA damage. *Proc Natl Acad Sci U S A*. 2007; 104:20759–63. [PubMed: 18077395]

88. Li L, Halaby MJ, Hakem A, Cardoso R, El Ghamrasni S, Harding S, et al. Rnf8 deficiency impairs class switch recombination, spermatogenesis, and genomic integrity and predisposes for cancer. *J Exp Med*. 2010; 207:983–97. [PubMed: 20385750]
89. Lu LY, Wu J, Ye L, Gavrilina GB, Saunders TL, Yu X. RNF8-dependent histone modifications regulate nucleosome removal during spermatogenesis. *Dev Cell*. 2010; 18:371–84. [PubMed: 20153262]
90. Print CG, Loveland KL. Germ cell suicide: new insights into apoptosis during spermatogenesis. *Bioessays*. 2000; 22:423–30. [PubMed: 10797482]
91. Chang L, Kamata H, Solinas G, Luo JL, Maeda S, Venuprasad K, et al. The E3 ubiquitin ligase itch couples JNK activation to TNF α -induced cell death by inducing c-FLIP(L) turnover. *Cell*. 2006; 124:601–13. [PubMed: 16469705]
92. Lee J, Richburg JH, Younkin SC, Boekelheide K. The Fas system is a key regulator of germ cell apoptosis in the testis. *Endocrinology*. 1997; 138:2081–8. [PubMed: 9112408]
93. Richburg JH, Nanez A, Gao H. Participation of the Fas-signaling system in the initiation of germ cell apoptosis in young rat testes after exposure to mono-(2-ethylhexyl) phthalate. *Toxicol Appl Pharmacol*. 1999; 160:271–8. [PubMed: 10544061]
94. Richburg JH, Nanez A, Williams LR, Embree ME, Boekelheide K. Sensitivity of testicular germ cells to toxicant-induced apoptosis in gld mice that express a nonfunctional form of Fas ligand. *Endocrinology*. 2000; 141:787–93. [PubMed: 10650961]
95. Nishito Y, Hasegawa M, Inohara N, Nunez G. MEX is a testis-specific E3 ubiquitin ligase that promotes death receptor-induced apoptosis. *Biochem J*. 2006; 396:411–7. [PubMed: 16522193]
96. Embree-Ku M, Venturini D, Boekelheide K. Fas is involved in the p53-dependent apoptotic response to ionizing radiation in mouse testis. *Biol Reprod*. 2002; 66:1456–61. [PubMed: 11967210]
97. Pentikäinen V, Erkkilä K, Dunkel L. Fas regulates germ cell apoptosis in the human testis *in vitro*. *Am J Physiol*. 1999; 276:E310–E6. [PubMed: 9950791]
98. Richburg JH, Nanez A. Fas- or FasL-deficient mice display an increased sensitivity to nitrobenzene-induced testicular germ cell apoptosis. *Toxicol Lett*. 2003; 139:1–10. [PubMed: 12595153]
99. Wang S, Zheng H, Esaki Y, Kelly F, Yan W. Cullin3 is a KLHL10-interacting protein preferentially expressed during late spermiogenesis. *Biol Reprod*. 2006; 74:102–8. [PubMed: 16162871]
100. Zhao B, Ito K, Iyengar PV, Hirose S, Nakamura N. MARCH7 E3 ubiquitin ligase is highly expressed in developing spermatids of rats and its possible involvement in head and tail formation. *Histochem Cell Biol*. 2013; 139:447–60. [PubMed: 23104140]

Highlights

This review evaluates the E3 ligase ubiquitination of target proteins in the testis.

- An overview of the hierarchical process by which ubiquitin is transferred to its target protein and the key role of the E3 ligase for conferring substrate specificity is provided.
- The role that protein ubiquitination plays in protein degradation as well the various cellular functions such as protein trafficking, cell signaling, DNA repair, chromatin modifications, cell-cycle progression and cell death is discussed.
- Specific examples of E3 ligases in the testis and the differing roles that they play in maintaining functional spermatogenesis are highlighted.

Figure 1.

The process of ubiquitination. **(A)** Conjugation of ubiquitin (Ub) to its target protein requires the hierarchical transfer of ubiquitin from an E1 ubiquitin-activating enzyme (ATP-dependent) to an E2 ubiquitin conjugating enzyme and finally to an E3 ubiquitin ligase that confers the substrate specificity to ubiquitinate the target protein. HECT or Ring E3 ligases are the two major E3 ligase family members. A family of deubiquitinating (DUB) enzymes act to modulate the ubiquitination of proteins. **(B)** The specific lysine (K) amino acid of ubiquitin that links to the protein and the length of the ubiquitin chain determines if the ubiquitinated protein is targeted to the proteasome for degradation or if the protein has an altered function. Polyubiquitinated proteins linked to the K48 of ubiquitin are directed to the proteasome for degradation. The mammalian 26S proteasome is composed of the 19S regulatory particle and the 20S particle containing the α and β subunits that create four stacked rings of proteins with the active protease sites inside on the face of the β subunits. Proteins linked to monomers of ubiquitin at K48 or chains of ubiquitin linked by K63 have a non-proteolytic influence on the proteins activity and function.

Table 1

IDENTIFIED E3 LIGASES AND THEIR PROPOSED TESTICULAR TARGETS AND PHENOTYPE

PHYSIOLOGICAL PROCESS	E3 LIGASE	SPECIES	CELLULAR FUNCTIONS/ TARGETS	REFS
Spermiogenesis & Sperm DNA				
	Bruce	<i>Drosophila</i>	• cleavage of intracellular bridges and sperm individualization.	[46, 51, 52]
	Cullin3	mouse	• present in elongating and elongated spermatids to influence spermiogenesis.	[99]
	Cullin3 ^{testis}	<i>Drosophila</i>	• role in regulating Bruce and sperm individualization	[51, 52]
	E3 ^{Histone} /LASU1	rat, bovine	• degradation of spermatid histones.	[40]
	MARCH7	rat	• role in the shaping of the head and tail of spermatids.	[100]
	RNF8	mouse	• role in the removal of histones in spermatids.	[89]
Junctional Complexes				
	Itch	mouse	• expressed in the mouse Sertoli cell line TM4.	[64]
		mouse	• suggested to play a role in the uptake and degradation of spermatid residual cytoplasm by the Sertoli cell.	[67]
		mouse	• ubiquitinates occludin and disrupt TJ structure and function.	[63]
	β-TrCP	mouse	• controls the stability of the transcriptional regulator snail and testis E cadherin levels and AJ architecture.	[76]
Germ Cell Meiosis				
	CUL4A	mouse	• regulates meiotic prophase I of germ cells.	[81, 82]
	Itch	mouse	• regulates meiotic germ cell progression.	[67]
	RNF8	mouse	• histone ubiquitination during meiotic DNA double strand breaks.	[83, 88]
	Siah1a	mouse	• controls meiosis I progression in germ cells.	[79]
Germ Cell Apoptosis				
	Cullin3 ^{testis}	<i>Drosophila</i>	• regulates caspase activation in spermatids.	[51, 52]
	MEX	mouse	• promotes germ cell apoptosis by aiding death receptor signaling	[95]
	Itch	mouse	• loss of <i>Itch</i> gene results in activation of caspase-9 in an age-dependent manner & results in decreased sperm numbers.	[67]