

## Developmental Regulation of Ubiquitin C-Terminal Hydrolase Isozyme Expression During Spermatogenesis in Mice

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### ABSTRACT

The ubiquitin pathway functions in the process of protein turnover in eukaryotic cells. This pathway comprises the enzymes that ubiquitinate/deubiquitinate target proteins and the proteasome that degrades ubiquitin-conjugated proteins. Ubiquitin C-terminal hydrolases (UCHs) are thought to be essential for maintaining ubiquitination activity by releasing ubiquitin (Ub) from its substrates. Mammalian UCH-L1 and UCH-L3 are small proteins that share considerable homology at the amino acid level. Both of these UCHs are highly expressed in the testis/ovary and neuronal cells. Our previous work demonstrated that UCH-L1-deficient gracile axonal dystrophy (*gad*) mice exhibit progressively decreasing spermatogonial stem cell proliferation, suggesting that UCH isozymes in the testis function during spermatogenesis. To analyze the expression patterns of UCH isozymes during spermatogenesis, we isolated nearly homogeneous populations of spermatogonia, spermatocytes, spermatids, and Sertoli cells from mouse testes. Western blot analysis detected UCH-L1 in spermatogonia and Sertoli cells, whereas UCH-L3 was detected in spermatocytes and spermatids. Moreover, reverse transcription-polymerase chain reaction analysis of UCH isozymes showed that UCH-L1 and UCH-L4 mRNAs are expressed in spermatogonia, whereas UCH-L3 and UCH-L5 mRNAs are expressed mainly in spermatocytes and spermatids. These results suggest that UCH-L1 and UCH-L3 have distinct functions during spermatogenesis, namely, that UCH-L1 may act during mitotic proliferation of spermatogonial stem cells whereas UCH-L3 may function in the meiotic differentiation of spermatocytes into spermatids.

*male reproductive tract, meiosis, Sertoli cells, spermatogenesis, testis*

### INTRODUCTION

Ubiquitination of proteins is mediated by specific enzymes, namely E1 (ubiquitin-activating), E2 (ubiquitin-con-

jugating), and E3 (ubiquitin ligase) [1]. In this pathway, polyubiquitinated proteins are translocated to the proteasome and proteolytically degraded in an energy-dependent manner. The ubiquitin pathway plays important roles in regulating numerous cellular processes, including the degradation of intracellular proteins, cell-cycle regulation, stress responses, and programmed cell death [2–6]. Ubiquitin can be released from polyubiquitin chains or ubiquitin-protein conjugates via the action of deubiquitinating enzymes. These enzymes are divided into two families: ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific proteases (UBPs). UCHs remove ubiquitin from peptides or small C-terminal ubiquitin adducts only, whereas UBPs are thought to disassemble polyubiquitin chains [7, 8].

Recent studies show that there are at least four mammalian UCH isozymes, among which the residues surrounding the active site share a high degree of homology [8, 9]. Mouse *Uchl1* and *Uchl3* encode proteins of similar size that share 52% amino acid sequence identity [8, 10]. However, the distribution of these isozymes is quite distinct; UCH-L1 mRNA is selectively and highly expressed in the testis/ovary and neuronal cells [10–12], whereas UCH-L3 mRNA is expressed in all tissues, including the testis/ovary and brain [13, 14]. UCH-L1-specific antibodies stain the testis, especially spermatogonia and Sertoli cells [15–18]. In addition, UCH-L1-deficient gracile axonal dystrophy (*gad*) mutant mice exhibit pathological changes, such as progressively decreasing spermatogonial stem cell proliferation [18]. These results led us to postulate that UCH isozymes in the testis function in the development of spermatogonia into mature sperm. Spermatogenesis is a complex, highly organized process that is divided into three phases: proliferation, meiosis, and spermiogenesis [19], each of which may require the activity of specific UCH isozymes. However, our understanding of the functional roles and the localization of UCH isozymes during spermatogenesis is limited.

In the present study, we generated peptide-specific antibodies against sequences within UCH-L1 or UCH-L3 and purified developing sperm cells at each stage from testes using immunomagnetic beads followed by discontinuous Percoll gradient centrifugation [20–23]. We found that the expression level of each UCH isozyme increased during the first round of spermatogenesis, and that the isozymes exhibited differential expression in the mouse testis [24]. Our results suggest that UCH isozymes play an important role in the regulation of spermatogenesis.

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## MATERIALS AND METHODS

### Animals

The *gad* [11] and *Uchl3* knockout [13] mice were used as controls. The *gad* mouse is an autosomal recessive mutant that was obtained from the cross between CBA and RFM mice. The mutant line was maintained by intercross for more than 20 generations. The *Uchl3* knockout mouse was generated by the standard method using homologously recombinant ES cells from 129SV mice. The knockout line was back-crossed several times to C57BL/6J mice. Male Balb/c mice were purchased from Nihon CLEA, Inc. (Tokyo, Japan), and all animals were maintained at the National Institute of Neuroscience, National Center of Neurology and Psychiatry (Japan).

All mouse experiments were performed in accordance with the institution's regulations for animal care and with the approval of the Animal Investigation Committee.

### Isolation of Type A Spermatogonia and Sertoli Cells

Sequential enzymatic digestion of testicular tubules was performed as previously described [21]. After treatment with erythrocyte lysing buffer, testes from ten 2-wk-old and two 8-wk-old Balb/c mice were incubated twice for 5 min at 34°C with medium containing 0.5 mg/ml collagenase IV-S (Sigma-Aldrich, St. Louis, MO) and digested for 10 min at 34°C with medium containing 1 mg/ml trypsin (Sigma-Aldrich).

After the sequential enzymatic digestion, type A spermatogonia were isolated using immunomagnetic beads. The cells were incubated at room temperature for 15 min with biotin-conjugated rat anti-mouse CD117 (1  $\mu$ g/10<sup>6</sup> cells), which recognizes the extracellular domain of the c-kit receptor (clone 2B8; Pharmingen, San Diego, CA). The cell suspension was then centrifuged at 300  $\times$  g for 5 min and washed with Dulbecco modified Eagle medium to remove excess antibody. The cell pellet was then resuspended in 80  $\mu$ l buffer and incubated with 20  $\mu$ l MACS anti-biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) per 10<sup>7</sup> total cells at 6–12°C for 15 min. The cell suspension was then washed carefully and resuspended in 500  $\mu$ l buffer per 10<sup>8</sup> total cells. The c-kit-positive cells (type A spermatogonia) were separated with a MACS separator (Miltenyi Biotec) and collected. The suspension containing Sertoli cells was resuspended at a concentration of  $1 \times 10^6$  cells/ml in tissue culture medium containing 10% fetal calf serum.

### Sertoli Cell Culture and Purification

The suspension containing Sertoli cells obtained from testes of ten 2-wk-old mice was plated on lectin- (Datura stramonium agglutinin; Sigma, St. Louis, MO) coated dishes as described [25, 26] and incubated for 3 days at 37°C. Alkaline phosphatase activity was visualized according to the procedure of Cox and Singer [27]. Cultured cells were incubated in reaction buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl<sub>2</sub>, pH 9.5) containing 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.33 mg/ml nitro blue tetrazolium chloride.

### Isolation of Spermatocytes and Spermatids

After isolating type A spermatogonia from testes of 8-wk-old mice using immunomagnetic beads, the testicular cell suspension was separated by discontinuous Percoll (Amersham Biosciences, Piscataway, NJ) gradient centrifugation [20]. The recovered cell populations were analyzed by phase-contrast microscopy [28], fluorescence-activated cell sorting (FACS) [29], and reverse transcription-polymerase chain reaction (RT-PCR).

### RT-PCR Analysis of Isolated Cells Expressing Specific Marker Genes

From each population of spermatogonia, spermatocytes, spermatids, and Sertoli cells isolated from testicular cell suspensions, mRNAs were extracted using the QuickPrep micro mRNA purification kit (Amersham Biosciences) and subjected to reverse transcription with a first-strand cDNA synthesis kit (Amersham Biosciences) according to the manufacturer's instructions. PCR was performed with the following primers; c-kit [30], 5'-AAGATTTGCGATTTCGGGC-3' and 5'-CTGAAAATGCTCTCTGGTGCC-3'; Histone H1t [7], 5'-GTCCAGCTCTTGACCATGTGCG-3' and 5'-GCTTTTCCCTCGCCTTTTAG-3'; SP-10 [31], 5'-TTTATCTGCTTGATCTGCCC-3', and 5'-GCTTGAAAGTTGCTGAACCG-3'; stem cell factor (SCF) [32], 5'-ATAGGAAAGCCGCAAAGGC-3' and 5'-TTACAAGCGAAATGAGAGCCG-3'; and glyceraldehyde-3-phosphate

dehydrogenase [33]. Each sample was amplified using the AmpliTaq Gold GeneAmp system (Applied Biosystems, Foster City, CA).

### FACS Analysis of Isolated Cells

FACS analysis was performed as described by Malkove et al. [29, 34]. Briefly, isolated spermatogenic cells were fixed in 70% ethanol overnight at 4°C and then incubated in propidium iodide staining solution (50  $\mu$ g/ml and 100 U/ml RNase A in PBS) for 30 min at room temperature. Within 2 h poststaining, the isolated spermatogenic cells were analyzed by FACS (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). Excitation was at 488 nm and emission was at  $\sim$ 600 nm.

### Quantitative mRNA Analysis of UCH Isozyme Genes by Real-Time PCR

SYBR Green-based real-time quantitative RT-PCR (ABI PRISM 7700 Sequence detection system, Columbia, MD) was performed [33, 35] in SYBR Green Master mix using the following primers; UCH-L1, 5'-TTCTGTTCACAACGTGGACG-3' and 5'-TCACTGGAAAGGGCATTCG-3'; UCH-L3, 5'-TGAAGGTGACTGAGGCACC-3' and 5'-AATTGGAAATGGTTTCCGTCC-3'; UCH-L4, 5'-AAACAACCATCAGCAATGCC-3' and 5'-GACCCTGATTCAAAGTGCACC-3'; UCH-L5, 5'-TTTTCTTTTCAAGTGGCAGCC-3' and 5'-GATAGCCTGAGTGGCACAAGC-3'; and  $\beta$ -actin, 5'-CGTGCCTGACATCAAAGAGAA-3' and 5'-CAATAGTGATGACCTGGCCGT-3'. For comparing relative UCH isozyme gene expression in isolated germ cells and Sertoli cells, the formula  $2^{-\Delta\Delta C_t}$  was used to calculate relative expression levels compared with spermatogonia of two-week-old mice. For comparing the expression level of UCH isozyme genes in the time course of testicular maturation, the formula  $2^{-\Delta\Delta C_t}$  was used to calculate relative expression levels compared with the testes of 5-day-old mice.

### Western Blotting of UCH-L1 and UCH-L3 from Isolated Cells

Total protein was extracted [24] from isolated testicular cells or whole testes of 5-, 7-, 15-, 19-, 21-, 23-, 26-, and 33-day-old Balb/c mice. Control extracts were obtained from testes of *gad* and *Uchl3* knockout mice. For preparation of antibodies, we designed two specific peptides, AQHEN-FRKKQIEELKGQEVSPK (R891A, GenBank no. NP\_057932, residues 57–78) and EKYEVFRTEEEIKISQGGQDVTS (R837A, GenBank no. NP\_035800, residues 60–83) corresponding to mouse UCH-L1 and UCH-L3, respectively. Polyclonal antibodies against R891A and R837A were raised in rabbits and the IgG fraction was isolated (Tana Laboratories, L.C., Houston, TX). Each sample was adjusted to 5  $\mu$ g protein/10  $\mu$ l and subjected to SDS-PAGE (15% acrylamide; XV Pantera gel; DRC, Tama, Japan). After transferring the proteins to a nitrocellulose membrane and blocking with 5% skim milk, the membranes were incubated at 4°C overnight with the primary antibody to UCH-L1 (1:1000) or UCH-L3 (1:400). The membranes were then incubated with peroxidase-conjugated goat anti-rabbit IgG (H+L) (1:10000; Pierce, Rockford, IL) for 60 min at room temperature. Immunoreactivity was visualized using the SuperSignal detection kit (Pierce) and analyzed with a ChemiImager (Alpha Innotech, San Leandro, CA).

### Immunohistochemistry of UCH-L1 and UCH-L3

Tissues were fixed in vivo with 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin, and sections (4  $\mu$ m thickness) were treated with absolute methanol containing 3% H<sub>2</sub>O<sub>2</sub> for 30 min to block endogenous peroxidase activity. After blocking with 10% goat serum for 1 h at room temperature, the sections were incubated at 4°C overnight with antibodies to UCH-L1 (1:500) or UCH-L3 (1:200) diluted in PBS containing 1% BSA. The sections were then incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:200, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature and examined by confocal laser scanning microscopy (Olympus, Tokyo, Japan).

## RESULTS

### Isolation of Spermatogenic Cells and Sertoli Cells

Using the c-kit antibody, we isolated an average of  $17 \times 10^5$  type A spermatogonia from ten 2-wk-old juvenile mice and an average of  $3.5 \times 10^5$  type A spermatogonia

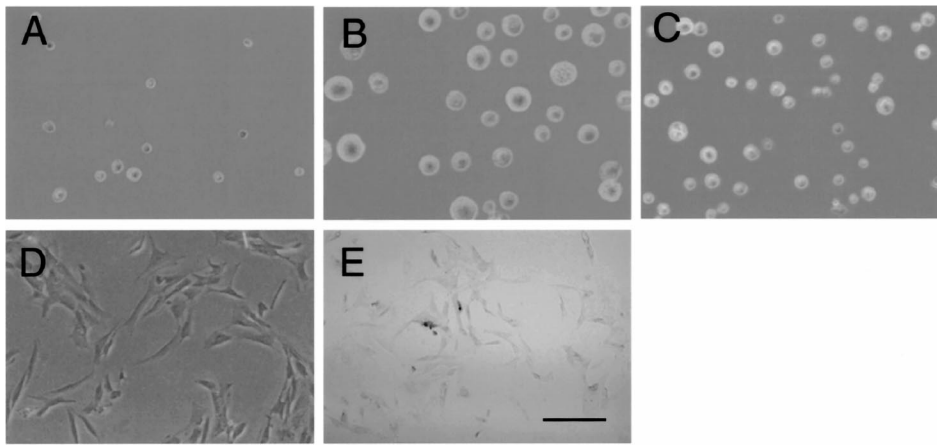


FIG. 1. Phase-contrast micrographs showing isolated germ cells and Sertoli cells from a testicular cell suspension. **A)** Type A spermatogonia. **B)** Spermatocytes. **C)** Spermatids. **D)** Sertoli cells. **E)** Alkaline phosphatase activity of Sertoli cell cultures. Germ cells in **A–C** were from testes of 8-wk-old mice. Sertoli cells in **D** and **E** were from testes of 2-wk-old mice. Scale bar = 30  $\mu$ m.

from two 8-wk-old adult mice. The purity of each isolated population was <84.5% (Fig. 1A and Table 1). Using phase-contrast microscopy, we determined that these cells were 14–16  $\mu$ m in diameter. Following elimination of the remaining spermatogonia using magnetic microbeads, the remaining cells were separated into two principal visible bands by centrifugation through a discontinuous Percoll gradient. Phase-contrast microscopy revealed that these two bands consisted primarily of pachytene spermatocytes and round spermatids (Fig. 1, B and C). We isolated an average of  $3 \times 10^5$  spermatocytes and  $200 \times 10^5$  spermatids from two 8-wk-old adult mice. We also isolated an average  $20 \times 10^5$  Sertoli cells from ten 2-wk-old juvenile mice (Fig. 1D). Peritubular myoid cells were identified by alkaline phosphatase staining. Although >95% of these cells were unstained, ~5% of the Sertoli cells showed considerable alkaline phosphatase activity (Fig. 1E).

#### RT-PCR Analysis of Isolated Cells Expressing Specific Marker Genes

RT-PCR using extracts of isolated cells confirmed the expression of mRNAs from *c-kit*, Histone H1t, *SP-10*, and *SCF*. Each gene was expressed in only one cell type, as follows: *c-kit* in spermatogonia, the Histone H1t gene in spermatocytes, *SP-10* in spermatids, and *SCF* in Sertoli cells (Fig. 2).

#### Characterization of Isolated Cells by FACS

To determine the purity of isolated germ cells, we used FACS analysis to follow the differentiation-dependent acquisition of stage-specific patterns during spermatogenesis and monitored the percentage of cells with differing DNA content. The histogram in Figure 3 presents the number of

cells at each fluorescence level (FL2-A). Diploid (2n DNA) cells were observed in 84.5% of the isolated spermatogonia, tetraploid (4n DNA) cells were observed in 79.6% of the isolated spermatocytes, and haploid (1n DNA) cells were observed in 85.3% of the isolated spermatids (Table 1). These results demonstrate that the majority of cells in each population exhibited the expected ploidy.

#### Expression of UCH Isozymes During Spermatogenesis

We characterized the expression pattern of each UCH gene in isolated testicular cell populations during spermatogenesis using SYBR Green-based real-time quantitative RT-PCR (Fig. 4). The  $2^{-ddCt}$  values indicate the relative mRNA expression levels compared with spermatogonia from 2-wk-old mice (Sg2). The genes encoding UCH-L1 and UCH-L4 were expressed mainly in spermatogonia (Fig. 4, A and C). UCH-L1 was also expressed significantly in the Sertoli population, and UCH-L4 was expressed to a lesser degree in spermatocytes and spermatids. UCH-L3 and UCH-L5 genes were expressed primarily in the spermatid population and to a much lesser extent in spermatocytes (Fig. 4, B and D). We further examined the expression of UCH mRNAs during testicular maturation (Fig. 5A) in whole testes from 5-, 7-, 15-, 19-, 21-, 23-, 26-, and 33-

TABLE 1. The percentage of 1n, 2n, and 4n cells in isolated germ cells from a testicular cell suspension from 8-wk-old mice, as determined by FACS.<sup>a</sup>

Ploidy <sup>b</sup>	Total cells (%) <sup>c</sup>	Isolated germ cells (%)		
		Spermatogonia	Spermatocytes	Spermatids
1n	56.2 $\pm$ 4.2	9.4 $\pm$ 1.2	5.3 $\pm$ 1.8	85.3 $\pm$ 3.3
2n	25.2 $\pm$ 3.6	84.5 $\pm$ 2.7	9.3 $\pm$ 1.3	6.2 $\pm$ 0.9
4n	18.6 $\pm$ 3.1	12.7 $\pm$ 0.9	79.6 $\pm$ 2.5	7.7 $\pm$ 2.2

<sup>a</sup> Calculated after eliminating cell debris (FL2-A < 50 in Fig. 2).

<sup>b</sup> 1n, haploid cells (one copy of genome); 2n, diploid cells (two copies of genome); 4n, tetraploid cells (four copies of genome).

<sup>c</sup> Each percentage represents the mean  $\pm$  SD of 4–6 measurements.

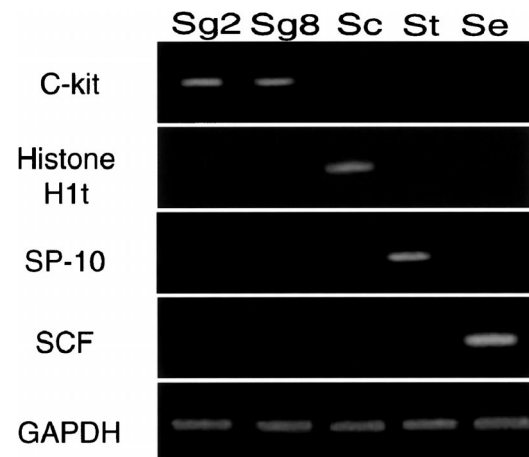


FIG. 2. Expression of *c-kit*, Histone H1t, *SP-10*, and *SCF* mRNAs determined by RT-PCR of extracts of isolated germ cells and Sertoli cells from a testicular cell suspension. Sg2, Spermatogonia of 2-wk-old mice; Sg8, spermatogonia from 8-wk-old mice; Sc, spermatocytes from 8-wk-old mice; St, spermatids from 8-wk-old mice; Se, Sertoli cells from 2-wk-old mice.



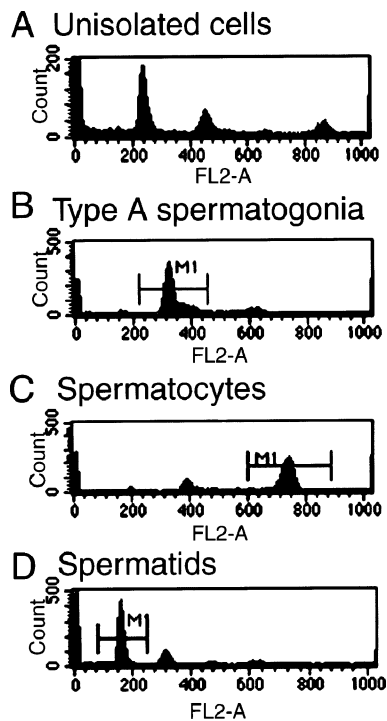


FIG. 3. FACS analysis of isolated germ cells from a testicular cell suspension obtained from 8-wk-old mice. Each window is a histogram representing the number of cells at each fluorescence level (FL2-A). **A)** Unisolated cells. **B)** Type A spermatogonia. **C)** Spermatocytes. **D)** Spermatids.

day-old mice. The UCH-L1 and UCH-L4 mRNAs were expressed similarly during development and likewise for the UCH-L3 and UCH-L5 genes. The expression of UCH-L1 mRNA appears relatively high on Postnatal Day 15. However, the percentage of spermatogonia and Sertoli cells would have become diluted by meiotic and postmeiotic germ cells after Day 15, thereby accounting for the relatively lower levels of UCH-L1 at subsequent time points. RT-PCR data suggest that UCH-L1 mRNA expression in spermatogonia and Sertoli cells increased continuously even after Postnatal Day 15 (Fig. 5B). We also analyzed the protein expression patterns of UCH-L1 and UCH-L3 in

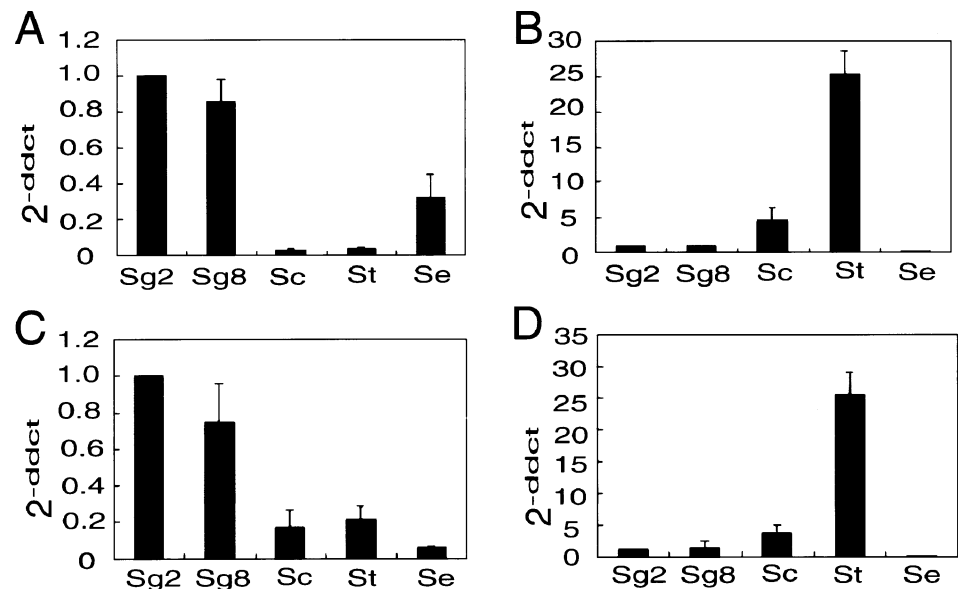
the mouse testis using peptide-specific antibodies that recognize regions in mouse UCH-L1 or UCH-L3. Western blot analysis detected UCH-L1 in spermatogonia and Sertoli cells, and UCH-L3 was detected primarily in spermatocytes and spermatids, as expected, although it was also found to a lesser extent in spermatogonia (Fig. 6A). Also, UCH-L3 expression increased in a differentiation-dependent manner during juvenile spermatogenesis (Fig. 5C). Immunohistochemistry detected homogeneous UCH-L1 expression in both spermatogonia and Sertoli cells from wild-type (Balb/c) and *Uchl3* knockout mice, whereas UCH-L3 was detected mainly in spermatocytes and round spermatids of wild-type (Balb/c) and *gad* mice (Fig. 6, B and C).

## DISCUSSION

The ubiquitin pathway plays critical roles in the progression of spermatogenesis through the mitotic, meiotic, and postmeiotic phases [2, 3, 18, 36]. Because numerous proteins are regulated by ubiquitination, mutations that affect the ubiquitin pathway result in the dysregulation of multiple cellular processes and induce apoptosis during spermatogenesis [3, 37–39]. For example, mutation of HR6B, a ubiquitin-conjugating enzyme, affects both meiosis and postmeiotic germ cell development [40–43].

Ubiquitin C-terminal hydrolases catalyze the hydrolysis of C-terminal esters and amides of ubiquitin. These enzymes are believed to play a key role in processing polyubiquitin and ubiquitylated proteolytic peptides [8]. The genes for at least four UCHs, UCH-L1 and UCH-L3–5, have been identified in the mouse. Although the specificity and function of these isozymes in spermatogenesis remains elusive, each of these enzymes contains conserved residues that are critical for enzymatic activity [8–10, 44, 45]. The predominant mouse isozymes, UCH-L1 and UCH-L3, share 52% amino acid sequence identity [10, 13, 45]. However, UCH-L1 mRNA is selectively expressed in the mouse testis and nervous systems [11], whereas UCH-L3 mRNA is expressed in nearly every tissue tested, with high levels in the testis [13]. Intracellular localization of UCH-L1 was reported to be closely associated with the proliferative activity of spermatogonia and Sertoli cells [15–18]. In contrast, the expression of UCH-L3 has not been examined in the testis. To address this question, we first generated poly-

FIG. 4. Comparison of the relative UCH isozyme gene expression levels ( $2^{-ddct}$ ) in isolated germ cells and Sertoli cells using RT-PCR. The formula  $2^{-ddct}$  indicates the relative expression level in isolated testicular cells compared with Sg2 cells. Sg2 gene expression in each case was set to 1.0. **A)** *Uchl1*. **B)** *Uchl3*. **C)** *Uchl4*. **D)** *Uchl5*. Sg2, Spermatogonia from 2-wk-old mice; Sg8, spermatogonia from 8-wk-old mice; Sc, spermatocytes from 8-wk-old mice; St, spermatids from 8-wk-old mice; Se, Sertoli cells from 2-wk-old mice.



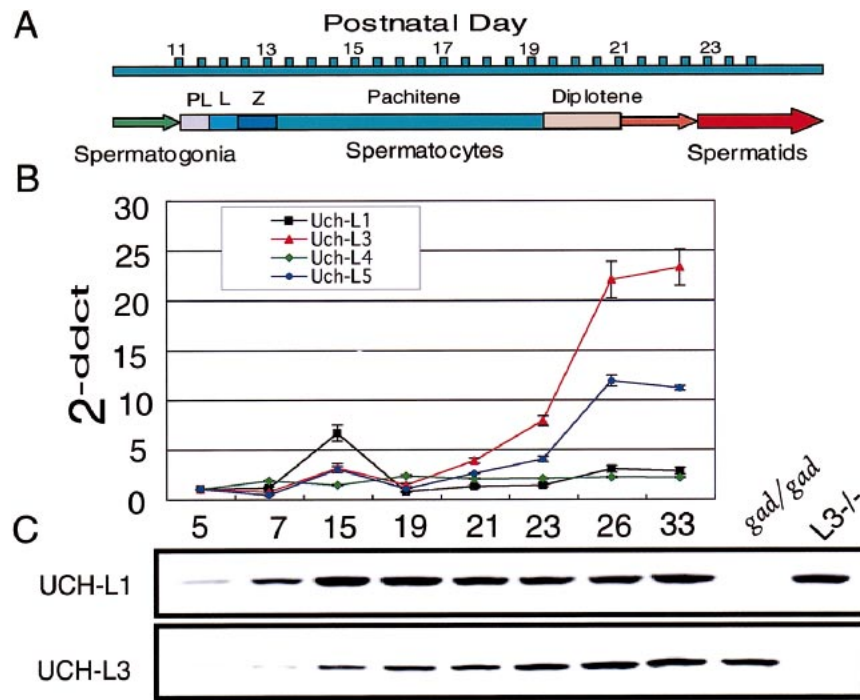


FIG. 5. Expression of UCH isozymes during testicular maturation. **A**) Timetable for juvenile spermatogenesis. PL, Preleptotene spermatocyte; L, leptotene spermatocyte; Z, zygotene spermatocyte. **B**) Comparison of the relative UCH isozyme gene expression levels ( $2^{-ddct}$ ) by SYBR Green-based real-time quantitative RT-PCR. The value for gene expression from the testes of 5-day-old mice was set to 1.0. **C**) Comparison of UCH-L1 and UCH-L3 expression by Western blotting. Each lane represents the testes of 5-, 7-, 15-, 19-, 21-, 23-, 26-, or 33-day-old Balb/c mice, *gad* mice, and *Uchl3* knockout mice (*L3<sup>-/-</sup>*) (**B**, **C**).

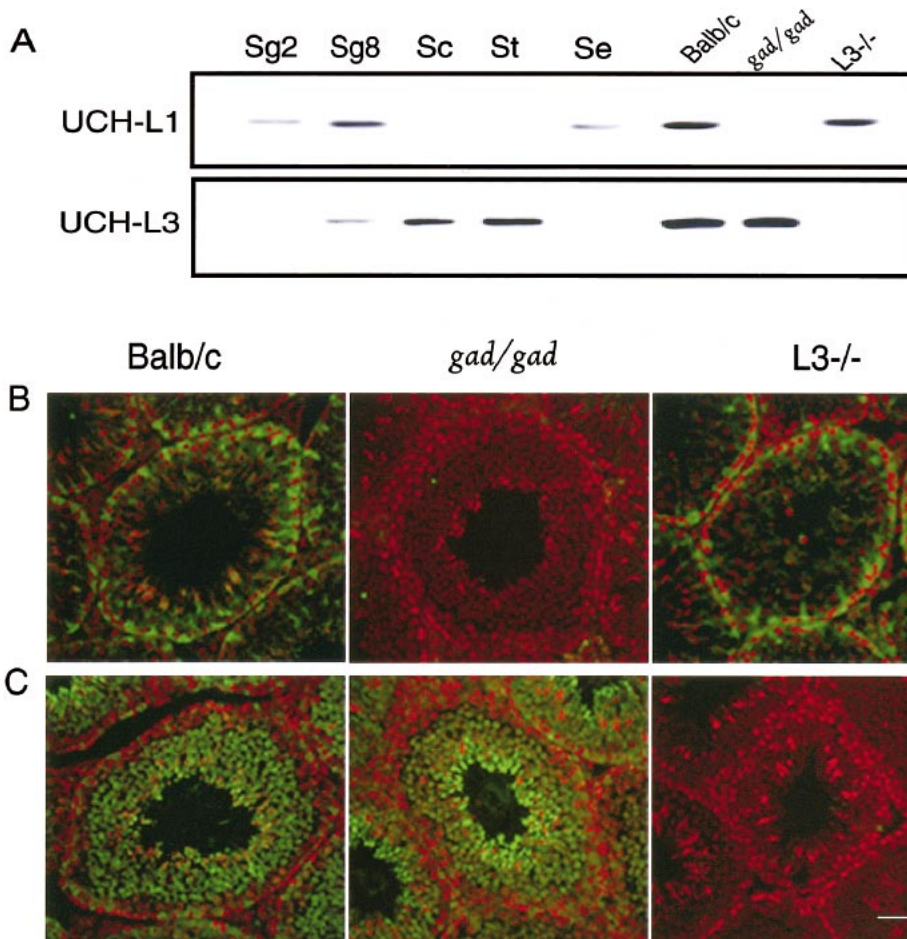


FIG. 6. Analysis of UCH-L1 and UCH-L3 expression by western blotting and immunohistochemistry. **A**) UCH-L1 and UCH-L3 expression in isolated germ cells and Sertoli cells. Sg2, Spermatogonia from 2-wk-old mice; Sg8, spermatogonia from 8-wk-old mice; Sc, spermatocytes from 8-wk-old mice; St, spermatids from 8-wk-old mice; Se, Sertoli cells from 2-wk-old mice. Balb/c, testis from a Balb/c mouse; *gad/gad*, testis from a *gad* mouse (*Uchl1* knockout mouse); *L3<sup>-/-</sup>*, testis from a *Uchl3* knockout mouse. Immunohistochemistry of UCH-L1 (**B**) and UCH-L3 (**C**) in the testis of wild type, *gad*, and *Uchl3* knockout mouse. Stages V–VIII of seminiferous epithelium predominate in each panel as visualized by PAS staining of serial sections. Green fluorescence represents UCH-L1 (**B**) and UCH-L3 (**C**); red fluorescence represents staining of cell nuclei by propidium iodide (PI). Magnification,  $\times 200$ . Scale bar = 30  $\mu$ m.

clonal antibodies that specifically react with mouse UCH-L3. Using RT-PCR and western blotting, we detected high levels of UCH-L3 mRNA and protein in meiotic pachytene spermatocytes and postmeiotic spermatids during spermatogenesis (Figs. 4B and 6A). These results suggest that UCH-L1 and UCH-L3 may play distinct roles in spermatogenesis, in that UCH-L1 may function in mitotic proliferation, whereas UCH-L3 may function in the meiotic differentiation of spermatocytes into spermatids.

Around Postnatal Day 15, the higher expression of UCH-L1 mRNA suggests that UCH-L1 might have certain functions during testicular maturation. Furthermore, the increased expression of UCH-L1 after Postnatal Day 15 suggests that it might play an active role in mitotic proliferation. In addition to the *Uchl1* genes, we also analyzed transcription from two other UCH isozyme genes, *Uchl3* and *Uchl5*, in isolated testicular cells. UCH-L3 and UCH-L5 mRNAs were found in meiotic pachytene spermatocytes and postmeiotic spermatids (Fig. 4, B and D) and showed similar expression patterns during the course of testicular maturation (Fig. 5B). These results suggest that the *Uchl3* and *Uchl5* genes may have overlapping functions during spermatogenesis. The ubiquitin pathway is very active during the postmitotic phase of spermatogenesis [3, 36]. Thus, UCH-L3 may function to regulate the cell cycle and chromatin structure during the meiotic phase but may facilitate the en masse degradation of cytoplasmic proteins as well as organelle/nuclear remodeling during the postmeiotic phase. The present study demonstrates for the first time that UCH-L3 is expressed mainly in meiotic spermatocytes and postmeiotic spermatids during spermatogenesis. Although UCH-L3 shares considerable sequence homology with UCH-L1, the hydrolytic activity of UCH-L3 in vitro differs substantially from that of UCH-L1. The rate of UCH-L3-mediated catalysis ( $K_{cat}$ ) is more than 200 times greater than UCH-L1 using ubiquitin amide as a substrate [46]. This relatively high activity of UCH-L3 is consistent with its expression during the postmitotic phase of spermatogenesis, in that maturation from spermatocytes to spermatids may be critically dependent on the ubiquitin pathway despite the fact that *Uchl3* knockout mice exhibit normal fertility [13].

In conclusion, our results demonstrate that the expression of UCH isozymes is differentially and developmentally regulated during spermatogenesis and that UCH-L1 and UCH-L3 likely have distinct functions during different developmental phases. These results enhance our understanding of how the ubiquitin pathway is regulated by UCH isozymes during spermatogenesis. Moreover, isolation of mouse germ cells and Sertoli cells from testes may afford the opportunity to assess UCH isozyme function during spermatogenesis in vitro. UCH-L1 has been suggested to associate with monoubiquitin and thereby increase the half-life of ubiquitin in neurons [47]. In addition, a UCH-L1 ubiquitinyl ligase-like activity has also been proposed [46]. Further biochemical and genetic analyses of UCH family members will help elucidate the role of UCHs in the complex molecular mechanisms involved in spermatogenesis.

## REFERENCES

- Weissman AM. Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* 2001; 2:169–178.
- Baarends WM, Roest HP, Grootegoed JA. The ubiquitin system in gametogenesis. *Mol Cell Endocrinol* 1999; 151:5–16.
- Baarends WM, van der Laan R, Grootegoed JA. Specific aspects of the ubiquitin system in spermatogenesis. *J Endocrinol Invest* 2000; 23:597–604.
- Ciechanover A, Orian A, Schwartz AL. Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays* 2000; 22:442–451.
- Varshavsky A. The ubiquitin system. *Trends Biochem Sci* 1997; 22:383–387.
- Wilkinson KD. Roles of ubiquitinylation in proteolysis and cellular regulation. *Annu Rev Nutr* 1995; 15:161–189.
- Grimes SR, Wilkerson DC, Noss KR, Wolfe SA. Transcriptional control of the testis-specific histone H1t gene. *Gene* 2003; 304:13–21.
- Wilkinson KD. Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *FASEB J* 1997; 11:1245–1256.
- Mayer AN, Wilkinson KD. Detection, resolution, and nomenclature of multiple ubiquitin carboxyl-terminal esterases from bovine calf thymus. *Biochemistry* 1989; 28:166–172.
- Kurihara LJ, Kikuchi T, Wada K, Tilghman SM. Loss of Uch-L1 and Uch-L3 leads to neurodegeneration, posterior paralysis and dysphagia. *Hum Mol Genet* 2001; 10:1963–1970.
- Saigoh K, Wang YL, Suh JG, Yamanishi T, Sakai Y, Kiyosawa H, Harada T, Ichihara N, Wakana S, Kikuchi T, Wada K. Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice. *Nat Genet* 1999; 23:47–51.
- Wilkinson KD, Deshpande S, Larsen CN. Comparisons of neuronal (PGP 9.5) and non-neuronal ubiquitin C-terminal hydrolases. *Biochem Soc Trans* 1992; 20:631–637.
- Kurihara LJ, Semenova E, Levorse JM, Tilghman SM. Expression and functional analysis of Uch-L3 during mouse development. *Mol Cell Biol* 2000; 20:2498–2504.
- Zhang N, Wilkinson K, Bownes M. Cloning and analysis of expression of a ubiquitin carboxyl terminal hydrolase expressed during oogenesis in *Drosophila melanogaster*. *Dev Biol* 1993; 157:214–223.
- Wilson PO, Barber PC, Hamid QA, Kikuchi T, Wada K, Dhillon AP, Rode J, Day IN, Thompson RJ, Polak JM. The immunolocalization of protein gene product 9.5 using rabbit polyclonal and mouse monoclonal antibodies. *Br J Exp Pathol* 1988; 69:91–104.
- Tokunaga Y, Imai S, Torii R, Maeda T. Cytoplasmic liberation of protein gene product 9.5 during the seasonal regulation of spermatogenesis in the monkey (*Macaca fuscata*). *Endocrinology* 1999; 140:1875–1883.
- Kon Y, Endoh D, Iwanaga T. Expression of protein gene product 9.5, a neuronal ubiquitin C-terminal hydrolase, and its developing change in Sertoli cells of mouse testis. *Mol Reprod Dev* 1999; 54:333–341.
- 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. *Exp Anim* 2003; 52:1–9.
- Loveland KL, Schlatt S. Stem cell factor and c-kit in the mammalian testis: lessons originating from mother nature's gene knockouts. *J Endocrinol* 1997; 153:337–344.
- Lassalle B, Ziyat A, Testart J, Finaz C, Lefevre A. Flow cytometric method to isolate round spermatids from mouse testis. *Hum Reprod* 1999; 14:388–394.
- van der Wee KS, Johnson EW, Dirami G, Dym TM, Hofmann MC. Immunomagnetic isolation and long-term culture of mouse type A spermatogonia. *J Androl* 2001; 22:696–704.
- van Pelt AM, Morena AR, van Dissel-Emiliani FM, Boitani C, Gaemers IC, de Rooij DG, Stefanini M. Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. *Biol Reprod* 1996; 55:439–444.
- von Schonfeldt V, Krishnamurthy H, Foppiani L, Schlatt S. Magnetic cell sorting is a fast and effective method of enriching viable spermatogonia from Djungarian hamster, mouse, and marmoset monkey testes. *Biol Reprod* 1999; 61:582–589.
- Kato S, Kobayashi T, Kusuda K, Nishina Y, Nishimune Y, Yomogida K, Yamamoto M, Sakagami H, Kondo H, Ohnishi M, Chida N, Yanagawa Y, Tamura S. Differentiation-dependent enhanced expression of protein phosphatase 2C $\beta$  in germ cells of mouse seminiferous tubules. *FEBS Lett* 1996; 396:293–297.
- Anthony CT, Skinner MK. Cytochemical and biochemical characterization of testicular peritubular myoid cells. *Biol Reprod* 1989; 40:811–823.
- Scarpino S, Morena AR, Petersen C, Froya B, Soder O, Boitani C. A rapid method of Sertoli cell isolation by DSA lectin, allowing mitotic analyses. *Mol Cell Endocrinol* 1998; 146:121–127.
- Cox WG, Singer VL. A high-resolution, fluorescence-based method for localization of endogenous alkaline phosphatase activity. *J Histochem Cytochem* 1999; 47:1443–1456.



28. Bellve AR. Purification, culture, and fractionation of spermatogenic cells. *Methods Enzymol* 1993; 225:84–113.
29. Malkov M, Fisher Y, Don J. Developmental schedule of the postnatal rat testis determined by flow cytometry. *Biol Reprod* 1998; 59:84–92.
30. Sorrentino V, Giorgi M, Geremia R, Besmer P, Rossi P. Expression of the c-kit proto-oncogene in the murine male germ cells. *Oncogene* 1991; 6:149–151.
31. Kurth BE, Wright RM, Flickinger CJ, Herr JC. Stage-specific detection of mRNA for the sperm antigen SP-10 in human testes. *Anat Rec* 1993; 236:619–625.
32. Mauduit C, Hamamah S, Benahmed M. Stem cell factor/c-kit system in spermatogenesis. *Hum Reprod Update* 1999; 5:535–545.
33. Aoki K, Sun YJ, Aoki S, Wada K, Wada E. Cloning, expression, and mapping of a gene that is upregulated in adipose tissue of mice deficient in bombesin receptor subtype-3. *Biochem Biophys Res Commun* 2002; 290:1282–1288.
34. Janca FC, Jost LK, Evenson DP. Mouse testicular and sperm cell development characterized from birth to adulthood by dual parameter flow cytometry. *Biol Reprod* 1986; 34:613–623.
35. Aoki S, Su Q, Li H, Nishikawa K, Ayukawa K, Hara Y, Namikawa K, Kiryu-Seo S, Kiyama H, Wada K. Identification of an axotomy-induced glycosylated protein, AIGP1, possibly involved in cell death triggered by endoplasmic reticulum-Golgi stress. *J Neurosci* 2002; 22:10751–10760.
36. 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.
37. Wing SS. Deubiquitinating enzymes—the importance of driving in reverse along the ubiquitin-proteasome pathway. *Int J Biochem Cell Biol* 2003; 35:590–605.
38. Guardavaccaro D, Kudo Y, Boulaire J, Barchi M, Busino L, Donzelli M, Margottin-Goguet F, Jackson PK, Yamasaki L, Pagano M. Control of meiotic and mitotic progression by the F box protein beta-Trop1 in vivo. *Dev Cell* 2003; 4:799–812.
39. van Baren MJ, van der Linde HC, Breedveld GJ, Baarends WM, Rizu P, de Graaff E, Oostra BA, Heutink P. A double RING-H2 domain in RNF32, a gene expressed during sperm formation. *Biochem Biophys Res Commun* 2002; 292:58–65.
40. Escalier D, Bai XY, Silvius D, Xu PX, Xu X. Spermatid nuclear and sperm periaxonemal anomalies in the mouse Ube2b null mutant. *Mol Reprod Dev* 2003; 65:298–308.
41. Baarends WM, Wassenaar E, Hoogerbrugge JW, van Cappellen G, Roest HP, Vreeburg J, Ooms M, Hoeijmakers JH, Grootegoed JA. Loss of HR6B ubiquitin-conjugating activity results in damaged synaptonemal complex structure and increased crossing-over frequency during the male meiotic prophase. *Mol Cell Biol* 2003; 23:1151–1162.
42. Roest HP, van Klaveren J, de Wit J, van Gurp CG, Koken MH, Vermey M, van Roijen JH, Hoogerbrugge JW, Vreeburg JT, Baarends WM, Bootsma D, Grootegoed JA, Hoeijmakers JH. Inactivation of the HR6B ubiquitin-conjugating DNA repair enzyme in mice causes male sterility associated with chromatin modification. *Cell* 1996; 86:799–810.
43. Escalier D. New insights into the assembly of the periaxonemal structures in mammalian spermatozoa. *Biol Reprod* 2003; 69:373–378.
44. Hershko A, Ciechanover A. The ubiquitin system for protein degradation. *Annu Rev Biochem* 1992; 61:761–807.
45. Osawa Y, Wang YL, Osaka H, Aoki S, Wada K. Cloning, expression, and mapping of a mouse gene, Uchl4, highly homologous to human and mouse Uchl3. *Biochem Biophys Res Commun* 2001; 283:627–633.
46. Liu Y, Fallon L, Lashuel HA, Liu Z, Lansbury PT Jr. The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. *Cell* 2002; 111:209–218.
47. Osaka H, Wang YL, Takada K, Takizawa S, Setsue R, Li H, Sato Y, Nishikawa K, Sun YJ, Sakurai M, Harada T, Hara Y, Kimura I, Chiba S, Namikawa K, Kiyama H, Noda M, Aoki S, Wada K. Ubiquitin carboxy-terminal hydrolase L1 binds to and stabilizes monoubiquitin in neuron. *Hum Mol Genet* 2003; 12:1945–1958.