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### Permalink

<https://escholarship.org/uc/item/9jp6h383>

### Journal

Reproductive Biology and Endocrinology, 1(1)

### ISSN

1477-7827

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### Publication Date

2003-11-01

### DOI

10.1186/1477-7827-1-77

Peer reviewed

Research

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## Biochemical heterogeneity, migration, and pre-fertilization release of mouse oocyte cortical granules

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Published: 07 November 2003

Received: 19 September 2003

*Reproductive Biology and Endocrinology* 2003, **1**:77

Accepted: 07 November 2003

This article is available from: <http://www.rbej.com/content/1/1/77>

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### Abstract

**Background:** Oocyte cortical granules are important in the fertilization of numerous species including mammals. Relatively little is known about the composition, migration, and pre-fertilization release of mammalian oocyte cortical granules.

**Results:** Results obtained with confocal scanning laser microscopy indicated that mouse oocytes have at least two populations of cortical granules, one that bound both the lectin LCA and the antibody ABL2 and one that bound only LCA. Both types of granules were synthesized at the same time during oocyte maturation suggesting that the ABL2 antigen is targeted to specific granules by a sorting sequence. The distribution of both populations of cortical granules was then studied during the germinal vesicle to metaphase II transition. As the oocytes entered metaphase I, the first cortical granule free domain, which was devoid of both populations of cortical granules, formed over the spindle. During first polar body extrusion, a subpopulation of LCA-binding granules became concentrated in the cleavage furrow and underwent exocytosis prior to fertilization. Granules that bound ABL2 were not exocytosed at this time. Much of the LCA-binding exudate from the release at the cleavage furrow was retained in the perivitelline space near the region of exocytosis and was deduced to contain at least three polypeptides with approximate molecular weights of 90, 62, and 56 kDa. A second cortical granule free domain developed following pre-fertilization exocytosis and subsequently continued to increase in area as both, LCA and LCA/ABL2-binding granules near the spindle became redistributed toward the equator of the oocyte. The pre-fertilization release of cortical granules did not affect binding of sperm to the overlying zona pellucida.

**Conclusions:** Our data show that mouse oocytes contain at least two populations of cortical granules and that a subset of LCA-binding cortical granules is released at a specific time (during extrusion of the first polar body) and place (around the cleavage furrow) prior to fertilization. The observations indicate that the functions of the cortical granules are more complex than previously realized and include events occurring prior to gamete membrane fusion.

## Background

Cortical granules are membrane-bound organelles present in the cortex of mature unfertilized oocytes of most animal species. In mammals, several proteinases [1-5], heparin binding placental protein. [6,7], and tissue plasminogen activator [8-10] have been inferred to be in cortical granules since they are released from oocytes at fertilization when granules undergo exocytosis. Additionally, several proteins have been identified in mammalian cortical granules using cytochemical techniques. These include p62 and p56 that appear immunologically related to sea urchin hyalin [11], ovoperoxidase [12], n-acetylglucosaminidase [13], calreticulin [14], p75, [15] and p32 [16]. The total number of cortical granule proteins in mammals is not known, but appears from a metabolic labeling study to be about 14 [15].

At the ultrastructural level, mammalian cortical granules range in size from 0.2  $\mu\text{m}$  to 0.6  $\mu\text{m}$  in diameter and appear morphologically similar to each other [3,17-19]. The contents of granules are usually uniformly dense; however, light and dark granules have been reported based on differences in electron density in some species [3,19,20]. It has not yet been determined if the difference in ultrastructural density in these granules represents a difference in biochemical composition, different stages in granule maturation, or different stages in exocytosis [3,19].

The purpose of this study was to test the hypothesis that mouse oocytes contain more than one biochemically distinct population of cortical granules. To accomplish this, the lectin LCA (Lens culinaris agglutinin) and the ABL<sub>2</sub> (antiblastocyst IgG) antibody were used as probes to double label mouse cortical granules. Prior studies have shown that both LCA and ABL<sub>2</sub> antibody when applied separately to oocytes specifically label cortical granules and that they readily produce sufficient signal to be detected using confocal scanning laser microscopy (CSLM) [15,21,22]. Others have clearly shown that most mouse cortical granules label with LCA [19,21]. In contrast, the ABL<sub>2</sub> antibody specifically recognizes a protein (p75) in mouse cortical granules [15], but it is not known if p75 is in all cortical granules. Oocytes double labeled with LCA and ABL<sub>2</sub> were examined with a confocal scanning laser microscope, and 3-dimensional projections were analyzed to determine if the probes co-localized to all cortical granules. Our results indicated that mouse oocytes had at least two distinct populations of cortical granules. Using LCA and ABL<sub>2</sub> to distinguish the two populations of granules, we then studied their synthesis, migration to the cortex, subsequent redistribution in the cortex, and pre-fertilization release.

## Results

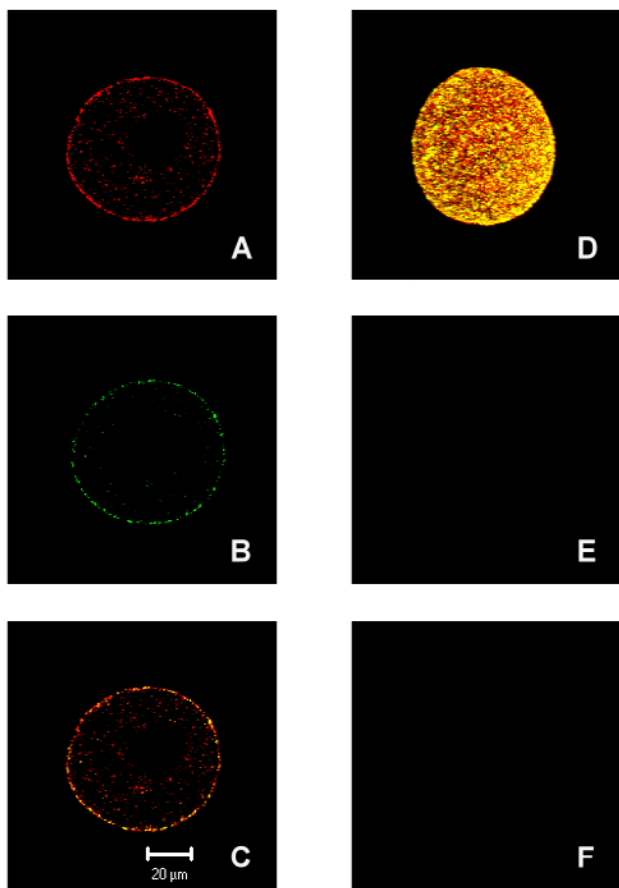
### **Mouse oocytes contain two biochemically distinct populations of cortical granules**

To determine if mouse oocytes contain biochemically distinct populations of cortical granules, the lectin LCA and the polyclonal antibody ABL<sub>2</sub> were used to double label fully-grown germinal vesicle intact oocytes. LCA recognizes mannosylated glycoconjugates and would be expected to interact with most or all cortical granule components, while the ABL<sub>2</sub> antibody reacts with only one or two cortical granule proteins in mouse oocytes [23-25]. Both probes were shown previously to recognize cortical granule components in mouse and hamster oocytes [15,21,22,26,27]. LCA and ABL<sub>2</sub> both labeled the cortical granules in germinal vesicle intact oocytes (Figs. 1A and 1B). The merged image of both staining patterns indicated that at least two types of cortical granules were present in mouse oocytes (Fig. 1C). One granule population (which was yellow in the merged image) contained both LCA-binding and ABL<sub>2</sub>-binding components, while the other granule population (which was red in the merged image) contained only LCA-binding components. Three-dimensional reconstructions of LCA and ABL<sub>2</sub> labeled oocytes further demonstrated the presence of two populations of mouse cortical granules (Fig. 1D). Oocytes stained first with ABL<sub>2</sub> followed by LCA showed identical labeling (data not shown). Control oocytes were not labeled by Texas Red-streptavidin (Fig. 1E) or goat anti-rabbit IgG conjugated to Alexa 488 alone (Fig. 1F). Control oocytes incubated with preimmune rabbit IgG, LCA preincubated with  $\alpha$ -D-methyl-mannopyranoside showed no labeling (data not shown). Non-permeabilized samples did not show any labeling with either LCA or ABL<sub>2</sub> (data not shown).

### **Biogenesis and temporal migration of LCA/ABL<sub>2</sub>-binding and LCA-binding granules before germinal vesicle breakdown**

To examine the biogenesis and migration of cortical granules, germinal vesicle intact oocytes between 30  $\mu\text{m}$  to 70  $\mu\text{m}$  in diameter were collected from ovaries of 12-day old pups. In 30  $\mu\text{m}$  and 40  $\mu\text{m}$  germinal vesicle intact oocytes, both LCA/ABL<sub>2</sub>-binding and LCA-binding granules were observed (Figs. 2A,2B,2C,2D,2E,2F). Both types of granules were present throughout the cytoplasm of the oocytes, and only a few granules had migrated to the cortex (Figs. 2C and 2F). LCA staining regions (arrow) larger than the size of cortical granules were often observed adjacent to the nuclei (Figs. 2A and 2D). Within these regions, yellow areas representing both LCA and ABL<sub>2</sub> binding were observed (Fig. 2F).

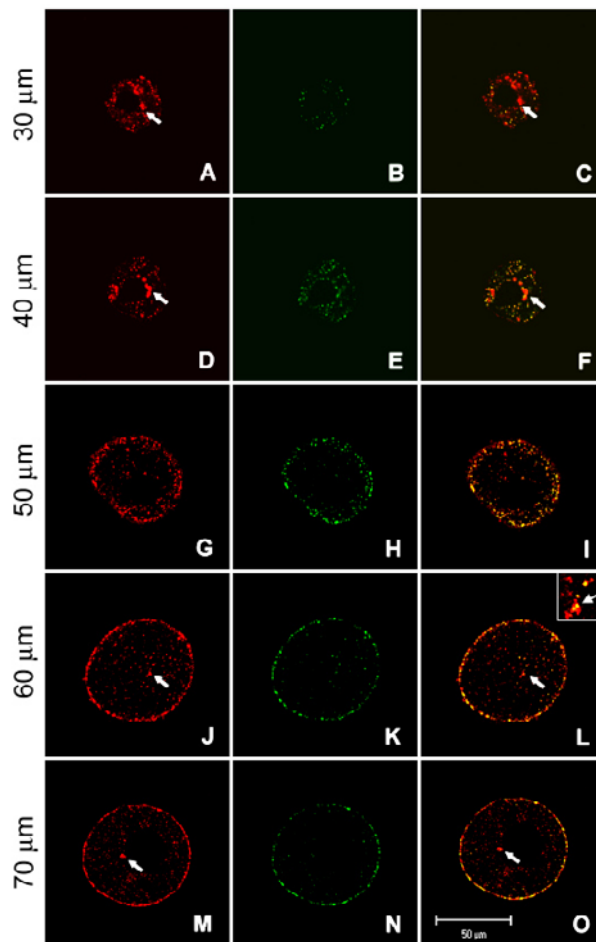
In 50  $\mu\text{m}$  germinal vesicle intact oocytes, both LCA/ABL<sub>2</sub>-binding and LCA-binding cortical granules were present (Figs. 2G,2H,2I). While some labeled granules were



**Figure 1**  
 Confocal laser scanning micrographs of fully-grown mouse germinal vesicle intact oocytes double labeled with ABL<sub>2</sub> and LCA. (A) LCA and (B) ABL<sub>2</sub> labeled the cortical granules of a germinal vesicle intact oocyte. (C) Merged images of both ABL<sub>2</sub> and LCA labeled germinal vesicle intact oocytes showed two populations of cortical granules (red and yellow). (D) Three-dimensional reconstruction of LCA and ABL<sub>2</sub> labeled oocytes showing the two populations of cortical granules. Control oocytes were not labeled by Texas Red-streptavidin (E) or goat anti-rabbit IgG conjugated to Alexa 488 (F) alone.

present toward the center of oocytes, many granules had migrated into subcortical and cortical regions at this stage (Fig. 2I). Although not shown in Figures 2G, 2I, the large perinuclear areas of LCA staining observed in the smaller oocytes were present in 50 µm oocytes.

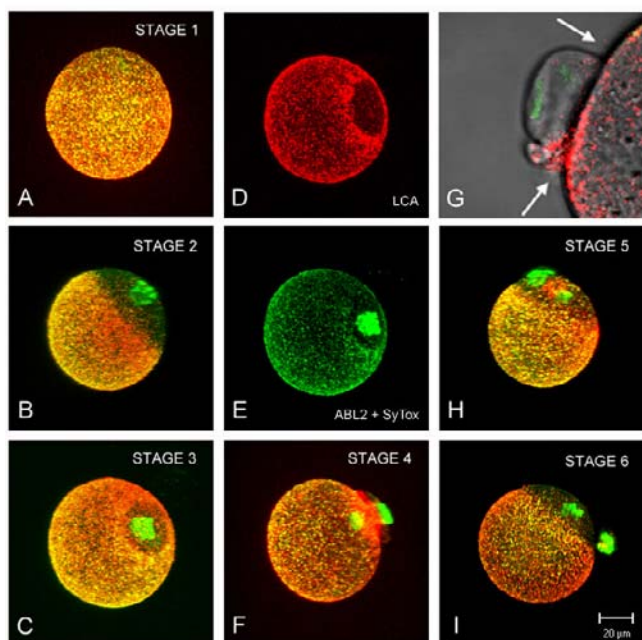
In 60 µm germinal vesicle intact oocytes, both types of cortical granules were observed (Figs. 2J, 2K, 2L). By this stage, most of LCA/ABL<sub>2</sub>-binding granules were located in the cortex (Figs. 2K and 2L), while the LCA-binding gran-



**Figure 2**  
 Confocal laser scanning micrographs of 30 µm to 70 µm mouse germinal vesicle intact oocytes. All oocytes were zona free and double labeled with ABL<sub>2</sub> (B, E, H, K, and N) and LCA (A, D, G, J, and M). Some images of ABL<sub>2</sub> and LCA labeled oocytes were merged (C, F, I, L, and O). Various sizes of germinal vesicle intact oocytes are shown as follows: 30 µm (A–C), 40 µm (D–F), 50 µm (G–I), 60 µm (J–L), and 70 µm (M–O). Fig. 2L insert shows a large LCA-stained perinuclear area with a subregion that was stained with both LCA and ABL<sub>2</sub> (yellow at the tip of the arrow).

ules were found throughout the cytoplasm including the cortex (Figs. 2J and 2L). Near the nuclei, large LCA labeled areas with yellow foci (arrow) were observed at this stage (Figs. 2J and 2L).

70 µm germinal vesicle intact oocytes had numerous LCA/ABL<sub>2</sub>-binding and LCA-binding cortical granules (Figs. 2M, 2N, 2O). The LCA/ABL<sub>2</sub>-binding granules were virtually all confined to the cortex of oocytes with only a few granules occasionally found elsewhere in the cytoplasm



**Figure 3**  
 Three-dimensional projections of mouse follicular oocytes collected at different times after the hCG injection. All oocytes were zona free and triple labeled with ABL<sub>2</sub>, LCA, and SyTox to see the DNA. Each oocyte sample was optically sectioned, and all optical sections of each oocyte were used to compile 3D projections which were animated. **(A)** Stage 1: oocyte in metaphase I shortly after germinal vesicle break down. **(B)** Stage 2: metaphase I oocyte showing the first cortical granule free domain over the spindle. **(C–E)** Stage 3: metaphase I to metaphase II transition showing a subset of LCA-binding cortical granules concentrated in a band around the cleavage furrow. **C** is a three-dimensional reconstruction while **D** and **E** show the LCA and ABL<sub>2</sub> labels separately. **(F–G)** Stage 4: Later stage of polar body extrusion. LCA-binding cortical granules in the cleavage furrow have undergone pre-fertilization release. **(G)** Stage 4: Metaphase II oocyte with the first polar body extruded. LCA-binding cortical granule material has been released into the perivitelline space during pre-fertilization exocytosis (arrows). **(H, I)** Stages 5 and 6: oocytes in metaphase II showing the formation of the second cortical granule free domain.

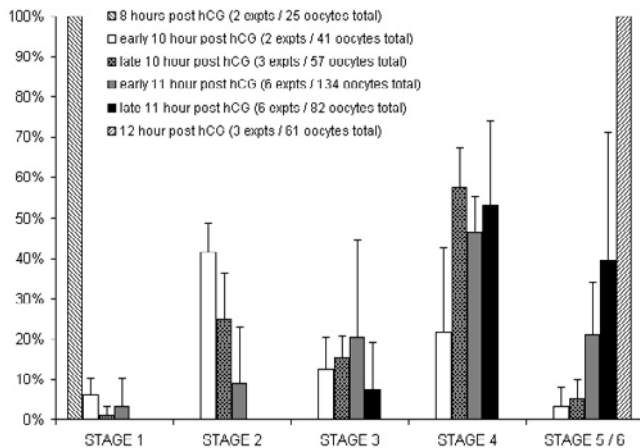
(Figs. 2N and 2O). The LCA-binding granules were abundant in the cortex, but were also found throughout the cytoplasm of the oocyte (Figs. 2M and 2O). The perinuclear LCA staining (arrow) was still present (Figs. 2M and 2O).

**Mouse cortical granule distribution after germinal vesicle breakdown**

To examine mouse cortical granule redistribution in the cortex after germinal vesicle breakdown, metaphase I to metaphase II oocytes were collected from ovaries prior to ovulation at different times after the hCG injection. Results obtained with confocal scanning laser microscopy indicated that both the LCA/ABL<sub>2</sub>-binding and LCA-binding cortical granules were continuously distributed in the cortex of germinal vesicle intact oocytes (Fig. 1D). After germinal vesicle breakdown, both types of cortical granules were still evenly distributed in the cortical region for a short period of time (stage 1; Fig. 3A). However, as the oocytes progressed into metaphase I, the first cortical granule free domain appeared over the spindle and lacked both LCA/ABL<sub>2</sub>-binding and LCA-binding cortical granules (stage 2; Fig. 3B). No evidence of cortical granule exocytosis was observed this time. When the polar body started to extrude, a subset of LCA-binding cortical granules became concentrated around the cleavage furrow (stage 3; Fig. 3C and 3D). In contrast, granules that bound ABL<sub>2</sub> did not become concentrated around the furrow (Fig. 3E). As the polar body started to pinch off, the subpopulation of LCA-binding cortical granules in the cleavage furrow underwent pre-fertilization exocytosis (stage 4; Figs. 3F and 3G). As the oocytes matured into metaphase II, both the LCA/ABL<sub>2</sub>-binding and LCA-binding cortical granules remaining near the spindle became redistributed toward the equator of the oocyte and created a second cortical granule-free domain (stages 5 and 6; Fig. 3H and 3I).

Figure 4 shows the percentage of oocytes in each of the above mentioned stages at various times after the hCG injection. All the oocytes collected at 8 hours after hCG had granules evenly distributed in the cortex (stage 1) (Fig. 4). At early 10 (10.25) hours after hCG injection, cortical granules in most oocytes redistributed (stage 2) and a cortical granule free-domain over the metaphase I spindle was developed (Fig. 4). At late 10 (10.75) hours, early 11 (11.25) hours, and late 11 (11.75) hours after the hCG injection, most of the oocytes had started to extrude their polar bodies and underwent pre-fertilization exocytosis in the area of the cleavage furrow (stage 4) (Fig. 4). 12 hours after hCG injection, cortical granules above the metaphase II spindle redistributed toward the equator, and a second cortical granule free-domain (CGFD) was formed (stage 5 / 6) (Fig. 4).

As a result of this final redistribution, the density of cortical granules around the equator increased in ovulated metaphase II mature oocytes. This increase in density of granules was best visualized by rotating three-dimensional reconstructions of metaphase II oocytes (Fig. 5, "see Additional file 1"). The belt of granules around the equator was particularly rich in LCA/ABL<sub>2</sub>-binding

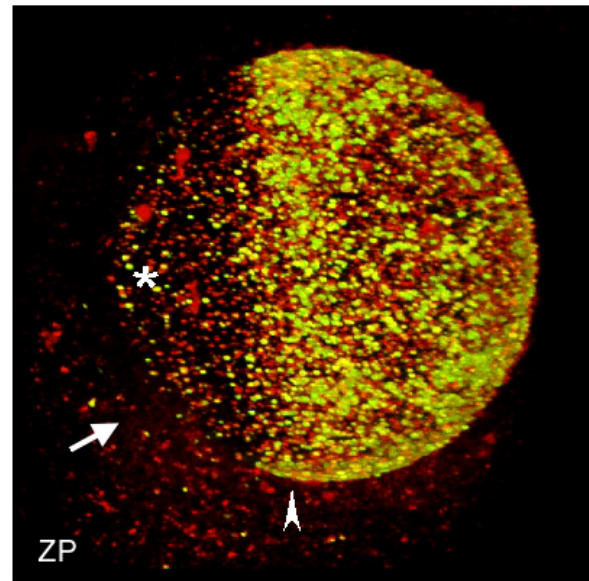


**Figure 4**  
The percentage of oocytes in stages 1 to 6 at various time after the hCG injection. The number of experiments and the total number of oocytes for all experiments are shown in the figure for each injection group. For each individual experiment, the mean percentage of oocytes in each stage was determined. Then the means for the individual experiments were averaged and are plotted as the means + SD for each injection group.

granules, while granules that bound only LCA were also present in areas away from the equator. This polar distribution of cortical granules established in metaphase II oocytes was maintained until the oocytes were fertilized and the remaining granules underwent exocytosis. The three-dimensional reconstructions of zona intact metaphase II oocytes recovered from oviduct also showed that the LCA-binding cortical granule components released prior to fertilization were present in the perivitelline space in the area adjacent to the cortical granule-free domain (Fig. 5). These results do not preclude the possibility that some of the pre-fertilization exudate entered and modified the zona.

**Sperm binding to the zona pellucida after the pre-fertilization release of cortical granules**

Given the above observations, we hypothesized that some of the pre-fertilization exudate enters and modifies the zona to prevent sperm from binding to and penetrating the zona near the metaphase II spindle. To test this hypothesis, we performed a sperm-binding assay as described in Materials and Methods. If our hypothesis is correct, we would expect to see an area on the zona devoid of sperm binding. When sperm were incubated with oocytes at a concentration that maximized binding to the zona pellucida, sperm bound to all regions of the zona,



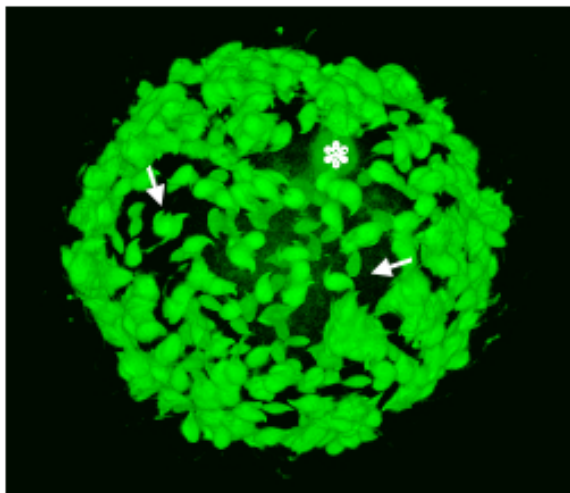
**Figure 5**  
Three-dimensional projection showing an ovulated unfertilized metaphase II zona intact oocyte collected 14 hours after the hCG injection and stained with ABL<sub>2</sub> and LCA. arrow-head = equator; asterisk = cortical granule free domain; arrow = perivitelline space. ZP = indicates the zona pellucida which does not actually show as it is not fluorescently labeled. Entire oocyte was optically sectioned, and all optical sections were used to compile the 3D projection, which was animated. A movie of this oocyte can be viewed by double clicking the movie file Fig5Movie.avi ("see Additional file 1").

and only very small zones without bound sperm were occasionally observed randomly positioned on the zona surface (Fig. 6). The small size and random distribution of these zones suggest that they were not due to zona modification by per-fertilization release of cortical granules.

**Identification of LCA binding mouse cortical granule components released during metaphase I**

To identify cortical granule component(s) that were released during polar body formation, western blots of zona free germinal vesicle intact and zona free unfertilized metaphase II mouse oocytes were probed with LCA, and the bands in the two groups of oocytes were compared (Fig. 7A and 7B). Numerous bands were present in both groups of oocytes. However, three LCA binding glycoconjugates with molecular weights of 90, 62, and 52–56 kDa were detected in germinal vesicle intact oocytes (lane 1 in Fig. 7A), but not in unfertilized metaphase II oocytes (lane 2 in Fig. 7A). To better resolve the region containing the





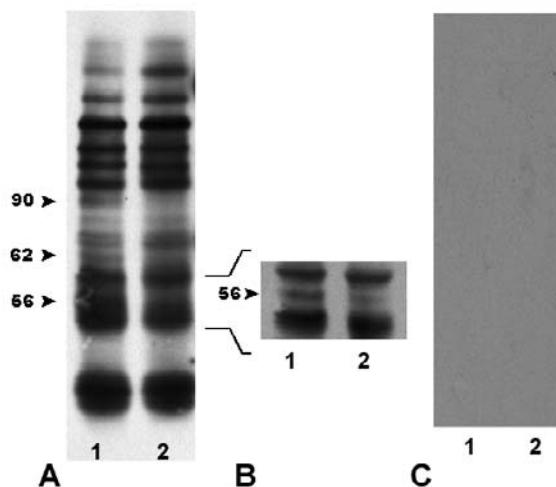
**Figure 6**

The sperm-binding assay showing a three-dimensional projection of an oocyte incubated in vitro with a saturating number of sperm. Small areas devoid of bound sperm were found randomly on the zona (arrows). Occasionally, similar "bald spots" were observed above the spindle (\*), as shown in this figure.

52–56 kDa band, additional gels were run for a longer time and probed with LCA (Fig. 7B), revealing that a band at 56 kDa decreased in amount in the unfertilized metaphase II oocytes. No bands were detected on blots probed with LCA preabsorbed with  $\alpha$ -D-methyl-mannopyranoside (Fig. 7C).

**Discussion**

The composition, migration, and the time and place of release of cortical granules from oocytes have important significance with respect to their functions in fertilization. Oocytes from several invertebrates have multiple populations of cortical granules [28,29]. For example, lobster oocytes have four morphologically distinct types of cortical granules [30], while sea urchin oocytes have at least two populations of cortical granules that differ biochemically [31,32]. Mammalian cortical granules are generally similar in size and ultrastructural appearance [3,33-36]. Although mammalian cortical granules contain a number of proteins [11,13,14,16,22,27,37,38], biochemically distinct populations of granules have not previously been demonstrated in mammals. In one prior study [16], both LCA and an antibody to a specific cortical granule protein (3E10) labeled more than 90% of mouse cortical granules, while fewer than 10% labeled with LCA only. It was not determined in this study if the LCA-posi-



**Figure 7**

(A, B) Western blots with 100 zona free germinal vesicle intact oocytes (lane 1) and 100 zona free unfertilized oviductal oocytes (lane 2) probed with 10  $\mu$ g/ml of LCA and (C) western blot with 50 zona free germinal vesicle intact oocytes (lane 1) and 50 zona free unfertilized oviductal oocytes (lane 2) probed with 10  $\mu$ g/ml of LCA preincubate with  $\alpha$ -D-methyl-mannopyranoside followed by HRP-streptavidin. (A) Same LCA blotting pattern was found in six separate experiments. (B) Same LCA blotting pattern was found in two separate experiments. (C) This control was run separately three times.

tive/3E10-negative granules were a distinct population that were only labeled with LCA, or an organelle other than cortical granules, or granules that failed to label with 3E10 antibody. Our data demonstrate that mouse oocytes do contain two biochemically distinct populations of cortical granules, and several lines of evidence support this conclusion. First, LCA/ABL<sub>2</sub> binding granules were more concentrated around the equator in metaphase II oocytes than in other regions of the oocyte, and LCA-binding granules were the only type of granules concentrated around the cleavage furrow during polar body extrusion. It is improbable that these distinct patterns of cortical granule distribution could be attributed to failure of the ABL<sub>2</sub> antibody to label granules. Second, the exudate released around the furrow bound only LCA. It is very improbable that the ABL<sub>2</sub> antibody would not have had access to the ABL<sub>2</sub> antigen in the exudate if the antigen were there.

Ultrastructurally, cortical granules in some mammals differ in their electron density and have been characterized as

"light" and "dark" [3,19,20]. The significance of this difference in density is not known, but may be due to decondensation of granules prior to or during exocytosis [3]. It is not likely that the electron dark and light granules reported by others correspond to the two populations of granules that we observed, since the distribution of the light and dark granules versus the LCA/ABL<sub>2</sub> and LCA-binding granules was different.

There are at least two mechanisms that could account for the formation of two populations of cortical granules in mice. First, the ABL<sub>2</sub>-binding protein may be synthesized only during a finite window of oocyte development. During this window, granules would be produced that could bind ABL<sub>2</sub> and LCA, while at times outside the window, granules that bind only LCA would be produced. An alternative possibility is that the ABL<sub>2</sub>-binding protein is directed to specific cortical granules via a sorting signal, as has been shown for melanosomal membrane proteins, neuropeptides, and hormones [39,40]. To distinguish between these possibilities, we examined cortical granule formation at various times during oocyte development prior to germinal vesicle breakdown. We found that LCA/ABL<sub>2</sub>-binding cortical granules were produced in 30  $\mu$ m to 70  $\mu$ m oocytes, in agreement with previous work in which ABL<sub>2</sub>-binding proteins were studied using metabolic labeling [41]. Simultaneous production of both LCA/ABL<sub>2</sub>-binding and LCA-binding granules supports the idea that ABL<sub>2</sub>-binding proteins were directed to specific granules via a sorting signal. Because of their position, size and LCA staining [26], the large perinuclear regions we observed in 30  $\mu$ m to 70  $\mu$ m oocytes were interpreted to be Golgi bodies. While most of these regions stained mainly with LCA, there were foci within these regions that stained with both LCA and ABL<sub>2</sub>. This indicates that the contents of the two granule types were segregated within the Golgi bodies and further supports a sorting mechanism. Our observations on the 30  $\mu$ m to 70  $\mu$ m diameter oocytes also showed that migration of granules to the cortex was a continuous process in the mouse, in agreement with others [42]. In contrast, cortical granule translocation occurred only in the periovulatory period or right after germinal vesicle breakdown in porcine [43] and in sea urchin [44,45] oocytes.

Translocation of the cortical granules to the cortex resulted in an uniform distribution of the LCA/ABL<sub>2</sub>-binding and LCA-binding granules in germinal vesicle intact oocytes. However, as the germinal vesicle broke down, the two populations of granules underwent significant changes in their cortical location. Initially, a large cortical granule free domain formed around the metaphase I spindle, in agreement with other reports on the mouse [26,46]. Formation of this domain was most likely due to redistribution of both populations of granules since we did not observe evidence of exocytosis during its

formation. Alternative interpretations (e.g., cortical granules above the spindle could be selectively degraded) are also possible but less plausible. Our conclusion that redistribution of granules is the main factor contributing to formation of the first cortical granule free domain is in agreement with another study on mice in which the formation of this domain in metaphase I oocytes was not inhibited in the presence of BAPTA, a Ca<sup>2+</sup> chelator that prevents cortical granule exocytosis [47].

While the formation of the first cortical granule free domain appears to occur by redistribution of cortical granules, exocytosis has been shown previously using transmission electron microscopy to occur during the metaphase I to metaphase II transition [48,49]. In addition, it was recently shown that during the metaphase I to metaphase II transition, ZP<sub>2</sub> to ZP<sub>2f</sub> conversion increased, and this increase was inhibited by BAPTA treatment [47]. Our CSLM results confirm that some cortical granules are released by exocytosis during the metaphase I to metaphase II transition. However, we only observed evidence of exocytosis during polar body extrusion and only in the cleavage furrow. Our data further showed that mainly LCA-binding granules are released from the cleavage furrow during polar body formation and that some of the released LCA-binding exudate is retained in the perivitelline space following exocytosis. In addition, our data clarify that the first cortical granule free domain had formed prior to exocytosis of cortical granules during the metaphase I and metaphase II transition and that this exocytosis took place before formation of the second cortical granule free domain. Formation of this large cortical granule free domain allowed the first polar body to be extruded with few cortical granules in it. In pigs and hamsters, no apparent or a much smaller cortical granule free domain forms in oocytes at this stage, and these species both have a substantial number of cortical granules in their first polar body [43,48]. These observations suggest that the size of the first cortical granule free domain determines the number of cortical granules in the polar body.

In metaphase II oocytes, a second cortical granule free domain formed over the spindle. This domain appeared to form largely due to redistribution of both populations of cortical granules as their density at the edge of the granule-containing domain increased. Our results are in agreement with the study by Deng et al. [47] who demonstrated that cortical granule redistribution is responsible for the formation of the second cortical granule free domain in metaphase II oocytes. However, these authors proposed that some exocytosis contributes to the enlargement of this domain. Because their experiments were done during the metaphase I to metaphase II transition, they may have been observing the release of granules at the cleavage furrow.



Several mechanisms could explain why pre-fertilization exocytosis is confined to the cleavage furrow. Cortical granule exocytosis following sperm-oocyte fusion is a calcium-dependent secretory event [50]. Although it is not known if pre-fertilization exocytosis results from a calcium increase during polar body extrusion, in sea urchin eggs and rat epithelial cells, there is an increase in calcium during cytokinesis [51-53]. It is possible that a similar increase in calcium occurs at the cleavage furrow of mouse oocytes and induces pre-fertilization exocytosis during polar body extrusion. Alternatively, at the time of polar body extrusion, LCA-binding granules concentrated around the cleavage furrow may be the only cortical granules equipped with a full complement of SNARE proteins [54-56], and therefore capable of undergoing exocytosis.

The pre-fertilization exocytosis occurring during polar body extrusion involved a subpopulation of LCA-binding cortical granules. Our lectin blots with LCA support the idea that at least three proteins (90, 62, and 56 kDa) were released from the LCA-binding cortical granules concentrated around the metaphase I cleavage furrow, although alternative interpretations are possible (e.g., the 90, 62, and 56 kDa proteins may have been degraded during maturation). Although mouse and hamster cortical granules have been reported to contain proteinases [1-5], heparin binding placental protein [6,7], tissue plasminogen activator [8-10], p62/56 [11], calreticulin [14], ovoperoxidase [12], n-acetylglucosaminidase [13], p75 [23], and p32 [16], it is not likely that any of these correspond to the 90, 62, and 56 kDa bands in our blot for several reasons. First, the proteinases, heparin binding placental proteins, and tissue plasminogen activator were inferred to be cortical granule proteins because they were released from oocytes at fertilization. The 90 and 62 kDa proteins would not be present at the time of fertilization since they were absent in mature unfertilized oocytes. Secondly, the molecular weights of calreticulin, ovoperoxidase, n-acetylglucosaminidase, p75, or p32 do not correspond to the weights of these three proteins.

Previous studies have shown that sperm-oocyte fusion occurs less frequently in the cortical granule free-domain [19,57]. We hypothesized that the pre-fertilization release modified the zona pellucida directly above the site of the release, thereby minimizing the opportunity for sperm to bind and penetrate the zona pellucida near the cortical granule free domain. Although our sperm binding experiment did not support this hypothesis, it is possible that the pre-fertilization release establishes a block to sperm binding and fusion at the level of the perivitelline space or plasma membrane and such a block would not be detected by our assay. In any case, it is highly probable that the pre-fertilization release performs a biological function since it occurs at a specific time, in a specific loca-

tion, and involves only a subpopulation of cortical granules.

## Conclusion

Our data show that mouse oocytes have at least two biochemically distinct types of cortical granules that display complex redistribution in the cortex during the germinal vesicle breakdown to metaphase II transition and that a subset of LCA-binding granules is released from the cleavage furrow of the first polar body prior to fertilization. Taken together, these data indicate that mammalian cortical granules are more complex and appear to have more functions than previously realized. These functions could include modifying the oolemma to prevent sperm fusion near the meiotic spindle or modifying the zona pellucida to prevent induction of acrosome reaction or zona penetration.

## Materials and Methods

### Chemicals and Supplies

The sources of the chemicals used in this study have been given previously [27], except for Alexa-488 conjugated to goat anti-rabbit IgG (Molecular Probes, Eugene, OR).

### Animals

NIH Swiss white female (6-12 weeks old) and male (10 weeks old) mice were purchased from Harlan Sprague-Dawley (San Diego, CA). Animals were housed in the Spieth Hall vivarium with a 14:10 light and dark cycle and fed water and Purina rodent chow (Ralston-Purina, St Louis, MO) ad libitum. Animal maintenance and usage were covered by a proposal approved by the UCR IACUC.

### Media and Fixatives

Earle's balance salt solution with 28.18 mM of sodium bicarbonate and 24.98 mM of HEPES free acid (EBSS-H) was made as previously described [22]. EBSS-H, pH 7.4 supplemented with 0.2% of polyvinylpyrrolidone (EBSS-H/0.2% PVP) was used for dissection and oocyte collection. Brinster Ca/Mg-free solution [58] was used to collect growing germinal vesicle intact oocytes. For the sperm-binding assay, Whittingham's solution, pH 7.2 at room temperature supplemented with 20 mM HEPES and 30 mg/ml of bovine serum albumin (HWS/3%BSA) was used to prepare gametes. Solution was gassed in humidified 37°C incubator (5% CO<sub>2</sub>, 95% air) for 24 hours before use. For CSLM, Dulbecco's phosphate buffered saline (DPBS), pH 7.4 was prepared [22]. To fix oocytes for CSLM, 3.7% paraformaldehyde or 4% glutaraldehyde were made immediately prior to use in DPBS, pH 7.4. Blocking solution was made by supplementing DPBS, pH 7.4, with 7.5 mg/ml glycine and 3 mg/ml BSA. 10 mM Citrate buffer pH 6.0 was made with 3.78 g of citric acid and 2.411 g of sodium citrate in 1 L of H<sub>2</sub>O. The labeling solution was made by supplementing DPBS, pH 7.4 with

30 mg/ml BSA (DPBS/3% BSA). For lectin blotting, Tris-buffered saline (TBS), pH 7.6 was used (147 mM NaCl; 20 mM Tris-base).

### **Gametes Collection**

Oocytes were handled under light mineral oil to maintain the pH and osmolarity of the solutions. For CSLM and gel electrophoresis, oocytes were collected in collection medium, EBSS-H supplemented with 0.2% of PVP unless specified otherwise. Germinal vesicle intact oocytes were collected either from 12 day-old females or 10 week-old females 60 hours after they were primed with 10 IU of PMSG injection. Germinal vesicle intact oocytes were mechanically denuded of their cumulus cells using a thin-bore glass pipette. To collect follicular oocytes, females were primed with 10 IU of PMSG at 0300 hours on day 1 followed by 10 IU of hCG 47 hours later, and oocytes ranging in stage from germinal vesicle break down to metaphase II were collected 8 to 12 hours after the hCG injection. To collect mature metaphase II unfertilized oocytes, females were superovulated by injecting 10 IU of PMSG at 2200 hour on day 1 followed by 10 IU of hCG 46 hours later and unfertilized oocytes were collected from the oviduct 14–16 hours after the hCG injection. Unfertilized oocytes were denuded of their cumulus cells in collection medium containing 100 IU of hyaluronidase for 5 minutes at room temperature. For LCA blotting of germinal vesicle intact oocytes and unfertilized oocytes, the zona pellucida was removed with 0.25% pronase in collection medium. For CSLM, the zona pellucida was removed by heat treatment (75 °C) following fixation and permeabilization (see the section on CSLM).

Gametes for the sperm binding assay were collected in sperm binding medium, HWS/3%BSA at room temperature. Mature metaphase II oocytes were collected with the same procedures as described above for CSLM and electrophoresis. Sperm were collected from the cauda epididymis and vas deferens of fertile male mice in sperm binding medium and capacitated in under light mineral oil at 37 °C for 1.5 hours.

### **Confocal Scanning Laser Microscopy**

All steps used to prepare samples for CSLM were carried out at room temperature unless otherwise specified. Oocytes were fixed with 3.7% paraformaldehyde (for CSLM) or with 4% glutaraldehyde (for sperm binding assay) in DPBS, pH 7.4 for 30 minutes. After washing in blocking solution, oocytes were permeabilized with 0.1% Triton X-100 in blocking solution for 5 minutes and washed 6 times in blocking solution for 30 minutes. In some experiments, oocytes were treated with 10 mM citrate buffer for 30 minutes at 75 °C for antigen retrieval following the permeabilization. To label with ABL<sub>2</sub>, the oocytes were incubated for 30 minutes with a 1:300 dilu-

tion (40 µg/ml) of ABL<sub>2</sub>. The samples were then incubated in goat anti-rabbit IgG conjugated to Alexa 488 at a 1:300 dilution (6.6 µg/ml) for an additional 30 minutes. Control oocytes were incubated with a 1:1000 (28.3 µg/ml) of preimmune rabbit IgG for 30 minutes. For LCA labeling, the oocytes were incubated with 10 µg/ml of biotinylated LCA for 30 minutes and subsequently incubated in 5 µg/ml of Texas Red-streptavidin for 30 minutes. For the control, 10 µg/ml of LCA was first preincubated with 100 mM α-D-methyl-mannopyranoside for 30 minutes, and the solution was then used to label the oocytes for 30 minutes followed by 20 minutes of incubation with 5 µg/ml of Texas Red-streptavidin. For double labeling experiments, samples were first incubated with ABL<sub>2</sub> followed by the goat anti-rabbit IgG conjugated to Alexa 488, then incubated with LCA followed by the Texas Red-streptavidin as mentioned above. In some instances, LCA labeling preceded ABL<sub>2</sub> labeling. For follicular oocytes and sperm binding assay, oocytes were labeled with SYTOX green nucleic acid stain at a 1:2,000 dilution (2.5 µM) for 15 minutes to visualize meiotic spindles. Controls were done by labeling the non-permeabilized samples or permeabilized samples with goat anti-rabbit IgG conjugated to Alexa 488 or Texas Red-streptavidin only. All labeled oocyte samples were then washed with labeling solution overnight at 4 °C. Samples were washed, mounted in Vectashield and examined using Zeiss LS 510 confocal scanning laser microscope. Samples were optically sectioned with a space interval determined optimally according to the pinhole setting. 180 to 190 optical sections were obtained from each oocyte, and all optical sections were used to compile animated 3D projections. All oocytes were also examined with Zeiss DIC optics. Both image acquisition and processing were done on a Microsoft Windows NT 4.0 workstation with the LSM 510 Software, version 2.3.

### **Gel Electrophoresis and Lectin Blotting**

Germinal vesicle intact and metaphase II oocytes were made zona free with 0.25% pronase, then solubilized in Laemmli sample buffer. Protein samples were loaded and run in one-dimensional SDS-PAGE Doucet gels [22] that consisted of 4% stacking and 7.5% separating gels at 70 V and 140 V respectively, and separated proteins were blotted onto nitrocellulose at 100 V for 1 hour. Blots were blocked with Tris-buffered saline with 0.5% Tween-20 (TBT) for 30 minutes at room temperature. 10 µg/ml of the biotinylated LCA in TBT was used to incubate the blots at 4 °C overnight. For the control, biotinylated LCA was preincubated with 100 mM of the α-D-methyl-mannopyranoside for 1 hour at room temperature. On the following day, blots were incubated with a 1:20,000 dilution of HRP-streptavidin in TBT incubation for 30 min at room temperature. Glycoconjugates of interest were detected by enhanced chemiluminescence. Biotinylated standards

were used to calculate molecular weights of detected glycoconjugates.

### Sperm Binding Assay

Oviductal metaphase II oocytes were added to capacitated sperm in sperm binding medium under light mineral oil to give a final sperm concentration in the range  $1 \times 10^7$  to  $2 \times 10^7$  sperm/ml. Gametes were incubated for 10 to 15 minutes at 37 °C. Oocytes were picked out with large-bore glass pipettes and washed in fresh sperm binding medium to remove loosely bound sperm. Oocytes were then handled and labeled as described in confocal scanning laser microscopy section.

### Authors Contributions

ML performed all the experiments and prepared the manuscript. DS provided technical assistance with some experiments. PC provided the ABL<sub>2</sub> antibody and critically reviewed the manuscript. PT supervised all the work and helped prepare the manuscript.

### Additional material

#### Additional File 1

Animated three-dimensional projection showing an unfertilized metaphase II zona intact oocyte collected 14 hours after hCG injection and stained with ABL<sub>2</sub> and LCA. Avi movie corresponding to Figure 5 (see Figure 5 legend for details)

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### Acknowledgement

We are grateful to Frank Li for his help in obtaining Figure 5 and to Trish Steen for her assistance in training on the confocal microscope. We would like to thank Paul Jaegu Kim and Regina Wright for their invaluable technical assistance in obtaining Figure 7. This study was supported by a grant from NIH.

### References

- Gwatkin RBL: **Fertilization Mechanisms in Man and Mammals.** New York, Plenum Press; 1977.
- Wolf DP, Hamada M: **Induction of zonal and egg plasma membrane blocks to sperm penetration in mouse eggs with cortical granule exudate.** *Biol Reprod* 1977, **17**:350-354.
- Cherr GN, Drobnis EZ, Katz DF: **Localization of cortical granule constituents before and after exocytosis in the hamster egg.** *J Exp Zool* 1988, **246**:81-93.
- Moller CC, Wassarman PM: **Characterization of a proteinase that cleaves zona pellucida glycoprotein ZP2 following activation of mouse eggs.** *Dev Biol* 1989, **132**:103-112.
- Tawia SA, Lopata A: **The fertilization and development of mouse oocytes following cortical granule discharge in the presence of a protease inhibitor.** *Hum Reprod* 1992, **7**:1004-1009.
- Sinosich MJ, Lanzendorf SE, Hodgen HG: **Immunocytochemical studies of hamster oocyte activation.** *Mol Reprod Dev* 1990, **26**:331-336.
- Sinosich MJ, Lanzendorf SE, Hodgen HG: **Immunocytochemical studies of hamster oocyte activation.** *Mol Reprod Dev* 1990, **26**:331-336.
- Zhang X, Rutledge J, Khamsi F, Armstrong DT: **Release of tissue-type plasminogen activator by activated rat eggs and its possible role in the zona reaction.** *Mol Reprod Dev* 1992, **32**:28-32.
- Huarte J, Vassalli JD, Belin D, Sakkas D: **Involvement of the plasminogen activator/plasmin cascade in fertilization.** *Dev Biol* 1993, **157**:539-546.
- Rekkas CA, Besenfelder U, Havlicek V, Vainas E, Brem G: **Plasminogen activator activity in cortical granules of bovine oocytes during in vitro maturation.** *Theriogenology* 2002, **57**:1897-1905.
- Hoodbhoy T, Carroll EJ, Talbot P: **The Relationship Between p62 and p56, Two Proteins of the Mammalian Cortical Granule Envelope, and Hyalin, the Major Component of the Echinoderm Hyaline Layer.** *Biol Reprod* 2000, **62**:979-987.
- Gulyas BJ, Schmell ED: **Ovoperoxidase activity in ionophore treated mouse eggs. I. Electron microscopic localization.** *Gam Res* 1980, **3**:267-277.
- Miller DJ, Gong X, Decker G, Shur BD: **Egg cortical granule N-acetylglucosaminidase is required for the mouse zona block to polyspermy.** *J Cell Biol* 1993, **123**:1431-1440.
- Munoz-Gotera RJ, Hernandez-Gonzalez EO, Mendoza-Hernandez G, Contreras RG, Mujica A: **Exocytosis of a 60 kDa protein (calreticulin) from activated hamster oocytes.** *Mol Reprod Dev* 2001, **60**:405-413.
- Pierce KE, Siebert MC, Kopf GS, Schultz RM, Calarco PG: **Characterization and localization of a mouse egg cortical granule antigen prior to and following fertilization or egg activation.** *Dev Biol* 1990, **141**:381-392.
- Gross VS, Wessel G, Florman HM, Ducibella T: **A Monoclonal Antibody That Recognizes Mammalian Cortical Granules and a 32-Kilodalton Protein in Mouse Eggs.** *Biol Reprod* 2000, **63**:575-581.
- Gulyas BJ: **Cortical granules of mammalian eggs.** *Int Rev Cytol* 1979, **63**:357-392.
- Cran DG: **Cortical granules during oocyte maturation and fertilization.** *J Reprod Fertil Suppl* 1989, **38**:49-62.
- Nicosia SV, Wolf DP, Inoue M: **Cortical granule distribution and cell surface characteristics in mouse eggs.** *Dev Biol* 1977, **57**:56-74.
- Hinduja IN, Kumar A, Anand Kumar TC: **Ultrastructure of the cortex in the human egg.** *Hum Reprod* 1990, **5**:66-70.
- Ducibella T, Anderson E, Albertini DF, Aalberg J, Rangarajan S: **Quantitative studies of changes in cortical granule number and distribution in the mouse oocyte during meiotic maturation.** *Devel Biol* 1988, **130**:184-197.
- Hoodbhoy T, Dandekar P, Calarco P, Talbot P: **p62/p56 are cortical granule proteins that contribute to formation of the cortical granule envelope and play a role in mammalian preimplantation development.** *Mol Reprod Dev* 2001, **59**:78-89.
- Pierce KE, Calarco PG: **Establishment and maintenance of a regionalized glycoprotein distribution during early mouse development.** *Dev Biol* 1990, **137**:56-67.
- Polak-Charcon S, Calarco-Gillam P, Johnson L: **Intracellular localization and surface expression of a stage-specific embryonic glycoprotein.** *Gamete Res* 1985, **12**:329-343.
- Johnson LV, Calarco PG: **Stage-specific embryonic antigens detected by an antiserum against mouse blastocysts.** *Dev Biol* 1980, **79**:224-231.
- Ducibella T, Duffy P, Reindollar R, Su B: **Changes in the distribution of mouse oocyte cortical granules and ability to undergo the cortical reaction during gonadotropin-stimulated meiotic maturation and aging in vivo.** *Biol Reprod* 1990, **43**:870-876.
- Hoodbhoy T, Talbot P: **Characterization, fate, and function of hamster cortical granule components.** *Mol Reprod Dev* 2001, **58**:223-235.
- Brummett AR, Dumont JN: **Cortical vesicle breakdown in fertilized eggs of Fundulus heteroclitus.** *J Exp Zool* 1981, **216**:63-79.
- Hart NH, Yu SF: **Cortical granule exocytosis and cell surface reorganization in eggs of Brachydanio.** *J Exp Zool* 1980, **213**:137-159.
- Talbot P, Goudeau M: **A complex cortical reaction leads to formation of the fertilization envelope in the lobster, Homarus.** *Gamete Res* 1988, **19**:1-18.
- Matese JC, Black S, McClay DR: **Regulated exocytosis and sequential construction of the extracellular matrix surrounding the sea urchin zygote.** *Dev Biol* 1997, **186**:16-26.

32. Anstrom JA, Chin JE, Leaf DS, Parks AL, Raff RA: **Immunocytochemical evidence suggesting heterogeneity in the population of sea urchin egg cortical granules.** *Dev Biol* 1988, **125**:1-7.
33. Szollosi D: **Development of cortical granules and the cortical reaction in rat and hamster eggs.** *Anat Rec* 1967, **159**:431-446.
34. Selman K, Anderson E: **The formation and cytochemical characterization of cortical granules in ovarian oocytes of the golden hamster (*Mesocricetus auratus*).** *J Morphol* 1975, **147**:251-274.
35. Gulyas BJ: **Cortical granules in artificially activated (parthenogenetic) rabbit eggs.** *Am J Anat* 1974, **140**:577-582.
36. Gulyas BJ: **Ultrastructural observations on rabbit, hamster and mouse eggs following electrical stimulation in vitro.** *Am J Anat* 1976, **147**:203-218.
37. Hoodbhoy T, Talbot P: **Mammalian cortical granules: contents, fate, and function.** *Mol Reprod Dev* 1994, **39**:439-448.
38. Miyazaki S, Igusa Y: **Fertilization potential in golden hamster eggs consists of recurring hyperpolarizations.** *Nature* 1981, **290**:702-704.
39. Setaluri V: **Sorting and targeting of melanosomal membrane proteins: signals, pathways, and mechanisms.** *Pigment Cell Res* 2000, **13**:128-134.
40. Glombik MM, Gerdes HH: **Signal-mediated sorting of neuropeptides and prohormones: secretory granule biogenesis revisited.** *Biochimie* 2000, **82**:315-326.
41. Pierce KE, Grunvald EL, Schultz RM, Kopf GS: **Temporal pattern of synthesis of the mouse cortical granule protein, p75, during oocyte growth and maturation.** *Dev Biol* 1992, **152**:145-151.
42. Ducibella T, Duffy P, Buetow J: **Quantification and localization of cortical granules during oogenesis in the mouse.** *Biol Reprod* 1994, **50**:467-473.
43. Cran DG, Cheng WT-K: **Changes in cortical granules during porcine oocyte maturation.** *Gamete Res* 1985, **11**:311-319.
44. Wessel GM, Conner SD, Berg L: **Cortical granule translocation is microfilament mediated and linked to meiotic maturation in the sea urchin oocyte.** *Development* 2002, **129**:4315-4325.
45. Berg LK, Wessel GM: **Cortical granules of the sea urchin translocate early in oocyte maturation.** *Development* 1997, **124**:1845-1850.
46. Ducibella T, Kurasawa S, Rangarajan S, Kopf GS, Schultz RM: **Precocious loss of cortical granules during mouse oocyte meiotic maturation and correlation with an egg-induced modification of the zona pellucida.** *Dev Biol* 1990, **137**:46-55.
47. Deng M, Kishikawa H, Yanagimachi R, Kopf GS, Schultz RM, Williams CJ: **Chromatin-mediated cortical granule redistribution is responsible for the formation of the cortical granule-free domain in mouse eggs.** *Dev Biol* 2003, **257**:166-176.
48. Okada A, Yanagimachi R, Yanagimachi H: **Development of a cortical granule-free area of cortex and the perivitelline space in the hamster oocyte during maturation and following ovulation.** *J Submicrosc Cytol* 1986, **18**:233-247.
49. Okada A, Inomato K, Nagae T: **Spontaneous cortical granules release and alteration of zona pellucida properties during and after meiotic maturation of mouse oocytes.** *Anat Rec* 1993, **237**:518-526.
50. Abbott AL, Ducibella T: **Calcium and the control of mammalian cortical granule exocytosis.** *Front Biosci* 2001, **6**:D792-806.
51. Ratan RR, Maxfield FR, Shelanski ML: **Long-lasting and rapid calcium changes during mitosis.** *J Cell Biol* 1988, **107**:993-999.
52. Poenie M, Alderton J, Tsien RY, Steinhardt RA: **Changes of free calcium levels with stages of the cell division cycle.** *Nature* 1985, **315**:147-149.
53. Ratan RR, Shelanski ML, Maxfield FR: **Transition from metaphase to anaphase is accompanied by local changes in cytoplasmic free calcium in Pt K2 kidney epithelial cells.** *Proc Natl Acad Sci U S A* 1986, **83**:5136-5140.
54. Masumoto N, Sasaki T, Tahara M, Mammoto A, Ikebuchi Y, Tasaka K, Tokunaga M, Takai Y, Miyake A: **Involvement of Rabphilin-3A in cortical granule exocytosis in mouse eggs.** *J Cell Biol* 1996, **135**:1741-1747.
55. Ikebuchi Y, Masumoto N, Matsuoka T, Yokoi T, Tahara M, Tasaka K, Miyake A, Murata Y: **SNAP-25 is essential for cortical granule exocytosis in mouse eggs.** *Am J Physiol* 1998, **274**:C1496-500.
56. Conner S, Leaf D, Wessel G: **Members of the SNARE hypothesis are associated with cortical granule exocytosis in the sea urchin egg.** *Mol Reprod Dev* 1997, **48**:106-118.
57. Johnson MH, Eager D, Muggleton-Harris A, Grave HM: **Mosaicism in organisation concanavalin A receptors on surface membrane of mouse egg.** *Nature* 1975, **257**:321-322.
58. Daniel Joseph C. Jr.: **Methods in Mammalian Embryology.** Edited by: Daniel Joseph C Jr. San Francisco, W. H. Freeman and Company; 1971.

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