American Journal of Pathology, Vol. 165, No. 4, October 2004

Cell Injury, Repair, Aging and Apoptosis

Two Closely Related Ubiquitin C-Terminal Hydrolase Isozymes Function as Reciprocal Modulators of Germ Cell Apoptosis in Cryptorchid Testis

Jungkee Kwon,*† Yu-Lai Wang,* Rieko Setsuie,*‡ Satoshi Sekiguchi,† Yae Sato,*‡ Mikako Sakurai,*‡ Mami Noda,‡ Shunsuke Aoki,* Yasuhiro Yoshikawa,† and Keiji Wada*

From the Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo; the Department of Biomedical Science,† Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo; and the Laboratory of Pathophysiology,‡ Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan*

The experimentally induced cryptorchid mouse model is useful for elucidating the *in vivo* molecular mechanism of germ cell apoptosis. Apoptosis, in general, is thought to be partly regulated by the ubiquitin-proteasome system. Here, we analyzed the function of two closely related members of the ubiquitin C-terminal hydrolase (UCH) family in testicular germ cell apoptosis experimentally induced by cryptorchidism. The two enzymes, UCH-L1 and UCH-L3, deubiquitinate ubiquitin-protein conjugates and control the cellular balance of ubiquitin. The testes of gracile axonal dystrophy (*gad*) mice, which lack UCH-L1, were resistant to cryptorchid stress-related injury and had reduced ubiquitin levels. The level of both anti-apoptotic (Bcl-2 family and XIAP) and prosurvival (pCREB and BDNF) proteins was significantly higher in *gad* mice after cryptorchid stress. In contrast, *Uchl3* knockout mice showed profound testicular atrophy and apoptotic germ cell loss after cryptorchid injury. Ubiquitin level was not significantly different between wild-type and *Uchl3* knockout mice, whereas the levels of Nedd8 and the apoptotic proteins p53, Bax, and caspase3 were elevated in *Uchl3* knockout mice. These results demonstrate that UCH-L1 and UCH-L3 function differentially to regulate the cellular levels of anti-apoptotic, prosurvival, and apoptotic proteins during testicular germ cell apoptosis. *(Am J Pathol 2004, 165:1367–1374)*

In the ubiquitin-proteasome system, the levels of poly- and monoubiquitin are strictly controlled by the balance of two groups of specific enzymes: ubiquitinating enzymes (E1, E2, and E3) and deubiquitinating enzymes (DUBs).[1,2] DUBs are subdivided into ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific proteases (UBPs).[3,4] The genes for at least four UCHs, UCH-L1 and UCH-L3, UCH-L4, and UCH-L5, have been identified in mice.[5,6] Among them, UCH-L1 and UCH-L3 predominate; these isozymes have 52% amino acid identity and share significant structural similarity;[7] however, the distribution of these two isozymes is quite distinct in that UCH-L3 mRNA is expressed ubiquitously whereas UCH-L1 mRNA is selectively expressed in the testis/ovary and neuronal cells.[7–10] Despite the high-sequence homology, the *in vitro* hydrolytic activities of these two enzymes differ significantly. The activity (Kcat) of UCH-L3 is more than 200-fold higher than UCH-L1 when a fluorogenic ubiquitin substrate is used.[11] In addition to its relatively weak hydrolase activity, UCH-L1 exhibits dimerization-dependent ubiquityl ligase activity.[11] In contrast, UCH-L3 has little or no ligase activity compared with UCH-L1.[11] It was recently suggested that UCH-L1 has anti-proliferative activity in tumor cells, and that its expression is induced in response to tumor growth.[12] Furthermore, UCH-L1 associates with monoubiquitin and prolongs ubiquitin half-life in neurons.[13] Other work demonstrated that UCH-L3 binds to Nedd8 and subsequently processes its C-terminus.[14] Nedd8 is a small ubiquitin-like protein that shares with ubiquitin the ability to be conjugated to a lysine residue in a substrate protein.[15] Covalent conjugation of proteins by Nedd8 is an important form of the posttranscriptional modification and plays a critical role in many cellular processes.[16] These conju-

Supported by Grants-in-Aid for Scientific Research from the Ministry of Health, Labor, and Welfare of Japan; Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; a grant from Pharmaceuticals and Medical Devices Agency of Japan; and a grant from Japan Science and Technology Agency.

Accepted for publication June 24, 2004.

Address reprint requests to Keiji Wada, Department of Degenerative Neurological Disease, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, 187-8502, Japan. E-mail: wada@ncnp.go.jp.

gates are regulated by a large number of deconjugating enzymes. This activity is unique to UCH-L3 because UCH-L1 is relatively weak to cleave the C terminus of Nedd8.[14–16] Collectively, these data suggest that the two mouse isozymes, UCH-L1 and UCH-L3, have distinct but overlapping functions. In addition, we recently found that *gad* mice, which lack UCH-L1 expression, show reduced retinal cell apoptosis in response to ischemia, suggesting that UCH-L1 may promote apoptosis.[17]

Our previous work focused on the possibility that UCH-L1 and UCH-L3 exhibit functional diversity during spermatogenesis. We showed that both UCH-L1 and UCH-L3 are strongly but reciprocally expressed in the testis during spermatogenesis,[18] suggesting that each isozyme may have a distinct function in the testis. To elucidate the pathophysiological roles of these two isozymes in the testis, our present work examines the extent of heat-induced stress using experimentally induced cryptorchidism in *Uchl3* knockout[7] and *gad* mice.[8] Normally, the testes are maintained in the scrotum at a temperature lower than that of the abdomen. Exposure of a testis to higher body temperature via experimentally induced cryptorchidism results in rapid degeneration of testicular germ cells.[19–22] Recent studies show that testicular germ cell degeneration in cryptorchid testes occurs via apoptosis, and that protein and lipid oxidation, along with p53 promote germ cell death.[23–25] The ubiquitin-proteasome system is required for the subsequent degradation of the damaged testicular germ cells.[26–28] Here, we show that both UCH-L1 and UCH-L3 have reciprocal functions in testicular germ cells during cryptorchid-induced apoptosis. Our data show that the absence of UCH-L1 causes resistance to cryptorchid-induced testicular germ cell apoptosis, and that the knockout of UCH-L3 promotes germ cell apoptosis after cryptorchid injury.

Materials and Methods

Animals

We used 8-week-old *Uchl3* knockout (C57BL/6J)[7,18] and *gad*[8,18,29] (CBA/RFM) male mice. *Uchl3* knockout mice were generated by the standard method using homologously recombinant ES cells, and the knockout line was back-crossed several times to C57BL/6J mice.[7] The *gad* mouse is an autosomal recessive mutant that was obtained by crossing CBA and RFM mice.[8] The *gad* line was maintained by intercrossing for more than 20 generations.[8,29] Both strains were maintained at our institute. Animal care and handling were in accordance with institutional regulations for animal care and were approved by the Animal Investigation Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan.

Unilateral Experimental Cryptorchidism

Unilateral cryptorchidism was experimentally induced under pentobarbital anesthesia (Abbott Laboratories, North Chicago, IL).[20,22] Briefly, a midline abdominal incision was made, and the left testis was displaced from scrotum and fixed to the upper abdominal wall. The right testis remained in the scrotum as an intact control within the same animal. At 0, 4, 7, and 14 days after the operation, four control and four cryptorchid testes were harvested to determine testis weight.

Histological and Immunohistochemical Assessment of Testes

Testes were embedded in paraffin wax after fixation in 4% paraformaldehyde, sectioned at 4-μm thickness, and stained with hematoxylin and eosin.[29] Light microscopy was used for routine observations. For immunohistochemical staining, the sections were incubated with 10% goat serum for 1 hour at room temperature, followed by incubation overnight at 4°C with a rabbit polyclonal antibody against ubiquitin (1:500; DakoCytomation, Glostrup, Denmark) or Nedd8 (1:500; Alexis Biochemicals, San Diego, CA) diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. Sections were then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature and examined by confocal laser-scanning microscopy (Olympus, Tokyo, Japan).

Apoptotic cells in testicular tissues were identified by terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labeling (TUNEL) using the DeadEnd Fluorimetric TUNEL system kit (Promega, Madison, WI) and the anti-PARP p85 fragment pAb (Promega) according to the manufacturer's instructions.

Quantitative Analysis of Apoptotic Germ Cells

The number of apoptotic cells was determined by counting the positively stained nuclei in 30 circular seminiferous tubule cross-sections per testis section.[23,29] The proportion of seminiferous tubules containing apoptotic germ cells was calculated by dividing the number of seminiferous tubules containing apoptotic cells by the total number of seminiferous tubules. The incidence of apoptotic cells per apoptotic cell-containing seminiferous tubule was categorized into three groups, defined as 1 to 5, 6 to 10, and >11 positive cells.

Western Blotting

Western blots were performed as previously reported.[8,18,29] Total protein (5 μg/lane) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 15% gels (Perfect NT Gel; DRC, Japan). Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and blocked with 5% nonfat milk in TBS-T [50 mmol/L Tris base, pH 7.5, 150 mmol/L NaCl, 0.1% (w/v) Tween-20]. The membranes were incubated individually with one or more primary antibodies to UCH-L1 and UCH-L3 (1:1000; peptide antibodies[18]), Bcl-2, Bcl-xL, Bax, p53, and caspase-3, (1:1000; all from Cell Signaling Technology, Beverly, MA), phosphorylated cyclic AMP response element-binding protein (pCREB, 1:500; Upstate Biotech-

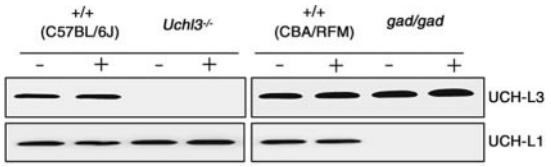

Figure 1. Western blotting analyses of both UCH-L3 and UCH-L1 in the testes of *gad* and *Uchl3* knockout mice, respectively, on day 4 after cryptorchid injury. Scrotal and cryptorchid testes did not differ significantly with respect to protein expression (−, scrotal testes; +, cryptorchid testes).

nology, Waltham, MA), brain-derived neurotrophic factor (BDNF, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), XIAP (1:500; Transduction Laboratories, Franklin Lakes, NJ), polyubiquitin (1:1000, clone FK-2; Medical & Biological Laboratories, Nagoya, Japan), monoubiquitin (1:1000, u5379; Sigma-Aldrich, St. Louis, MO), and Nedd8 (1:1000; Alexis Biochemicals, San Diego, CA). Blots were further incubated with peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:5000; Pierce, Rockford, IL) for 1 hour at room temperature. Immunoreactions were visualized using the SuperSignal West Dura extended duration substrate (Pierce) and analyzed with a ChemiImager (Alpha Innotech, San Leandro, CA). Each protein level was relatively quantificated after analysis with a ChemiImager using AlphaEase software.

Statistical Analysis

The mean and SD were calculated for all data (presented as mean ± SD). One-way analysis of variance was used for all statistical analyses.

Results

Level of Two UCH Isozymes in Scrotal and Cryptorchid Testes from Uchl3 Knockout and Gad Mice

We first confirmed the lack of UCH-L3 protein in the testes from *Uchl3* knockout mice by Western blotting (Figure 1). Similarly, we did not detect UCH-L1 protein in the testes of *gad* mice (Figure 1), as we previously observed.[13] Thus, in a biochemical sense, *gad* mice are analogous to *Uchl1*-null mice.[8,13] Compensatory level of UCH-L3 and UCH-L1 in *gad* and *Uchl3* knockout mice, respectively, was not observed (Figure 1; compare UCH-L3/UCH-L1 level with that of wild-type control mice). Experimental cryptorchidism did not affect UCH-L3 level in *gad* or wild-type control mice. Similarly, cryptorchidism did not affect UCH-L1 level in *Uchl3* knockout and wild-type control mice (Figure 1). Quantitative reverse transcriptase-polymerase chain reaction analysis showed that transcription from the *Uchl3* and *Uchl1* in both scrotal and cryptorchid testes from *gad* and *Uchl3* knockout mice was not significantly different from that measured in the corresponding wild-type control mice (data not shown). These results suggest that the level of UCH-L3 is regulated independently of UCH-L1 in the mouse testis, and that cryptorchid injury does not affect the level of either protein.

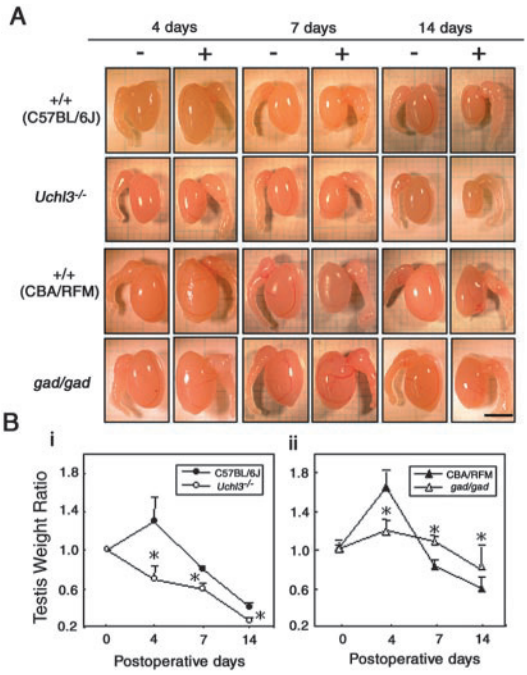

Figure 2. Comparison of testicular sizes and weights after experimental cryptorchidism. **A:** Gross images of changes in testicular size throughout time in two wild-type (C57BL/6J and CBA/RFM), *Uchl3* knockout, and *gad* mice (−, scrotal testes; +, cryptorchid testes). **B:** Ratio of cryptorchid to scrotal testis weight on days 0, 4, 7, and 14 after injury. i: Throughout time, the ratio for *Uchl3* knockout mice (**open circles**) differed significantly compared with wild-type mice (**filled circles**). ii: The ratio for *gad* mice (**open triangles**) did not differ significantly throughout time compared with wild-type mice (**closed triangles**). ($n = 4$; *, $P < 0.05$). Scale bar, 5 mm. Original magnifications, ×40.

Changes in Testicular Weight and Structure in Cryptorchid Uchl3 Knockout and Gad Mice

Unilateral cryptorchidism was surgically induced in *Uchl3* knockout and *gad* mice, and testes were evaluated on days 0, 4, 7, and 14 after the operation (Figure 2). Nonoperated (scrotal) testes served as controls for the evaluation of testicular weight and histochemistry. Cryptorchid testes from *Uchl3* knockout mice appeared smaller than the nonoperated controls at each time point, whereas the size of the cryptorchid testes from *gad* mice was similar to the controls (Figure 2A). Figure 2B shows the time course of the ratio of testicular weight of cryptorchid testes to scrotal testes. In wild-type mice (C57BL/6J and CBA/RFM), the ratio transiently increased 4 days after cryptorchid injury, most likely a consequence of inflammation-induced fluid accumulation[22,23] and biochemical changes observed. The ratio for these mice subsequently decreased below 1.0 by day 7. The ratio remained ~1.0 in *gad* mice (range,1.15 ~ 0.85), whereas it decreased significantly in *Uchl3* knockout mice compared with wild-type mice (Figure 2B). These results demonstrate that testes from *Uchl3* knockout and *gad* mice differ in their response to experimental cryptorchidism.

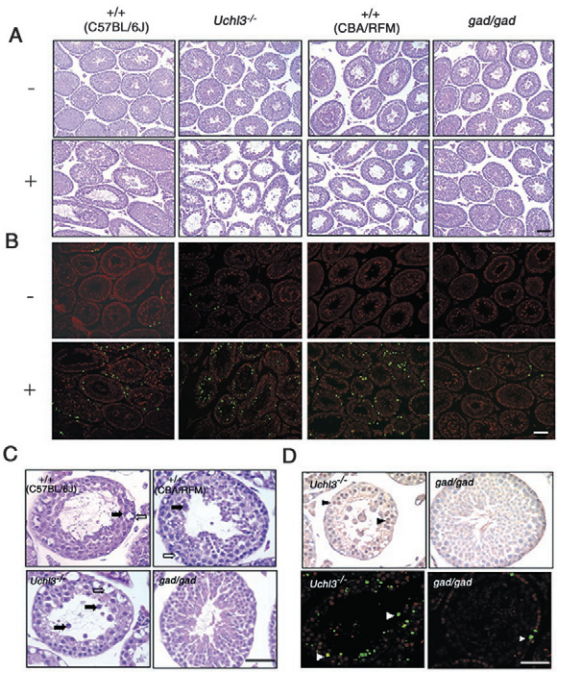

Figure 3. Histology and TUNEL staining of testicular cross sections after experimental cryptorchidism. **A:** Morphological analysis of seminiferous tubules on day 7 after cryptorchid injury. Note the germ cell loss and atrophy in cryptorchid testes compared with uninjured controls. (−, scrotal testes; +, cryptorchid testes). **B:** TUNEL staining of testicular cross-sections on day 7 after cryptorchid injury. Green fluorescence, TUNEL-positive cells; red fluorescence, nuclei stained with propidium iodide. **C:** Magnified cryptorchid testes sections. Pyknotic bodies (**filled arrows**) and Sertoli cell vacuolization (**open arrows**) were evident in cryptorchid testes of *Uchl3* knockout and the two wild-type (C57BL/6J and CBA/RFM) mice on day 7 after injury. **D:** PARP analysis to detect apoptotic germ cells in cryptorchid testes of *Uchl3* knockout and *gad* mice on day 7 after injury. The detection of apoptotic germ cells (**arrowheads**, **top**) by PARP analysis was consistent with that of apoptotic germ cells (**arrowheads**, **bottom**) by TUNEL analysis. Scale bar, 50 μm. Original magnifications: **A** and **B** ×100; **C** and **D** ×200.

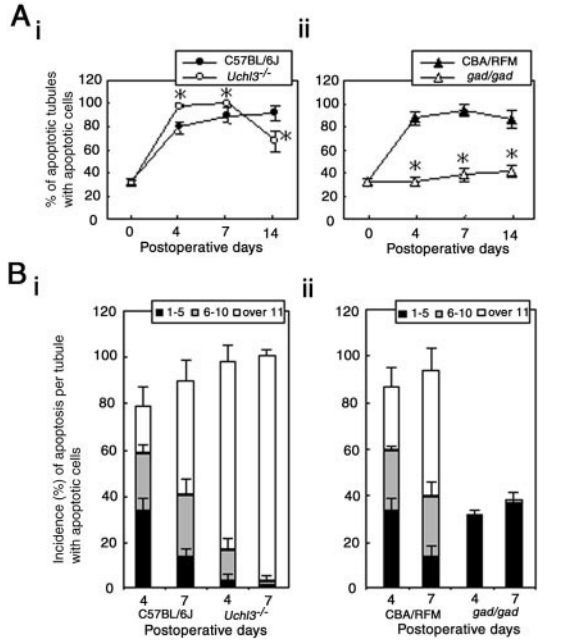

Figure 4. Quantitation of testicular germ cell apoptosis in testes after experimental cryptorchidism. **A:** The percentage of seminiferous tubules containing apoptotic germ cells in cryptorchid testes on days 0, 4, 7, and 14 after injury. **i:** The increase in the percentage of tubules containing apoptotic cells in the cryptorchid testes of *Uchl3* knockout mice is statistically significant compared with wild-type mice on days 4, 7, and 14. Each value represents the mean ± SD; *, $P < 0.05$. **ii:** The percentage of apoptotic tubules in cryptorchid testes of *gad* mice is significantly different on days 4, 7, and 14 after injury. Each value represents the mean ± SD; *, $P < 0.01$. **B:** Incidence of apoptosis per seminiferous tubule with apoptotic germ cells on days 4 and 7 after injury. The incidence of seminiferous tubules containing >11 apoptotic germ cells is significantly increased ($P < 0.05$) in cryptorchid testes of *Uchl3* knockout mice compared with wild-type mice. **i:** Comparison with *Uchl3* knockout mice. **ii:** Comparison with *gad* mice. Each value represents the mean ± SD.

Testicular Germ Cell Apoptosis in Cryptorchid Uchl3 Knockout and Gad Mice

To explore the mechanism underlying the observed differences between *Uchl3* knockout and *gad* cryptorchid testes, we prepared histological cross-sections on day 7 after testicular injury (Figure 3, A and C). The presence of nuclear pyknosis, multinucleated giant cells, and Sertoli cell vacuolization with germ cell loss in the germinal epithelium is indicative of cryptorchid testes.[22,23] These hallmarks of testicular injury were the most remarkable characteristics of cryptorchid testes from *Uchl3* knockout mice, demonstrating profound testicular atrophy and germ cell loss compared with wild-type mice (Figure 3, A and C). In contrast, no nuclear pyknosis, cellular shrinkage, or germ cell loss was observed in cryptorchid testes from *gad* mice. Spermatocytes and early spermatids comprised the majority of affected cell types in cryptorchid testes (Figure 3, A and C).

Germ cell apoptosis was further examined by TUNEL and PARP assays in tissue sections from postoperative day 7 mice (Figure 3, B and D). All but the *gad* cryptorchid testes showed a time-dependent increase in germ cell apoptosis during experimental cryptorchidism; germ cell apoptosis was always found in tubules that had germ cell loss on days 4, 7, and 14 (Figure 3, B and D, and Figure 4). Compared to wild-type mice, the cryptorchid testes of *Uchl3* knockout mice showed a marked increase in apoptotic germ cells in response to testicular injury, whereas *gad* mice lacked cryptorchid-induced germ cell apoptosis (Figure 3B and Figure 4). By postoperative days 4 and 7, the percentage of seminiferous tubules containing apoptotic germ cells increased with statistical significance ($n = 4$) ($P < 0.05$) in cryptorchid testes of *Uchl3* knockout mice as compared with wild-type mice (Figure 4A). In addition, cryptorchid testes of *Uchl3* knockout mice showed a high incidence of seminiferous tubules containing >11 apoptotic germ cells on days 4 and 7 days as compared with wild-type mice (Figure 4B); however, germ cell apoptosis did not increase in cryptorchid testes of *gad* mice during postoperative days 4 to 14 ($P < 0.01$) (Figure 4, A and B).

Cellular Mono- and Polyubiquitin Level in Cryptorchid Uchl3 Knockout and Gad Mice

Ubiquitin is required for energy-dependent degradation of structurally altered proteins.[26] We previously reported that UCH-L1 binds ubiquitin and stabilizes ubiquitin turnover in neurons, and that the level of monoubiquitin is decreased in *gad* mice.[13] In a model of ischemic insult in the retina, ubiquitin induction was unexpectedly lower and ischemic

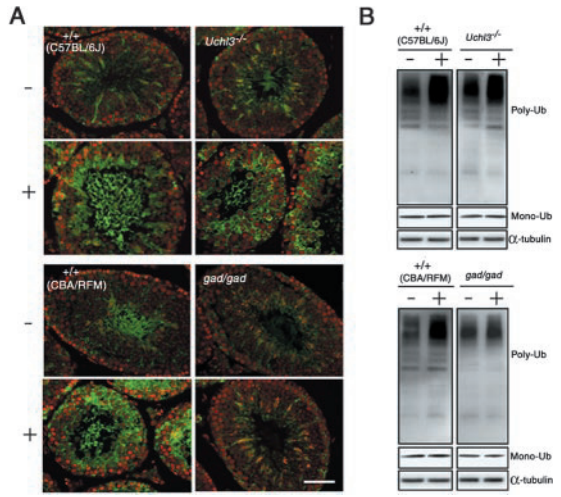

Figure 5. Immunohistochemical and Western blotting analyses of mono- and polyubiquitin in testes on day 7 after experimental cryptorchidism. **A:** Ubiquitin induction was not different between cryptorchid testes from *Uchl3* knockout and wild-type mice, whereas cryptorchid-induced ubiquitin induction in *gad* mice was reduced. Green fluorescence, ubiquitin-positive cells; red fluorescence, nuclei stained with propidium iodide. **B:** Polyubiquitin level in *Uchl3* knockout mice and the two wild-type (C57BL/6J and CBA/RFM) mice substantially increased after injury, whereas that in *gad* mice did not change significantly. Monoubiquitin level did not change after injury. Representative images from four independent experiments are shown (−, scrotal testes; +, cryptorchid testes). Scale bar, 50 μm. Original magnifications, ×200.

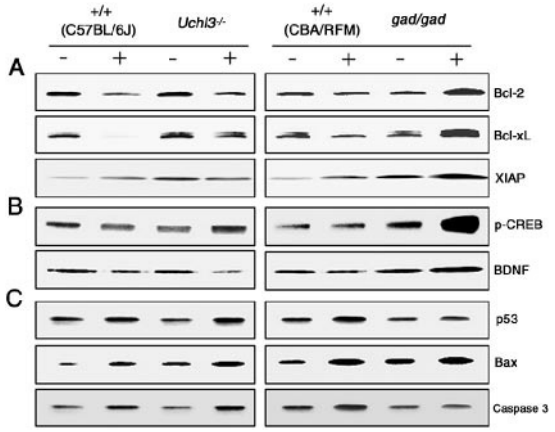

Figure 6. Western blotting analysis of anti-apoptotic, prosurvival, and apoptotic proteins in testes after experimental cryptorchidism. Total protein (5 μg per lane) was prepared from scrotal and cryptorchid testes on day 4 after cryptorchid injury. The expression levels of anti-apoptotic (**A**), prosurvival (**B**), and apoptotic (**C**) proteins in the cryptorchid testes of *Uchl3* knockout, *gad*, and the two wild-type (C57BL/6J and CBA/RFM) mice were significantly different compared with control mice. Representative images from four independent experiments are shown (−, scrotal testes; +, cryptorchid testes).

damage was weaker in the retina of *gad* mice (compared with wild-type mice) after ischemic insult.[17] To determine whether the increase in germ cell apoptosis in cryptorchid testes is associated with ubiquitin induction, we performed immunohistochemical analysis of testes from postoperative day 7 mice. Ubiquitin immunoreactivity increased substantially in cryptorchid testes from *Uchl3* knockout mice and the two wild-type mice, whereas those from *gad* mice showed only minor ubiquitin induction (Figure 5A). The scrotal testes of *Uchl3* knockout and *gad* mice did not show significant differences in ubiquitin induction compared with corresponding controls (Figure 5A). Interestingly, most of the increased ubiquitin induction was detected in spermatocytes and spermatids, consistent with the data on germ cell apoptosis after cryptorchid injury (Figure 3D and Figure 5A). Cryptorchid-induced polyubiquitin levels in the testes from *Uchl3* knockout and the two wild-type mice also increased substantially after injury, whereas the cryptorchid testes of *gad* mice showed no significant difference compared with scrotal testes (Figure 5B); however, the expression levels of monoubiquitin did not change significantly in any of the mice after cryptorchid injury.

Level of Anti-Apoptotic and Apoptotic Proteins in Cryptorchid Uchl3 Knockout and Gad Mice

We previously showed that anti-apoptotic proteins such as Bcl-2 and prosurvival proteins including phosphorylated cyclic AMP response element-binding protein (pCREB) are up-regulated in degenerated retina of *gad* mice.[17] These proteins are degraded by ubiquitination-mediated proteolysis.[30] We examined the expression of the Bcl-2 family proteins, XIAP, pCREB, and caspases to determine their role in testicular germ cell apoptosis after experimental cryptorchidism 4 days after injury in *Uchl3* knockout and *gad* mice. The level of anti-apoptotic proteins such as Bcl-2, Bcl-xL, and XIAP was up-regulated (323.8 ± 57.5, 262.3 ± 22.1, and 209.9 ± 11.7, respectively, as compared with wild type, 100) in the cryptorchid testes of *gad* mice compared with wild-type mice (Figure 6A). Additionally, pCREB, which is normally degraded in a ubiquitination-mediated manner,[30] was apparently highly up-regulated (259.0 ± 22.6, as compared with wild type, 100) in the cryptorchid testes of *gad* mice (Figure 6B). It has been demonstrated that pCREB activates genes that up-regulate trophic factors including BDNF.[31,32] Consistent with pCREB up-regulation, BDNF level also increased (203.0 ± 19.6, as compared with wild type, 100) in cryptorchid testes of *gad* mice (Figure 6B). Level was variable for anti-apoptotic, prosurvival, and apoptotic proteins in the cryptorchid testes of *Uchl3* knockout mice. The level of pCREB, p53, Bax, and caspase3 was slightly increased (169.9 ± 15.2, 152.6 ± 12.9, and 157.3 ± 14.0, respectively, as compared with scrotal testes, 100) in cryptorchid testes of *Uchl3* knockout mice compared with scrotal testes (Figure 6, B and C). Wild-type control mice had a similar expression level pattern except for pCREB. Because p53 acts as an upstream activator of Bax expression,[33] the observed Bax up-regulation after cryptorchid injury was consistent with the elevated p53 level in *Uchl3* knockout and wild-type control mice (Figure 6C). In contrast, BDNF was down-regulated (74.3 ± 7.7 as compared with wild type, 100) in cryptorchid testes of *Uchl3* knockout mice (Figure 6B). The down-regulation of BDNF combined with the up-regulation of pCREB suggests that BDNF might be regulated by another pathway that involves UCH-L3 but not pCREB.[34] Compared with scrotal testes, the expression of anti-apoptotic proteins decreased or was unchanged in cryptorchid testes of *Uchl3* knockout mice (Figure 6A).

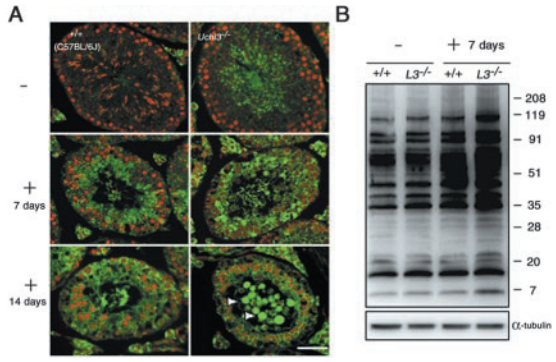

Figure 7. Immunohistochemical and Western blotting analyses of Nedd8 in testes from *Uchl3* knockout mice on days 7 and 14 after experimental cryptorchidism. **A:** Nedd8 induction in *Uchl3* knockout mice increased in both scrotal and cryptorchid testes. The shedding germ cells (**arrowheads**) in the cryptorchid testes of *Uchl3* knockout mice showed strong Nedd8 induction (−, scrotal testes; +7 days and + 4 days, cryptorchid testes). Green fluorescence, Nedd8-positive cells; red fluorescence, nuclei stained with propidium iodide. **B:** On day 7, the expression levels of Nedd8-conjugated proteins in *Uchl3* knockout mice were higher than in wild-type mice. Representative images of four independent experiments are shown. Scale bar, 50 μm. Original magnifications, ×200.

Nedd8 Level in Cryptorchid Uchl3 Knockout Mice

The varied expression levels of ubiquitin, anti-apoptotic, and apoptotic proteins in cryptorchid testes did not adequately explain the relatively exacerbated testicular atrophy and germ cell loss in *Uchl3* knockout mice compared with wild-type mice. We explored the underlying mechanism of this observation using the fact that UCH-L3 cleaves Nedd8.[14,16] We tested whether any change in Nedd8 expression correlated with greater testicular atrophy and germ cell loss in *Uchl3* knockout mice. Nedd8 immunoreactivity was highly detected in scrotal and cryptorchid testes from *Uchl3* knockout mice compared with wild-type mice (Figure 7A). The increased Nedd8 induction was mainly observed in spermatocytes and spermatids, and its expression pattern was similar to that of UCH-L3 during spermatogenesis.[18] These results suggest that Nedd8 may interact closely with UCH-L3 during testicular atrophy and germ cell loss. The cryptorchid testes of *Uchl3* knockout mice showed time-dependent and rapid Nedd8 induction compared with wild-type mice throughout the period 7 to 14 days after injury (Figure 7A). Moreover, the cryptorchid testes of *Uchl3* knockout mice showed strong Nedd8 induction in luminal shedding germ cells on day 14. An immunoblot of scrotal and cryptorchid testes proteins on day 7 confirmed the higher expression levels of Nedd8-conjugated proteins in *Uchl3* knockout mice as compared with wild-type mice (Figure 7B).

Discussion

During spermatogenesis, apoptosis controls germ cell numbers and eliminates defective germ cells to facilitate testicular homeostasis.[35–37] Recent studies indicate that ubiquitination targets proteins for degradation and modulates the turnover of various classes of short-lived signaling proteins.[28,38] Germ cell apoptosis after cryptorchid stress involves genes for various factors, such as Bcl-2 family proteins, p53, and caspases;[39–44] however, the impact of the ubiquitin system on the regulatory mechanisms of germ cell apoptosis is not fully understood. In a previous study, we used *gad* mice, which lack UCH-L1 expression, to show that neural cell apoptosis is suppressed after ischemic retinal injury *in vivo*.[17] These results suggest that UCH-L1 is involved in apoptosis-inducing pathways after stress. UCH-L1 and UCH-L3 are highly similar in sequence; however, UCH-L3 is expressed ubiquitously,[7] whereas UCH-L1 is selectively expressed in neurons and testes/ovaries.[8,9] We recently demonstrated that the expression of these UCH isozymes is differentially and developmentally regulated during spermatogenesis, and that UCH-L1 and UCH-L3 likely have distinct functions during different developmental phases.[18]

To understand the pathophysiological roles of UCH-L1 and UCH-L3 *in vivo*, two mutant mice, *Uchl3* knockout and *gad* mice, were examined after cryptorchid injury. The cryptorchid testes of the two mutant mice had fundamental differences after injury, in that testes of *Uchl3* knockout mice showed profound apoptosis-mediated germ cell loss, whereas *gad* mice were relatively resistant to injury (Figures 3 and 4). In addition, cryptorchid testes of *Uchl3* knockout mice showed greater testicular atrophy and germ cell loss than wild-type mice.

There are several proposed mechanisms for germ cell loss after experimental cryptorchidism.[21–23,45] The tumor suppressor protein, p53, is highly expressed in the testis and regulates both cell proliferation and apoptosis.[23,28,37] A role for p53 in experimental cryptorchidism has been demonstrated convincingly. The higher temperature of the testis caused by cryptorchidism induces p53-mediated apoptosis in the testis, and p53 overexpression results in increased germ cell apoptosis and decreased spermatozoa production.[23,46] In addition to p53, the Bcl-2 family and IAP (inhibitor of apoptosis protein) family are other major classes of intracellular apoptosis regulators.[47,48] The Bcl-2 family can be divided into anti-apoptotic members, such as Bcl-2, Bcl-xL, and Bcl-w, and proapoptotic members, such as Bax and Bak.[49] It has been suggested that the ratio of proapoptotic to anti-apoptotic Bcl-2 family members is important in determining whether a cell will undergo apoptosis.[49] A major function of the Bcl-2 family members appears to be the regulation of mitochondrial events, such as the release of proapoptotic factors.[50] The IAP family inhibits apoptosis primarily by inactivating and degrading proapoptotic proteins.[51] XIAP, a member of IAP family, can bind to and inhibit the proteinase activity of cellular caspase-3 and caspase-9, and thereby block the apoptotic process.[44,52,53]

With regard to cryptorchid injury, the balance between the expression of apoptosis-inducing and apoptosis-protecting proteins constitutes one possible mechanism underlying the observed germ cell apoptosis and protection from apoptosis in *Uchl3* knockout and *gad* mice, respectively. In *gad* mice, cryptorchid injury caused a large increase in the anti-apoptotic proteins Bcl-2, Bcl-xL, and XIAP, consistent with our previous report using retina.[17]

In addition, the expression levels of the prosurvival proteins pCREB and BDNF also increased in *gad* mice. Consistent with these results, caspase-3 expression was suppressed in *gad* mice. Cryptorchid testes of *Uchl3* knockout mice showed slightly increased expression of the apoptotic proteins p53, Bax, and caspase-3 after injury, although similar increases were also observed in wild-type control mice. In total, these results suggest that UCH-L1 plays a role in balancing the expression of apoptosis-inducing and apoptosis-protecting proteins. In contrast, UCH-L3 seems to resist germ cell apoptosis after cryptorchid injury.

Recent studies demonstrate that many molecules in the cellular apoptosis machinery, such as p53,[39,41] Bcl-2 family,[42,43,54] XIAP,[52] and caspase[44] members, are targets for ubiquitination.[28] This suggests that ubiquitination is one of the major mechanisms by which apoptotic cell death is regulated. UCH-L1 has been suggested to associate with monoubiquitin,[13] and the monoubiquitin pool is reduced in *gad* mice relative to wild-type mice. Protection from cryptorchid injury was reported in testes of mice expressing a mutant K48R ubiquitin,[22] suggesting that ubiquitin plays a critical role in processing or modulating testicular insults. Normally, damaged proteins are polyubiquitinated and degraded via the ubiquitin-proteasome system; however, if damaged proteins are not degraded as easily when monoubiquitin is either depleted or mutated, then germ cell death could be delayed.[17,22] Our results with the *gad* mouse suggest that ubiquitin induction plays a critical role in regulating cell death during cryptorchid injury-mediated germ cell apoptosis.

Uchl3 knockout mice exhibit severe retinal degeneration, suggesting that the UCH-L3-mediated ubiquitin pathway is involved in retinal homeostasis.[55] In the cryptorchid testes of *Uchl3* knockout mice, however, the profound testicular weight reduction and germ cell apoptosis after injury cannot be explained by ubiquitin induction alone. Our present results show that *Uchl3* knockout and wild-type mice have similar ubiquitin expression level in the testes, suggesting that UCH-L3 has another nonhydrolase activity in the ubiquitin-proteasome system. UCH-L3 also binds and cleaves the C-terminus of the ubiquitin-like protein, Nedd8.[14,56] This activity is unique to UCH-L3 because UCH-L1 does not cleave Nedd8. Thus, UCH-L3 appears to have dual affinities for ubiquitin and Nedd8. Our present results show that Nedd8 is strongly induced in scrotal testes of *Uchl3* knockout mice compared with those of wild-type mice (Figure 7). Cryptorchid testes of both *Uchl3* knockout and wild-type mice showed Nedd8 induction after injury, although the induction was higher in *Uchl3* knockout mice. These observations suggest that UCH-L3 may function as a deneddylating enzyme[16] *in vivo*, although further studies are necessary to clarify whether UCH-L3 interacts with Nedd8 during spermatogenesis.

In the present study, we demonstrate apparent reciprocal functions for the two deubiquitinating enzymes, UCH-L1 and UCH-L3, with respect to mediating injury after experimental cryptorchidism (Figure 8). Our results advance our understanding of the role of the ubiquitin-proteasome system in regulating apoptosis, and provide a unique opportunity for effective therapeutic intervention.

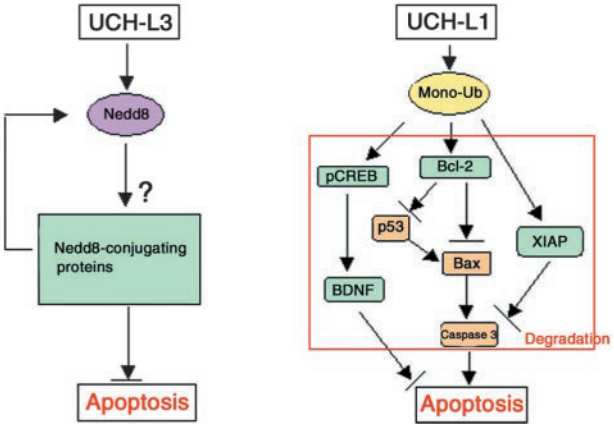

Figure 8. Differential function of the two UCH isozymes in response to experimental cryptorchidism. UCH-L3 has specificity for Nedd8. Cryptorchid injury results in protein damage and the accumulation of Nedd8-conjugated proteins. The accumulation of Nedd8-conjugated proteins in *Uchl3* knockout mice may contribute to profound germ cell loss via apoptosis. Hence, UCH-L3 might function as an anti-apoptotic regulator. UCH-L1 is involved in the maintenance of monoubiquitin levels. A deficiency in monoubiquitin results in delayed polyubiquitination and the accumulation of short-lived proteins after cryptorchid injury. Hence, UCH-L1 may function as a regulator of apoptosis.

Acknowledgments

We thank Dr. S.M. Tilghman for providing *Uchl3* knockout mice, H. Kikuchi for technical assistance with tissue sections, and M. Shikama for the care and breeding of animals.

References

1. Weissman AM: Themes and variations on ubiquitylation. Nat Rev Mol Cell Biol 2001, 2:169–178
2. Ciechanover A: The ubiquitin-proteasome pathway: on protein death and cell life. EMBO J 1998, 17:7151–7160
3. Pickart CM, Rose IA: Ubiquitin carboxyl-terminal hydrolase acts on ubiquitin carboxyl-terminal amides. J Biol Chem 1985, 260:7903–7910
4. Baker RT, Tobias JW, Varshavsky A: Ubiquitin-specific proteases of Saccharomyces cerevisiae. Cloning of UBP2 and UBP3, and functional analysis of the UBP gene family. J Biol Chem 1992, 267:23364–23375
5. 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
6. 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
7. 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
8. 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
9. 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
10. 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
11. Liu Y, Fallon L, Lashuel HA, Liu Z, Lansbury Jr PT: The UCH-L1 gene

encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. Cell 2002, 111:209–218
12. Liu Y, Lashuel HA, Choi S, Xing X, Case A, Ni J, Yeh LA, Cuny GD, Stein RL, Lansbury Jr PT: Discovery of inhibitors that elucidate the role of UCH-L1 activity in the H1299 lung cancer cell line. Chem Biol 2003, 10:837–846
13. Osaka H, Wang YL, Takada K, Takizawa S, Setsuie 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
14. Wada H, Kito K, Caskey LS, Yeh ET, Kamitani T: Cleavage of the C-terminus of NEDD8 by UCH-L3. Biochem Biophys Res Commun 1998, 251:688–692
15. Hemelaar J, Borodovsky A, Kessler BM, Reverter D, Cook J, Kolli N, Gan-Erdene T, Wilkinson KD, Gill G, Lima CD, Ploegh HL, Ovaa H: Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins. Mol Cell Biol 2004, 24:84–95
16. Gong L, Kamitani T, Millas S, Yeh ET: Identification of a novel isopeptidase with dual specificity for ubiquitin- and NEDD8-conjugated proteins. J Biol Chem 2000, 275:14212–14216
17. Harada T, Harada C, Wang YL, Osaka H, Amanai K, Tanaka K, Takizawa S, Setsuie R, Sakurai M, Sato Y, Noda M, Wada K: Role of ubiquitin carboxy terminal hydrolase-L1 in neural cell apoptosis induced by ischemic retinal injury in vivo. Am J Pathol 2004, 164:59–64
18. Kwon J, Wang YL, Setsuie R, Sekiguchi S, Sakurai M, Sato Y, Lee WW, Ishii Y, Kyuwa S, Noda M, Wada K, Yoshikawa Y: Developmental regulation of ubiquitin C-terminal hydrolase isozyme expression during spermatogenesis in mice. Biol Reprod 2004, 71:515–521
19. Boekelheide K, Hall SJ: 2,5-Hexanedione exposure in the rat results in long-term testicular atrophy despite the presence of residual spermatogonia. J Androl 1991, 12:18–26
20. Ohta Y, Nishikawa A, Fukazawa Y, Urushitani H, Matsuzawa A, Nishina Y, Iguchi T: Apoptosis in adult mouse testis induced by experimental cryptorchidism. Acta Anat (Basel) 1996, 157:195–204
21. Yin Y, Hawkins KL, DeWolf WC, Morgentaler A: Heat stress causes testicular germ cell apoptosis in adult mice. J Androl 1997, 18:159–165
22. Rasoulpour RJ, Schoenfeld HA, Gray DA, Boekelheide K: Expression of a K48R mutant ubiquitin protects mouse testis from cryptorchid injury and aging. Am J Pathol 2003, 163:2595–2603
23. Yin Y, DeWolf WC, Morgentaler A: Experimental cryptorchidism induces testicular germ cell apoptosis by p53-dependent and -independent pathways in mice. Biol Reprod 1998, 58:492–496
24. Peltola V, Huhtaniemi I, Ahotupa M: Abdominal position of the rat testis is associated with high level of lipid peroxidation. Biol Reprod 1995, 53:1146–1150
25. Ahotupa M, Huhtaniemi I: Impaired detoxification of reactive oxygen and consequent oxidative stress in experimentally cryptorchid rat testis. Biol Reprod 1992, 46:1114–1118
26. Morimoto RI, Santoro MG: Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. Nature Biotechnol 1998, 16:833–838
27. Wojcik C: Proteasomes in apoptosis: villains or guardians? Cell Mol Life Sci 1999, 56:908–917
28. Yang Y, Yu X: Regulation of apoptosis: the ubiquitous way. EMBO J 2003, 17:790–799
29. Kwon J, Kikuchi T, Setsuie 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
30. Taylor CT, Furuta GT, Synnestvedt K, Colgan SP: Phosphorylation-dependent targeting of cAMP response element binding protein to the ubiquitin/proteasome pathway in hypoxia. Proc Natl Acad Sci USA 2000, 97:12091–12096
31. Park C, Choi WS, Kwon H, Kwon YK: Temporal and spatial expression of neurotrophins and their receptors during male germ cell development. Mol Cells 2001, 12:360–367
32. Finkbeiner S: CREB couples neurotrophin signals to survival messages. Neuron 2000, 25:11–14
33. Selvakumaran M, Lin HK, Miyashita T, Wang HG, Krajewski S, Reed JC, Hoffman B, Liebermann D: Immediate early up-regulation of bax expression by p53 but not TGF beta 1: a paradigm for distinct apoptotic pathways. Oncogene 1994, 9:1791–1798
34. Liu L, Cavanaugh JE, Wang Y, Sakagami H, Mao Z, Xia Z: ERK5 activation of MEF2-mediated gene expression plays a critical role in BDNF-promoted survival of developing but not mature cortical neurons. Proc Natl Acad Sci USA 2003, 100:8532–8537
35. Matsui Y: Regulation of germ cell death in mammalian gonads. AP-MIS 1998, 106:142–148
36. Gosden R, Spears N: Programmed cell death in the reproductive system. Br Med Bull 1997, 53:644–661
37. Print CG, Loveland KL: Germ cell suicide: new insights into apoptosis during spermatogenesis. Bioessays 2000, 22:423–430
38. Lee JC, Peter ME: Regulation of apoptosis by ubiquitination. Immunol Rev 2003, 193:39–47
39. Haupt Y, Maya R, Kazaz A, Oren M: Mdm2 promotes the rapid degradation of p53. Nature 1997, 387:296–299
40. Oren M: Regulation of the p53 tumor suppressor protein. J Biol Chem 1999, 274:36031–36034
41. Ryan KM, Phillips AC, Vousden KH: Regulation and function of the p53 tumor suppressor protein. Curr Opin Cell Biol 2001, 13:332–337
42. Dimmeler S, Breitschopf K, Haendeler J, Zeiher AM: Dephosphorylation targets Bcl-2 for ubiquitin-dependent degradation: a link between the apoptosome and the proteasome pathway. J Exp Med 1999, 189:1815–1822
43. Marshansky V, Wang X, Bertrand R, Luo H, Duguid W, Chinnadurai G, Kanaan N, Vu MD, Wu J: Proteasomes modulate balance among proapoptotic and antiapoptotic Bcl-2 family members and compromise functioning of the electron transport chain in leukemic cells. J Immunol 2001, 166:3130–3142
44. Suzuki Y, Nakabayashi Y, Takahashi R: Ubiquitin-protein ligase activity of X-linked inhibitor of apoptosis protein promotes proteasomal degradation of caspase-3 and enhances its anti-apoptotic effect in Fas-induced cell death. Proc Natl Acad Sci USA 2001, 98:8662–8667
45. Yin Y, Stahl BC, DeWolf WC, Morgentaler A: P53 and Fas are sequential mechanisms of testicular germ cell apoptosis. J Androl 2002, 23:64–70
46. Ohta H, Aizawa S, Nishimune Y: Functional analysis of the p53 gene in apoptosis induced by heat stress or loss of stem cell factor signaling in mouse male germ cells. Biol Reprod 2003, 68:2249–2254
47. Beumer TL, Roepers-Gajadien HL, Gademan IS, Lock TM, Kal HB, De Rooij DG: Apoptosis regulation in the testis: involvement of Bcl-2 family members. Mol Reprod Dev 2000, 56:353–359
48. Oldereid NB, Angelis PD, Wiger R, Clausen OP: Expression of Bcl-2 family proteins and spontaneous apoptosis in normal human testis. Mol Hum Reprod 2001, 7:403–408
49. Borner C: The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. Mol Immunol 2003, 39:615–647
50. Yamamoto CM, Sinha Hikim AP, Huynh PN, Shapiro B, Lue Y, Salameh WA, Wang C, Swerdloff RS: Redistribution of Bax is an early step in an apoptotic pathway leading to germ cell death in rats, triggered by mild testicular hyperthermia. Biol Reprod 2000, 63: 1683–1690
51. Deveraux QL, Reed JC: IAP family proteins—suppressors of apoptosis. Genes Dev 1999, 13:239–252
52. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD: Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science 2000, 288:874–877
53. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC: IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. EMBO J 1998, 17:2215–2223
54. Li B, Dou QP: Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression. Proc Natl Acad Sci USA 2000, 97:3850–3855
55. Semenova E, Wang X, Jablonski MM, Levorse J, Tilghman SM: An engineered 800 kilobase deletion of Uchl3 and Lmo7 on mouse chromosome 14 causes defects in viability, postnatal growth and degeneration of muscle and retina. Hum Mol Genet 2003, 12:1301–1312
56. Dil Kuazi A, Kito K, Abe Y, Shin RW, Kamitani T, Ueda N: NEDD8 protein is involved in ubiquitinated inclusion bodies. J Pathol 2003, 199:259–266