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Two PAR6 Proteins Become Asymmetrically Localized during Establishment of Polarity in Mouse Oocytes

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Summary

Meiotic maturation in mammals is characterized by two asymmetric divisions, leading to the formation of two polar bodies and the female gamete. Whereas the mouse oocyte is a polarized cell, molecules implicated in the establishment of this polarity are still unknown. PAR proteins have been demonstrated to play an important role in cell polarity in many cell types, where they control spindle positioning and asymmetric distribution of determinants. Here we show that two PAR6-related proteins have distinct polarized distributions in mouse oocytes. mPARD6a is first localized on the spindle and then accumulates at the pole nearest the cortex during spindle migration. In the absence of microtubules, the chromosomes still migrate to the cortex, and mPARD6a was found associated with the chromosomes and was facing the cortex. mPARD6a is the first identified protein to associate with the spindle during spindle migration and to relocalize to the chromosomes in the absence of microtubule behavior, suggesting a role in spindle migration. The other protein, mPARD6b, was found on spindle microtubules until entry into meiosis II and relocalized to the cortex at the animal pole during metaphase II arrest. mPARD6b is the first identified protein to localize to the animal pole of the mouse oocyte and likely contributes to the polarization of the cortex.

Results and Discussion

Cellular diversity during development of many organisms is generated in part through asymmetric divisions, in which a cell produces two phenotipically different daughter cells. These daughter cells have unequal sizes and/or different molecular contents and have different developmental potentials. Many asymmetric divisions require the establishment of asymmetries in the cell prior to division, which implies the existence of polarity-establishing molecules. Some of these asymmetries are set up already in the oocyte, before fertilization. During female meiosis, two successive asymmetric divisions occur, leading to the formation of two small cells, the polar bodies, and one big cell, the mature oocyte, and thereby limiting the loss of maternal stores. The mouse mature oocyte, arrested in metaphase of second meiosis (MII), is polarized along an animal/vegetative axis. The positions of the second metaphase spindle and its overlying cortical region define the animal pole. This polarity is established during the first meiotic division. In immature oocytes arrested in prophase I, meiotic maturation resumes with the breakdown of the nucleus (GVBD: germinal vesicle breakdown), chromatin condensation, and reorganization of the microtubule network into a bipolar metaphase I spindle. This spindle initially forms in the center of the cell and then migrates to the cortex along its long axis toward the nearest part of the cortex [1]. During spindle migration, an area devoid of microvilli and enriched in actin microfilaments begins to form in the cortex overlying the spindle [1–4]. When the spindle reaches the cortex, the first polar body is extruded in this actin-rich cortical domain. The spindle then reforms parallel to the actin-rich cortical domain, and the oocyte remains arrested in metaphase II. It is only after fertilization that meiosis II resumes, resulting in a second asymmetric division and the extrusion of the second polar body. Some experimental evidence has suggested that factors located to the oocyte animal pole influence the cleavage pattern of the mouse embryo [5]. As yet, however, no protein involved in this process has been described. In many organisms the conserved PAR (*par*titioning-defective) proteins are essential mediators of cell polarity [6]. Here we show that two mPARD6 proteins are asymmetrically localized during meiosis, indicating that they could be responsible for the patterning of mouse oocytes.

Three genes encoding PAR6 are present in the mouse genome. We found that mPARD6b and mPARD6a (mouse homolog of the PAR6 gene of *C. elegans*) were expressed in both immature (GV) and mature (MII) oocytes at the mRNA (Figure 1A) and protein (Figures 1B and 1C) levels. Both proteins presented similar localization patterns until the spindle began to migrate (Figure 1D); they did not exhibit any specific localization in the GV (data not shown), but they associated with the developing spindle after GVBD (GVBD + 4 hr; n = 20 for mPARD6b, n = 13 for mPARD6a). When the spindle started to migrate, mPARD6b remained uniformly distributed on the spindle (n = 33), whereas mPARD6a accumulated at the spindle pole closest to the cortex (n = 20) (GVBD + 6 hr). Just before polar body extrusion (GVBD + 8 hr), a stronger accumulation of mPARD6a was observed at the pole nearest to the cortex. After extrusion of the first polar body, mPARD6b showed no specific distribution (n = 26), whereas a part of mPARD6a was found around the chromatin (n = 9) (GVBD + 8 hr + PB). In oocytes arrested in metaphase II, mPARD6b became excluded from the spindle and was localized at the cortex overlying the spindle (n = 101), whereas mPARD6a was still found on the spindle and accumulated at both poles (n = 36). GFP-tagged proteins localized as the endogenous mPARD6b (at the cortex, n = 53) and mPARD6a (on the spindle and at

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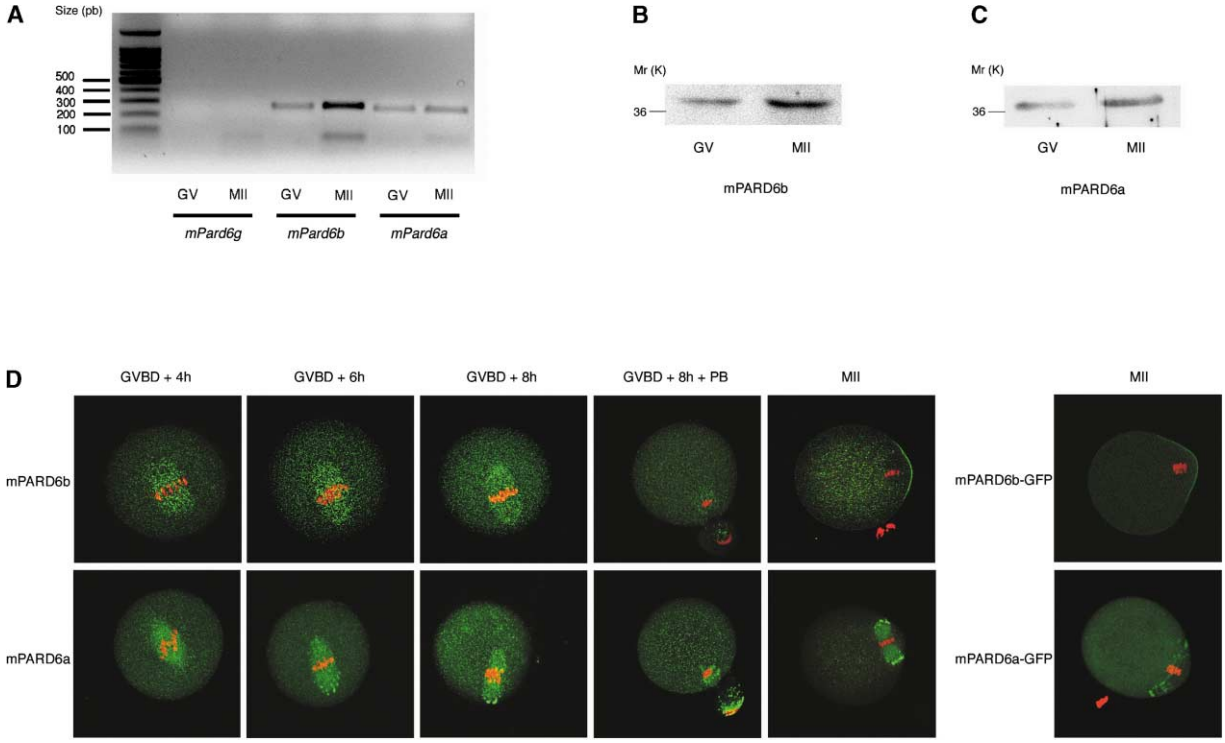

Figure 1. mPARD6b and mPARD6a Show Asymmetric Localization Patterns in Mouse Oocytes

(A) RT-PCR performed on 20 immature (GV) and 20 mature (MII) oocytes with primers specific for *mPard6g*, *mPard6b*, and *mPard6a*. Only *mPard6b* and *mPard6a* were present in mouse oocytes (this experiment was repeated three times).

(B) Western blot performed on 200 GV and MII oocytes with the anti-PARD6b antibody (see Experimental Procedures).

(C) Western blot performed on 400 GV and MII oocytes with the anti-mPARD6a antibody (see Experimental Procedures). The two proteins are expressed in both GV and MII stages (results are from two separate experiments).

(D) Localization of mPARD6b and mPARD6a during mouse meiotic maturation. Oocytes at different stages of meiotic maturation were stained with antibodies raised against PARD6b (green, upper panels) or mPARD6a (green, lower panels) and with propidium iodide for chromatin visualization (red).

Both proteins were homogeneously localized on the spindle until it began to migrate. After GVBD + 6 hr, mPARD6b remained uniformly distributed, whereas mPARD6a accumulated at the spindle pole closest to the cortex. In oocytes arrested in metaphase II, mPARD6b was found at the cortex overlying the spindle, whereas mPARD6a was still present on the spindle and on both poles. In MII oocytes, exogenous GFP-tagged proteins (green, right panels) localized similarly to the endogenous proteins: at the cell cortex overlying the spindle for mPARD6b-GFP and on the spindle and at the poles for mPARD6a-GFP.

the poles, n = 25), attesting to the specificity of the antibodies (Figure 1D, right panels). The staining seemed to be weaker for the GFP-tagged protein than for the endogenous protein. This could be due to the overexpression of the exogenous protein; the cytoplasmic unlocalized GFP-tagged protein could mask the staining on the spindle (compare the cytoplasmic staining in injected MII and uninjected MII). Alternatively, competition between the endogenous protein and the GFP-tagged one for localization on the spindle might render some sites inaccessible to the GFP-tagged protein. All samples analyzed showed the staining pattern reported in Figure 1D. Our data show that mouse oocytes express two mPARD6 proteins, which show asymmetric localization patterns. Moreover, these proteins colocalize only partially, suggesting distinct roles during meiotic maturation.

In order to assess the roles of these two proteins, we attempted to perform RNA interference experiments. We were not able to deplete these proteins from the oocyte, probably because they are already present at the GV stage and do not accumulate significantly during maturation (Figures 1B and C).

Because mPARD6b was detected on the spindle before first polar-body extrusion and located at the cortex in mature oocytes, we investigated more precisely the time at which it first became localized to the cell cortex. Surprisingly, mPARD6b was still localized on the spindle 9, 10, 11, and 12 hr after GVBD, during which time spindle migration is completed and the actin-rich cortical domain forms (data not shown and Figure 2, GVBD + 12 hr). From GVBD + 13 hr onward, mPARD6b was localized at the cortex. This result indicates that, whereas the actin-rich domain is formed during spindle migration [1], its molecular composition is modified after completion of meiosis I. In order to determine whether the cytoskeleton was involved in the switch of mPARD6b localization from the spindle to the cell cortex, we used the microtubule- and microfilament-depolymerizing drugs nocodazole (NZ) and cytochalasin D (CCD), respectively. The drugs were added just after polar-body extrusion during prometaphase II, when mPARD6b is still localized on

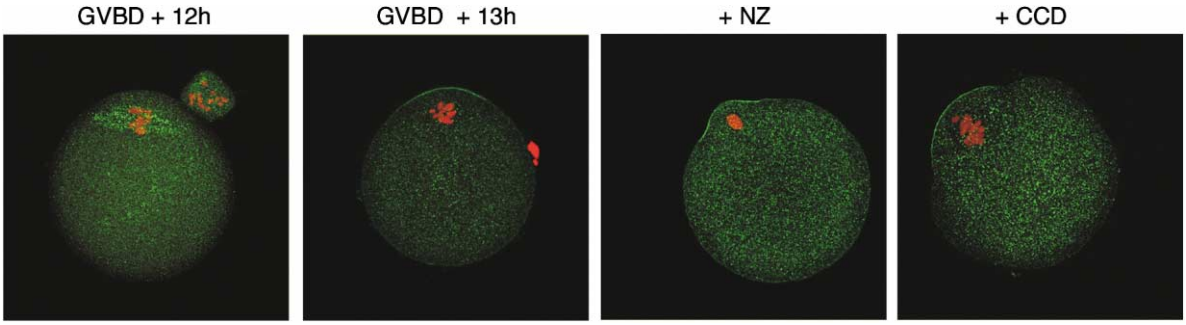

Figure 2. mPARD6b Switches from the Spindle to the Cortex, Independently of Cytoskeleton

Oocytes were stained with the anti-PARD6b antibody (green) and with propidium iodide (red) at GVBD + 12 hr GVBD + 13 hr, or after treatment with nocodazole 10 μM (+NZ) or 1 μg/ml cytochalasin D (+ CCD) from GVBD + 9 hr during 6 hr, just after polar body extrusion during prometaphase II. At that time, mPARD6b is still localized on the spindle. Thus, neither microtubules nor microfilaments were necessary for targeting mPARD6b to the cortex. Sixty-eight oocytes treated with NZ and 68 oocytes treated with CCD were examined in three independent experiments.

the spindle. These treatments did not prevent the localization of mPARD6b to the cortex, indicating that neither microfilaments nor microtubules alone are required to target mPARD6b to the cortex (Figure 2, right panels).

To determine whether spindle migration was required for the asymmetric distribution of mPARD6a at spindle poles, we examined the localization of mPARD6a in *mos*$^{-/-}$ oocytes (oocytes lacking mitogen-activated protein kinase activity), in which spindle migration does not occur [1]. In *mos*$^{-/-}$ oocytes, the spindle elongates at anaphase and only the pole closest to the cortex moves [1]. In these oocytes, depending on the position of the spindle within the cell, mPARD6a was observed along the spindle (Figure 3A, *mos*$^{-/-}$, central spindle) or with a slight accumulation at the pole closest to the cortex (Figure 3A, *mos*$^{-/-}$, noncentral spindle). The same result was obtained with wild-type oocytes treated with CCD (Figure 3A, +CCD), which inhibits spindle migration but not anaphase [1]. These experiments indicate that spindle migration and the actin cytoskeleton are neither required for localizing mPARD6a to the spindle nor for its accumulation at the poles. Moreover, they suggest that the mechanism responsible for the asymmetric accumulation of mPARD6a to one pole requires proximity between this pole and the cortex.

Next, we examined the localization of mPARD6a in MII oocytes in the absence of microtubules. After a short treatment with nocodazole (15 min), mPARD6a was found associated with the chromatin (Figure 3B, +NZ 15min), as has been previously shown for the remaining microtubules [7]. Later, when microtubules were totally depolymerized [7] (Figure 3B, + NZ 3,5 hr), mPARD6a was sometimes observed near the chromatin. After release from the drug, mPARD6a was able to reassociate with each reformed spindle (Figure 3B, release). These results show that mPARD6a follows the distribution of microtubules and suggest that this protein may have a weak affinity for chromatin. It has been demonstrated that in the absence of a spindle in MI oocytes, the chromatin is still able to migrate to the cortex [1], indicating that the mechanism involved in migration is independent of microtubules and may require molecules that associate with chromatin in the absence of the spindle. We observed that in oocytes treated with nocodazole during spindle migration, mPARD6a was always concentrated in a few clusters located in the vicinity of the chromatin (Figure 3C). These clusters were found between the cell cortex and the chromatin (upper panel) or behind the chromatin when it adhered to the cortex (lower panel), suggesting that mPARD6a could mediate interactions between the cell cortex and the chromatin in the absence of a spindle. The behavior of this protein is consistent with an involvement of mPARD6a in MI spindle migration. Moreover, the distribution of the protein at the MII spindle poles suggests that mPARD6a is also involved in the anchorage of the MII spindle parallel to the cortex.

In all species for which they have been described, PAR proteins accumulate at the cell cortex [6]. We present here the first evidence of an association of two mPARD6 proteins with spindle microtubules (Figure 4). Moreover, in mouse oocytes, mPARD6 does not colocalize with mPARD3 (our unpublished data), as has been described for astrocytes [8], which suggests new roles for these proteins.

Interactions between chromosomes and microfilaments at the cell cortex are involved in migration of the MI spindle [1, 4]. Recently, it has been shown that Formin-2, a microfilament binding protein, is essential for this process because the spindle does not migrate in oocytes from *formin-2*$^{-/-}$ mice [9]. It has been suggested that a network of microfilaments is nucleated from the cell cortex by Formin-2 and interacts with chromosome-associated proteins [10]. mPARD6a is a good candidate for this role because it accumulates on the spindle pole closest to the cell cortex and associates with the chromatin in the absence of microtubules.

Conclusions

We have described a switch in the localization of mPARD6b from the spindle to the cortex. This is the first such relocalization to be described, and mPARD6b is the first protein to be found localized only in the actin-rich, microvilli-devoid region induced by the presence of chromatin [4]. Our results show that the molecular composition of this region changes between MI and MII.

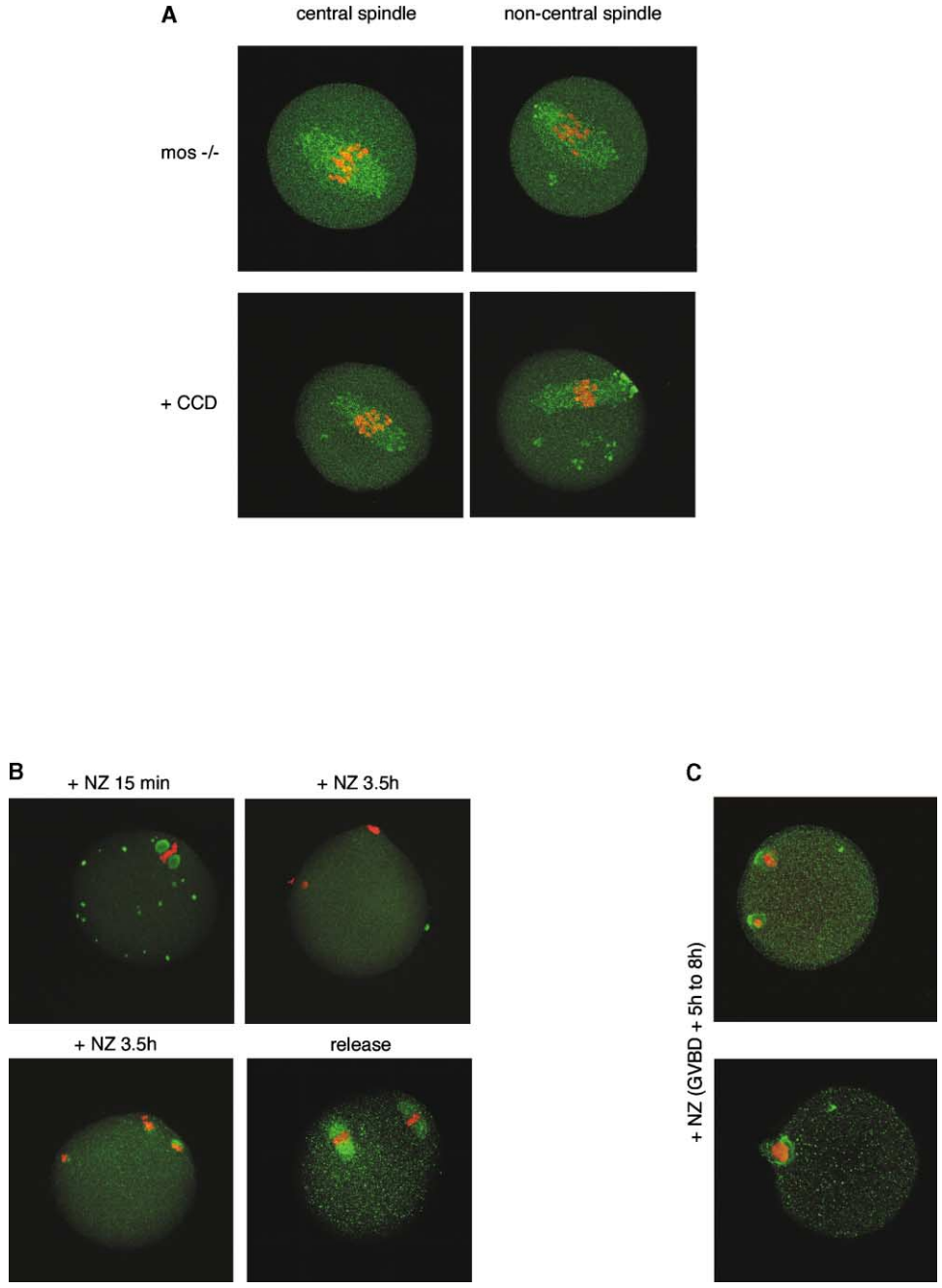

Figure 3. Role of the Cortex and Cytoskeleton on the mPARD6a Localization

(A–C) oocytes were stained with the mPARD6a antibody (green) and propidium iodide (red).

(A) *mos*$^{-/-}$: mPARD6a localization in *mos*$^{-/-}$ oocytes. +CCD: MI oocytes were incubated in medium containing 1 μg/ml cytochalasin D from 4.5 hr to 8 hr after GVBD and fixed and stained 8 hr after GVBD (43 oocytes in two independent experiments were observed). Two different staining patterns were observed. mPARD6a uniformly distributed along the spindle (central spindle) or accumulated at one spindle pole (noncentral spindle). mPARD6a accumulation to one pole requires proximity between this pole and the cortex.

(B) MII oocytes were stained after different times of incubation in a medium containing 10 μM nocodazole. Fifteen minutes after treatment, mPARD6a was localized with the remaining microtubules [7] (+NZ 15 min). After a 3.5 hr treatment, no staining was observed in 47% of the cases (right upper panel), and in the remaining 53%, mPARD6a was observed near the chromatin (left lower panel). Four hours after release (release), the protein relocalized on each reformed spindle. mPARD6a localization in mouse oocytes was dependent on microtubules. Oocytes (108) were examined in two independent experiments.

(C) MI oocytes stained after incubation in nocodazole from GVBD + 5–8 hr. mPARD6a remained associated with the chromatin. Clusters of mPARD6a were found between the cortex and DNA (upper panel) or behind chromatin when it adhered to the cortex (lower panel). Forty-one oocytes were examined in two separate experiments.

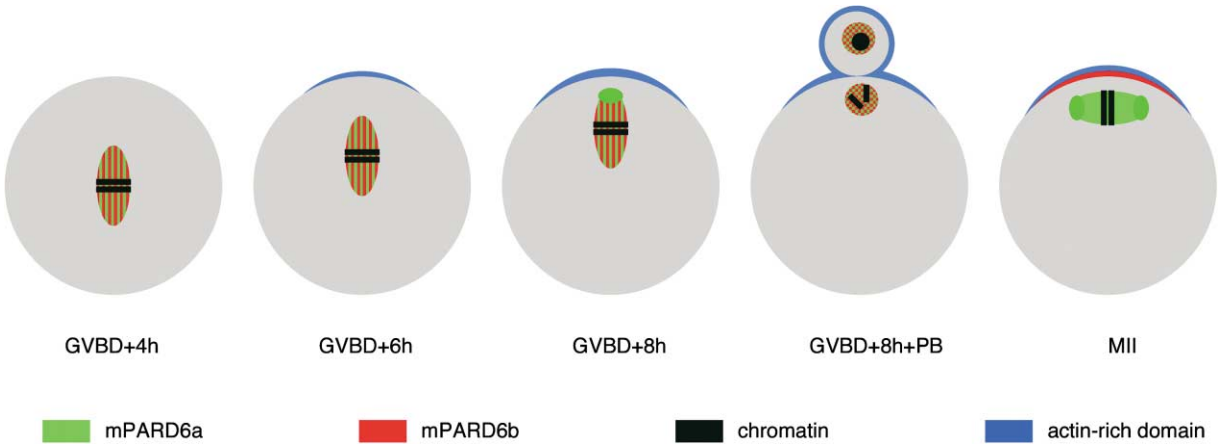

Figure 4. Schematic Model for mPARD6b and mPARD6a Localization Patterns during Establishment of Polarity in the Mouse Oocyte

mPARD6a is in green, and mPARD6b is in red. The cortical actin-rich region is in blue, and chromatin is in black. GVBD: germinal vesicle breakdown. PB: Polar Body. MII: Metaphase II oocyte.

The cleavage plane of the first mitotic division, after fertilization, is oriented with respect to factors present around the animal pole [5]. The presence of mPARD6b at the animal pole and the role of PAR6 proteins in spindle orientation in *C. elegans* zygote or *Drosophila* neuroblasts suggest that this protein plays a similar role in mouse oocytes.

The two differentially distributed mPARD6 proteins in mouse oocytes could be crucial for directing spindle migration and stabilizing the animal pole. Thus, they would contribute to the establishment of the axis of polarity of the mouse oocyte and influence the orientation of the first embryonic cleavage plane.

Experimental Procedures

Recovery and Culture of Mouse Oocytes

Immature oocytes arrested in prophase I or arrested in metaphase II of meiosis were obtained and cultured as previously described [11]. When necessary, zonae pellucidae were removed by a brief incubation in Tyrode's acid solution [12].

RT-PCR

Reverse transcription was processed with the RT kit (Invitrogen) according to the manufacturer's instructions. PCR amplification was performed with primers specific for each *mPard6* gene: 5′-TTC ATAAGTCTCAGACCCTAC-3′ and 5′-GACGCCGACGCAGACCGT CAT-3′ primers for *mPard6g*, 5′-GAGTTTTACGGACTGCTGCAA-3′ and 5′-GCTTCTTCCGGTGGTTGTCG-3′ primers for *mPard6b* and 5′-TCGTCGAGGTGAAGAGCAAA-3′ and 5′-TGCCCGTTTCTGAAC CAAGA-3′ primers for *mPard6a*.

In Vitro Synthesis and Microinjection of Messenger RNA

Plasmids containing cDNA encoding full-length mouse mPARD6b and mPARD6a were a gift from I.G. Macara (Virginia University) and P. Aspenström (Biomedical Center, Sweden). They were inserted between a T3 promoter and a 3′UTR/polyA sequence in a pRN3 plasmid, allowing synthesis of polyadenylated mRNAs. We produced mRNAs encoding full-length mPARD6b tagged with GFP at the C-terminal position (denoted mPARD6b-GFP) and mPARD6a tagged with GFP at the N-terminal position (denoted mPARD6a-GFP). In vitro synthesis of mRNAs was performed on linearized constructs with the mMessage mMachine kit (Ambion) according to the manufacturer's instructions. mRNAs were then purified with an Rneasy kit (Qiagen) and eluted in DEPC water at a final concentration of 0.1 to 0.5 μg/μl. Aliquots of 4 μl were then stored at -80°C. The in vitro synthesized mRNAs were microinjected into the cytoplasm of the oocytes at the germinal vesicle stage via an Eppendorf microinjector connected to a micromanipulator system (Leitz). Oocytes were kept in M2+BSA+dbcAMP (100 mg/ml) medium during injection and then cultured in M2+BSA medium at 37°C.

Antibodies

The rabbit polyclonal antibody raised against the PDZ domain of mPARD6a was a gift from T. Pawson (Toronto University) and was previously described [13]. The rabbit polyclonal antibody raised against the last 14 amino acids of the human homolog of PARD6b was a gift from S. Ohno (Yokohama University) and was previously described [14]. The FITC-conjugated anti-rabbit Ig antibody was purchased from Jackson Laboratories.

Immunoblotting

The total oocyte protein content was separated by SDS-PAGE (10%) and transferred electrophoretically onto a nitrocellulose membrane. The membrane was blocked in TBS/0,1% Tween 20 containing 3% dry milk (for mPARD6a) or 3% BSA (for mPARD6b), and incubation with the anti-mPARD6a- or anti-PARD6b-specific antibodies (diluted in TBS/Tween containing 3% milk or BSA) followed. After several washes in TBS/Tween, membranes were incubated with an anti-rabbit Ig antibody linked to peroxydase (Amersham, diluted in TBS/Tween containing 3% milk or BSA). Membranes were developed with the Super Signal Western blotting detection system (Pierce) according to the manufacturer's instructions.

Oocyte Fixation and Immunocytological Staining

Oocytes and embryos were placed in specially designed chambers as previously described [3]. After centrifugation at 450 g for 10 min at 37°C, samples were fixed in 3.7% formaldehyde (BDH) in PBS for 30 min at 37°C, neutralized with 50 mM NH4Cl in PBS for 10 min, and postpermeabilized in 0.25% Triton X-100 in PBS for 10 min. Immunocytological staining was performed on fixed samples by incubation with the anti-mPARD6a or the anti-PARD6b antibody in PBS/0.1% Tween 20/1.5% BSA for 1 hr, and subsequent incubation in FITC-conjugated anti-rabbit Ig antibody (KPL) in PBS/Tween for 30 min. DNA was revealed by an incubation in propidium iodide (5 μg/ml) for 3 min. Samples were observed under a Leica TCS-SP confocal microscope.

Acknowledgments

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mPARD6a plasmids, respectively. We thank M.H. Verlhac, K. Wassmann, E. Houliston, and M. Gho's lab members for critical reading of the paper and discussion and R. Schwartzmann for technical assistance. This work was supported by grants from the Association pour la Recherche contre le Cancer and La Ligue contre le Cancer to B.M.

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