

Effects of ubiquitin C-terminal hydrolase L1 deficiency on mouse ova

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Abstract

Maternal proteins are rapidly degraded by the ubiquitin–proteasome system during oocyte maturation in mice. Ubiquitin C-terminal hydrolase L1 (UCHL1) is highly and specifically expressed in mouse ova and is involved in the polyspermy block. However, the role of UCHL1 in the underlying mechanism of polyspermy block is poorly understood. To address this issue, we performed a comprehensive proteomic analysis to identify maternal proteins that were relevant to the role of UCHL1 in mouse ova using UCHL1-deficient *gad*. Furthermore, we assessed morphological features in *gad* mouse ova using transmission electron microscopy. NACHT, LRR, and PYD domain-containing (NALP) family proteins and endoplasmic reticulum (ER) chaperones were identified by proteomic analysis. We also found that the ‘maternal antigen that embryos require’ (NLRP5 (MATER)) protein level increased significantly in *gad* mouse ova compared with that in wild-type mice. In an ultrastructural study, *gad* mouse ova contained less ER in the cortex than in wild-type mice. These results provide new insights into the role of UCHL1 in the mechanism of polyspermy block in mouse ova.

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Introduction

Maternal proteins are synthesized and degraded dynamically during oocyte maturation. Intracellular protein degradation plays important roles in modulating the levels of specific proteins and eliminating damaged proteins to achieve fertilization and early embryo development. The ubiquitin–proteasome system is a major pathway for selective intracellular protein degradation in ova. Ubiquitination of proteins is recognized to target proteins for degradation by proteasomes and for internalization into the lysosomal system. Deubiquitinating enzymes (DUBs) regulate ubiquitination and regenerate free ubiquitin after proteins have been targeted to the proteasome or lysosome.

While other DUB members are ubiquitously expressed, ubiquitin C-terminal hydrolase L1 (UCHL1), one of the DUBs, is selectively expressed in neurons and germ cells (Wilson *et al.* 1988, Wilkinson *et al.* 1989). Recent studies suggest that UCHL1 associates with monoubiquitin and prolongs the ubiquitin half-life in neurons (Osaka *et al.* 2003) and that UCHL1 regulates apoptosis in testicular germ cells (Kwon *et al.* 2004,

2005). However, the role of UCHL1 in ova is largely unknown. We previously reported that UCHL1 is exclusively localized on the plasma membrane of mouse ova and plays an important role in the polyspermy block (Sekiguchi *et al.* 2006). Polyspermic fertilization refers to the penetration of ova by two or more spermatozoa, resulting in the developmental failure of the zygote (Sun 2003). It is necessary to block polyspermy for successful fertilization. At the time of fertilization, polyspermy is prevented primarily by the zona pellucida (ZP) block (also known as the zona reaction) following cortical granule exocytosis (CGE) in mammalian ova (Hatanaka *et al.* 1992). Susor *et al.* (2010) showed that UCHL1 regulates CG reorganization and contributes to the ZP block using UCHL1 inhibitors. In contrast, ‘gracile axonal dystrophy’ (*gad*) female mice, which are autosomal recessive, spontaneous mutants carrying an intragenic deletion of the gene encoding UCHL1, show a significantly increased *in vitro* polyspermy rate and a decrease in litter size, even though *gad* mouse ova undergo a normal zona reaction (Saigoh *et al.* 1999, Sekiguchi *et al.* 2006). Thus, the role of

UCHL1 in the mechanism of inhibiting polyspermy in mouse ova remains unclear.

To address these issues, we sought to identify the difference in protein expression induced by *Uchl1* deletion, based on a comprehensive proteomic analysis using *gad* mice, and identified maternal-specific proteins and endoplasmic reticulum (ER) chaperones that were relevant to the role of UCHL1 in mouse ova. Further, we observed morphological changes in *gad* mouse ova using transmission electron microscopy (TEM). These findings have important implications for understanding the role of UCHL1 in mouse ova.

Results

Normal expression of F-actin and cofilin in *gad* mouse ova

F-actin is necessary for CGE, changes membrane structure at fertilization, and consequently blocks polyspermy of ova (Tsaadon *et al.* 2006). We investigated the protein expression and localization of F-actin and its depolymerizing factor, cofilin, in wild-type and *gad* mouse ova (Fig. 1). Cofilin belongs to a family of F-actin depolymerizing factors that are highly conserved throughout the animal kingdom. The cofilin protein severs actin filaments and promotes actin dynamics by accelerating the treadmilling of actin filaments, a process in which monomers are removed from the pointed end of the filaments and are added to the barbed end (Carlier *et al.* 1997). However, no difference in the protein level or localization was observed between the wild-type and *gad* mouse ova.

Identification of differentially expressed ovum proteins by proteomic analysis

A typical proteomic analysis involves three fundamental steps: 1) proteins of a complex mixture are separated and digested into peptide units, 2) peptide masses are determined using mass spectroscopy, and 3) proteins are identified by searching databases for matching peptides (Kirkpatrick *et al.* 2005). In this study, liquid chromatography–tandem mass spectrometry (LC–MS/MS) was used. The list of proteins that were

downregulated or upregulated with statistical significance (one-sided P value <0.025) in *gad* mouse ova is presented in Table 1. ZP1 is a component of the ZP, consisting of three glycoproteins (ZP1, ZP2, and ZP3), and cross-links ZP2 and ZP3 (Wassarman & Litscher 2008). TLE6 belongs to the *Groucho/Tle* superfamily of transcriptional corepressors that plays critical roles in a range of developmental processes (Bajoghli 2007, Buscarlet & Stifani 2007). ENPL, HSPA5 (GRP78), and CALR are ER chaperones and regulators of the unfolded protein response (UPR) in the ER (Qiu & Michalak 2009, Eletto *et al.* 2010, Pfaffenbach & Lee 2011). NLRP14 (NALP14) is a member of the NALP family, which consists of 14 members. The family members have quite similar domain structures and expression during oogenesis and embryogenesis. Thus, they are assumed to have common functions during oocyte maturation and early embryo development (Zhang *et al.* 2008). However, at present, there is little information about NLRP14 and its function remains to be determined. In contrast, NLRP5 (NALP5), one of the NALP family members, has been well studied. Furthermore, the NLRP5 protein has many characteristics in common with UCHL1. Their expression levels and subcellular localization are strongly correlated throughout oogenesis and embryogenesis (Li *et al.* 2008, Ohsugi *et al.* 2008). NLRP5 localizes in the subcortex of ova and is excluded from the region of cell–cell contact at the two-cell stage. Correspondingly, UCHL1 shows the similar expression pattern in the ovum and at the two-cell stage (Fig. 2). Thus, in this study, we focused on the NLRP5 protein for its interesting similarities to UCHL1.

Expression and localization of the NLRP5 protein in mouse ova

We conducted a western blot analysis to confirm the expression levels of the NLRP5 protein in ova obtained from *gad* mice with those from wild-type mice. Levels of NLRP5 in *gad* mouse ova were significantly higher than those in wild-type mouse ova ($P=0.022$; Fig. 3A and B). Furthermore, to assess the relationship between UCHL1 and NLRP5, we analyzed their localization in wild-type and *gad* mouse ova. The two proteins colocalized on the plasma membrane and in the subcortex of wild-type

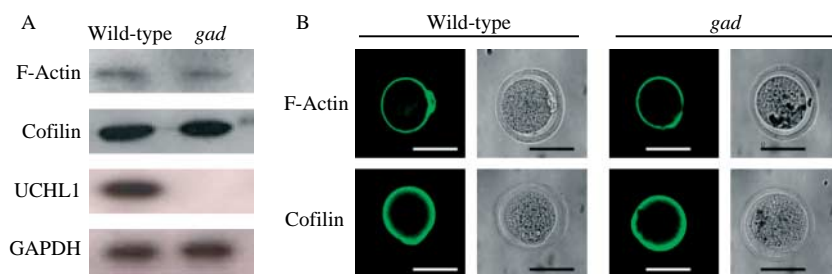


Figure 1 Expression and localization of F-actin and cofilin proteins in mouse ova. (A) Western blot analysis of F-actin, cofilin, ubiquitin C-terminal hydrolase L1 (UCHL1), and GAPDH in ova from wild-type ($n=6$) and *gad* ($n=7$) mice. No significant difference was observed in the F-actin and cofilin protein levels. UCHL1 was not detected in *gad* mouse ova. (B) F-actin and cofilin immunoreactivity are seen on the plasma membrane of ova from wild-type and *gad* mice. Bars, 50 μm.

Table 1 Differentially expressed oocyte proteins in wild-type and *gad* mice by liquid chromatography–mass spectrometry analysis.

Identified proteins	Molecular weight (kDa)	Expression level (wild-type (wt) vs <i>gad</i>)	P value (one-sided)
Zona pellucida sperm-binding protein 1 precursor (ZP1)	69	wt < <i>gad</i>	0.022
Transducin-like enhancer protein 6 (TLE6)	65	wt < <i>gad</i>	0.025
Endoplasmic precursor (ENPL)	92	wt < <i>gad</i>	0.022
78 kDa glucose-regulated protein precursor (HSPA5)	72	wt < <i>gad</i>	0.023
Calreticulin precursor (CALR)	48	wt < <i>gad</i>	0.002
NACHT, LRR, and PYD domain-containing protein 14 (NLRP14)	113	wt < <i>gad</i>	0.023
Ubiquitin C-terminal hydrolase L1 (UCHL1)	25	wt > <i>gad</i>	0.002

mouse ova (Fig. 4). However, the localization of NLRP5 in *gad* mouse ova was not different from that in wild-type mice (Fig. 4).

Ultrastructural observations of *gad* mouse ova

To assess whether *gad* mouse ova have a morphologically normal cortex structure, we analyzed them ultrastructurally by TEM (Fig. 5). Although basic structures such as CGs, microvilli, and cytoplasmic lattices (CPLs) were observed in both wild-type and *gad* mouse ova, there were no obvious structures that can be identified with ER cluster in the cortex of *gad* mouse ova.

Discussion

This study provided important findings on the role of UCHL1 in the mechanism of polyspermy block in mouse ova. It is necessary to block polyspermy for successful fertilization, which primarily involves a ZP block in mammalian ova. The ZP block comprises CGE and modification of the ZP so that the plasma membrane cannot support sperm binding by the enzymes released from CGs. F-actin regulates the movement of CGs toward the cortex during oocyte maturation and anchors them at the cortex (Connors *et al.* 1998). F-actin depolymerization occurs at CGE, and the protein level of F-actin decreases after fertilization (Eliyahu *et al.* 2005). We observed that the F-actin and cofilin protein expression levels and distribution on the plasma membrane were similar between the wild-type and *gad* mouse ova. We also found decreased F-actin protein levels, but no significant difference between the wild-type and *gad* mouse zygotes was observed after fertilization (data not shown). These results confirm our results of the CGE and ZP reaction in *gad* mouse ova (Sekiguchi *et al.* 2006). However, each protein is recognized differentially according to various post-translational modifications, such as phosphorylation and

ubiquitination (Kirkpatrick *et al.* 2005). In fact, cofilin proteins are thought to be regulated through phosphorylation and activated by dephosphorylation at the serine residue (Meberg & Bamberg 2000). Thus, these findings suggest that cofilin proteins in *gad* mouse oocytes might be degenerated or modified differently from those in wild-type ova. Further experiments are needed to confirm this.

We then conducted a comprehensive proteomic analysis to identify any UCHL1-related proteins using *gad* mouse ova and identified NALP family proteins. The NALP family has 14 members with similar domain structures, and thus members are assumed to have common functions (Zhang *et al.* 2008). Almost all NALP family genes are expressed during oogenesis and embryogenesis (Zhang *et al.* 2008). We then focused on NLRP5 and confirmed protein expression and localization in wild-type and *gad* mice. The specificity and quantification of the LC–MS/MS method are greater than or equal to immunoassays including western blot and immunocytochemical staining. However, the high specificity enables us to reduce false-positive, but, on the other hand, it also reduces sensitivity (Kruse *et al.* 2008, Krone *et al.* 2010). Hence, this is the reason why NLRP5 was not detected by LC–MS/MS analysis in this study. The NLRP5 protein has many characteristics in common with UCHL1 (Sekiguchi *et al.* 2006, Ohsugi *et al.* 2008). First, UCHL1 and NLRP5 protein expression persist from immature oocytes in a primary follicle until the blastocyst stage.

Secondly, UCHL1 and NLRP5 may play an important role in oocyte maturation or embryogenesis because both *gad* and NLRP5-deficient females attain normal sexual maturity with intact ovarian folliculogenesis and the ability to ovulate fertilizable ova, but they have severe defects in reproduction. Thirdly, the most interesting analogy is the subcellular localization; neither protein is detected in the region of contact between the blastomeres, and they are restricted to the periphery through embryogenesis. This shared and unique expression pattern appears to indicate an interaction, such as regulation of NLRP5 protein levels by the ubiquitin–proteasome system including UCHL1. In fact, we found a significant increase in NLRP5 protein levels in *gad* mice with localization on the plasma membrane and subcortex.

The NLRP5 protein was identified abundantly and is an excellent candidate for a maternal-effect protein for the maturation and ovulation of ova (Zhang *et al.* 2009). The NLRP5 protein and transcript accumulate during oogenesis, and the transcript is almost completely degraded during meiotic maturation (Su *et al.* 2007, Li *et al.* 2008, Ohsugi *et al.* 2008). In contrast to the dramatic decrease in the transcript, the protein remains largely unchanged until the early blastocyst stage, suggesting that the protein level is properly regulated by degradation for the maintenance and control of

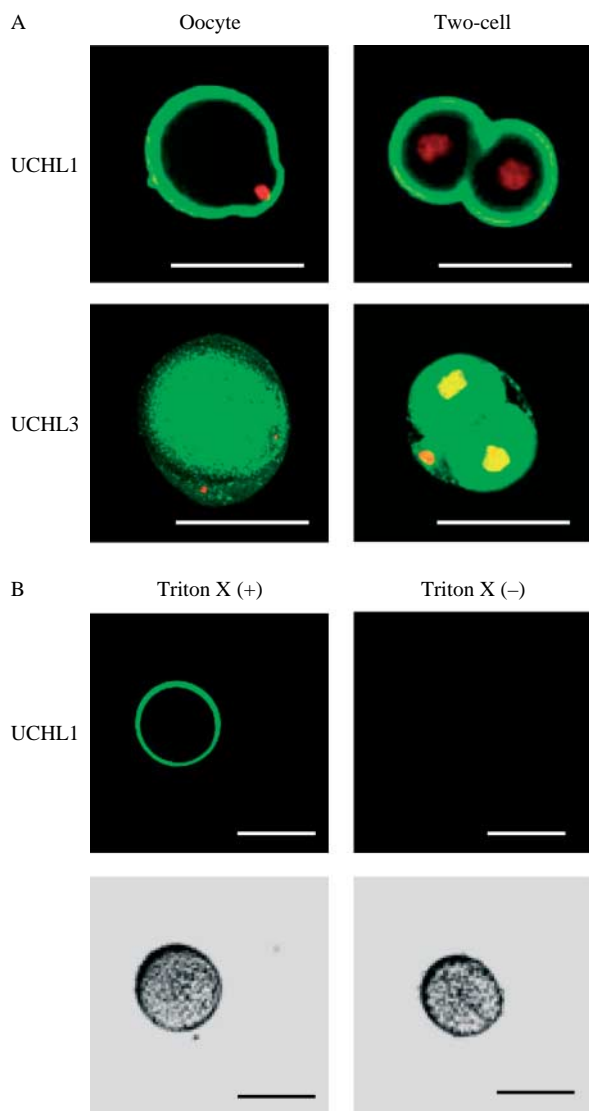


Figure 2 Localization of ubiquitin C-terminal hydrolase L1 (UCHL1) and UCHL3 in wild-type mouse ova. (A) UCHL1 immunoreactivity is seen on the plasma membrane of the ovum and at the two-cell stage. On the other hand, UCHL3, one of the DUBs, is seen in the cytoplasm of the ovum and at the two-cell stage. (B) While UCHL1 immunoreactivity is seen on the plasma membrane when treated with Triton X-100, it is not seen in the ova when not treated with Triton X-100. Bars, 50 μ m.

maturation, fertilization, and early embryo development. Degradation of maternal proteins and transcripts is carried out rapidly in many organisms and plays a critical role in oocyte maturation. Most protein degradation is regulated by the ubiquitin–proteasome system and any impairment in degradation results in the failure of oocyte maturation (DeRenzo & Seydoux 2004, Huo *et al.* 2004, Ryu *et al.* 2008, Beall *et al.* 2010). UCHL1 is essential for maintaining the ubiquitin–proteasome system and is abundantly expressed in mouse ova (Sekiguchi *et al.* 2006). Similarly, previous studies have identified UCHL1 as a maternal protein necessary for oocyte maturation

(Ellederova *et al.* 2004, Massicotte *et al.* 2006, Susor *et al.* 2007). Thus, a logical potential conclusion would be that the NLRP5 protein level is potentially regulated by the ubiquitin–proteasome system for oocyte maturation. In fact, ubiquitin mRNA levels are upregulated when the NLRP5 protein decreases during oogenesis and embryogenesis (Robert *et al.* 2002, Pennetier *et al.* 2006, Ohsugi *et al.* 2008). Furthermore, a UCHL1 inhibitor prevents germinal vesicle (GV) breakdown and the metaphase I–anaphase transition in porcine oocytes by downregulating the ubiquitin–proteasome system (Susor *et al.* 2007). Thus, excess NLRP5 protein resulting from downregulating the ubiquitin–proteasome system prevents complete oocyte maturation. Recent studies have reported that NLRP5 forms a subcortical maternal complex (SCMC) with maternally encoded proteins, an oocyte-specific binding protein of NLRP5 (FILIA), ‘a factor located in oocytes permitting embryonic development’ (FLOPED), and a putative transcriptional corepressor (TLE6; Li *et al.* 2008, Ohsugi *et al.* 2008). Considering that TLE6 was significantly upregulated in *gad* ova (Table 1), it seems likely that SCMC of *gad* mouse ova had difficulty in regulating the component balance. Thus, further research is required to understand the relationship between maternal proteins and UCHL1 in mouse ova. Taken together, we hypothesized that *gad* mouse ova polyspermy is caused by oocyte maturation failure (Fig. 6).

To test this, we investigated whether *gad* mouse ova showed morphological characteristics of immaturity using TEM. As expected, the CG of *gad* mouse ova localized normally. This result provides further support for our previous work (Sekiguchi *et al.* 2006). Recent reports have shown that NLRP5 also localizes to the CPLs, and *Mater*^{tm/tm} mouse ova lack CPLs (Kim *et al.* 2010, Tashiro *et al.* 2010). Unlike *Nlrp5* hypomorphic mice, *gad* mouse ova containing excess NLRP5 protein showed normal CPLs. Interestingly, there were no obvious structures that can be identified with ER cluster in the cortex of *gad* mouse ova. The ER is a multifunctional and highly dynamic organelle involved in lipid and protein synthesis and is a major internal store of calcium ions that must be properly mobilized at fertilization (Baumann & Walz 2001, Berridge 2002). It is well established that fertilization triggers an increase and oscillations in intracellular calcium concentration, resulting in the induction of CGE (Ducibella & Fissore 2008). The mechanisms of intracellular calcium release from the ER develop during oocyte maturation, and an ER cluster appears in the cortex of mature mouse ova (Mehlmann *et al.* 1995). Cortical reorganization of the ER occurs in many species before fertilization, and the presence of ER cortical clusters is thought to result in a high susceptibility of ova to initiate calcium oscillations following sperm entry (Ducibella & Fissore 2008). The sensitivity of the system to calcium release increases after maturation. A previous study reported differences

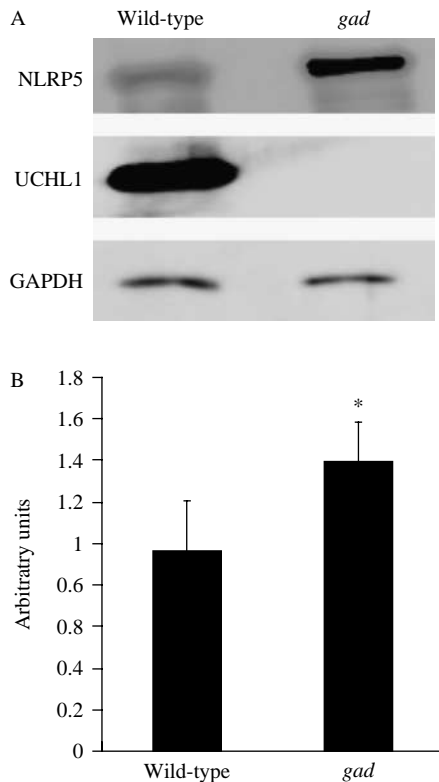


Figure 3 Expression of 'maternal antigen that embryos require' (NLRP5 (MATER)) and 'ubiquitin C-terminal hydrolase L1' (UCHL1) proteins in mouse ova. (A) Western blot analysis of NLRP5, UCHL1, and GAPDH in ova from wild-type ($n=5$) and *gad* ($n=4$) mice. UCHL1 was not detected in *gad* mouse ova. (B) NLRP5 protein levels in *gad* mouse ova were significantly higher than those in wild-type mice ($P=0.02$). Bars represent mean \pm s.d. Significant difference ($P<0.05$) is indicated by an asterisk.

regarding fertilization-induced calcium signals between immature oocytes and ova (Stricker 1999). Immature oocytes have less ER in the cortex compared with ovulated ova, and calcium release in response to fertilization is substantially less and slower than mature ova, resulting in polyspermy (Ducibella *et al.* 1993). Polyspermy in immature oocytes was attributed to complete failure or a CGE delay following calcium signaling (Ducibella *et al.* 1993). These findings suggest that polyspermy observed in *gad* mouse ova may have been caused by a delay in CGE resulting from its immaturity, although CGE and ZP react (Fig. 6). Furthermore, a recent study showed that calcium signaling plays a role in establishing the membrane block to polyspermy using ZP-free mouse ova (McAvey *et al.* 2002). Thus, *gad* mouse ova polyspermy was also considered to be due to the failure of the membrane block (Fig. 6). However, the mechanism for a polyspermy membrane block is largely unknown. Further examination is required to confirm this.

Additionally, we identified ER chaperones (ENPL, HSPA5, and CALR) by proteomic analysis, which were

upregulated significantly in *gad* mouse ova. These chaperone proteins are constitutively expressed in the ER, and expression is regulated by the UPR, known as the ER signal transduction pathway (Rutkowski & Hegde 2010). Unfolded or malformed proteins accumulating in the ER (ER stress) activate the UPR, which degrades the proteins via ER-associated degradation involving the ubiquitin–proteasome system (Yoshida 2007). Given that the ubiquitin–proteasome system is impaired due to the lack of UCHL1 in *gad* mouse ova, ER chaperones may be upregulated by the accumulation of abnormal proteins. Indeed, ER stress is induced by a UCHL1 inhibitor in neuronal cells (Tan *et al.* 2008). Thus, it is also possible that ER stress following an excess of abnormal protein causes ER dysfunction, and *gad* mouse ova showed an impaired polyspermy block (Fig. 6). However, further investigations are necessary to reveal the relationship between polyspermy and a UCHL1 deficiency in ova.

In conclusion, we identified UCHL1-related proteins by proteomic analysis using *gad* mouse ova. We also found that the NLRP5 protein level increased significantly in *gad* mouse ova vs wild-type mice. Furthermore, we observed that *gad* mouse ova contained less ER in the cortex. These results suggest that UCHL1 regulates the NLRP5 protein level in mouse ova maturation.

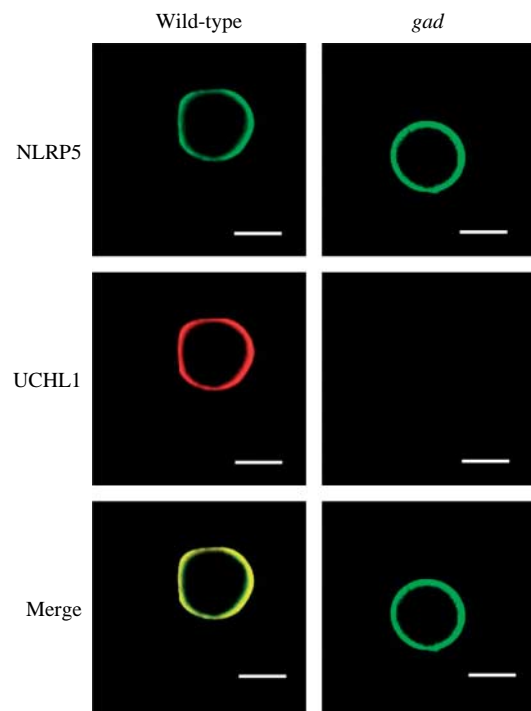


Figure 4 Localization of 'maternal antigen that embryos require' (NLRP5 (MATER)) and 'ubiquitin C-terminal hydrolase L1' (UCHL1) proteins in mouse ova. Immunocytochemical analysis of NLRP5 and UCHL1 in ova from wild-type and *gad* mice. NLRP5 immunoreactivity is seen on the plasma membrane of ova from wild-type and *gad* mice. NLRP5 and UCHL1 are co-localized on the plasma membrane of ova from wild-type mice. Bars, 50 μ m.

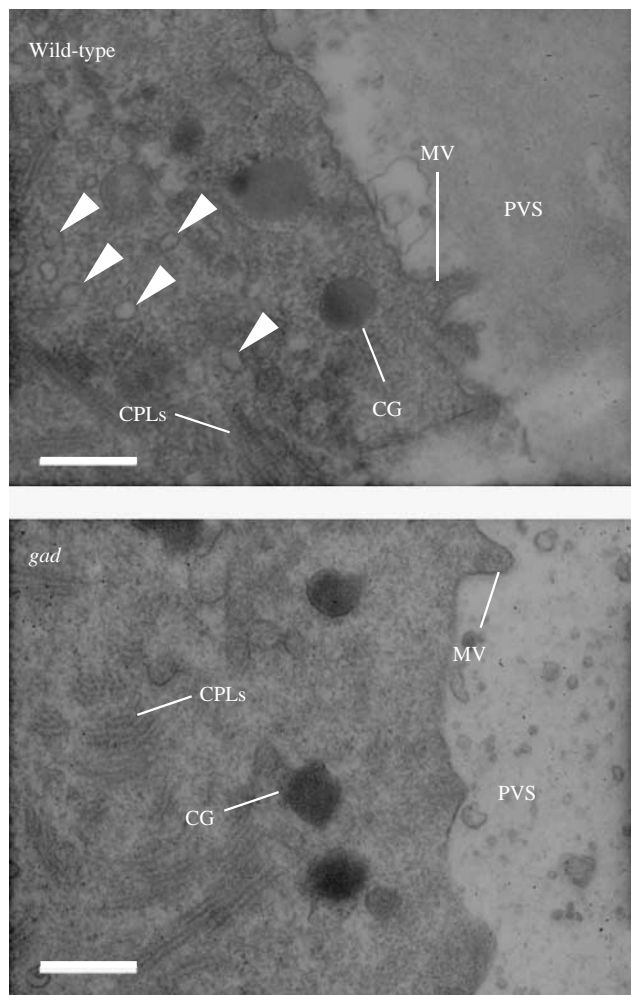


Figure 5 Ultrastructural study of ova from wild-type and *gad* mice by transmission electron microscopy. Basic cellular structures and cortical granule (CG), microvilli (MV), cytoplasmic lattices (CPLs), and perivitelline space (PVS) are observed in wild-type and *gad* mice. *gad* ova appear to have less endoplasmic reticulum (ER) than wild-type mice (arrowheads). Bars, 0.5 μm .

Materials and Methods

Animals

Nine-week-old BDF1 and *gad* female mice were used in this study. BDF1 mice were purchased from Nihon SLC, Inc. (Hamamatsu, Japan). The *gad* mouse is an autosomal recessive mutant obtained by cross-breeding CBA and RFM mice (Saigoh *et al.* 1999). The *gad* line is maintained at our institute. Genotyping was conducted using PCR with the following primers: F1, 5'-AGCTTGGAGCCTGTGGTTTCAACTC-3'; R1, 5'-TGGCAGCATCCTGAAAAGGAGAGAGGTG-3'; R2, 5'-TACAGATGGCCGTCCACGTTGTTGA-3', as described elsewhere (Sakurai *et al.* 2008).

Animal care and handling were conducted in accordance with institutional regulations. These experiments were approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Ova collection

Female mice were superovulated with i.p. injections of 5 IU equine chorionic gonadotropin for 48 h, followed by 5 IU human chorionic gonadotropin (hCG). Ovulated ova were collected from the ampullae of the oviducts using the scratching method 15 h after hCG injection and placed in 400 μl droplets of HTF medium containing 0.4 mg/ml BSA. Then, 0.05% hyaluronidase was added to the medium to remove the cumulus cells.

Liquid chromatography–tandem mass spectrometry

The capillary reversed-phase μLC –MS/MS system (ZAPLOUS System, AMR, Inc., Tokyo, Japan) consisted of a Paradigm MS4 dual solvent delivery system (Michrom BioResources, Inc., Auburn, CA, USA) for HPLC, an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), and Finnigan LTQ linear ion-trap mass spectrometers (ITMS, Thermo Fischer, San Jose, CA, USA) equipped with XYZ nanoelectrospray ionization (NSI) sources (AMR, Inc.). We used three wild-type mice and three *gad* mice for LC–MS/MS analysis and collected 20 ova from each mouse. Samples (20 ova each) were prepared using digestion solution to a final volume of 20 μl . Then, digested samples of 1.0 μl (equivalent to 1-ovum proteins) were automatically injected into a peptide L-trap column (Chem. Eval. Res. Inst., Saitama, Japan) on an injector valve for concentrating and desalting. Sample handling and injection were conducted with the HTC-PAL autosampler (CTC Analytics).

An injection volume was 1/20 equivalents. After desalting with 0.1% TFA (trifluoro acetic acid; aq.) containing 2% acetonitrile, the sample was loaded into a MAGIC C18 capillary reversed-phase column (3 μm , 200 \AA , 150 \times 0.2 mm i.d.; Michrom BioResources, Inc.) for separation. The mobile phase was as follows: A) 98% H_2O /2% CH_3CN /0.1% HCOOH and B) 10% H_2O /90% CH_3CN /0.1% HCOOH . The gradient conditions in the chromatographic run were as follows: B from 5% B (0 min) to 55% B (100 min), and then 95% B (1 min), 95% B (9 min), 5% B (1 min), and 5% B (9 min). Effluent at a flow rate of 0.5 ml/min was introduced into the mass spectrometer by the NSI interface via an injector valve with the L-trap column and the reversed phase (RP) column. The NSI needle (150/20 FortisTip; OmniSeparo-TJ, Hyogo, Japan), connected directly to the reverse-phase column outlet, was used as the NSI interface and the voltage was 2.0 kV, while the capillary was heated to 200 $^\circ\text{C}$. No sheath or auxiliary gas was used.

Furthermore, the mass spectrometer was operated in a data-dependent acquisition mode in which MS acquisition with a mass range of 450–1800 m/z was automatically switched to MS/MS acquisition under the automated control of Xcalibur Software (Thermo Fisher, San Jose, CA, USA). The MS measurement sequence was Full MS (enhanced/profile), MS/MS (Top1: normal/centroid), MS/MS (Top2: normal/centroid), and MS/MS (Top3: normal/centroid). The parent ions were subjected to MS/MS scans with an isolation width of 2.0 m/z ; the activation amplitude parameter was set at 35%. The trapping time was 50 ms under the auto gain control mode. Data were acquired using the dynamic mass exclusion windows that had an exclusion of 2.0 min duration and an exclusion mass width of -0.5 and $+1.5$ Da.

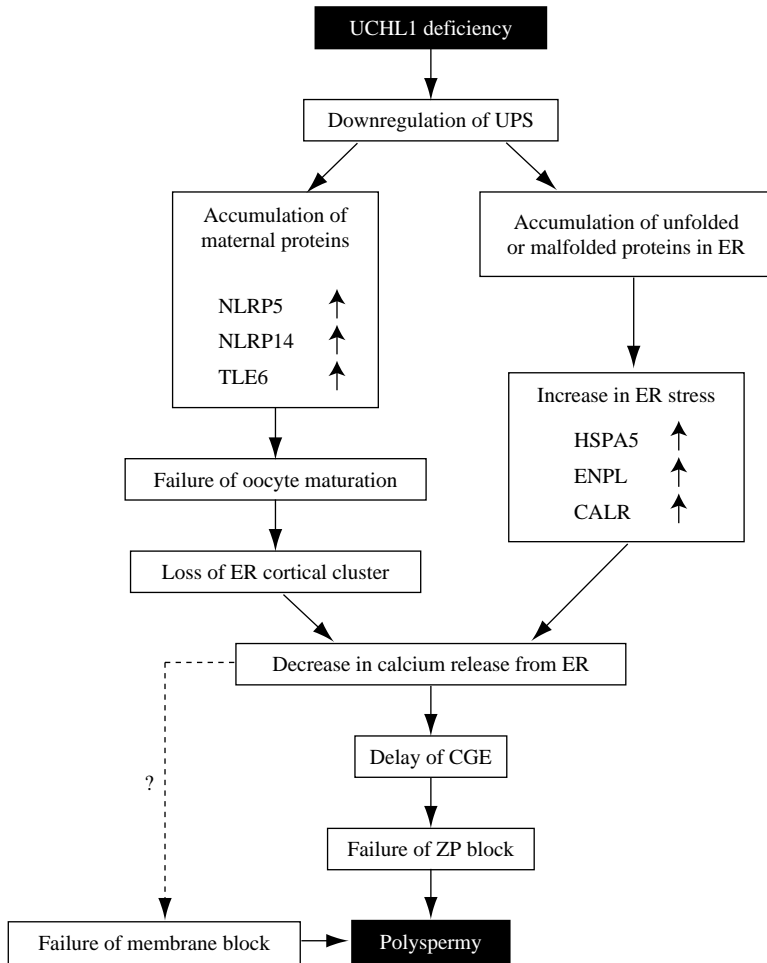


Figure 6 Consequences of UCHL1 deficiency in *gad* mouse ova. UCHL1 deficiency results in the downregulation of UPS and accumulation of maternal proteins in the cytoplasm and unfolded or malformed proteins in the ER. These accumulations of protein in the cytoplasm and ER may contribute to the failure of oocyte maturation and increase in ER stress, which in turn decrease calcium release from the ER after fertilization. Thus, UCHL1 deficiency might downregulate CGE, ZP block, and membrane block and consequently induce polyspermy. UPS, ubiquitin–proteasome system; ER, endoplasmic reticulum; CGE, cortical granule exocytosis; ZP, zona pellucida.

Spectral analysis and database searching

All MS/MS data were investigated using the Bioworks (version 3.1; Thermo Fischer) against the NCBI mouse (*Mus musculus*) database. The database searches allowed for variable modification of the methionine residue (oxidation, 116 Da), oxidation (M), peptide mass tolerance at 2.0 Da, and fragment mass tolerance at 1.0 Da. The identified proteins resulted from triplicate LC–MS runs for each sample and included one peptide identification. We performed label-free quantification on ovum proteins from *gad* mouse and wild-type mice (Kawamura *et al.* 2010).

Antibodies

Rabbit polyclonal anti-UCHL1 and UCHL3 antibodies were provided by Dr Kwon (Tyun-Puk University, South Korea). Mouse monoclonal anti-PGP9.5 antibody was obtained from Neuromics (Northfield, MN, USA). Rabbit polyclonal anti-cofilin and mouse monoclonal anti-GAPDH antibodies were purchased from Abcam. Rabbit anti-MATER antiserum was donated by the Laboratory of Cellular and Developmental Biology, NIDDK, National Institutes of Health (NIH), Bethesda, MD, USA.

Western blot analysis

Ova were subjected to SDS–PAGE using 8% gel for cofilin and 15% gel for NLRP5 proteins. The proteins were electrophoretically transferred to a PVDF membrane and blocked with 5% nonfat dried milk in PBS–Tween 80. The membranes were incubated individually with primary antibodies (cofilin, 1:10 000; MATER, 1:15 000; UCHL1, 1:10 000; and GAPDH, 1:10 000) at 4 °C overnight. Then, the membrane was incubated with primary antibody against GAPDH as an internal control for 1.5 h and further incubated with peroxidase-conjugated secondary antibody (goat antirabbit IgG, 1:10 000–15 000; goat antisheep IgG, 1:3000; and goat antimouse IgG, 1:10 000) for 1 h at room temperature. Immunoreactions were visualized by ECL plus (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Each immunoreactive band was quantified using Scion Image Software (NIH).

Phalloidin and immunohistochemical staining

For immunocytochemical staining, whole ova were fixed for 45 min with 4% paraformaldehyde in PBS and permeabilized for 20 min with 0.2% Triton X-100 in PBS. Nonspecific binding of immunoglobulins was blocked by incubating with Block

Ace for 45 min at room temperature. The sections were incubated with Alexa 488-phalloidin (1:2000) for 20 min at room temperature or with rabbit polyclonal anti-cofilin (1:10 000) antibody at 4 °C overnight. Then, they were further incubated with Alexa 488-conjugated anti-rabbit IgG for 1 h at room temperature.

For double-immunohistochemical staining, the sections were incubated with anti-MATER antiserum (1:10 000) and mouse monoclonal anti-UCHL1/PGP9.5 (1:40) antibody at 4 °C overnight. Then, they were further incubated with Alexa 488-conjugated anti-rabbit IgG and Alexa568-conjugated anti-mouse IgG for 1 h at room temperature. Stained sections were observed under a confocal laser microscope (Laser Scanning Microscope 510; Carl Zeiss, Oberkochen, Germany).

Transmission electron microscopy

Isolated ova were fixed in 2.5% glutaraldehyde/0.1 M phosphate buffer (PB) for 4 h at 4 °C. After washing with PBS, they were postfixed in 1% OsO₄ in 0.1 M PB for 2 h at 4 °C. They were then dehydrated and embedded in araldite M. Ultrathin sections were examined under a JEOL-1010 transmission electron microscope at 80 kV.

Statistical analyses

The mean and s.d. were calculated for all data. Student's *t*-test was used for all statistical analyses. A *P* value <0.05 was considered to indicate statistical significance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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