Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 280 (2005) 38–47

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

PAR-3 defines a central subdomain of the cortical actin cap in mouse eggs

Francesca E. Duncan[a], Stuart B. Moss[a], Richard M. Schultz[a,b,1], Carmen J. Williams[a,*,1]

[a]*Center for Research on Reproduction and Women's Health and Department of Obstetrics and Gynecology, Philadelphia, PA 19104, USA*
[b]*Department of Biology, University of Pennsylvania Philadelphia, PA 19104, USA*

Received for publication 27 July 2004, revised 2 December 2004, accepted 23 December 2004
Available online 29 January 2005

Abstract

The evolutionarily conserved partitioning defective (PAR) protein PAR-3 is pivotal for establishing and maintaining cell polarity. During mammalian oocyte maturation, the radially symmetric oocyte is transformed into a highly polarized metaphase II (MII)-arrested egg. We therefore examined several aspects of PAR-3 expression during oocyte maturation. We cloned two novel PAR-3 transcripts from an oocyte library that likely encode proteins of M_r = 73 K and 133 K that are phosphorylated during maturation. PAR-3, which is found throughout the GV-intact oocyte, becomes asymmetrically localized during meiosis. Following germinal vesicle breakdown, PAR-3 surrounds the condensing chromosomes and associates with the meiotic spindles. Prior to emission of the first and second polar bodies, PAR-3 is located within a central subdomain of the polarized actin cap, which overlies the spindle. This cortical PAR-3 localization depends on intact microfilaments. These results suggest a role for PAR-3 in establishing asymmetry in the egg and in defining the future site of polar body emission.

Keywords: Mouse egg; PAR-3; Cortical actin; Meiosis

Introduction

During maturation of mouse oocytes, the radially symmetric oocyte is transformed into a highly polarized metaphase II (MII)-arrested egg, one hallmark of which is a cortical MII spindle and its overlying actin-rich domain. This polarization is necessary to produce two highly asymmetric cell divisions (emission of the polar bodies) that maximize retention of maternal components required for early development. This polarity has also been suggested to contribute to early axis determination in the preimplantation mouse embryo (Zernicka-Goetz, 2002), although this proposal is controversial (Hiiragi and Solter, 2004).

The molecular basis for how mammalian oocytes become polarized is poorly understood. Other than formin-2 (Leader et al., 2002) and MAP kinase (Verlhac et al., 2000) that are required for microfilament-based migration of the MI spindle to the cortex, there is a paucity of information regarding how other proteins are involved in establishing and/or maintaining mammalian egg polarity. In lower species, three evolutionarily conserved and interacting proteins, PAR-3, PAR-6, and aPKC (atypical PKC), are essential for development and maintenance of cell polarity (Henrique and Schweisguth, 2003; Knoblich, 2001; Macara, 2004; Ohno, 2001; Pellettieri and Seydoux, 2002). In *Caenorhabditis elegans*, these proteins are critical in establishing anterior–posterior polarity and regulating spindle orientation and asymmetric cell division in the 1-cell embryo. In *Drosophila,* PAR-3/PAR-6/aPKC homologs function to (1) regulate the anterior–posterior axis in the presumptive oocyte, (2) establish apical–basal polarity in embryonic epithelial cells, and (3) generate asymmetry in mitotic neuroblasts. In mammalian cells, this complex is critical for establishing

* Corresponding author. Center for Research on Reproduction and Women's Health, 1313 BRBII/III, 421 Curie Blvd., Philadelphia, PA 19104, USA. Fax: +1 215 537 7627.
E-mail address: cjwill@mail.med.upenn.edu (C.J. Williams).
[1] These authors contributed equally to this work.

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2004.12.034

and maintaining apical–basal polarity in epithelial cells via tight junctions. Because of their documented roles, these three proteins are logical candidates for participating in establishing and/or maintaining egg polarity in mammals.

In *Xenopus* and mouse, PAR proteins become asymmetrically distributed during meiotic maturation. In *Xenopus* oocytes, aPKC and the frog PAR-3 homolog, XASIP, become localized to the animal hemisphere during maturation (Nakaya et al., 2000). In mouse oocytes, two PAR-6 proteins, PAR-6a and PAR-6b, also become asymmetrically distributed towards the animal pole during meiotic maturation in vitro (Vinot et al., 2004). Changes in the localization during maturation of PAR-3 and aPKC, however, have not been reported in the mouse. We report here that mouse oocytes express at least two novel forms of PAR-3 that are post-translationally modified by phosphorylation. Following germinal vesicle breakdown, PAR-3 becomes enriched in the region of the chromosomes. At MI and MII, PAR-3 is associated with the spindle and enriched in the central subdomain of the actin cap overlying the meiotic spindles; this localization depends on intact actin microfilaments. The finding that PAR-3 occupies the central subdomain of the actin cap as early as MI and can interact with chromosomes suggests a role for PAR-3 in defining the future site of polar body emission.

Materials and methods

Antibodies and reagents

A rabbit polyclonal antibody made against the second and third PDZ domains of mPAR-3 was obtained from Upstate Biotechnology (Lake Placid, NY) and will be referred to as anti-PAR-3 (UBI) in the text. The anti-ASIP C2-2 antibody was a generous gift of S. Ohno (Yokohama City University School of Medicine, Yokohama, Japan) and was made against the aPKC binding region. The Cy5-conjugated anti-rabbit IgG was from Jackson Immunoresearch (West Grove, PA), Alexa Fluor 488-conjugated anti-rabbit IgG was from Molecular Probes (Eugene, OR), and FITC-conjugated phalloidin, Latrunculin A (Lat A), and nocodazole were from Sigma (St. Louis, MO).

Collection and culture of mouse oocytes and eggs

Female CF-1 mice (6–8 weeks old) were obtained from Harlan Sprague–Dawley (Indianopolis, IN). Fully-grown, germinal vesicle (GV)-intact oocytes and metaphase II-arrested (MII) eggs were collected from gonadotropin-treated females as previously described (Manejwala et al., 1986). The collection medium used was modified Whitten's medium (Whitten, 1971) containing 15 mM Hepes, pH 7.2, 7 mM $NaHCO_3$, 10 μg/ml gentamicin, and 0.01% polyvinyl alcohol. Unless otherwise stated, cells were cultured either in Whitten's medium supplemented with 10 μg/ml gentamicin and 0.01% polyvinyl alcohol or in CZB (Chatot et al., 1989) at 37°C in a humidified atmosphere of 5% CO_2 in air. For collecting and culturing germinal GV-intact oocytes, the medium was supplemented with 0.3 mM dibutyryl cAMP to inhibit resumption of meiosis (Cho et al., 1974). For maturation in vivo, mice were injected with 0.5 IU eCG and 48 h later with 0.5 IU hCG. Cells were then harvested from ovaries 3 h post-hCG for GVBD, 8 h post-hCG for MI, 10 h post-hCG for telophase I, and from oviducts 13–15 h post-hCG for MII. One-cell embryos were collected 14–15 h after hCG injection and mating to $B6D2F_1$/J males (Jackson Laboratory, Bar Harbor, ME).

Immunoblotting

Oocyte and egg protein extracts and control 3T3/A31 cell lysate were separated by SDS–PAGE using either 8% or 10% gels and then transferred electrophoretically onto nitrocellulose membranes. For use with the PAR-3 (UBI) antibody, membranes were immunoblotted according to the instructions included with the antibody, except for the following modifications: the membranes were incubated with 2 μg/ml of anti-PAR-3 (UBI) overnight at 4°C and phosphate-buffered saline (PBS) was used instead of Tris-buffered saline. As a control, a membrane containing identical samples was immunoblotted with 2 μg/ml of non-immune rabbit IgG. For use with the C2-2 antibody, membranes were blocked overnight at 4°C in PBS containing 5% nonfat milk and 0.1% Tween-20 (PBST). The membranes were then incubated in the C2-2 antibody diluted to a final of concentration of 4 μg/ml in PBST containing 3% BSA for 2 h at room temperature. A secondary anti-rabbit HRP-conjugated antibody was used to detect the primary antibody. Membranes probed with both antibodies were developed using ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

For phosphatase treatment, MII protein extracts were treated with 45.3 units of calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA) for 30 min at 37°C. As a control, the untreated MII extract was also incubated for 30 min under the same conditions. These samples were then separated on a 10% SDS–PAGE gel that was run such that the 47 kDa molecular weight marker reached the bottom of the gel, and were then processed for immunoblotting as described above.

Screening of an oocyte library with a PAR-3 probe and sequencing of complete transcripts

An mPAR-3 cDNA fragment (nt# 664–1393, Accession # NM_033620) was labeled with [$\alpha-^{32}$P]dCTP (3000 Ci/mmol) by random priming (Invitrogen, Piscataway, NJ). This probe was then used to screen a total of 600,000 plaques from an oocyte cDNA library (a gift of John J.

Eppig, Jackson Laboratory, Bar Harbor, ME) in λTriplEx2 (Clontech, Palo Alto, CA). The library was transferred to Hybond N+ membranes (Amersham Biosciences) and the membranes were soaked in DNA denaturing solution (1.5 M NaCl, 0.5 M NaOH) and DNA neutralizing solution (1.5 M NaCl, 0.5 M Tris–HCl, pH 8.0). Membranes were pre-hybridized in Rapid-hybridization buffer (Amersham Biosciences) for 15 min at 65°C and then incubated overnight at 42°C in Rapid-hybridization buffer containing the radiolabeled probe. The membranes were washed with 2× SSPE/0.1% SDS at room temperature and then with 0.1× SSPE/0.1% SDS at 65°C, and transferred to auto-radiography cassettes. Positive λ clones were purified by two additional screenings and were converted to plasmids according to the manufacturer's directions. Of the seven clones analyzed, one was 3.7 kb and six were 3.3 kb. Both size clones were sequenced in both directions and the sequences were analyzed using MacVector (Accelrys, Madison, WI) and Sequencher (Gene Codes, Ann Arbor, MI) software. The sequences correspond to two novel PAR-3 splice variants, which have been named PAR-3o1 and PAR-3o2 for oocyte PAR-3 form 1 and 2, respectively. These sequences have been deposited in GenBank (Accession number AY856082 and AY856081).

Immunocytochemical analysis

Prior to fixation, the *zonae pellucidae* of oocytes and eggs were removed by a brief incubation in acid Tyrodes, pH 1.6. For PAR-3 detection, cells were fixed in freshly prepared 3.8% paraformaldehyde in PBS for 1 h at room temperature, permeabilized in PBS containing 0.3% BSA and 0.1% Triton X-100 for 15 min, and then blocked in PBS containing 0.3% BSA and 0.01% Tween-20 (blocking solution). Cells were incubated in 20 μg/ml anti-PAR-3 (UBI) or in 10 μg/ml anti-ASIP C2-2 antibody for 1 h and then washed in blocking solution. Both primary antibodies were detected using either a Cy5- or Alexa Fluor 488-conjugated secondary antibody for 1 h at room temperature. Actin was detected using FITC-conjugated phalloidin (final concentration 2 μg/ml). Cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 2 μg/ml propidium iodide for chromatin visualization, and observed with a laser-scanning confocal microscope (Leica DMRE, Leica Microsystems, Inc., Exton, PA). Cells were observed using a 40× Plan APO objective, N.A. 0.75. Images were acquired with Leica confocal software and processed using Photoshop software (Adobe Systems Inc., San Jose, CA).

Drug treatments

To disrupt the microtubule cytoskeleton at MI, GV-intact oocytes were matured for 6 h in CZB and then transferred to medium containing 10 μM nocodazole and cultured an additional 2 h. To disrupt the microtubule cytoskeleton at MII, MII-arrested eggs were incubated in CZB containing 10 μM nocodazole for 4 h. In all cases, control cells were left in CZB for the duration of the experiment. To disrupt the cortical actin cytoskeleton, MII-arrested eggs were incubated for 10 min in medium alone or medium containing 10 μM Latruculin A (Lat A). Half of the Lat A-treated eggs were washed free of the drug and cultured for an additional 4 h to allow the actin cytoskeleton to reform.

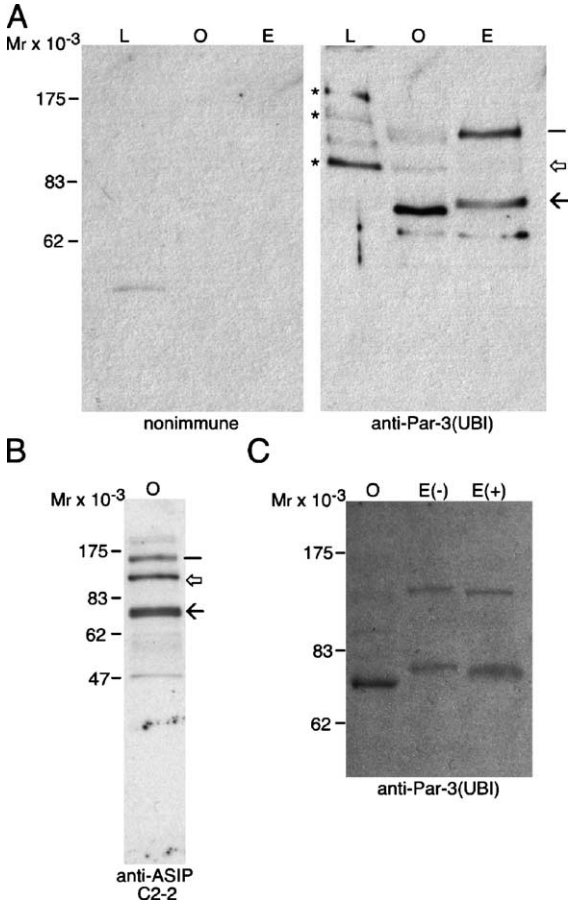

Fig. 1. Immunoblot analysis of PAR-3 protein in mouse oocytes and MII-arrested eggs. (A) Immunoblots using extracts derived from 500 GV-intact oocytes, 500 MII-arrested eggs, or 20 μg of 3T3 cell lysate was probed with either anti-PAR-3 (UBI) antibody (right panel) or non-immune rabbit IgG (left panel). L, 3T3 cell lysate; O, oocytes; E, eggs. Of note, the bands in oocyte and egg extract at M_r = 68,000 were not consistently observed and are likely non-specific. The three major bands in the control lysate are marked with asterisks (the band that is unmarked was not consistently observed). The line highlights the M_r = 133 K/135 K forms of PAR-3o1, the block arrow highlights the M_r = 100 K form, and the solid arrow marks the M_r = 73 K/76 K forms of PAR-3o2. The experiment was performed three times and representative immunoblots are shown. (B) Immunoblot using extract derived from 500 GV-intact oocytes was probed with the anti-ASIP C2-2 antibody. Immunoreactive bands are designated as in panel A. (C) Immunoblot of proteins treated with calf alkaline phosphatase. A protein extract of 500 untreated oocytes and protein extracts of 500 eggs that were incubated in a buffer either with (+) or without (−) phosphatase were subjected to immunoblot analysis using the anti-PAR-3 (UBI) antibody. O, oocytes; E, eggs. This experiment was performed twice.

Results

PAR-3 expression and isoforms in mouse oocytes and eggs

To determine whether PAR-3 was expressed in mouse oocytes and eggs, immunoblot analysis was performed on total protein extracts of GV-intact oocytes and MII eggs using a polyclonal antibody raised against the second and third PDZ domains of mouse PAR-3 (Fig. 1A). In the control lysate, the bands observed corresponded to three previously published mammalian splice forms of PAR-3 that encode proteins of M_r = 180 K, M_r = 150 K, and M_r = 100 K (Fig. 1A, see asterisks) (Lin et al., 2000). In oocyte extracts, a predominant band of M_r = 73 K was observed as well as additional bands of M_r = 133 K and 100 K. In egg extracts, two bands of M_r = 135 K and 76 K were identified. Antibody specificity was established by the lack of signal when non-immune rabbit IgG was used (Fig. 1A). Furthermore, an independent polyclonal PAR-3 antibody, raised against the aPKC binding domain of rat PAR-3 (anti-ASIP C2-2 antibody), recognized the same three bands of M_r = 133 K, 100 K, and 73 K in oocyte extracts (Fig. 1B).

Although multiple spliced variants of PAR-3 are found in mammalian cell lines and tissues (Gao et al., 2002), cDNA sequences consistent with the M_r = 73 K and 133 K forms observed in oocytes have not been reported. A screen of an oocyte cDNA library yielded complete transcripts for two novel splice variants of PAR-3 that likely account for the uncharacterized immunoreactive PAR-3 bands in oocytes (Fig. 2, PAR-3o1 and PAR-3o2). The full-length transcript of mammalian PAR-3 (mPAR-3) is predicted to have 25 exons and is reported to encode a protein of 1337 amino acids which migrates at M_r = 180 K (Lin et al., 2000). The library screen we performed revealed the presence of three additional exons in the PAR-3 gene (Fig. 2A; Exons 3b, 15b, and 20b). Both the novel transcripts share an identical novel 5′ untranslated region and use a start methionine that is downstream and in-frame to the start codon used by mPAR-3. However, PAR-3o1 and PAR-3o2 utilize different stop codons and vary in the sequences of their 3′ untranslated regions. As a result, PAR-3o1 encodes a protein of 896 residues, whereas PAR-3o2 encodes a 606 amino acid protein. Because mPAR proteins seem to migrate anomalously slower than would be predicted by their amino acid composition (Lin et al., 2000), it is likely that PAR-3o1 and PAR-3o2 encode the M_r = 133 K and 73 K forms observed on immunoblots of oocyte and egg extracts, respectively. Henceforth, we refer to these forms as PAR-3o1 and PAR-3o2.

Similar to mPAR-3, both novel transcripts encode proteins that contain three conserved PDZ domains (Fig. 2B). Although the PAR-3o1 protein contains the entire

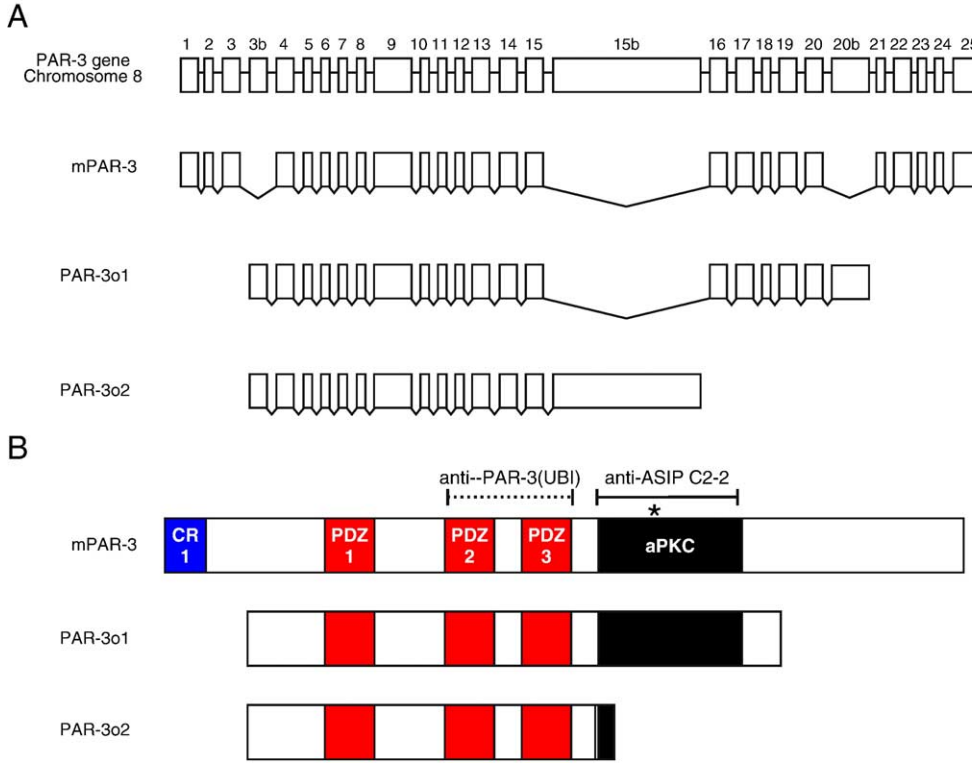

Fig. 2. (A) Genomic structure of PAR-3 and diagrams of mPAR-3, PAR-3o1, and PAR-3o2 transcripts (Accession numbers AY856082 and AY856081). Exons are represented by boxes and are drawn to scale. Exons 3b, 15b, and 20b are novel and were identified in the oocyte library screen. Introns are not drawn to scale. (B) Protein domain organization of mPAR-3, PAR-3o1, and PAR-3o2. The asterisk indicates the position of the consensus aPKC phosphorylation site. The dotted line highlights the region of the protein used to generate the anti-PAR-3 (UBI) antibody, and the solid line marks the region used to generate the anti-ASIP C2-2 antibody.

aPKC-binding domain, the PAR-3o2 protein contains only about 30 amino acid residues of this domain (Fig. 2B). The aPKC-binding ability of this 30 amino acid portion of the protein has not been determined. Of note, neither PAR-3o1 or PAR-3o2 have the CR1 domain that is important for PAR-3 oligomerization and previously was thought to be conserved among all PAR-3 proteins (Benton and St. Johnston, 2003; Mizuno et al., 2003).

Both PAR-3o1 and PAR-3o2 migrated with decreased electrophoretic mobility in egg extracts as compared to oocyte extracts, which suggested that these isoforms were post-translationally modified during maturation (Figs. 1A and C). To determine whether the shifts in mobility were due to phosphorylation, MII egg extracts were treated with calf intestinal alkaline phosphatase. After treatment, PAR-3o1 migrated as the M_r = 133 K form found in oocytes, demonstrating that phosphorylation was completely responsible for the size increase observed in MII eggs (Fig. 1C). Phosphatase treatment also caused PAR-3o2 to migrate slightly faster as compared to the protein in untreated egg extract. However, the PAR-3o2 in the dephosphorylated MII egg extract still migrated more slowly than the M_r = 73 K form found in oocytes (Fig. 1C). An additional modification of PAR-3o2, besides phosphorylation, could explain the decreased mobility that persists even after phosphatase treatment.

In addition to changes in PAR-3o1 and PAR-3o2 phosphorylation, differences in the relative amounts of the two isoforms also occurred during meiotic maturation (Figs. 1A and C). Although there was a decrease in the amount of PAR-3o2 in the egg as compared to the oocyte, there was a dramatic increase in the amount of PAR-3o1. This increase occurred steadily throughout oocyte maturation (data not shown), consistent with recruitment of a maternal mRNA. Note that this increase could also be due to differences in antibody affinity towards the phosphorylated form.

Asymmetric localization of PAR-3 during oocyte maturation

The presence of PAR-6 isoforms in the reorganized cortex and spindle of the MII egg suggested that PAR-3 might be similarly localized because of its ability to associate with PAR-6 and modulate PAR-6's localization (Ohno, 2001). Accordingly, we determined changes in PAR-3 localization during meiotic maturation. In GV-intact oocytes, PAR-3 was distributed uniformly throughout the cytoplasm and GV, with slightly lower signal intensity in the GV (Fig. 3A). Following GVBD, the intensity of the punctate PAR-3 signal increased around the condensing chromatin and the developing spindle (Fig. 3B). Optical sectioning revealed that PAR-3 completely surrounded the spindle (data not shown). At MI, PAR-3 was enriched in the cytoplasm surrounding the spindle, throughout the spindle, and preferentially at the spindle ends. Moreover, intense PAR-3 staining was observed in the cortex overlying the spindle (Fig. 3C). At MII, the overall PAR-3 signal intensity

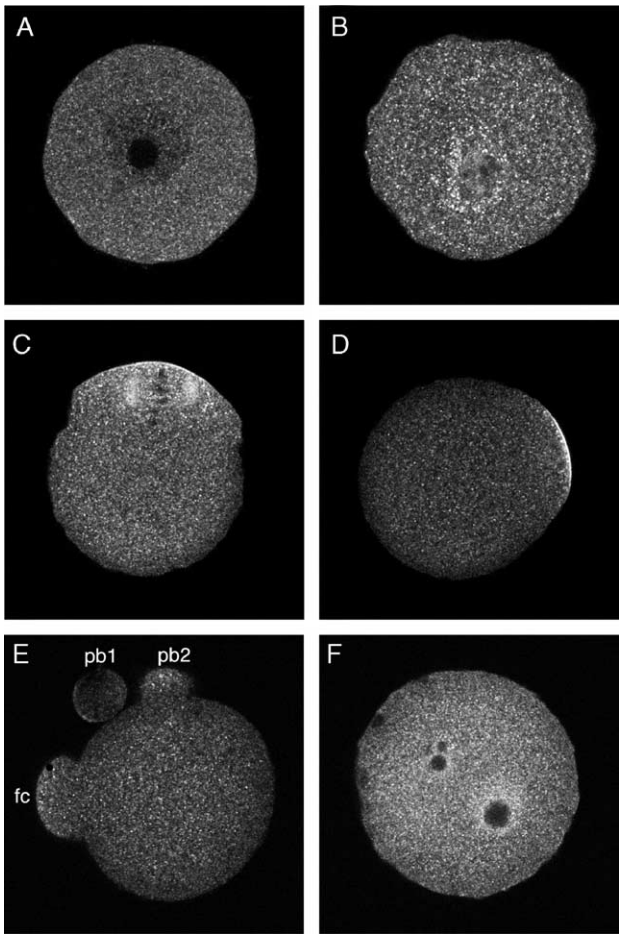

Fig. 3. PAR-3 localization during meiotic maturation and following fertilization. (A) GV-intact, (B) GVBD, (C) MI, (D) MII, (E) early 1-cell embryo, and (F) pronuclear embryo. fc, fertilization cone; pb1, first polar body; pb2, second polar body. The anti-PAR-3 (UBI) antibody was used. The experiment was performed three times and at least 15 cells were analyzed at each stage per experiment; representative images are shown.

was decreased, however PAR-3 was highly enriched in a narrow band at the cell cortex overlying the meiotic chromosomes (Fig. 3D; note that the spindle is oriented perpendicular to the plane of the confocal section.), and was enriched in the MII spindle (Fig. 4A). These maturation-associated changes in PAR-3 localization also were observed when oocytes were matured in vitro (data not shown). Following fertilization, the high degree of PAR-3 asymmetry in the cortex was not observed, although there was a slight enrichment of PAR-3 staining in the fertilization cone and second polar body (Fig. 3E). In the 1-cell embryo, PAR-3 was uniformly distributed in the cytoplasm and was enriched in both male and female pronuclei (Fig. 3F).

To confirm the immunostaining specificity, PAR-3 localization was also determined with the C2-2 antibody (Fig. 4). Similar to the antibody used for Fig. 3 (PAR-3 (UBI) antibody), the C2-2 antibody showed polarized PAR-3 staining most intense in the region of the MII spindle. Nevertheless, the staining patterns were not identical in that the intense PAR-3 (UBI) staining was restricted to the cortex

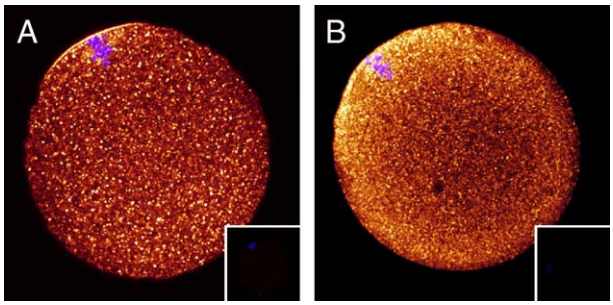

Fig. 4. PAR-3 localization in MII eggs using two different antibodies. (A) Anti-PAR-3 (UBI) antibody, (B) anti-ASIP C2-2 antibody. The insets in each panel are the corresponding controls performed using only the secondary antibody. PAR-3 immunostaining is shown using a glowover lookup table (high signal white, moderate signal orange, low signal red); the DNA staining is blue. The experiment was performed four times and at least 15 cells were analyzed at each stage per experiment; representative images are shown.

and the MII spindle, whereas the staining pattern obtained with the C2-2 antibody was more diffuse. These differences are not surprising because the two antibodies, which were raised against different regions of PAR-3 (Fig. 2B), differentially detect the PAR-3 isoforms by immunoblotting (Figs. 1A and B). For example, the PAR-3 (UBI) antibody detects predominantly the fastest migrating PAR-3 isoform, whereas the C2-2 antibody readily recognizes all three isoforms. These results suggest that the immunostaining signal detected with the PAR-3 (UBI) antibody in Fig. 4A mainly reflects localization of the fastest migrating PAR-3 isoform, whereas the pattern observed in Fig. 4B may be attributed to all three isoforms.

Because of the distinct asymmetric localization of PAR-3 that was observed at MI and MII, we focused on its localization during the transition between these stages of oocyte maturation. PAR-3 was observed in oocytes obtained from ovaries 10 h after hCG injection; due to their asynchronous maturation, oocytes were caught at different stages of the MI to MII transition. PAR-3 was localized to the spindle ends at early telophase, similar to its localization at MI (Figs. 3C and 5). At telophase, however, PAR-3 was immediately adjacent to the DNA (Figs. 5B and C). Between early and late telophase, PAR-3 localization changed in reference to the DNA so that in late telophase it was located between the egg cortex and the condensed DNA (Fig. 5C).

Role of microtubules and microfilaments in PAR-3 localization

Although a dramatic change was observed in PAR-3 localization during maturation, how PAR-3 reached the cortex was unknown. The observation that PAR-3 surrounded the forming spindle and localized to the MI spindle suggested that translocation of the spindle to the cortex could provide a source of cortical PAR-3. When MI spindle formation was inhibited by nocodazole treatment during maturation, PAR-3 was located in a dense patch adjacent to the condensed chromatin (Fig. 6B). In cases in which the chromatin was localized in the cortex, PAR-3 was enriched in cortex in the vicinity of the chromatin (Fig. 6C). These results indicate that PAR-3 can associate with microtubules and in their absence with chromatin. Furthermore, these results are consistent with the spindle providing a source of cortical PAR-3.

To determine if maintenance of the cortical PAR-3 required the presence of an underlying spindle or chromatin, as does cortical actin (Longo and Chen, 1985; van Blerkom and Bell, 1986), the MII spindle was disrupted by nocodazole treatment, which frequently results in scattering clumps of chromatin to different cortical regions (van Blerkom and Bell, 1986). In these treated eggs, cortical clumps of chromatin exhibited an overlying PAR-3 cap (Figs. 7A, B). No cortical PAR-3 staining was observed in the absence of underlying chromatin, suggesting that the original MII spindle-associated PAR-3 disassembled, i.e., the continued presence of the spindle/chromatin is required to maintain polarized regions of cortical PAR-3.

As mentioned in the Introduction, cortical actin is enriched in the region overlying the MII spindle, in a pattern highly reminiscent of cortical PAR-3. When MII eggs were stained for both F-actin and PAR-3, PAR-3 was

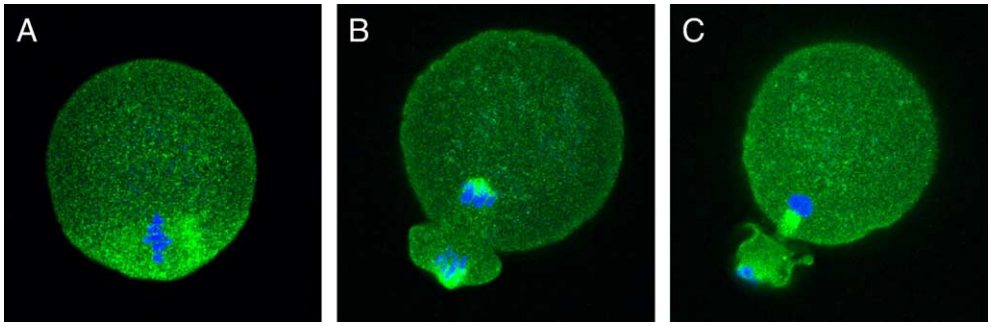

Fig. 5. PAR-3 localization during the MI to MII transition. Oocytes between MI and MII were stained for PAR-3 using the anti-PAR-3 (UBI) antibody (green) and DNA (blue). (A) MI, (B) early telophase, (C) late telophase. The experiment was performed twice and a total of 30 maturing oocytes were observed; representative images are shown. The confocal voltage settings were varied to highlight PAR-3 localization, which precludes directly comparing the signal intensities.

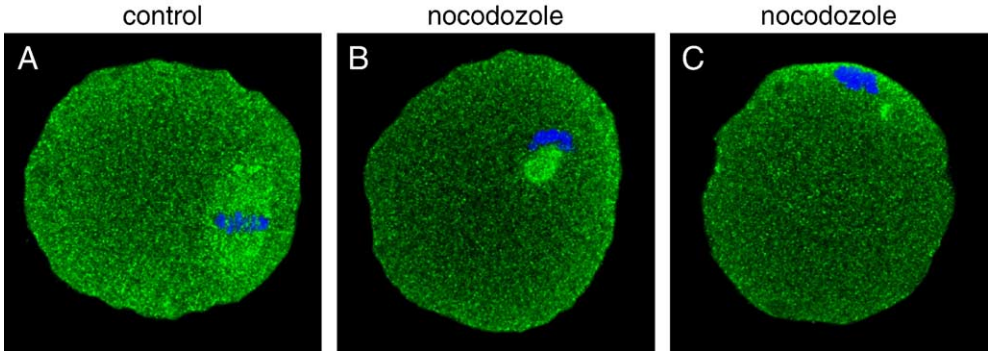

Fig. 6. PAR-3 localization in MI oocytes treated with nocodazole. In vitro matured oocytes at MI were either not treated (A) or treated with 10 μM nocodazole (B–C) and then stained with the anti-PAR-3 (UBI) antibody (green) and for DNA (blue). The experiment was performed three times and representative images are shown. A total of at least 60 oocytes in each treatment group were analyzed. The confocal voltage settings were varied to highlight PAR-3 localization, which precludes directly comparing the signal intensities.

only found in the central region of the actin cap (Figs. 8A, D, G). Cortical microfilaments likely mediated this association because treatment of MII eggs with the potent microfilament disruptor Lat A resulted in loss of cortical microfilaments and cortical PAR-3 (Figs. 8B, E, H). When cortical F-actin was permitted to reform by washing out Lat A, PAR-3 once again localized in the central domain of the actin cap (Figs. 8C, F, I). These results suggest that PAR-3 is directly or indirectly associated with F-actin.

Discussion

The results presented here demonstrate the presence of multiple forms of PAR-3 in mouse oocytes/eggs and document maturation-associated changes in PAR-3 phosphorylation and localization. The two clones characterized here likely represent oocyte-specific PAR-3 isoforms we named PAR-3o1 and PAR-3o2 because the 3b exon is only detected in oocyte/egg EST libraries. The presence of this exon creates splice forms that lack the conserved region 1 (CR1) domain. PAR-3o1 is identical to the 150 kDa mPAR-3 isoform reported in the literature, except PAR-3o1 lacks a portion of the amino terminus, including the CR1 domain, which accounts for its reduced size of 133 kDa (Lin et al., 2000, and mPAR-3 150 kDa sequence provided by Dr. S. Ohno). PAR-3o2 contains only the first 30 of 224 amino acids that comprise the aPKC-binding domain, which are likely sufficient for antibody recognition by the C2-2 antibody, and lacks the known aPKC phosphorylation site. If the fastest migrating PAR-3 isoform is in fact PAR-3o2, the maturation-associated phosphorylation must be due to phosphorylation of another residue(s). Because PAR-3o2 lacks most of the aPKC-binding domain, this spliced form could serve as a dominant negative protein by blocking PDZ domain interactions.

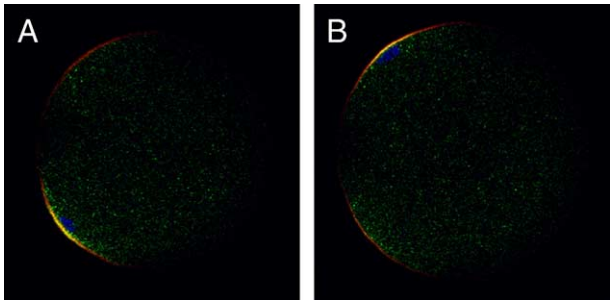

Fig. 7. PAR-3 localization in MII-arrested eggs treated with nocodazole. MII-arrested eggs were treated with 10 μM nocodazole and stained with the anti-PAR-3 (UBI) antibody (green), FITC-conjugated phalloidin (red), and propidium iodide to detect the scattered DNA (blue). The yellow signal is due to the co-localization of PAR-3 and actin. Shown in panels A and B are images of the same egg in two different planes. This experiment was performed twice and at least 10 eggs were observed in each experiment; representative images are shown.

Because the PAR-3 isoforms cannot be distinguished by the available antibodies, the following discussion uses the term PAR-3 to refer collectively to the various isoforms. PAR-3, which is initially uniformly localized throughout the cytoplasm, associates with MI and MII spindles. When either spindle is in the cortex, PAR-3 is highly enriched in the cortex overlying the spindle, i.e., the animal pole. A consequence of this restricted localization is loss of cortical PAR-3 during polar body emission, suggesting that cortical PAR-3 functions during this asymmetric cleavage and not in early post-fertilization events. A similar redistribution occurs during *Xenopus* oocyte maturation in which PAR-3 localizes to the animal hemisphere (Nakaya et al., 2000). Unlike the mouse, however, the boundaries of PAR-3 staining in the *Xenopus* oocyte extend throughout the animal hemisphere and are not restricted to the region overlying the MII spindle. Thus, it is likely that in *Xenopus* the bulk of PAR-3 is retained in the animal hemisphere following polar body emission. In *C. elegans*, PAR-3 (and PAR-6) asymmetry is established following fertilization in response to contact of microtubules that emanate from the sperm-derived centriole with the cortex of the future P1 blastomere (Ahringer, 2003). Thus, in contrast to the mouse egg, cortically located PAR-3 in both *Xenopus* and *C. elegans* is retained in the embryo and positioned to influence early development.

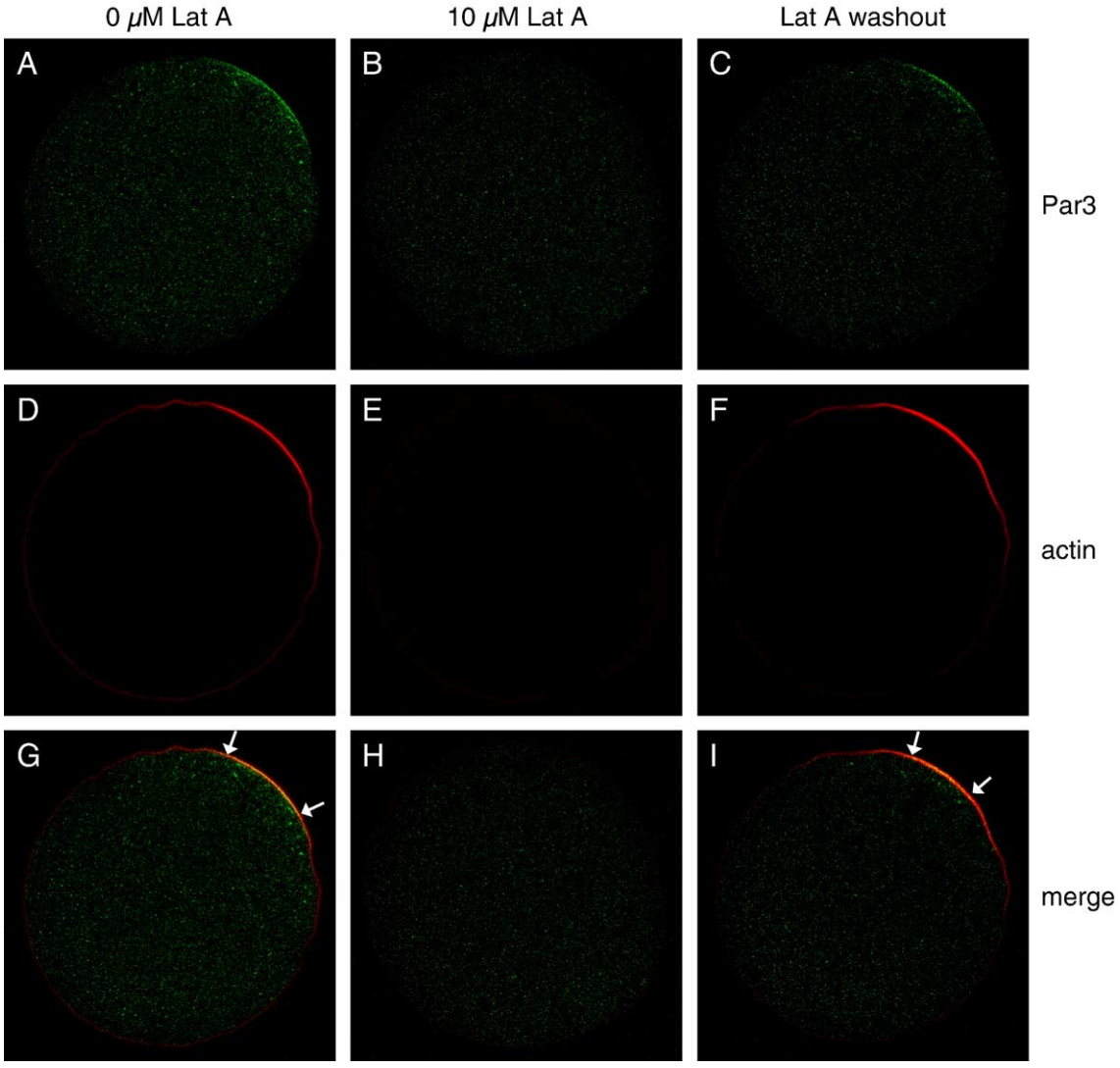

Fig. 8. PAR-3 association with cortical actin in MII-arrested eggs. MII-arrested eggs were either not treated with Lat A (A, D, G) or treated with 10 μM Lat A (B, E, H). Eggs were also washed free of Lat A (C, F, I) to allow cortical actin to repolymerize. Eggs were stained for PAR-3 using the anti-PAR-3 (UBI) antibody (A–C) and for actin (D–F), and the merged images are shown in panels G–I (where PAR-3 is green, actin is red, and yellow represents co-localization). The arrows indicate the borders of PAR-3 localization within the central subdomain of the actin cap. This experiment was performed 5 times, and at least 10 eggs in each treatment group were analyzed per experiment. Representative images are shown. The confocal voltage settings were varied to highlight PAR-3 localization, which precludes directly comparing the signal intensities.

Two forms of PAR-6, a and b, have been described in mouse oocytes/eggs and undergo changes in localization during maturation (Vinot et al., 2004) similar to what we observe for PAR-3. This is not surprising given that PAR-3 and PAR-6 are known to associate (Ohno, 2001). In contrast to PAR-3, which localizes to the cortex at MI, there is no indication that either PAR-6a or PAR-6b has a similar cortical localization until MII. Similar to the maturation-associated increase in the amount of both PAR-6a and PAR-6b (Vinot et al., 2004), the amount of the larger form of PAR-3, PAR-3o1, also apparently increases. Although the functional consequence of the up-regulation of both PAR-3 and PAR-6 is not known, one possibility is that the newly synthesized proteins associate with each other, whereas those in the existing pool cannot. Precedent for this possibility exists in mouse oocytes. Microinjection of meiotically incompetent oocytes with either cyclin B mRNA or CDK1 mRNA does not result in association of the newly synthesized protein with its endogenous counterpart, whereas injection of both mRNAs results in association of the newly synthesized cyclin B and CDK1 to form a functional complex that induces GVBD (de Vantéry et al., 1997).

The increased intensity of PAR-3 staining on the cortical side of the MI spindle (Fig. 3C) suggests that spindle-associated PAR-3 was translocating to the cortex. This cortical localization does not require intact microtubules because it occurs in the presence of nocodazole (Figs. 6 and 7). Moreover, microtubule disruption also reveals that PAR-3 can associate with chromosomes. This association is likely

weaker than that with microtubules because little, if any, PAR-3 staining is observed with chromosomes on the metaphase plate of either the MI or MII spindle. A similar association of PAR-6a with chromosomes is observed in the absence of microtubules (Vinot et al., 2004).

Following disruption of the MI or MII spindle, PAR-3 becomes localized to the region overlying chromosomes located in the cortex. This finding is reminiscent of the ability of chromosomes to induce cortical reorganization, the hallmark of which is formation of the actin cap (Longo, 1985). Like actin cap formation, the changes in PAR-3 localization also occur during maturation in vitro in the absence of associated granulosa/cumulus cells, implying that both are initiated by an intrinsic program. In fact, cortical actin is required for cortical PAR-3 localization because disrupting the actin cap leads to complete loss of cortical PAR-3 staining. Intact microfilaments are also required to maintain cortical PAR-3 localization in *C. elegans*, because cortically located PAR-3 is not observed in Lat A treated 1-cell embryos (Severson and Bowerman, 2003). A previous study reported that cytochalasin D treatment of mouse eggs does not disrupt the cortical localization of PAR-6b, that is, microfilaments are not required for its localization (Vinot et al., 2004). This may not be the case, however, because in contrast to Lat A, cytochalasin D does not completely disrupt cortical actin (our unpublished results).

Coupled with reformation of the actin cap is relocalization of PAR-3 to this region, suggesting a direct or indirect interaction between actin and PAR-3. Such an association may occur between these proteins in zebrafish retina (Wei et al., 2004). Of particular interest is that cortical PAR-3 is restricted to the central domain of the actin cap. To our knowledge, this is the first demonstration that the actin cap has subdomains. As described in the accompanying paper, there is a reciprocal subdomain at the circumference of the actin cap where the phosphorylated form of the myristoylated alanine-rich C-kinase substrate protein, p-MARCKS, is localized.

It is tempting to speculate that these subdomains regulate distinct processes in polar body emission. The circumferential localization of p-MARCKS may define the position of the contractile ring responsible for polar body abscission. The central localization of PAR-3 and its ability to associate preferentially with the ends of the MI or MII spindle suggest that PAR-3 could facilitate anchoring the spindle within the forming polar body. Furthermore, at telophase, when the chromosomes are distal to the microtubules, an interaction of PAR-3 with chromosomes may ensure their retention in the forming polar body.

The apparent co-localization of PAR-3 and PAR-6 is consistent with their forming a complex in mouse oocytes as they do in other systems (Vinot et al., 2004). Although two of the oocyte PAR-3 isoforms are phosphorylated during maturation, the role of aPKC is unclear, because PAR-3o2 lacks the known aPKC phosphorylation site and aPKC and PAR-3 immunostaining are not concordant (see accompanying paper). Attempts to clarify this issue by inhibiting aPKC using a myristoylated pseudo-substrate (Standaert et al., 1999) were unsuccessful because we found that concentrations of this inhibitor required to block autophosphorylation of aPKC were toxic (unpublished observations).

In summary, our findings suggest that in mouse oocytes/eggs PAR-3 is centrally involved in the initial establishment of the cortical subdomain containing PAR-3 and PAR-6, because PAR-3 is the first to localize to the cortex. Similarly, in *C. elegans* 1-cell embryos, PAR-3 is initially present in the anterior cortex in the absence of functional PAR-6 or aPKC, but the converse is not true (Hung and Kemphues, 1999; Tabuse et al., 1998; Watts et al., 1996). Thus, PAR-3 located in the cell cortex could serve as a platform for assembly of proteins involved in development of egg polarity.

Acknowledgments

This research was supported by grants from the NIH (HD22732 to R.M.S. and C.J.W. and HD06274 to S.B.M.). F.D. was supported by the National Science Foundation Graduate Research Fellowship. We thank Teri Ord and Kerry Krauss for technical assistance and advice. The oocyte cDNA library was a generous gift from John J. Eppig and the C2-2 antibody was a generous gift from S. Ohno. Portions of this work are being submitted by F.D. in partial fulfillment for the Ph.D. requirements at the University of Pennsylvania.

References

Ahringer, J., 2003. Control of cell polarity and mitotic spindle positioning in animal cells. Curr. Opin. Cell Biol. 15, 73–81.

Benton, R., St. Johnston, D., 2003. A conserved oligomerization domain in *Drosophila* Bazooka/PAR-3 is important for apical localization and epithelial polarity. Curr. Biol. 13, 1330–1334.

Chatot, C.L., Ziomek, C.A., Bavister, B.D., Lewis, J.L., Torres, I., 1989. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J. Reprod. Fertil. 86, 679–688.

Cho, W.K., Stern, S., Biggers, J.D., 1974. Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation in vitro. J. Exp. Zool. 187, 383–386.

de Vantéry, C., Stutz, A., Vassalli, J.D., Schorderet-Slatkine, S., 1997. Acquisition of meiotic competence in growing mouse oocytes is controlled at both translational and posttranslational levels. Dev. Biol. 187, 43–54.

Gao, L., Macara, I.G., Joberty, G., 2002. Multiple splice variants of Par3 and of a novel related gene, Par3L, produce proteins with different binding properties. Gene 294, 99–107.

Henrique, D., Schweisguth, F., 2003. Cell polarity: the ups and downs of the Par6/aPKC complex. Curr. Opin. Genet. Dev. 13, 341–350.

Hiiragi, T., Solter, D., 2004. First cleavage plane of the mouse egg is not predetermined but defined by the topology of the two apposing pronuclei. Nature 430, 360–364.

Hung, T.J., Kemphues, K.J., 1999. PAR-6 is a conserved PDZ domain-containing protein that colocalizes with PAR-3 in *Caenorhabditis elegans* embryos. Development 126, 127–135.

Knoblich, J.A., 2001. Asymmetric cell division during animal development. Nat. Rev., Mol. Cell Biol. 2, 11–20.

Leader, B., Lim, H., Carabatsos, M.J., Harrington, A., Ecsedy, J., Pellman, D., Maas, R., Leder, P., 2002. Formin-2, polyploidy, hypofertility and positioning of the meiotic spindle in mouse oocytes. Nat. Cell Biol. 4, 921–928.

Lin, D., Edwards, A.S., Fawcett, J.P., Mbamalu, G., Scott, J.D., Pawson, T., 2000. A mammalian PAR-3–PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. Nat. Cell Biol. 2, 540–547.

Longo, F.J., 1985. Fine structure of the mammalian egg cortex. Am. J. Anat. 174, 303–315.

Longo, F.J., Chen, D.-Y., 1985. Development of cortical polarity in mouse eggs: involvement of the meiotic apparatus. Dev. Biol. 107, 382–394.

Macara, I.G., 2004. Parsing the polarity code. Nat. Rev., Mol. Cell Biol. 5, 220–231.

Manejwala, F., Kaji, E., Schultz, R.M., 1986. Development of activatable adenylate cyclase in the preimplantation mouse embryo and a role for cyclic AMP in blastocoel formation. Cell 46, 95–103.

Mizuno, K., Suzuki, A., Hirose, T., Kitamura, K., Kutsuzawa, K., Futaki, M., Amano, Y., Ohno, S., 2003. Self-association of PAR-3-mediated by the conserved N-terminal domain contributes to the development of epithelial tight junctions. J. Biol. Chem. 278, 31240–31250.

Nakaya, M., Fukui, A., Izumi, Y., Akimoto, K., Asashima, M., Ohno, S., 2000. Meiotic maturation induces animal–vegetal asymmetric distribution of aPKC and ASIP/PAR-3 in *Xenopus* oocytes. Development 127, 5021–5031.

Ohno, S., 2001. Intercellular junctions and cellular polarity: the PAR–aPKC complex, a conserved core cassette playing fundamental roles in cell polarity. Curr. Opin. Cell Biol. 13, 641–648.

Pellettieri, J., Seydoux, G., 2002. Anterior–posterior polarity in *C. elegans* and *Drosophila*-PARallels and differences. Science 298, 1946–1950.

Severson, A.F., Bowerman, B., 2003. Myosin and the PAR proteins polarize microfilament-dependent forces that shape and position mitotic spindles in *Caenorhabditis elegans*. J. Cell Biol. 161, 21–26.

Standaert, M.L., Bandyopadhyay, G., Sajan, M.P., Cong, L., Quon, M.J., Farese, R.V., 1999. Okadaic acid activates atypical protein kinase C (zeta/lambda) in rat and 3T3/L1 adipocytes. An apparent requirement for activation of Glut4 translocation and glucose transport. J. Biol. Chem. 274, 14074–14078.

Tabuse, Y., Izumi, Y., Piano, F., Kemphues, K.J., Miwa, J., Ohno, S., 1998. Atypical protein kinase C cooperates with PAR-3 to establish embryonic polarity in *Caenorhabditis elegans*. Development 125, 3607–3614.

van Blerkom, J., Bell, H., 1986. Regulation of development of the fully grown mouse oocyte: chromosome-mediated temporal and spatial differentiation of the cytoplasm and plasma membrane. J. Embryol. Exp. Morphol. 93, 213–238.

Verlhac, M.H., Lefebvre, C., Guillaud, P., Rassinier, P., Maro, B., 2000. Asymmetric division in mouse oocytes: with or without Mos. Curr. Biol. 10, 1303–1306.

Vinot, S., Le, T., Maro, B., Louvet-Vallee, S., 2004. Two PAR6 proteins become asymmetrically localized during establishment of polarity in mouse oocytes. Curr. Biol. 14, 520–525.

Watts, J.L., Etemad-Moghadam, B., Guo, S., Boyd, L., Draper, B.W., Mello, C.C., Priess, J.R., Kemphues, K.J., 1996. PAR-6, a gene involved in the establishment of asymmetry in early *C. elegans* embryos, mediates the asymmetric localization of PAR-3. Development 122, 3133–3140.

Wei, X., Cheng, Y., Luo, Y., Shi, X., Nelson, S., Hyde, D.R., 2004. The zebrafish Pard3 ortholog is required for separation of the eye fields and retinal lamination. Dev. Biol. 269, 286–301.

Whitten, W.K., 1971. Nutrient requirements for the culture of preimplantation mouse embryo in vitro. Adv. Biosci. 6, 129–139.

Zernicka-Goetz, M., 2002. Patterning of the embryo: the first spatial decisions in the life of a mouse. Development 129, 815–829.