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# Localization of Ubiquitin C-Terminal Hydrolase L1 in Mouse Ova and Its Function in the Plasma Membrane to Block Polyspermy

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**Protein degradation is essential for oogenesis and embryogenesis. The ubiquitin-proteasome system regulates many cellular processes via the rapid degradation of specific proteins. Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) is exclusively expressed in neurons, testis, ovary, and placenta, each of which has unique biological activities. However, the functional role of UCH-L1 in mouse oocytes remains unknown. Here, we report the expression pattern of UCH-L1 and its isozyme UCH-L3 in mouse ovaries and embryos. Using immunocytochemistry, UCH-L1 was selectively detected on the plasma membrane, whereas UCH-L3 was mainly detected in the cytoplasm, suggesting that these isozymes have distinct functions in mouse eggs. To further investigate the functional role of UCH-L1 in mouse eggs, we analyzed the fertilization rate of UCH-L1-deficient ova of *gad* female mice. Female *gad* mice had a significantly increased rate of polyspermy in *in vitro* fertilization assays, although the rate of fertilization did not differ significantly from wild-type mice. In addition, the litter size of *gad* female mice was significantly reduced compared with wild-type mice. These results may identify UCH-L1 as a candidate for a sperm-oocyte interactive binding or fusion protein on the plasma membrane that functions during the block to polyspermy in mouse oocytes.** ***(Am J Pathol 2006, 169:1722–1729; DOI: 10.2352/ajpath.2006.060301)***

Ubiquitin C-terminal hydrolase L1 (UCH-L1) is one of many deubiquitinating enzymes and is selectively and abundantly expressed in the ovary, placenta, testis, and neuronal cells.[1–6] Recent studies suggest that UCH-L1 associates with monoubiquitin and prolongs ubiquitin half-life in neurons.[7] Our previous work on UCH-L1 function in *gad* mice suggests that these mice are resistant to apoptotic stress in retinal cells and testicular germ cells.[8,9] This observation is consistent with a recent report that the overexpression of UCH-L1 induces testicular germ cell apoptosis in *Uchl1* transgenic mice.[10] Furthermore, both UCH-L1 and UCH-L3, the predominant functional UCHs, are differentially expressed in testis during spermatogenesis. These results demonstrate that these enzymes have distinct functions in the testis and epididymis after apoptotic stress,[9,11] even though they have high (52%) amino acid sequence identity.[12]

The above data are in accordance with a number of studies that have linked inhibition of the ubiquitin-proteasome system (UPS) with suppression of apoptosis.[8,13–15] UCH-L1 is an important enzyme for UPS-dependent proteolysis and plays a regulatory role in the cell cycle and cellular proliferation. Thus, its expression in placenta is of

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considerable interest.[3,4] Recent studies reported that UPS controls the degradation of various substrates during gametogenesis and fertilization,[16–19] but relatively little is known about the functional role of the UPS in fertilization. UCH-L1 is expressed in oocytes in ovaries.[5,20] Oocytes, as well as spermatogonia in testis, have multiple potentials and activities for development. However, the function of UCH-L1 during oogenesis is unclear. RFPL4 (ret finger protein-like 4) and FAM (fat facets in mouse) are involved in regulating oogenesis.[21,22] RFPL4 is highly expressed during oogenesis and functions as an E3 ubiquitin ligase to target proteins for proteasomal degradation.[21] FAM is a developmentally regulated substrate-specific deubiquitinating enzyme that is required for preimplantation of the mouse embryo.[22] Thus, the UPS might be important during oocyte development and differentiation of the embryo after fertilization.

Here, we analyzed the functional role of UCH-L1 using mouse oocytes and embryos. Our results indicate that UCH-L1 is selectively expressed on the plasma membrane of mouse ova, where it may regulate membrane penetration by spermatozoa. In addition, the unique expression patterns of UCH-L1 and UCH-L3 suggest that these proteins have distinct functions during oogenesis and embryogenesis. Our results therefore provide strong evidence that UCH-L1 functions in the polyspermy block during mammalian fertilization.

## Materials and Methods

### Animals

We used 8-week-old BDF1, *gad* (CBA/RFM),[23,24] and *Uchl3* knockout (C57BL/6J)[12,25] female and male mice. BDF1 mice were purchased from Nihon SLC, Inc. (Hamamatsu, Japan). The *gad* mouse is an autosomal recessive mutant that was obtained by crossing CBA and RFM mice. The *gad* line was maintained by intercrossing for more than 20 generations.[23,24] The *Uchl3* knockout mouse was generated by standard methods using homologously recombinant ES cells from 129SV mice.[12,25] The knockout line was back-crossed several times with C57BL/6J mice. *gad* mice were maintained at our institute, and *Uchl3* knockout mice were maintained at the National Institute of Neuroscience, National Center of Neurology and Psychiatry (Tokyo, Japan). Animal care and handling were in accordance with institutional regulations and were approved by the Animal Care and Use Committee of the University of Tokyo.

### *Oocyte Collection and* in Vitro *Fertilization*

Female mice were superovulated by intraperitoneal injections with 5 IU of pregnant mare serum gonadotropin (Sankyo, Tokyo, Japan) for 48 hours, followed by 5 IU of human chorionic gonadotropin (Sankyo). Ovulated eggs were collected from the ampullae of oviducts by the scratching method 16 hours after human chorionic gonadotropin injection and placed in 400-$\mu$l droplets of Toyoda, Yokoyama, and Hoshi (TYH)[26] containing 0.4 mg/ml bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Spermatozoa were collected from the cauda epididymis of male mice and preincubated for 1 hour in 400 $\mu$l of TYH to allow capacitation before insemination. After capacitation, the sperms were introduced into the fertilization medium at a final concentration of 150 spermatozoa/$\mu$l. At 4 hours after insemination, 0.05% hyaluronidase (Sigma-Aldrich) was added to the medium for 5 minutes. The eggs were washed thoroughly three times and then cultured in potassium simplex optimized medium (KSOM).[26] After fertilization, all embryos were incubated in a humidified atmosphere of 5% $CO_2$ in air at 37°C in 100-$\mu$l drops of KSOM overlaid with mineral oil. To analyze fertilization in *gad* mice, *gad* ($n$ = 5) and wild-type (CBA/RFM) ($n$ = 5) female mice were superovulated. Ovulated oocytes of *gad* and wild-type mice were fertilized with wild-type spermatozoa.

### *Western Blotting*

Total protein of ovary extracts (10 $\mu$g/lane), oocytes, or embryos (20 oocytes or embryos per lane) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 12.5% gels. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and blocked with 1% bovine serum albumin in TBS-T [50 mmol/L Tris base, pH 7.5, 150 mmol/L NaCl, and 0.1% (w/v) Tween 20]. The membranes were incubated individually with primary antibodies against UCH-L1, UCH-L3,[2] monoubiquitin (U5379, Sigma-Aldrich), zona pellucida 2 (ZP2; Santa Cruz Biotechnology, Santa Cruz, CA), and zona pellucida 3 (ZP3) (Santa Cruz Biotechnology). After thorough rinsing, blots were further incubated with peroxidase-conjugated goat anti-rabbit IgG (DakoCytomation, Glostrup, Denmark) for 1 hour at room temperature. Immunoreactions were visualized by enhanced chemiluminescence (ECL Plus; GE Healthcare UK Ltd. Amersham Place, Little Chalfont, Buckinghamshire, UK). Each immunoreactive band was quantified using commercially available software (Quantity One; PDI, Upper Saddle River, NJ). Negative control extracts of ovary or oocytes were obtained from *gad* and *Uchl3* knockout mice.

### *Histological and Immunochemical Assessment*

Ovaries of BDF1 female and *gad* mice were fixed in 4% paraformaldehyde, embedded in paraffin wax, and then sectioned at 4-$\mu$m thickness. Ovary sections of *gad* mice were stained with hematoxylin and eosin (H&E). Light microscopy was used for routine observations. For immunohistochemical staining, the sections were incubated with Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) for 1 hour at room temperature followed by incubation overnight at 4°C with a rabbit polyclonal antibody against UCH-L1 and UCH-L3.[2] The sections were then incubated with biotinylated goat anti-rabbit IgG (DAKO), which was followed by incubation with streptavidin-biotin-horseradish peroxidase complex (sABC kit; DAKO). Immunoreactivity was visualized by treating the sections

with 3,3′-diaminobenzidine tetroxide (Dojin Kagaku, Kumamoto, Japan). Finally, the sections were counterstained with hematoxylin. Negative control ovaries were obtained from *gad* and *Uchl3* knockout mice.

For immunocytochemical staining, whole oocytes or embryos were fixed for 30 minutes with 4% paraformaldehyde in phosphate-buffered saline (PBS) and 0.2% (w/v) Triton X-100 (ICN Biomedicals, Aurora, OH) in PBS for 30 minutes. Nonspecific binding of immunoglobulins was blocked by incubation with Block Ace (Dainippon Pharmaceutical, Ltd.) for 1 hour at room temperature. The sections were then incubated with primary antibodies against UCH-L1, UCH-L3, and lectin with Rhodamine-Lens Culinaris Agglutinin (Vector Laboratories, Burlingame, CA). The sections were then incubated with Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) and propidium iodide (Molecular Probes). Stained sections were observed under a confocal laser microscope (Laser Scanning Microscope 510; Carl Zeiss, Jena, Germany). Negative control oocytes were obtained from *gad* and *Uchl3* knockout mice by superovulation.

### Quantitative Analysis

Normal oocytes were identified by the presence of the first polar body.[27,28] The frequency of normal oocytes was calculated by counting the normal oocytes in the total superovulated oocytes of *gad* ($n$ = 173) and wild-type ($n$ = 148) mice. To determine the fertilization rate, putative fertilized eggs (by *in vitro* fertilization) were fixed in acetic alcohol (1:3, glacial acetic acid/ethanol) and then stained with 1% aceto-orcein to visualize pronuclei and assess sperm penetration and incidence of monospermic and polyspermic fertilization. Polyspermic fertilization was defined as the presence of three or more pronuclei. The rate of polyspermic fertilization was calculated by counting the polyspermic eggs among the total fertilized eggs of *gad* ($n$ = 71) and wild-type ($n$ = 56) mice.

### Electron Microscopic Analysis

Ovulated mature oocytes and zygotes of BDF1 mice were fixed with 4% paraformaldehyde, and frozen sections were prepared for electron microscopy. The sections were incubated with an antibody against UCH-L1.[2] Subsequently, the ABC method was performed as indicated by the supplier, and the peroxidase reaction was developed in diaminobenzidine. Immunostained sections were fixed in 2.5% glutaraldehyde, postfixed in 1% $OsO_4$, dehydrated in a graded series of ethanol, and embedded in Epon 812.[29] Ultrathin sections were cut with an ultramicrotome, stained with uranyl acetate,[29] and examined with an electron microscope H-7100 (Hitachi, Hitachinaka, Japan).

### Breeding Test of gad Female Mice

*gad* ($n$ = 12) and wild-type (CBA/RFM) ($n$ = 15) female mice and wild-type (CBA/RFM) ($n$ = 9) male mice were subjected to a breeding study. Three female mice of the same genotype were housed with one male mouse per cage. Cages were monitored daily at midday, and the appearance of a vaginal plug was recognized as day 0.5 of gestation. The number of pups, litters, and litter size of *gad* and wild-type female mice were recorded.

### Statistical Analysis

The mean and SD were calculated for all data (presented as mean ± SD). The Student's $t$-test was used for all statistical analyses.

## Results

### Expression of UCH-L1 and UCH-L3 in Mouse Ovaries

We first used Western blotting to address whether both UCH-L1 and UCH-L3 levels are expressed in ovaries during proestrus, estrus, metestrus, and diestrus (Figure 1A). Both proteins were detected at all estrous cycle stages in wild-type mice. As expected, UCH-L1 was not detected in *gad* ovaries and UCH-L3 was not detected in *Uchl3* knockout ovaries. The levels of UCH-L1 and UCH-L3 in ovaries did not change throughout the estrous cycle (Figure 1A).

We next used immunohistochemistry to address whether both UCH-L1 and UCH-L3 are present in developing follicles (primordial, primary, secondary, tertiary, and mature follicles; Figure 1B). In the ovary, UCH-L1 staining was most intense at the plasma membrane of oocytes in developing follicles (Figure 1B, a–e), whereas UCH-L3 staining was concentrated in the cytoplasm of oocytes (Figure 1B, g–k). The oocytes of *gad* and *Uchl3* knockout mice ovaries were negative for UCH-L1 and UCH-L3, respectively (Figure 1B, f and l).

### Expression of UCH-L1 and UCH-L3 in Mouse Mature Oocytes and Preimplantation Embryos

Using Western blotting, we examined the levels of both UCH-L1 and UCH-L3 in mature oocytes and during embryogenesis (zygote, two cells, four cells, eight cells, morulas, and blastocysts). UCH-L1 and UCH-L3 were detected in mature oocytes and during all of the embryonic stages we tested; the level of UCH-L1 was essentially constant in all cases, but the level of UCH-L3 was lower in the blastocyst stage (Figure 2A).

We further analyzed the distribution of UCH-L1 and UCH-L3 in ovulated mature oocytes and during embryogenesis using immunocytochemistry (Figure 2B). UCH-L1 immunoreactivity was intense on the plasma membrane of oocytes and developing embryos but not in the cytoplasm of eggs (Figure 2B, a–g). Furthermore, UCH-L1 immunoreactivity was observed continuously during embryogenesis (Figure 2B, c–g). In the blastocyst stage, UCH-L1 was observed in the outer layer cells of the trophectoderm (Figure 2B, g). UCH-L3 immunoreac-

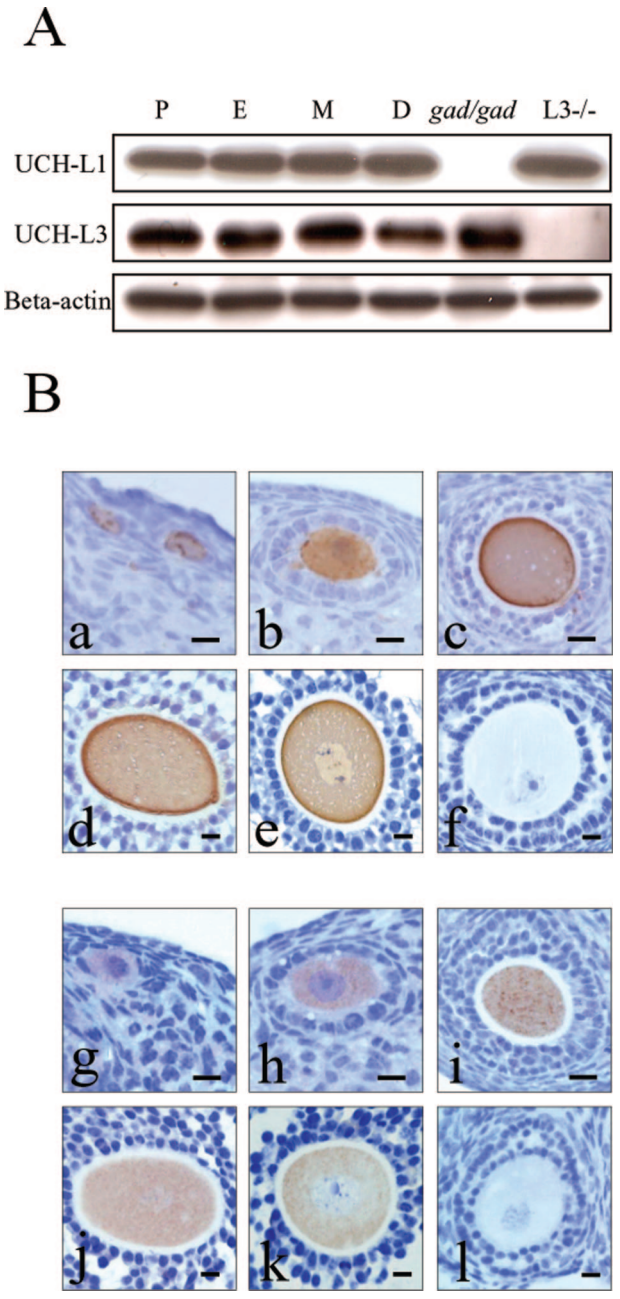


**Figure 1.** Expression of UCH-L1 and UCH-L3 in mouse ovaries. **A:** Western blotting analysis of UCH-L1 and UCH-L3 in ovaries during the estrous cycle. Proestrus (P), estrus (E), metestrus (M), and diestrus (D) ovaries; *gad/gad* and $L3^{-/-}$ represent *gad* and *Uchl3* knockout mouse ovaries, respectively. **B:** Immunohistochemical analyses of UCH-L1 (**a–e**) and UCH-L3 (**g–k**) in BDF1 mouse ovaries (**a–e**, **g–k**). Ovaries from *gad* (**f**) and *Uchl3* knockout mice (**l**) are negative controls. UCH-L1 is highly expressed on the plasma membrane of oocytes. UCH-L3 is diffusely expressed in the cytoplasm of oocytes. Follicle stages: primordial (**a**, **g**), primary (**b**, **h**), secondary (**c**, **i**), tertiary (**d**, **j**), and mature (**e**, **k**) from BDF1 mouse ovaries. Scale bars = 10 $\mu$m.

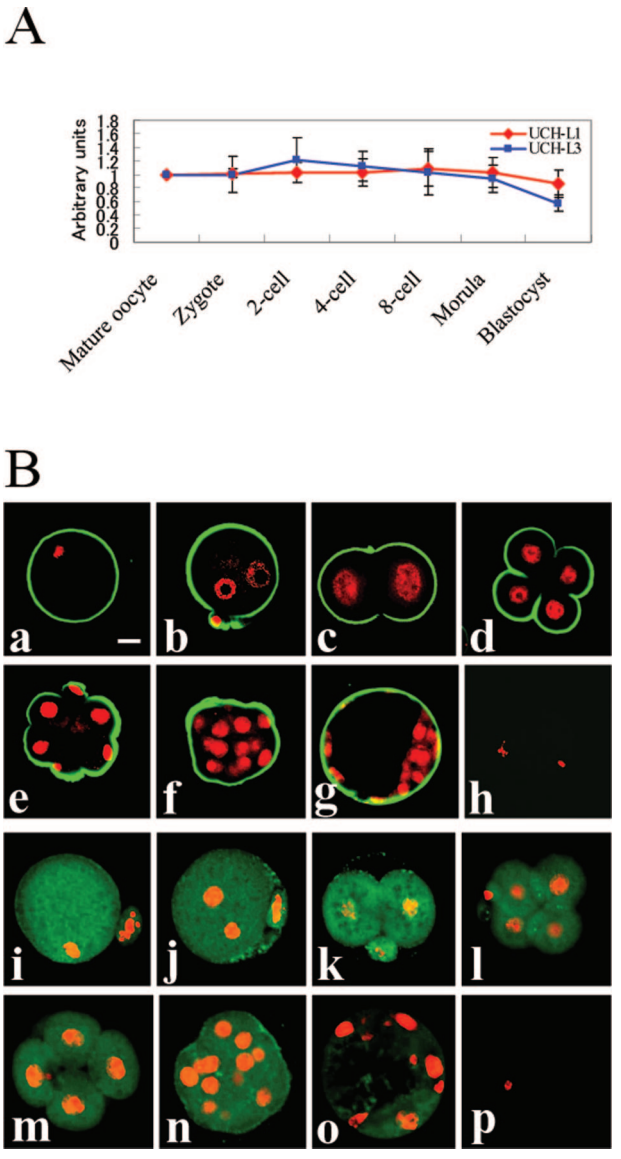


**Figure 2.** Expression of UCH-L1 and UCH-L3 in mature oocytes and fertilized eggs. **A:** Western blotting analysis of UCH-L1 and UCH-L3 in ovulated mature oocytes and developing eggs of BDF1 mice. **B:** Immunocytochemical analysis of UCH-L1 (**a–g**) and UCH-L3 (**i–o**) in ovulated mature oocytes and developing embryos of BDF1 mice (**a–g**, **i–o**). Ovulated mature oocytes of *gad* (**h**) and *Uchl3* knockout mice (**p**) are negative controls. UCH-L1 immunoreactivity was seen only on the plasma membrane of mature oocytes (**a**) and developing eggs (**b–g**). UCH-L3 immunoreactivity was seen in the cytoplasm of oocytes (**j**) and developing eggs (**j–o**). Stages: oocytes (**a**, **i**), zygotes (**b**, **j**), two cells (**c**, **k**), four cells (**d**, **l**), eight cells (**e**, **m**), morulas (**f**, **n**), and blastocyst (**g**, **o**). Nuclei are shown in red. Scale bar = 20 $\mu$m.

tivity was seen in the cytoplasm of oocytes and developing embryos (Figure 2B, i–o), as was detected in ovaries (Figure 2A). In the blastocyst stage, UCH-L3 was observed in the inner cells (Figure 2B, o). The oocytes and developing embryos of *gad* and *Uchl3* knockout mice were negative for UCH-L1 and UCH-L3, respectively (Figure 2B, h and p).

## *Immunoelectron Microscopy*

The cortical granule is located right under the plasma membrane in oocytes. This organelle is unique to oocytes and plays an important role in fertilization. At the light microscope level, it is difficult to determine whether UCH-L1 immunoreactivity localizes to the plasma membrane or cortical granule. To address this issue, we investigated the subcellular localization of UCH-L1 in ovulated mature oocytes and zygotes at the ultrastructural level (Figure 3). Intense UCH-L1 immunoreactivity was

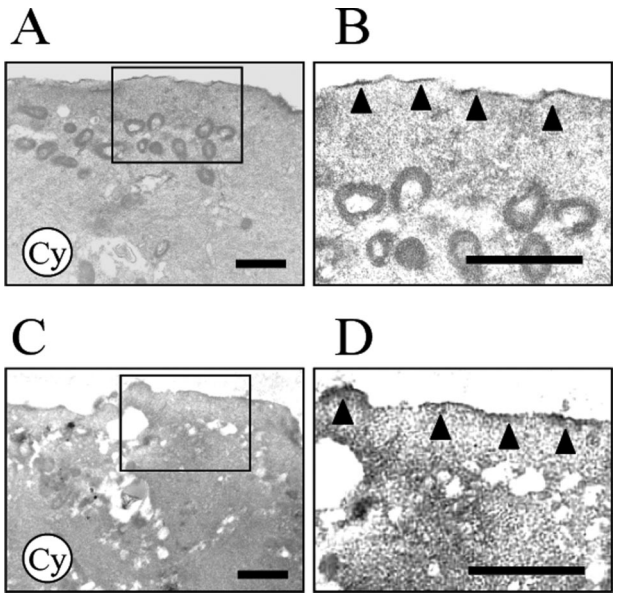


**Figure 3.** Localization of UCH-L1 on the plasma membrane in mature oocytes and zygotes by immunoelectron microscopy. **A–D:** Mature oocyte and zygote of BDF1 mouse are immunostained with UCH-L1. **B** and **D:** Magnified images of the region enclosed by small rectangles in **A** and **C**. UCH-L1 immunoreactivity is intense on the plasma membrane (**arrowheads**) of an ovulated mature oocyte (**B**) and zygote (**D**). The position of cytoplasm (Cy) is indicated. Scale bars = 1 μm.

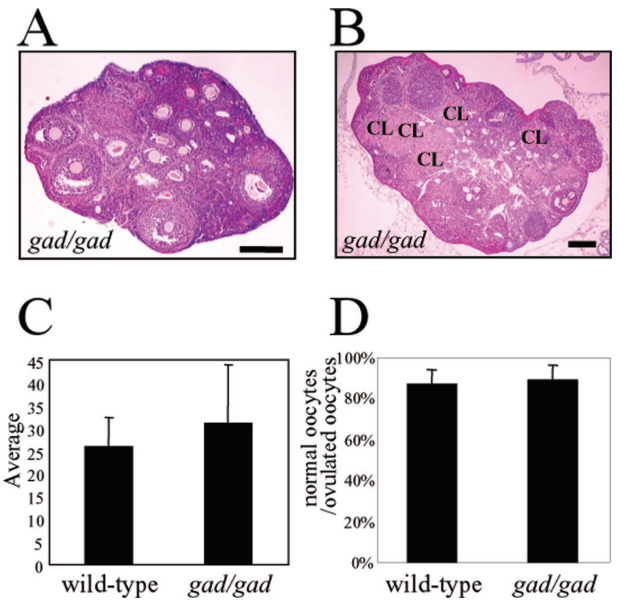


**Figure 4.** Histology of ovaries and the ovulation rate of *gad* mice. **A** and **B:** Ovarian sections of *gad* mice stained with H&E. *gad* mouse ovaries have morphologically normal oocytes, follicles, and corpora lutea (CL). **C** and **D:** The average number of superovulated oocytes (**C**) and the normal oocyte rate (normal oocytes/total ovulated oocytes) (**D**) in *gad* mice are not significantly different from wild-type mice. Scale bars = 200 μm.

observed on the plasma membrane of ovulated mature oocytes (Figure 3, A and B) and zygotes (Figure 3, C and D). However, the intensity of the plasma membrane immunoreactivity was greater in mature oocytes than in zygotes. Therefore, we concluded that the distribution of UCH-L1 may change after fertilization.

## *Morphology of the gad Ovaries and Ovulated Oocytes*

To assess whether *gad* mice have morphologically normal ovaries, we used histology to compare ovaries from *gad* and wild-type (CBA/RFM) mice. *gad* mouse ovaries had morphologically normal oocytes, follicles, and corpora lutea (Figure 4, A and B). In addition, *gad* mice had a normal estrous cycle (data not shown). We further assessed *gad* mouse ovarian function by comparison with wild-type mice using the ovulation test. The total number of ovulated oocytes and the normal oocyte ovulation rate of superovulated oocytes did not differ significantly between *gad* mice and wild-type mice (Figure 4, C and D).

## *High Polyspermy Rate in gad Mouse Oocytes*

To analyze the fertilization rate in UCH-L1-deficient embryos of *gad* female mice, we assessed fertilization by *in vitro* fertilization using wild-type spermatozoa. For mice, the presence of more than three or more pronuclei defines polyspermic fertilization. Fertilized eggs from *gad* mice had characteristics consistent with this definition of polyspermy (Figure 5, B and C). By contrast, wild-type eggs showed normal zygotic nuclei (Figure 5A). The fertilized eggs of *gad* mice had a significantly higher rate of polyspermy (27 ± 0.04%) compared with wild-type mice (2 ± 0.02%) (Figure 5E). However, the fertility of *gad* mouse eggs (46 ± 0.06%) did not differ significantly from that of wild-type mice (43 ± 0.09%) (Figure 5D).

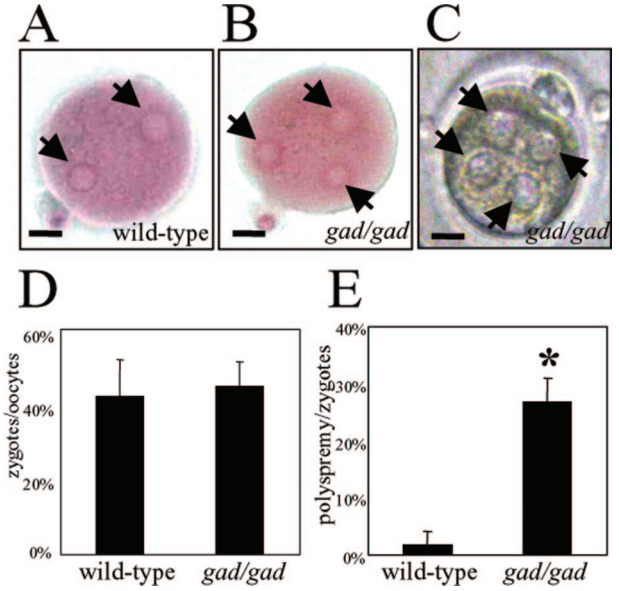


**Figure 5.** Incidence of polyspermy in *gad* mice. Fertilized eggs were stained with aceto-orcein (**A**, **B**) or visualized by inverted microscopy (**C**). **A:** Normal fertilized egg with two pronuclei (**arrows** in **A–C**) in wild-type mice. **B** and **C:** Polyspermic fertilized eggs in *gad* mice have three or four pronuclei. **D:** The fertility rate in *gad* mice is not significantly different from wild-type mice. **E:** Polyspermic rate in *gad* mice is significantly higher than that of wild-type mice. $^*P < 0.01$. Scale bars = 20 μm.

**Table 1.** Breeding Study of *gad* and Wild-Type Female Mice with Wild-Type Male Mice

| Genotype (*n*) | Litters | Pups | Litter size |
|---|---|---|---|
| Wild type (15) | 15 | 99 | 6.6 ± 1.3 |
| *gad/gad* (12) | 11 | 33 | 3 ± 2.0* |

Results represent the mean ± SD.
*Significantly different from wild-type mice, $P < 0.001$.

### *Low Breeding Rate of gad Female Mice*

To further evaluate the high polyspermic fertilization rate of *gad* female mice, we characterized the litter size of these mice after mating with wild-type male mice. *gad* female mice exhibited normal puberty and estrous cycle, as assessed by vaginal opening and vaginal smear, but they had reduced fertility, as evidenced by a significant decrease in litter size (3 ± 2.0) compared with wild-type mice (6.6 ± 1.3) (Table 1).

### *Monoubiquitin Level in gad Mouse Oocytes*

Our previous study demonstrated that UCH-L1 stabilizes monoubiquitin in testes and that the level of monoubiquitin is decreased in male *gad* mice.[8,9] To determine whether the high polyspermy rate in *gad* mouse oocytes correlated with a reduced level of monoubiquitin in the oocytes, we used Western blotting to measure monoubiquitin levels in oocytes from *gad* mice and wild-type mice. The monoubiquitin level was substantially lower in *gad* mouse oocytes (Figure 6A). However, wild-type and *gad* mouse oocytes showed the normal zona reaction (postfertilization proteolytic cleavage of ZP2) and normal cortical reaction (postfertilization exocytosis of cortical granules) (Figure 6, B and C).

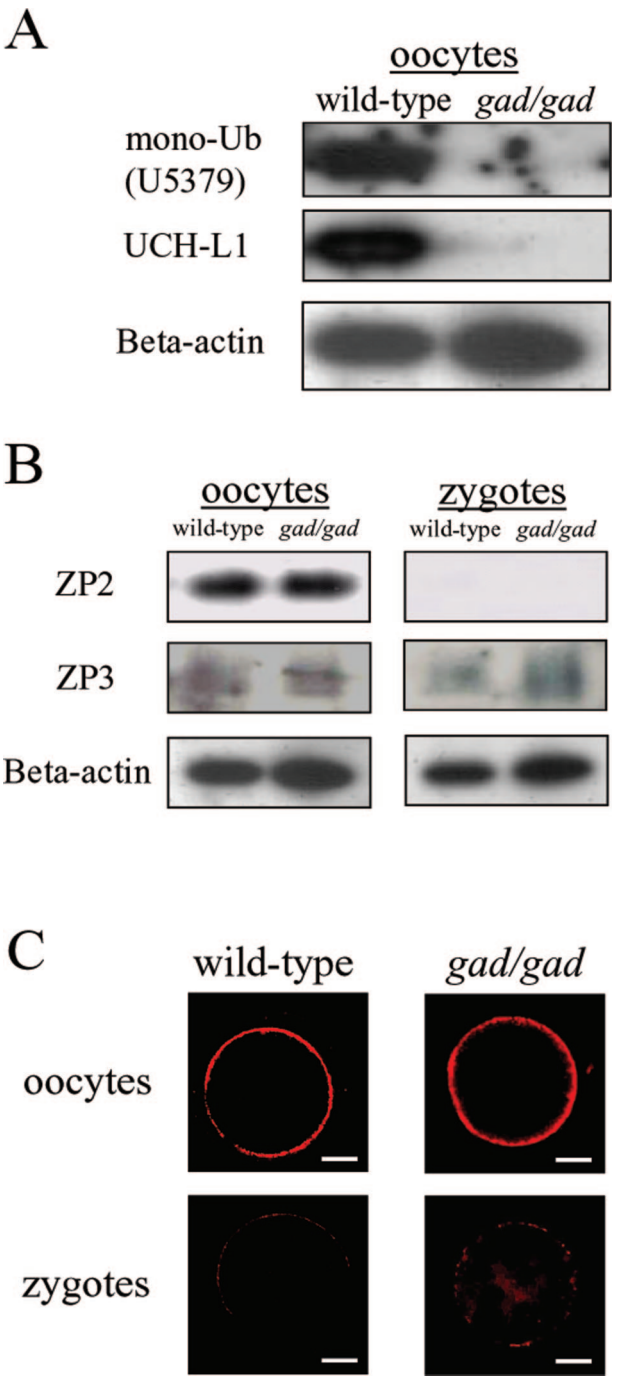


**Figure 6.** Expression of monoubiquitin, zona reaction, and cortical reaction in *gad* mice. **A:** Western blotting analysis of monoubiquitin of wild-type and *gad* mouse oocytes. The monoubiquitin protein level is substantially lower in *gad* than in wild-type. **B:** Western blotting analysis of ZP2 and ZP3 of wild-type and *gad* mouse oocytes and zygotes. Disappearance of ZP2 of zygotes because of postfertilization proteolytic cleavage is observed in wild-type and *gad* mice. **C:** Cortical reaction analysis of wild-type and *gad* mouse oocytes and zygotes. Disappearance of rhodamine-LCA of zygotes because of postfertilization cortical granule exocytosis is observed in wild-type and *gad* mice. Scale bars = 20 μm.

### *Discussion*

Polyspermy refers to the penetration of more than one sperm into oocytes during fertilization and is currently considered a pathological phenomenon in mammals.[30] However, an exceptionally high incidence of polyspermic fertilization has been revealed in pig fertilization.[31,32] The reasons for this higher incidence of polyspermy in oocytes are not clear, and relatively little is known about the exact mechanisms that prevent polyspermy. The mechanism that prevents polyspermy is classically illustrated by the zona reaction (cortical reaction) and subsequent plasma membrane block.[33,34] After penetration by sperm, the zona reaction occurs, and cortical contents are extruded into the perivitelline space, thereby preventing polyspermic penetration by hardening of the zona pellucida.[35] Plasma membrane block is assumed to result from changes (eg, the destruction of sperm receptors in the membrane) that preclude sperm adherence.[36,37]

Recent studies have demonstrated that the UPS is responsible for extracellular degradation of the sperm receptor on the outer face of zona pellucida and that proteasomal inhibitors block sperm penetration of the zona pellucida during fertilization.[17–19] These findings strongly support a role for the UPS in the sperm-oocyte interaction of the zona pellucida. However, our results showed the apparent contradiction that the high incidence of polyspermy was caused by down-regulation of the UPS function distinct from previous studies.[17–19] Our

present work shows that UCH-L1 is exclusively localized on the oocyte plasma membrane and that UCH-L1-deficient embryos of *gad* female mice have a significantly increased polyspermy rate *in vitro* (Figures 2B and 5). These results suggest that UCH-L1 may modify plasma membrane components, thereby preventing multiple sperm entry. In mammals, it has been suggested that UCH-L1 associates with monoubiquitin[7,10]; moreover, the monoubiquitin pool is reduced in *gad* mice relative to wild-type mice. We have shown that the monoubiquitin level in *gad* mouse oocytes is substantially reduced relative to that of wild-type mice (Figure 6A). These results suggest that the high incidence of polyspermy in *gad* mice may have its basis in the down-regulation of the UPS because of a reduction in available monoubiquitin at the plasma membrane, even though *gad* mouse oocytes undergo a normal zona reaction (Figure 6, B and C).[30,38] In addition, *gad* mice also have morphologically normal ovary development and a normal rate of ovulation compared with wild-type mice (Figure 4).

CD9 is an integral membrane protein associated with integrins and other membrane proteins.[39] CD9 is extensively localized on the oocyte plasma membrane.[39–41] Recent studies suggest that CD9 participates in sperm-oocyte fusion in the mouse.[39] CD9 knockout female mice ovulate normally, but the oocytes are rarely fertilized because CD9 deficiency on the plasma membrane inhibits sperm-oocyte fusion. Many plasma membrane channels, transporters, and receptors undergo ubiquitination at the extracellular face, which is required for internalization and subsequent entry into the endocytic pathway.[42] These studies indicate that ubiquitination (monoubiquitination, multiple monoubiquitination, or polyubiquitination) of plasma membrane proteins might facilitate plasma membrane block.

In the present study, we showed that both UCH-L1 and UCH-L3 are strongly expressed throughout all stages of oogenesis and embryogenesis (Figure 1A), even though ovarian and uterine functions change periodically because of fluctuations in hormone levels.[43–45] Our previous studies suggested that UCH-L1 and UCH-L3, despite their high sequence homology,[12] have distinct expression patterns and differential function in testis and epididymis.[9,11] Here we show that these two proteins have distinct distributions: UCH-L1 is exclusively expressed on the plasma membrane of oocytes and embryos, whereas UCH-L3 is diffusely expressed in the cytoplasm (Figures 1B and 2B). Therefore, it is conceivable that UCH-L1 and UCH-L3 have different functional roles in oocytes and embryos, as was shown in the testis/epididymis.[9,11] Although our studies show that UCH-L3 is highly expressed in the cytoplasm of oocytes and embryos, we found no difference in the fertilization rate and litter size in *Uchl3* knockout female mice compared with wild-type (C57BL/6J) female mice (data not shown). Thus, UCH-L3 may not impact these developmentally related processes; further research is necessary to elucidate the cytoplasmic function of this UCH.

Polyspermy in humans mostly results in embryonic lethality or triploid embryos, which usually mature into infertile offspring.[46,47] According to the World Health Organization, ~80 million people worldwide are infertile. Infertility may result from female- or male-derived factors, a combination of the two, or as yet unidentified biological factors.[48] Our present work shows that a deficiency in UCH-L1 may be a new cause of female infertility. Thus, *gad* mice may be a useful model for further studies of infertility.

In conclusion, we show that UCH-L1-deficient *gad* female mice oocytes have a significantly increased rate of polyspermy in an *in vitro* fertilization assay; consequently, these mice have significantly decreased litter size. These results suggest that UCH-L1 is a crucial factor in the plasma membrane block to polyspermy in mouse oocytes.

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