Specific deletion of *Cdc42* does not affect meiotic spindle organization/migration and homologous chromosome segregation but disrupts polarity establishment and cytokinesis in mouse oocytes

Zhen-Bo Wang[a], Zong-Zhe Jiang[a,b], Qing-Hua Zhang[a], Meng-Wen Hu[a,b], Lin Huang[a,b], Xiang-Hong Ou[a], Lei Guo[a], Ying-Chun Ouyang[a], Yi Hou[a], Cord Brakebusch[c], Heide Schatten[d], and Qing-Yuan Sun[a]

[a]State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China; [b]University of Chinese Academy of Sciences, Beijing 100049, China; [c]Molecular Pathology Section, Department of Biomedical Sciences, Biotech Research and Innovation Centre, University of Copenhagen, 2200 Copenhagen, Denmark; [d]Department of Veterinary Pathobiology, University of Missouri, Columbia, MO 65211

ABSTRACT Mammalian oocyte maturation is distinguished by highly asymmetric meiotic divisions during which a haploid female gamete is produced and almost all the cytoplasm is maintained in the egg for embryo development. Actin-dependent meiosis I spindle positioning to the cortex induces the formation of a polarized actin cap and oocyte polarity, and it determines asymmetric divisions resulting in two polar bodies. Here we investigate the functions of Cdc42 in oocyte meiotic maturation by oocyte-specific deletion of *Cdc42* through *Cre-loxP* conditional knockout technology. We find that *Cdc42* deletion causes female infertility in mice. *Cdc42* deletion has little effect on meiotic spindle organization and migration to the cortex but inhibits polar body emission, although homologous chromosome segregation occurs. The failure of cytokinesis is due to the loss of polarized Arp2/3 accumulation and actin cap formation; thus the defective contract ring. In addition, we correlate active Cdc42 dynamics with its function during polar body emission and find a relationship between Cdc42 and polarity, as well as polar body emission, in mouse oocytes.

INTRODUCTION

After birth, oocytes in mammalian ovaries are arrested at prophase of first meiosis, manifested by the germinal vesicle (GV) located at the center of the oocyte. Oocyte maturation, the final step of oogenesis, begins with germinal vesicle breakdown (GVBD) and ends with the first polar body emission, awaiting fertilization. During the program of oocyte maturation, two important processes must take place. First, during meiosis I, homologous chromosomes segregate, followed by second meiosis, which takes place after fertilization, to ensure haploid gamete production. Second, the two meiotic divisions must occur asymmetrically to allow almost all of the cytoplasm in the egg to support early embryogenesis (Fan and Sun, 2004). The asymmetric cell divisions depend on asymmetric positioning of the meiotic spindles, particularly the meiosis I (MI) spindle. During oocyte maturation, the centrally organized MI spindle migrates to the cortex, inducing formation of a polarized actin cap, as well as of oocyte polarity, and determines the position of two polar body emissions (Brunet and Verlhac, 2011). The migration of the MI spindle to the cortex is mainly dependent on microfilaments, which are regulated by actin nucleators formin-2 (Dumont *et al.*, 2007; Azoury *et al.*, 2008, 2011; Schuh and Ellenberg, 2008) and spire1/spire2 (Pfender *et al.*, 2011).

Cell division cycle 42 (Cdc42) is a member of the Rho family of small GTPase proteins and plays pivotal roles in the establishment of cellular polarity. Cdc42 cooperates with the polarity-related complex Par3/Par6/aPKC and regulates polarity establishment, spindle migration/orientation, and asymmetric cell division in several systems, including the one-cell embryo in *Caenorhabditis elegans* (Gotta *et al.*, 2001; Kay and Hunter, 2001), the neuroblast in *Drosophila* (Atwood *et al.*, 2007), and epithelial polarity and morphogenesis (Wu *et al.*, 2007; Jaffe *et al.*, 2008). Actin cytoskeletal dynamics takes place downstream of Cdc42, and the regulators of actin dynamics are main effectors of Cdc42. The direct relationship between Cdc42 and actin dynamics, as well as its regulators, including WASP/WAVE family and Diaphanous-related proteins Dia1–3, are mainly localized to membrane protrusions (reviewed in Ridley, 2006). Little is known about Cdc42 regulation of actin dynamics in the establishment of polarity in other systems, although it has been reported that cofilin, which enhances actin dynamics, is regulated by Cdc42 in the establishment of neuronal polarity (Garvalov *et al.*, 2007).

Cdc42 is required for coupling asymmetric spindle positioning with cytokinesis (Ma *et al.*, 2006) and regulates the Arp2/3 complex–mediated membrane protrusion and polar body extrusion in *Xenopus* oocyte maturation (Zhang *et al.*, 2008; Leblanc *et al.*, 2011). By using GTPase-defective Cdc42 mutants, it has been reported that Cdc42 might be required for asymmetric spindle migration, homologous chromosome segregation, and polar body extrusion in mouse oocytes (Na and Zernicka-Goetz, 2006). Another study showed disrupted meiotic progression in oocytes incubated with Toxin B, which inhibits the binding activity of Rho GTPases (Bielak-Zmijewska *et al.*, 2008).

In this study, we delete *Cdc42* specifically in oocytes by *Cre-loxP* conditional knockout (Hu *et al.*, 2012) and show that *Cdc42* deletion leads to female infertility in mice. In contrast to the aforementioned in vitro reports, we find that in vivo *Cdc42* deletion has little effect on spindle organization and migration to the cortex or homologous chromosome segregation, but it inhibits polar body emission by inhibiting the formation of the polarized actin cap and cytokinesis. In addition, we find a correlation of active Cdc42 with functions of Cdc42 during mouse oocyte maturation.

RESULTS
Generation of mutant mice with oocyte-specific deletion of *Cdc42*
To study how Cdc42 affects oocyte maturation in vivo, we generated mutant mice in which the *Cdc42* gene was deleted in oocytes of growing follicles. The mutant mice (referred to as *Cdc42*^loxP/loxP^; *ZCre*^+^ mice) were generated by crossing *Cdc42*^loxP/loxP^ mice (Wu *et al.*, 2006) with transgenic mice expressing *zona pellucida 3 (Zp3)*-promoter–mediated Cre recombinase (Supplemental Figure S1A). By Western blot, we found that the expression of Cdc42 protein was almost completely absent in oocytes of mutant mice (Supplemental Figure S1B), showing successful deletion of the *Cdc42* gene from oocytes. By reverse transcription (RT)-PCR, it was shown that exon 2 of *Cdc42* (154 base pairs) was deleted in oocytes from *Cdc42*^loxP/loxP^; *ZCre*^+^ ovaries (Supplemental Figure S2). The *Cdc42*^loxP/loxP^ mice combining transgenic mice expressing specific Cre have been used in many other systems (Wu *et al.*, 2006; van Hengel *et al.*, 2008; Kesavan *et al.*, 2009; Lammermann *et al.*, 2009), and they all show deletion of the *Cdc42* gene and absence of Cdc42 protein.

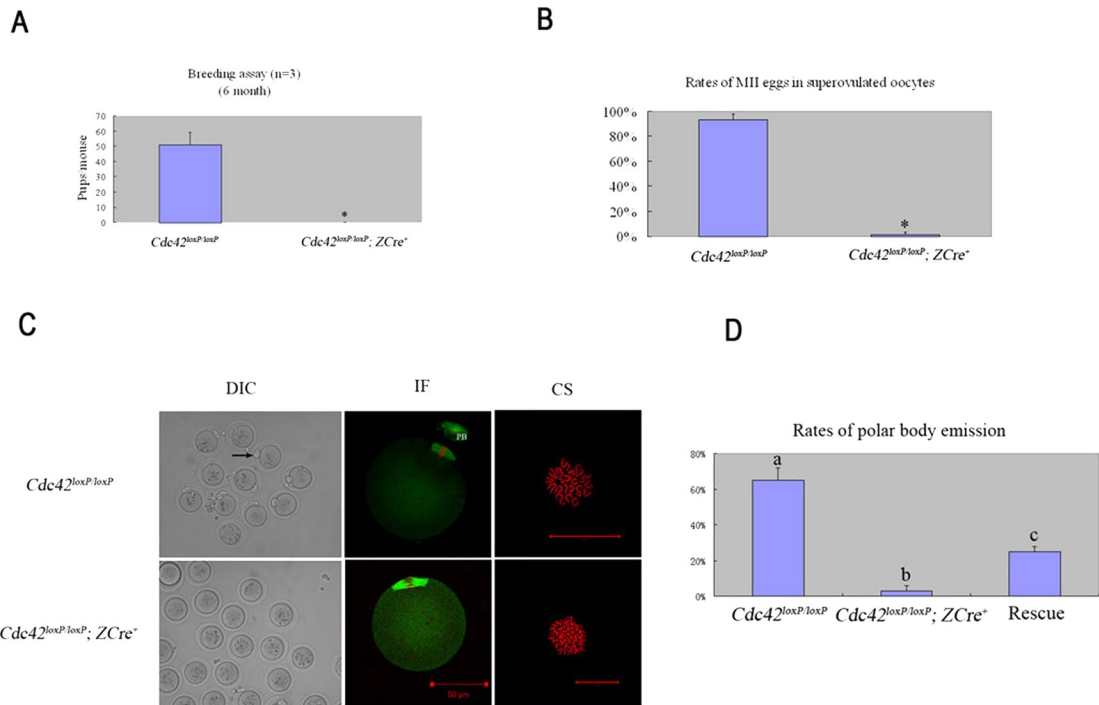### Infertility and impaired mature eggs in *Cdc42*^loxP/loxP^; *ZCre*^+^ mice
We found that the *Cdc42*^loxP/loxP^; *ZCre*^+^ females were completely infertile (Figure 1A). To investigate the possible reasons, we performed superovulation experiments. There was no difference between the numbers of ovulated oocytes from *Cdc42*^loxP/loxP^; *ZCre*^+^ and *Cdc42*^loxP/loxP^ females (unpublished data). The oocytes ovulated from *Cdc42*^loxP/loxP^ ovaries were normal mature eggs (Figure 1B) displaying the first polar body (Figure 1C, differential interference contrast, arrow, and immunofluorescence, PB) and a typical MII spindle. However, almost all of the ovulated oocytes from *Cdc42*^loxP/loxP^; *ZCre*^+^ ovaries had undergone GVBD but lacked the polar body (Figure 1C). Similar phenotypes were observed in in vitro maturation experiments (Figure 1D). We carried out chromosome-spread experiments (Hodges and Hunt, 2002) to analyze chromosome morphology in the ovulated oocytes. As expected, *Cdc42*^loxP/loxP^ eggs displayed the typical monovalent MII chromosome array, whereas ovulated oocytes from *Cdc42*^loxP/loxP^; *ZCre*^+^ ovaries exhibited twice as many monovalent MII chromosomes (Figure 1C). As expected, the impaired polar body emission in *Cdc42*^loxP/loxP^; *ZCre*^+^ oocytes could be partially rescued by Cdc42 mRNA microinjection (Figure 1D and Supplemental Figure S3).

Cdc42 deletion led to cytokinesis failure during oocyte maturation
To determine the nature of the oocytes ovulated without polar bodies, we carried out live oocyte imaging experiments in which we monitored chromosome and spindle dynamics during oocyte maturation. As shown in Figure 2A (also see Supplemental Movie S1A), oocytes from *Cdc42*^loxP/loxP^ ovaries underwent normal oocyte maturation. At 8 h of GVBD (Figure 2A, 8 h) the organized MI spindle had migrated and attached to the cortex. At 9.5 h, anaphase chromosomes were evident, associated with slight spindle elongation and membrane protrusion (Figure 2A, 9.5 h, arrowhead indicates protruding membrane; and Supplemental Movie S1A), followed by the first polar body emission. For *Cdc42*^loxP/loxP^; *ZCre*^+^ oocytes (Figure 2B and Supplemental Movie S1B), a well-formed and normal MI spindle (Supplemental Figures S4 and S5) reached the cortex at approximately the same time as control oocytes (Figure 2B, 7.5 h), and the oocytes proceeded to anaphase normally and timely (Figure 2B, 10.5 h, 21/30). In contrast, membrane protrusion did not occur as homologous chromosomes segregated (Figure 2B, 10.5 h), and the two separated sets of chromosomes remained inside the oocyte at all times (Figure 2B and Supplemental Movie S1B). Strikingly, two metaphase II spindles were organized around the two sets of separated homologous chromosomes, respectively (Figures B, 12 h, and B′, and Supplemental Movie S1B), and finally the two spindles incorporated into a single metaphase spindle (Figure 2B, 12.5 h, and Supplemental Movie S1B). For the *Cdc42*^loxP/loxP^; *ZCre*^+^ oocytes with GV near the cortex, the chromosomes stayed or moved slightly along the cortex (Supplemental Movie S5; 3/30) or moved to other areas near in the cortex (Supplemental Movie S6; 4/30) and then underwent a similar process as described.

Dynamics of active Cdc42 indicates its function during polar body emission
To determine Cdc42 functions in oocyte maturation, it is important to show the localization of Cdc42 during this process. Previous work showed that Cdc42 was mainly localized to meiotic spindles in oocytes during maturation (Bielak-Zmijewska *et al.*, 2008). Cdc42-GTP binding indicates its active and functional state; however, the methods used previously did not distinguish between the two forms

FIGURE 1: Deletion of *Cdc42* in mouse oocytes results in female infertility and impaired oocyte maturation. (A) Comparison of the total number of pups per female mouse during a period of 6 mo between *Cdc42[loxP/loxP]*; *ZCre[+]* (*n* = 3; 51 ± 8) and *Cdc42[loxP/loxP]* (*n* = 3; 0 ± 0) mice. *$P < 0.05$. The data are represented as mean ± SEM. (B) Comparison of the rates of MII eggs with the first polar body from superovulated *Cdc42[loxP/loxP]* females (*n* = 4; 94 ± 4%) and *Cdc42[loxP/loxP]*; *ZCre[+]* females (*n* = 4; 2 ± 2%). *$p < 0.05$. The data are represented as mean ± SEM. (C) differential interference contrast; representative images of ovulated oocytes; 20×. Arrow, the first polar body. IF, ovulated oocytes fixed and stained with anti–α-tubulin–FITC antibody (green) and counterstained with the fluorescent dye PI to visualize DNA (red). PB, the first polar body; bar, 50 μm. CS, chromosome spreads of ovulated oocytes, stained by PI; bar, 50 μm. Experiments were repeated at least three times; representative images are shown. (D) Comparison of the rates of MII eggs with the first polar body during in vitro maturation of *Cdc42[loxP/loxP]* GV oocytes, *Cdc42[loxP/loxP]*; *ZCre[+]* GV oocytes, and *Cdc42[loxP/loxP]*; *ZCre[+]* GV oocytes microinjected with Cdc42 mRNA(Rescue). Different letters (a, b, c) indicate statistically significance ($p < 0.05$).

of Cdc42, and thus it was difficult to determine its function. To visualize active Cdc42, we used the Cdc42-binding domain of N-WASP (wGBD) fused to enhanced green fluorescent protein (eGFP; Pertz and Hahn, 2004) in the present experiments. wGBD binds specifically to active Cdc42 in *Xenopus* brain extracts (Li *et al.*, 2002), and this has been used to visualize active Cdc42 in *Xenopus* eggs (Benink and Bement, 2005; Zhang *et al.*, 2008) and mammalian cells (Kim *et al.*, 2000).

For oocytes released from GV arrest, active Cdc42 appeared around the chromosomes and translocated to the cortex in synchrony with chromosomes (Figure 3A and Supplemental Movie S2A). In localizing close to the cortex, the ring-shaped active Cdc42 changed into an oval shape (Figure 3A, 3.5 h; i.e., 7.5 h after GVBD; also see Supplemental Movie S2A), and the leading edge gradually attached to the membrane to form a cap-like structure (18/20; Figure 3A, 4 h and 4 h 45 min, indicated by arrowhead and its enlarged image; also see Supplemental Movie S2A). When homologous chromosomes segregated, active Cdc42 appeared between the two sets of chromosomes (Figure 3A, 5 h 15 min). After polar body emission, the active Cdc42 was located near the chromosomes that were retained in the egg (Figure 3A, 5.5 h, and Supplemental Movie S2A). There was no specific signal of active Cdc42 throughout oocyte maturation in *Cdc42[loxP/loxP]*; *ZCre[+]* oocytes (Figure 3B and Supplemental Movie S2B). The signal of active Cdc42 was confirmed by microinjection of Cdc42[N17]

mRNAs (Zheng *et al.*, 2013) into control oocytes (Figure 3B'). As expected, the signal of active Cdc42 reappeared in *Cdc42[loxP/loxP]*; *ZCre[+]* oocytes microinjected with Cdc42 mRNAs (Supplemental Movie S7), which could recover the oocyte's ability to emit the first polar body (Figure 1D).

Disruption of the polarized actin cap and polar body emission in *Cdc42[loxP/loxP]*; *ZCre[+]* oocytes

On the basis of the dynamics of spindle/chromosomes and active Cdc42, we suspected that Cdc42 might play a role in the formation of the polarized actin cap and polar body emission. We carried out live oocyte imaging to determine actin dynamics especially in the actin cap by using monomeric G-actin: Alexa 568–G-actin. In *Cdc42[loxP/loxP]* oocytes, the chromosomes close to the cortex induced the formation of actin cap and indicated the establishment of polarity (Figure 4A, blue arrow, Supplemental Movie S3A, and Figure 4C). In contrast, there was no actin cap indicated by Alexa 568–G-actin when chromosomes became located at the cortex in mutant oocytes (11/12; Figure 4B, top, Supplemental Movie S3B, and Figure 4C). In addition, we fixed the oocytes from both *Cdc42[loxP/loxP]*; *ZCre[+]* and *Cdc42[loxP/loxP]* ovaries at appropriate time points during maturation, and similar phenotypes were shown after phalloidin staining (Figure 4D). As expected, the cortex localization of Arp2 (Figure 4E, blue arrowhead) was impaired in *Cdc42[loxP/loxP]*; *ZCre[+]* oocytes at MI stage (Figure 4E, yellow arrowhead).
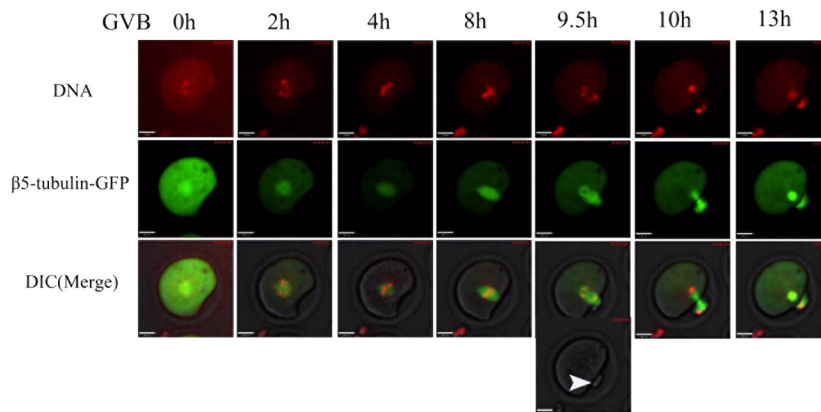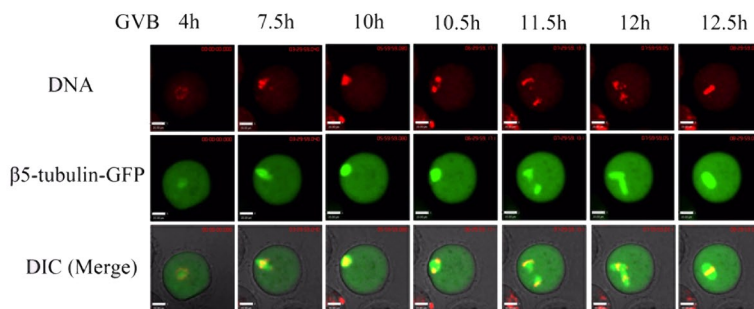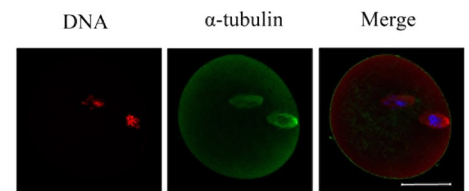
FIGURE 2: *Cdc42* deletion inhibits polar body emission but does not affect the dynamics of chromosomes and spindles during oocyte maturation. DNA (chromosomes) was stained in blue with Hoechst 33342 (1:500,000 dilution), and the color was changed to red. Spindle is shown by fluorescent tubulin expressed by microinjected β5-tubulin–GFP mRNAs. Bar, 20 μM. (A) Chromosomes and spindle dynamics during maturation of a representative oocyte from *Cdc42*^{loxP/loxP} female mice. Time points indicating the time lapse from GVBD occurrence in the oocyte are shown in Supplemental Movie S1A. Arrowhead indicates the protruding membrane accompanying homologue segregation. (B) Chromosomes and spindle images showing the phenotypes of *Cdc42*^{loxP/loxP}; *ZCre*⁺ oocyte maturation. Time points indicating the time lapse from GVBD occurrence in the oocyte are shown in Supplemental Movie S1B. (B') Representative images showing *Cdc42*^{loxP/loxP}; *ZCre*⁺ oocyte with two spindles during in vitro maturation. Oocytes were fixed and stained with anti–α-tubulin–FITC antibody (green) and counterstained with the fluorescent dye PI to visualize DNA (red). Bar, 50 μM.

It was clearly observed that homologous chromosome segregation occurred in both *Cdc42*^{loxP/loxP} and *Cdc42*^{loxP/loxP}; *ZCre*⁺ oocytes, whereas there was no polar body emission in the latter, so the process of cytokinesis needed further exploration. By Alexa 488–phalloidin microinjection and live oocyte imaging, we showed the effects on cytokinesis in *Cdc42*^{loxP/loxP}; *ZCre*⁺ oocytes. In control oocytes, the phalloidin signal was strong at the contractile ring (Figure 5A, white arrowhead, and Supplemental Movie S4A) between the two separated sets of chromosomes. For *Cdc42*^{loxP/loxP}; *ZCre*⁺ oocytes, there was no obvious contractile ring between the two sets of chromosomes (8/10; Figure 5B, blue arrowhead, and Supplemental Movie S4B). The results from fixed oocytes further confirmed our observations. Actin participated in cytokinesis in control oocytes (Figure 5C, *Cdc42*^{loxP/loxP}, arrow indicates contractile ring) and the final step of polar body emission. In *Cdc42*-deleted oocytes, the residual actin (Figure 5C, *Cdc42*^{loxP/loxP}; *ZCre*⁺, arrowhead) indicated the intent-to-initiate but failed cytokinesis.

DISCUSSION

In this study, we analyzed the functions of Cdc42 in mouse oocytes in vivo by conditional knockout technology. Oocyte-specific deletion

of *Cdc42* inhibits production of mature eggs and causes complete female infertility. Focusing on the process of oocyte maturation, we find that *Cdc42* deletion blocks polar body emission by inhibiting formation of the polarized actin cap, which defines the protrusion site of the first polar body. Unlike conclusions derived from in vitro studies, we find that *Cdc42* deletion has no effect on the initial steps of oocyte maturation, including spindle organization, spindle migration to the cortex, anaphase initiation, and homologous chromosome segregation. The inhibited polar body emission in *Cdc42*^{loxP/loxP}; *ZCre*⁺ oocytes could be partially rescued by Cdc42 mRNA microinjection, which confirms the function of Cdc42.

Cdc42 plays pivotal roles in cytokinesis and polar body emission during oocyte maturation

In cell division, Cdc42 and its related proteins have been reported to play potential roles in regulation of spindle/chromosome behavior. Cdc42 is required for microtubule attachment to kinetochores (Yasuda *et al.*, 2004) and subsequent chromosome segregation (Oceguera-Yanez *et al.*, 2005) in HeLa cells. In *Xenopus* egg extracts, Cdc42 is involved in spindle assembly (Tatsumoto *et al.*, 2003). Use of an in vitro approach showed

A. Cdc42$^{loxP/loxP}$
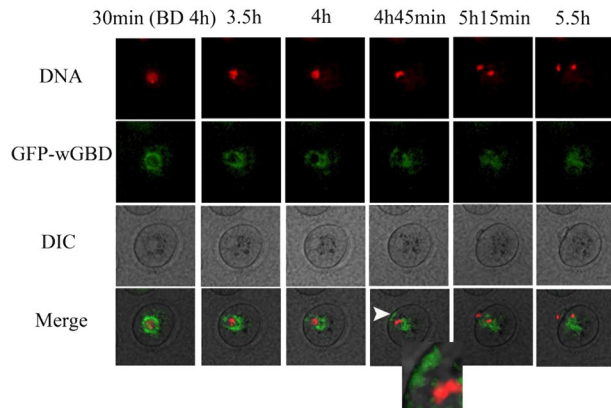
B. Cdc42$^{loxP/loxP}$; ZCre$^+$

B' Cdc42^{N17}
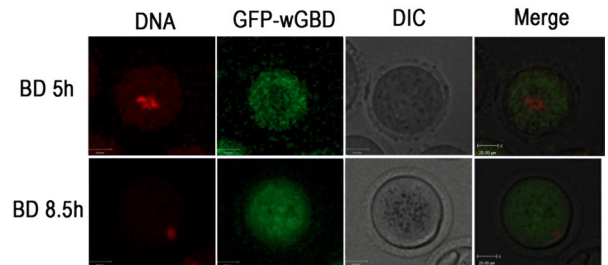
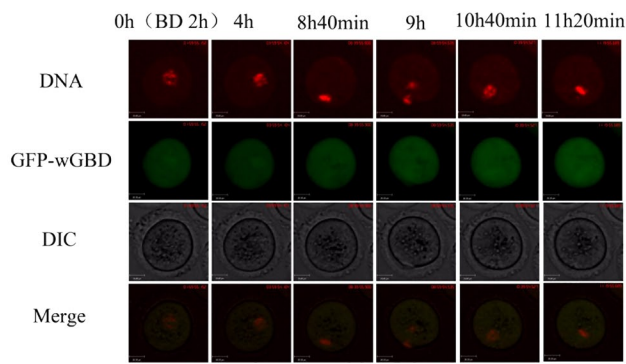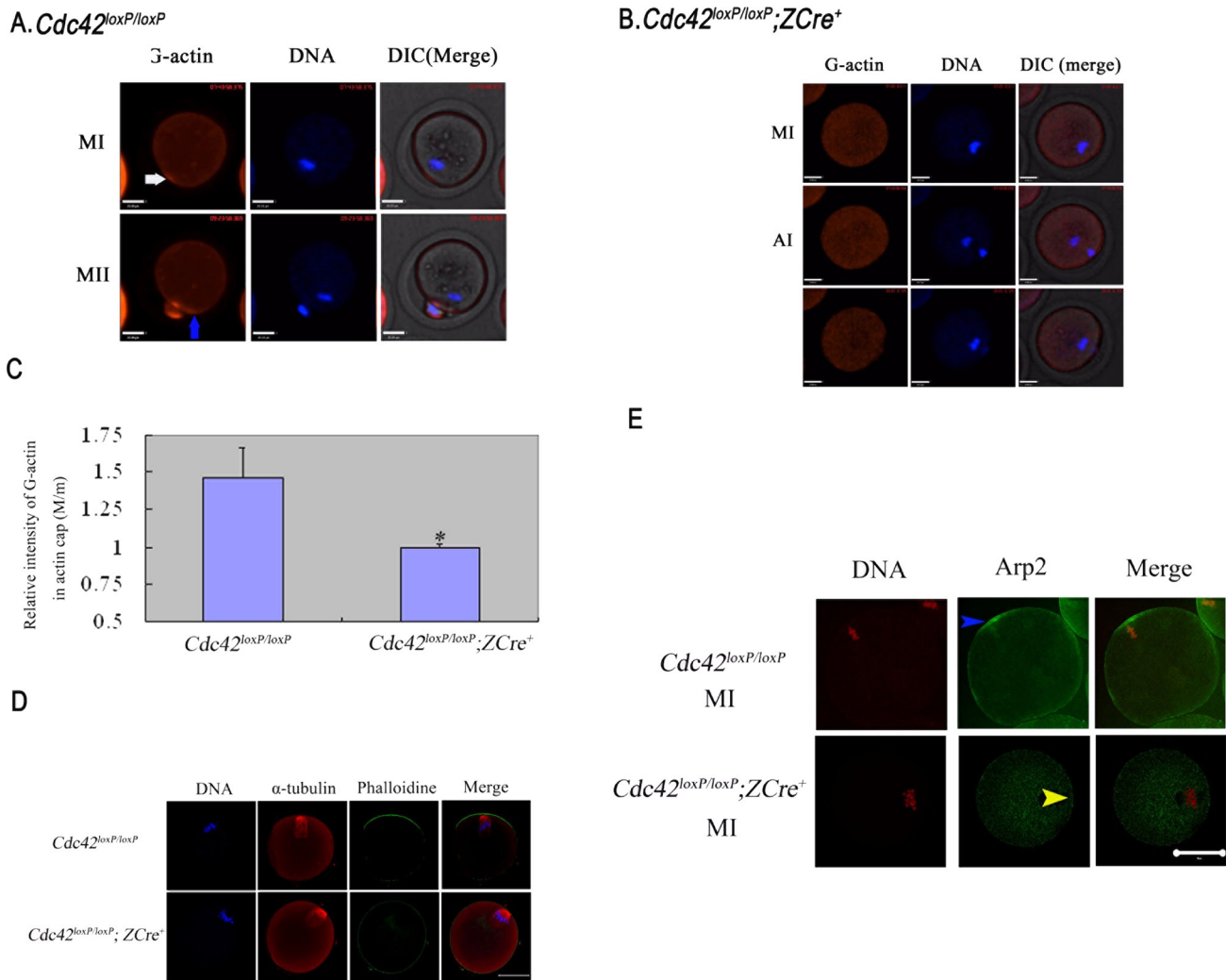**FIGURE 3:** Dynamics of active Cdc42 during oocyte maturation. DNA (chromosomes) was stained in blue with Hoechst 33342 (1:500,000 dilution), and the color was changed to red. Cdc42 is shown by binding to GFP-wGBD expressed through microinjected GFP-wGBD mRNAs. Bar, 20 μM. (A) Active Cdc42 dynamics during *Cdc42$^{loxP/loxP}$* oocyte maturation. Arrow, the active Cdc42 signal in the membrane overlying chromosomes. (B) The dynamics of GFP-wGBD in *Cdc42$^{loxP/loxP}$; ZCre$^+$* oocyte maturation. (B') Confirmation of the signal of active Cdc42 by comicroinjection of Cdc42^{N17} mRNAs and GFP-wGBD mRNAs into control oocytes. Snapshots were taken of microinjected oocytes at appropriate times after culture (BD 5 and 8.5 h, respectively); typical pictures are shown. DNA (chromosomes) was stained in blue with Hoechst 33342 (1:500,000 dilution), and the color was changed to red. Cdc42 is shown by binding to GFP-wGBD expressed through microinjected GFP-wGBD mRNAs. Bar, 20 μM.

(Na and Zernicka-Goetz, 2006) that disrupted Cdc42 causes spindle defects (abnormal bipolar spindles with multiple arrays of astral microtubules), impairs asymmetric spindle migration to the cortex, and inhibits homologous chromosome segregation. However, by using conditional knockout technology, we find that oocyte-specific deletion of *Cdc42* in vivo does not impair normal bipolar spindle formation (Figure 2B, Supplemental Movie S1B, Supplemental Figures S4 and S5, and Figure 4D later in this article), asymmetric spindle positioning near the cortex (Figure 2B and Supplemental Movie S1B), and homologue separation at anaphase (Figures 2B, 3B, 4B, and 5, B and C, and Supplemental Movies S1B, S2B, S3B, S4B, S5, and S6), whereas it plays a role in the process of polar body emission. The previous study was associated with the problem that the in vitro approach lacks the efficiency test of Cdc42 mutants and does not eliminate toxicity caused by microinjection of excess mRNAs. Another in vitro study (Bielak-Zmijewska *et al.*, 2008), in which inhibitor toxin B was used, supports our conclusion, but this inhibitor has binding activity to all the Rho-family proteins, which made it difficult to clarify the specific function of Cdc42.

Cdc42 plays a pivotal role in cytokinesis by regulating contractile ring formation through mediating the actin cytoskeleton, and this function is conserved in yeast (Merla and Johnson, 2000), *Xenopus* embryos (Drechsel *et al.*, 1997), and mammalian cells (Dutartre *et al.*, 1996; Zhu *et al.*, 2011). RhoA is one of the master keys for cytokinesis, and it is necessary for Cdc42 to collaborate with RhoA in the process of cytokinesis. During *Xenopus* oocyte maturation, Cdc42 cooperates with RhoA to complete cytokinesis (Zhang *et al.*, 2008). The residual actin at the center of the spindle (Figure 5C, *Cdc42$^{loxP/loxP}$; ZCre$^+$*, arrowhead) indicates the intent-to-initiate but failed cytokinesis, indicating that deleted Cdc42 alone could prevent polar body emission. Epithelial cell transforming protein 2 is the guanine exchange nucleotide factor that activates Rho GTPases (Tatsumoto *et al.*, 1999), and its depletion in mouse oocytes also results in inhibited polar body emission and produces oocytes with two MII spindles or one MI spindle (probably MII spindle with 40 univalents; Elbaz *et al.*, 2010), resembling the phenotypes observed in this study (Figure 2, B and B', and Supplemental Movie S1B). In addition, polar body emission resembles protrusion formation, and protrusion is closely related to the

FIGURE 4: *Cdc42* deletion inhibits the formation of the polarized actin cap by impairing cortical localization of the Arp2/3 complex. (A, B) DNA (chromosomes) was stained in blue with Hoechst 33342 (1:500,000 dilution). Alexa 568–G-actin was microinjected into GV oocytes to show the dynamics of actin during oocyte maturation. Bar, 20 μM. (A) The dynamics of Alexa 568–G-actin during *Cdc42*loxP/loxP oocyte maturation. White and blue arrows show the actin cap G-actin in MI and MII oocytes, respectively. MI, metaphase I; MII, metaphase II. (B) The dynamics of Alexa 568–G-actin during *Cdc42*loxP/loxP; *ZCre*+ oocyte maturation. MI, metaphase I; AI, anaphase I. (C) Comparison of the density of G-actin in the actin cap, measured by Volocity software. M, density of G-actin in the membrane above the MI chromosomes. We chose three other sites not above the MI chromosomes in every measured oocyte, measured the density of G-actin, and regarded their average value as *m*. *$p < 0.05$. (D) Representative images of oocytes with one pole of MI spindle attached to the cortex from three independent experiments. The oocytes from both *Cdc42*loxP/loxP and *Cdc42*loxP/loxP; *ZCre*+ females were cultured for 9.5 h before being fixed for spindle and actin analysis. The 9.5 h was chosen because a few of the *Cdc42*loxP/loxP oocytes started to extrude the first polar body. Fixed oocytes were stained with anti–α-tubulin antibody (red), Alexa 488–phalloidin (green), and Hoechst 33342 (1:1000 dilution, blue) and viewed with confocal microscopy. Bar, 50 μM. (E) Impaired cortex localization of Arp2 in *Cdc42*loxP/loxP; *ZCre*+ oocytes at the MI stage. Blue arrowhead, Arp2 localized to the cortex in *Cdc42*loxP/loxP oocytes at the MI stage. Yellow arrowhead, absence of cortex localization of Arp2. Bar, 50 μM.

establishment of cell polarity, which may be regulated by Cdc42. The relationships between protrusion, cell polarity, and Cdc42 will be discussed subsequently.

The dynamics of active Cdc42 during oocyte maturation and functions in the establishment of polarity

Oocyte maturation includes highly asymmetric cell divisions and polar body extrusion that is closely correlated with the establishment of polarity. The marker for polarity—the polarized actin cap—determines the sites for the first and second polar body emissions, and the actin cap is induced by the asymmetrically positioned MI spindle. Actin is required for asymmetric spindle positioning. Cdc42 is also associated with the spindle's asymmetric position/rotation in many systems; however, Cdc42 is not necessary for this process (Figures 2B and 5B and Supplemental Movies S1B and S4B), although active Cdc42 moves to the cortex along with chromosomes (Figure 3A and Supplemental Movies S2A) during mouse oocyte maturation, which is also reported in two recent articles (Dehapiot et al., 2013; Zheng et al., 2013). Our results indicate that the asymmetrically positioned spindle/chromosomes leads the distribution

A. Cdc42$^{loxP/loxP}$

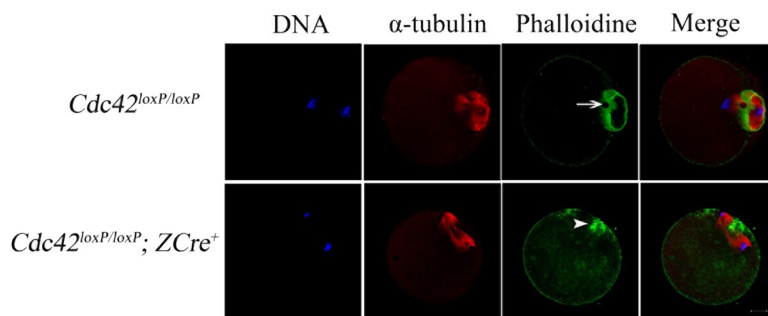Phalloidine   DNA   DIC(Merge)

B. Cdc42$^{loxP/loxP}$;ZCre$^+$
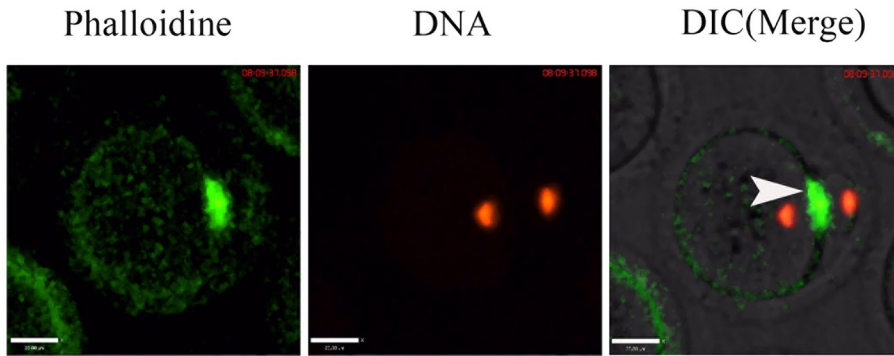
Phalloidine   DNA   DIC (Merge)
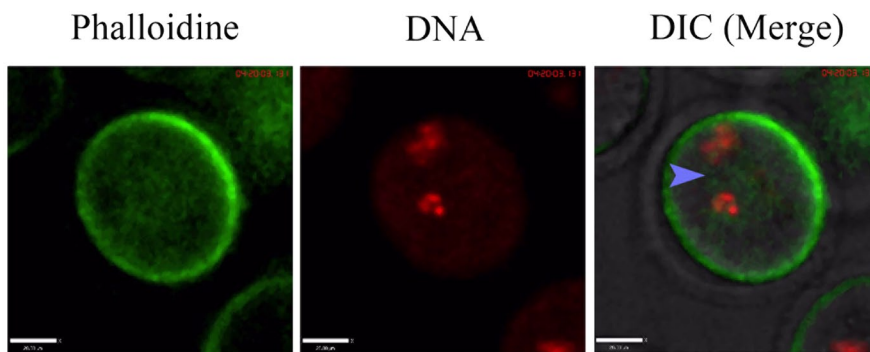
C.

FIGURE 5: *Cdc42* deletion causes cytokinesis failure in mouse oocytes. (A, B) DNA (chromosomes) was stained in blue by Hoechst 33342 (1:500,000 dilution), and the color was changed into red. Alexa 488–phalloidin was microinjected into GV oocytes to show the stable F-actin dynamics during oocyte maturation, especially in cytokinesis. Bar, 20 μM. (A) The dynamics of Alexa 488–phalloidin during *Cdc42$^{loxP/loxP}$* oocyte maturation. White arrowhead, contractile ring shown by Alexa 488–phalloidin. (B) The dynamics of Alexa 488–phalloidin during *Cdc42$^{loxP/loxP}$*; *ZCre$^+$* oocyte maturation. Blue arrowhead, nearly no phalloidin signal between two sets of separated homologues. (C) Representative images of oocytes undergoing cytokinesis from three repeated experiments. Oocytes were stained with anti–α-tubulin antibody (red), Alexa 488–phalloidin (green), and Hoechst 33342 (1:1000 dilution, blue) and viewed with confocal microscopy. Arrow, contractile ring during polar body emission in *Cdc42$^{loxP/loxP}$* oocyte; arrowhead, residual actin in the center of separating spindle in *Cdc42$^{loxP/loxP}$*; *ZCre$^+$* oocyte. Bar, 50 μM.

of active Cdc42 to the cortex and that the cause-and-effect relationship of Cdc42 and polarity establishment is contrary to that in several other important and widely studied systems, as mentioned in the *Introduction*, in which Cdc42 in combination with Par3/Par6/PKC establishes polarity, regulates asymmetric spindle position/orientation, and causes asymmetric cell division. During oocyte maturation, the localization of both Par3 (Duncan *et al.*, 2005) and Par6 (Vinot *et al.*, 2004) becomes asymmetric upon spindle migration and during polar body emission, similar to the dynamics of active Cdc42 (Figure 3A and Supplemental Movie 2A), indicating their function in the formation of the polarized actin cap but not in the regulation of spindle migration.

Polarity establishment by Cdc42 involves protrusion in several widely studied systems: Cdc42 controls the polarization of membrane protrusions in fibroblast migration (Cau and Hall, 2005), regulates epidermal growth factor–stimulated protrusions in MTLn3 carcinoma cells (El-Sibai *et al.*, 2007, 2008), and plays similar roles in other cell lines (Cox *et al.*, 2001). Of interest, Cdc42-regulated polarity mediates a special process of "protrusion" budding in yeast, resembling the protrusion in polar body extrusion during *Xenopus* oocyte maturation (Zhang *et al.*, 2008). During polar body extrusion, the protrusion is mediated by actin dynamics regulated by the Arp2/3 complex in *Xenopus* oocytes (Leblanc *et al.*, 2011), and the Arp2/3 protein complex has been widely reported as an actin nucleator downstream of Cdc42. In our study, *Cdc42* deletion leads to impaired cortex localization of Arp2 (Figure 4E, yellow arrowhead). However, the functions of Cdc42 and the Arp2/3 complex differ. During mouse oocyte maturation, Arp2/3 inhibition results in disruption of the actin cap by inhibiting spindle migration to the cortex (Sun *et al.*, 2011), which is in contrast to the phenotype resulting from *Cdc42* deletion (Figures 2B and 4B and Supplemental Movies S1B and S3B). In addition, Arp2/3 inhibition leads to failed cytokinesis in oocytes in which the migration of the spindle to the cortex is not affected, producing oocytes arrested at telophase (for the oocytes in which the migration is inhibited, symmetric division occurs; Sun *et al.*, 2011), which is different from the phenotypes resulting from Cdc42 deletion (Figures 2B, 3B, 4B, and 5B and Supplemental Movies S1B, S2B, S3B, S4B, S5, and S6). For MII eggs, Arp2/3 inhibition disrupts the actin cap and established polarity, causing MII spindle detachment from the cortex (Yi *et al.*, 2011). Although it appears

that active Cdc42 signal does not exist in the cortex overlying MII chromosomes in our study (Figure 3A and Supplemental Movie S2A), Dehapiot et al. (2013) report the existence of active Cdc42 in MII eggs and its function in the second polar body emission. In our study, we propose that Cdc42 may cooperate with downstream Arp2/3 to function in the formation of the actin cap, the marker of polarity establishment, which is conserved in mouse and *Xenopus* oocytes (Zhang et al., 2008).

In conclusion, our study for the first time clarifies the functions of Cdc42 in oocyte maturation by an in vivo approach. Cdc42 is required for the formation of the polarized actin cap induced by asymmetrically positioned spindle/chromosomes and for membrane protrusion, as well as for cytokinesis during polar body emission. The data provide new insights into the relationship between Cdc42 and polarity in an important polarity-related biological event—oocyte maturation and fertilization. It will be interesting to determine Cdc42-related proteins and networks that play a role in polarity events in the cortex during oocyte development.

MATERIALS AND METHODS
All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated.

Mice (*Mus musculus*)
Homologous *Cdc42*$^{loxP/loxP}$ mice were intercrossed with *Zp3-Cre* transgenic mice (C57BL6 background) from the Jackson Laboratory to generate *Cdc42*$^{loxP/loxP}$; *ZCre*$^+$ animals (C57BL6 and 129 mixed background). Control animals were littermates possessing two *loxP*-flanked alleles without Cre (*Cdc42*$^{loxP/loxP}$). Mice were maintained in alternating 12-h light/dark cycles. Animal care and use were carried out in accordance with the Animal Research Committee Guidelines of the Institute of Zoology, Chinese Academy of Sciences, China.

Oocyte collection and culture
The experiments used 4- to 8-wk-old female mice. For in vitro maturation, GV oocytes were collected and cultured in M2 medium under liquid paraffin oil at 37°C in an atmosphere of 5% CO_2 in air. To obtain MII eggs, mice were induced to superovulate by injection of 8 IU of pregnant mare's serum gonadotropin followed 48 h later by injection of 8 IU of human chorionic gonadotropin. At 14–18 h of human chorionic gonadotropin injection, mice were killed, and the oviductal ampullae were broken to release the cumulus–oocyte complexes. Eggs were freed of cumulus cells by exposure to 300 µg/ml hyaluronidase. Oocytes or MII eggs were collected for immunofluorescence staining, microinjection, or immunoblot analysis.

RT-PCR
One hundred fifty GV oocytes from *Cdc42*$^{loxP/loxP}$ or *Cdc42*$^{loxP/loxP}$; *ZCre*$^+$ ovaries were used to extract the total RNA using the RNeasy micro purification kit (Qiagen, Hilden, Germany), and first-strand cDNA generation was performed following the cDNA synthesis kit instructions (Takara, Otsu, Shiga, Japan). RT-PCR was carried out to assess the expression of Cdc42 mRNA based on the following primers: forward, TTCCGCGGGCACCCAACCAT; reverse, TGTGTGAGGGCAGAGCACTC.

Plasmids, mRNA transcription, microinjection, live oocyte imaging
The first-strand cDNA in the foregoing from *Cdc42*$^{loxP/loxP}$ oocytes was used for PCR cloning. The full length of Cdc42 cDNA was cloned by PCR using the following pair of primers: F1, CGCGGATCCATGCAGACAATTAAGTGTG; and R1, TGCTCTAGATCATAGCAG-

CACACACCT. For in vitro transcription reaction, the Cdc42 cDNA was subcloned into the pCS2+ vector. The pCS2+ -Cdc42 plasmid was linearized by SalI. The plasmids pCS2+ -Cdc42, pRN3-β5-tubulin-GFP, and pCS2+ -GFP-wGBD were used for the production of Cdc42 mRNA, β5-tubulin-GFP mRNA, and GFP-wGBD mRNA, respectively, by in vitro transcription kit (Ambion, Austin, TX). Microinjection of mRNAs, Alexa 568–G actin (Invitrogen, Carlsbad, CA), and Alexa 488–phalloidin (Invitrogen) into GV oocytes was performed using a Nikon Diaphot ECLIPSE TE 300 (Nikon UK, Kingston upon Thames, Surrey, United Kingdom) inverted microscope equipped with Narishige MM0202N hydraulic three-dimensional micromanipulators (Narishige, Sea Cliff, NY) and completed within 30 min. After 1–2 h of culture (except for Cdc42 mRNA) the microinjected oocytes were used for live oocyte imaging on an UltraVIEW VoX confocal imaging system (PerkinElmer, Waltham, MA).

Immunofluorescence and confocal microscopy
For single staining of α-tubulin, oocytes were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for at least 30 min at room temperature. After being permeabilized with 0.5% Triton X-100 at room temperature for 20 min, oocytes were blocked in 1% bovine serum albumin (BSA)–supplemented PBS for 1 h and incubated overnight at 4°C with 1:200 anti–α-tubulin–fluorescein isothiocyanate (FITC) antibody. After three washes in PBS containing 0.1% Tween-20 and 0.01% Triton X-100, the oocytes were stained with propidium iodide (PI; 10 µg/ml in PBS). Then the oocytes were mounted on glass slides and examined with a confocal laser scanning microscope (LSM 710; Carl Zeiss, Jena, Germany).

For double staining of α-tubulin and actin, after fixation, permeabilization, and blocking as described, oocytes were incubated overnight at 4°C with 1:100 rabbit anti–α-tubulin antibody (Cell Signaling Technology, Danvers, MA). After three washes in PBS containing 0.1% Tween- 20 and 0.01% Triton X-100 for 5 min each, the oocytes were labeled with 1:100 tetramethylrhodamine isothiocyanate–conjugated goat anti-rabbit immunoglobulin G (IgG) for 1 h at room temperature. After three washes, oocytes were blocked in 1% BSA-supplemented PBS for 1 h and incubated overnight at 4°C with 1:200 Alexa 488–phalloidin. After three washes in PBS, the oocytes were stained with Hoechst 33342 (1:1000 dilution). Then the oocytes were mounted on glass slides and examined with a confocal laser scanning microscope (Zeiss LSM 710).

For single staining of Arp2, oocytes were fixed in fixation solution (2% formaldehyde, 0.5% Triton X-100, 1 µM Taxol, 10 µg/ml aprotinin, 10 mM Tris, 150 mM NaCl) for 20 min at 37°C and then incubated in blocking solution (2% BSA, 2% fetal bovine serum, 0.1 M glycine, 0.01% Triton X-100, 10 mM Tris, pH 7.5, 150 mM NaCl). Rabbit anti-Arp2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) incubation was carried out at 4°C overnight after permeabilizing with 0.5% Triton X-100 for 20 min and blocking with 1% BSA in PBS. After three washes in PBS containing 0.1% Tween-20 and 0.01% Triton X-100 for 5 min each, the oocytes were labeled with 1:100 FITC-conjugated goat anti-rabbit IgG for 1 h at room temperature. After three washes in PBS containing 0.1% Tween-20 and 0.01% Triton X-100, the oocytes were stained with PI (10 µg/ml in PBS). Then the oocytes were mounted on glass slides and examined with a confocal laser scanning microscope (Zeiss LSM 710).

Immunoblot analysis
Samples each containing 200 mouse oocytes were collected in SDS sample buffer and heated for 5 min at 100°C. The proteins were separated by SDS–PAGE and then electrically transferred to

polyvinylidene fluoride membranes. After transfer, membranes were blocked in TBST buffer (TBS containing 0.1% Tween-20) containing 5% skimmed milk for 2 h, followed by incubation overnight at 4°C with 1:500 mouse monoclonal anti-Cdc42 antibody (BD Biosciences, Franklin Lakes, NJ) and 1:1000 mouse monoclonal anti-β-actin antibody (Proteintech, Chicago, IL). After three washes, 10 min each in TBST, the membranes were incubated for 1 h at 37°C with 1:1000 horseradish peroxidase–conjugated goat anti-mouse IgG. Finally, the membranes were processed using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ).

Chromosome spread

Briefly, the zona pellucida of oocytes was removed in Tyrode's solution, and then the oocytes were dispersed on a coverslip that contained the fixation solution (1% paraformaldehyde, 3 mM dithiothreitol, and 0.18% Triton X-100, pH 9.2), followed by slow drying of the coverslip in air. The spreads were stained with PI (10 μg/ml in PBS) and examined with a confocal laser scanning microscope (Zeiss LSM 710).

Statistics

All percentages from at least three repeated experiments were expressed as mean ± SEM. Data were analyzed by paired-samples t test. $p < 0.05$ was considered statistically significant.

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