The small GTPase Cdc42 promotes membrane protrusion during polar body emission via ARP2-nucleated actin polymerization

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ABSTRACT: Polar body emission is a specialized cell division throughout the animal kingdom, serving to reduce chromosome ploidy while preserving the egg cytoplasm. Critical to polar body emission are the asymmetric positioning of the meiotic spindle prior to anaphase, with one pole attached to the oocyte cortex, and the simultaneous membrane protrusion during subsequent cytokinesis. We have shown that, during Xenopus oocyte maturation, the small GTPase Cdc42 promotes membrane protrusion while a classical RhoA contractile ring forms and constricts at the base of the protrusion. We report here that treating oocytes with low concentrations of nocodazole diminished the size of metaphase I spindles and prevented polar body emission, and yet an active Cdc42 cap of correspondingly diminished size still developed, on time, atop of the spindle pole. Conversely, treating oocytes with low concentrations of taxol resulted in a spindle with multiple poles attached to the cortex, but still each of these poles were associated with activated cortical Cdc42 at the appropriate time. Therefore, the asymmetric positioning of the meiotic spindle with one pole anchored to the cortex is a prerequisite for Cdc42 activation. Furthermore, we demonstrated that the Cdc42-regulated F-actin nucleator ARP2/3 complex was similarly localized at the cortex of the protruding polar body membrane, suggesting that Cdc42 promotes membrane protrusion through an F-actin meshwork mechanism. Finally, we demonstrated that Cdc42 and RhoA formed similarly complementary activity zones during egg activation and that inhibition of Cdc42 prevented second polar body emission. Therefore, Cdc42 activation likely promotes membrane protrusion during polar body emission in widespread systems.

Key words: Cdc42 / cytokinesis / polar body / RhoA / ARP2

Introduction

Polar body emission is an extreme form of asymmetrical cytokinesis that occurs during female meiosis. The biological function of polar body emission is to reduce ploidy of the eggs while preserving the cytoplasm. In all animal species studied, polar body emission is preceded by asymmetric positioning of the meiotic spindle such that one pole lies apposed or anchored to the egg cortex and the other projected into the cytoplasm (Verlhac et al., 2000; Pielak et al., 2004; Ma et al., 2006; Dorn et al., 2010). Live cell imaging in several species have indicated that shortly after the initiation of anaphase, the membrane directly overlaying the spindle pole forms a bulge rich with F-actin (Pielak et al., 2004; Zhang et al., 2008; Deng and Li, 2009; Dorn et al., 2010). As the bulge grows outward, it pulls the whole spindle apparatus with it. At the base of this bulge, a classical actomyosin contractile ring develops (Bement et al., 2005; Zhang et al., 2008; Dorn et al., 2010) and the small GTPase RhoA is responsible for building the contractile ring and its constriction (Bement et al., 2005; Zhang et al., 2008; Elbaz et al., 2010). Using Xenopus oocytes as a model, we have recently established that a closely related GTPase Cdc42 promotes polar body membrane protrusion (Zhang et al., 2008). Therefore, active Cdc42 is first detected as a circular cap directly overlaying the spindle pole shortly after the initiation of anaphase I. The active Cdc42 zone rapidly expands as a membrane bulge to form the nascent polar body enclosure. Bordering this zone of active Cdc42 at the base of the membrane bulge is the active RhoA zone representing the contractile ring. As the ring constricts, active Cdc42 maintains its complementary but non-overlapping
relationship with the active RhoA contractile ring. Inhibition of Cdc42 abolishes membrane protrusion, and yet an even more robust RhoA activity zone still forms and constricts—except that it constricts underneath a flat membrane, hence producing no polar body (Zhang et al., 2008)

This highly restricted Cdc42 activation suggests a regulatory mechanism with both spatial and temporal elements. Spatially, Cdc42 activation is restricted to the cortex directly overlaying the spindle pole. However, contact of the spindle pole to the cortex alone does not lead to Cdc42 activation, since metaphase I spindle becomes asymmetrically anchored to the cortex at least 1 h before cortical Cdc42 activation and membrane protrusion. Temporally, Cdc42 activation occurs shortly after the initiation of anaphase during oocyte maturation (Zhang et al., 2008). To begin our quest to understand the mechanism that controls Cdc42 activation, we have examined the role of spindle microtubules by employing microtubule-destabilizing and microtubule-stabilizing drugs. We have also examined the activation, and the role, of Cdc42 in second polar body emission following parthenogenetic activation of metaphase II eggs. Since emission of the second polar body is immediately downstream to intracellular calcium signaling, we hypothesize that intracellular calcium signaling may be the crucial temporal element in Cdc42 activation during this unique form of asymmetric cytokinesis.

Materials and Methods

Plasmids and mRNA synthesis

Plasmids for eGFP-H2B (histone 2B) (Erhardt et al., 2008) were a gift from Dr. Aaron Straight (Stanford University). Plasmids for mRFP-H2B (Miller and Bement, 2009), eGFP-rGBD (rhotekin GTPase Binding Domain) and RFP- or eGFP-wGBD (WASP GTPase Binding Domain) (Benink and Bement, 2005) were gifts from Dr. William Bement (University of Wisconsin, Madison). Plasmid Cdc42N17 has been described previously (Ma et al., 2006). All cDNAs were in pcDNA3+ background and were linearized by NotI, then in vitro transcribed using the Ambion mMessage™ kit.

Oocyte isolation, incubation and imaging setup

Sexually mature Xenopus laevis females were purchased from Nasco (Fort Atkinson, Wisconsin). Although we have used oocytes isolated by collagenase treatment for many years, we typically use oocytes manually defolliculated nowadays. Similarly, instead of OR2 medium (83 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, 5 mM HEPES, pH 7.8), we now use OCM [oocyte culture medium: 60% of L-15 medium (Sigma) supplemented with 1.07 g BSA per litre, mixed with 40% autoclaved water to yield the appropriate isotonic solution for amphibian oocytes] for routine oocyte culture, unless otherwise indicated. Microinjection was carried out in Ca²⁺+-free OR2 (OR2 minus 1 mM CaCl₂) medium using a Medical System 100 microinjector.

Oocytes injected with mRNA were typically incubated in OCM for at least 6 h to allow for probe expression before the addition of progesterone (1 µM). We have observed germinal vesicle breakdown (GVBD) to occur ~3 h following progesterone stimulation, although this can be as long as 7 h. Oocytes that are undergoing GVBD (appearance of white spot at the animal pole) are transferred to fresh OCM. Anaphase I occurs abruptly 2 h (~±15 min) following GVBD. Therefore, typically 90 min following GVBD (Fig. 1F), the oocyte is positioned in a glass bottom culture dish (MetTak Corporation) filled with OCM (Fig. 1I).

With the help of a halved microscope cover slip and a pipette tip, the oocyte is flipped in between the two cover slips such that the animal pole (GVBD spot) faces downwards (Fig. 1J–K). We use a 60X oil objective on a Zeiss Axiovert (inverse) microscope and scanning is done using a BioRad 1024 laser scanning confocal imaging system. Once anaphase I starts (evident with the rapid and synchronous chromosome separation), first polar body emission usually takes 10 min to complete. Volume rendering (3D reconstruction) of the image sequence is completed with the Velocity software (Improvision). Most of the images shown in this paper are 3-D z-stacks in transverse section (side view) (Fig. 1L).

To study second polar body emission, the oocyte must first arrest in metaphase of meiosis-II. To ensure that the oocyte is properly arrested, we wait 1 h following first polar body emission, or at least 3 h after GVBD (Fig. 1G). Longer incubation (overnight) does not compromise the ability of the metaphase II oocytes to be activated, as long as oocytes appear healthy. The metaphase II arrested oocyte is parthenogenetically activated by pricking at the animal hemisphere with a fine needle (tip width ~10 µm) in OCM (Fig. 1H). Immediately following pricking, the oocyte is placed under the confocal microscope in the same manner as the oocyte undergoing first polar body emission (Fig. 1I-K). Sister chromatids separate ~10–12 min following pricking and scanning continues for another 20 min to ensure that the second polar body has been properly emitted.

Fluorescent probes

Rhodamine tubulin (Cytoskeleton, Inc.) was divided into single use aliquots and stored at ~70 °C. Each aliquot was diluted using the buffer provided by the supplier to 2 mg per ml and between 10 and 20 µl was injected per oocyte. The fluorescent probe for active Cdc42, eGFP-wGBD, contains the GTPase-binding domain of N-WASP (Neural Wiscott Aldrich Syndrome Protein, a Cdc42 effector protein) and therefore specifically binds and tracks Cdc42-GTP. This probe has been shown to specifically identify active Cdc42 in mammalian cells (Kim et al., 2000), in X. laevis brain extracts (Li et al., 2002), and in X. laevis eggs Sokac et al., 2003; Ma et al., 2006). Similarly, active RhoA is tracked using eGFP-rGBD, containing the GTPase-binding domain of rhotekin, a RhoA effector, which has been used to probe for active RhoA in mammalian cells (Ren et al., 1999), X. laevis brain extracts (Li et al., 2002) and X. laevis eggs (Bement et al., 2005; Ma et al., 2006). While these domain probes can amass vital information about a process, it is important to note that these probes could potentially inhibit the function of the target GTPases when overexpressed in a system. One therefore must empirically titrate the probe to levels that do not interfere with the given process.

Results

Spindle microtubule-dependent activation of Cdc42

We have previously demonstrated that Cdc42 activation coincides with the attachment of metaphase I spindle with the animal pole cortex in a perpendicular configuration, suggesting that microtubule spindle pole may be involved in Cdc42 activation (Zhang et al., 2008). We wished to investigate the relationship between spindle microtubules and Cdc42 activation by employing various concentrations of the microtubule disrupting drug nocodazole. We first treated GVBD oocytes with increasing concentrations of nocodazole followed, 3 h later, by staining with Hoechst dye to determine the effect of nocodazole on oocyte maturation (Fig. 2A). These experiments revealed that if GVBD oocytes were added (at GVBD = 0)
Figure 1 Time-lapse imaging during polar body emission in *X. laevis* oocytes. Steps A–H are explained in the text. Oocyte to be imaged is placed in a glass bottom dish with another piece of cover slip placed across the well (I). Using a MultiFlex round tips (Sorenson BioScience, Inc.) that had been melted slightly at the tip to avoid puncturing oocytes, the oocyte is flipped into the well (J) in such a way the oocyte is placed between the two cover slips with its animal pole facing down (K). With practice, this operation can be accomplished within 1 min. Our typical setup focuses on a 75 μm square centered around the spindle which is attached to the plasma membrane cortex with one pole and with the other pole projected into the cytoplasm (L). Confocal z-stacks (white lines; our single photon system is capable of imaging up to 50 μm deep into the cytoplasm, beyond the spindle length of 30–40 μm) are acquired at 1–3 min intervals during polar body emission and the images are 3-D reconstructed using Volocity (Improvision). Most of the time series in this paper are 3-D images in transverse direction (‘side view’) such that the readers are looking ‘side-on’ at the plasma membrane (L). ‘Top view’ refers to the direct view as seen through the microscope eye piece (L).
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(A) % first polar body

Concentration of Noc. (nM)

(B) control, tubulin/Cdc42-GTP

(C) 75 nM Nocodazole, tubulin/Cdc42-GTP

(D) 100 nM Nocodazole, tubulin/Cdc42-GTP

(E) Taxol, tubulin/Cdc42-GTP

DNA, top view

meiosis I

metaphase II
Cdc42 regulates polar body emission

...to medium containing nocodazole at 75 nM or higher concentrations, virtually no oocytes would emit the first polar body (Fig. 2A). On the other hand, if GVBD oocytes were placed in medium containing 30 nM nocodazole, no inhibition was observed, compared with control (Fig. 2A).

Live cell imaging indicated that GVBD oocytes treated with 75 nM nocodazole assembled a spindle of severely reduced size (top panels of Fig. 2C), compared with that in the control oocytes (top panel of Fig. 2B). However, at about the same time that control oocytes exhibited cortical Cdc42 activation atop the spindle pole (Fig. 2B, top panels), as we have previously reported (Ma et al., 2006; Zhang et al., 2008), oocytes treated with 75 nM nocodazole exhibited similar cortical Cdc42 activation (Fig. 2C, top panels). Strikingly, the area of Cdc42 activity in nocodazole-treated oocytes was much smaller, corresponding to the much thinner spindle. Parallel analyses indicated that at around the same time of Cdc42 activation, there was at least partial chromosome separation in sibling oocytes (Fig. 2C, bottom row, 122 and 126 min). These oocytes however did not extrude the first polar body, suggesting a failure of cytokinesis (Fig. 2C, top row, 145 min). The separated chromosomes eventually re-congressed and formed a single metaphase spindle (Fig. 2C, bottom row, 142 min). In the presence of higher concentrations (100 nM) of nocodazole, oocytes failed to assemble any spindle structure, as evident by the collapsing of chromosomes into a chromatin mass (compare the DNA image of Fig. 2D, 100 min to that of Fig. 2C, 110 min). No Cdc42 activation was observed. These results suggest that activation of Cdc42 required a spindle structure and microtubule-associated mechanism is responsible for Cdc42 activation.

Conversely, we also treated oocytes with various concentrations of the microtubule-stabilizing drug, taxol (10–100 nM added together with progesterone), and found that 25 nM was the lowest concentration that was required to inhibit first polar body emission in 100% of the oocytes treated (not shown). Time-lapse imaging of oocytes treated with 25 nM taxol revealed that these oocytes exhibited meiosis I spindles with multiple poles attached to the cortex (Fig. 2E). Interestingly, we could find cortical Cdc42 activation directly above each of these poles when the oocytes were examined between 120 and 150 min after GVBD (Fig. 2E, 140 min), but not when the oocytes were examined at 90 min after GVBD (Fig. 2E, 90 min) or following overnight incubation. Imaging sibling oocytes labeled with Hoechst dye indicated these oocytes did not undergo chromosome segregation (not shown), nor protruded first polar body. Instead, after overnight incubation, they were stably arrested with a single abnormal metaphase spindle that lay parallel to the cortex (Fig. 2E, two examples), evident by their typical linear chromosome array (Fig. 2E, DNA). These results clearly suggest that microtubules or a microtubule-associated mechanism is responsible for Cdc42 activation.

ARP2/3 complex localizes at the protruding polar body

We have previously shown the presence of two classes of F-actin during polar body emission. The protruding polar body cortex is enriched with F-actin readily labeled by Alexa-G actin but the contractile ring can only be labeled by fluorescent phalloidin; the latter also contains phosphorylated (active) myosin light chain (p-MLC) and therefore represents a classical actomyosin contractile ring (Zhang et al., 2008). These suggest that the two GTPases regulate two distinct classes of F-actin. Specifically, Cdc42 promotes branched F-actin nucleated by the ARP 2/3 complex at the protruding polar body cortex. In contrast, RhoA builds the contractile ring with formin-nucleated straight F-actin, which cannot be easily labeled with fluorescent tubulin (Kovar et al., 2006). To determine if ARP2/3 complex is indeed present at the protruding polar body, we carried out immunofluorescence microscopy of oocytes fixed between 120

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**Figure 2** Spindle microtubules and Cdc42 activation. (A) Oocytes isolated by collagenase treatment were incubated with progesterone in OR2. GVBD oocytes were transferred (at GVBD = 0) to OR2 containing the indicated concentrations of nocodazole. Three hours later, oocytes were stained with Hoechst dye and determined if they have successfully emitted the first polar body and reached metaphase II (a typical metaphase II image is shown in (B), DNA, 134 min). Shown are summaries (means with errors) of six determinations for each concentration with an average of 31 oocytes per determination (5 × 6 × 31 = 930 oocytes). The 100 nM group has no error bar because none of the oocytes in this group had emitted the first polar body. Typical DNA images in the 75 and 100 nM groups are shown in (C) (142 min) and (D) (155 min), respectively. (B) Oocytes injected with Rhodamine tubulin and eGFP-wGBD mRNA were incubated in OR2 containing 1 µM progesterone. Oocytes were subjected to time-lapse confocal imaging. Data were volume-rendered and presented as time series in side view. Time (min) indicates time lapse from GVBD. Shown are images from a single oocyte, which is representative of several dozens of oocytes in more than 10 experiments. Shown at the bottom are images of a sibling oocyte stained with Hoechst dye, 1 µg per ml OR2, and imaged live by epifluorescence microscopy. First polar body (first PB) is marked. (C) Oocytes injected with the Rhodamine tubulin and eGFP-wGBD mRNA were incubated in OR2 containing progesterone. GVBD oocytes were transferred to OR2 containing 75 nM nocodazole. Oocytes were subjected to time-lapse confocal imaging and volume rendering. Shown are time series (side view) of a typical oocyte (out of a total of more than 20), depicting spindle pole-associated activation of Cdc42 at the appropriate time following GVBD but failed polar body emission. Insets are top view of the respective time points. Note the much smaller active Cdc42 zone in this oocyte, corresponding to the reduced spindle size. Shown at the bottom are images of a sibling oocyte stained with Hoechst dye and imaged live by epifluorescence microscopy. Note the lack of polar body (142 min after GVBD). (D) GVBD oocytes were transferred to OR2 containing 100 nM nocodazole followed by time-lapse imaging. Note the lack of Cdc42 activation. Parallel imaging of sibling oocytes stained with Hoechst dye showed the lack of distinguishable chromosomes indicating the lack of spindle structure (more than 10 oocytes were imaged with very similar results). (E) Oocytes injected with the same two probes as in A were incubated in OR2 containing progesterone and 25 nM taxol. Oocytes were confocal-imaged live at either around 90 min after GVBD (before anaphase of control oocytes) or between 120–140 min after GVBD (during polar body emission of control oocytes). None of the oocytes (total of 10) imaged around 90 min exhibited any Cdc42 activation. In contrast, we found 5 oocytes (out of 40) with active Cdc42 when imaged between 120 and 150 min after GVBD. Sibling oocytes were stained with Hoechst dye, 1 µg per ml, in OR2 and imaged live by epifluorescence microscopy with only two time points shown.
Examining these oocytes confirmed that they were properly matured and arrested in metaphase II in the presence of first polar body. We then treated these metaphase II oocytes with calcium ionophore A23187 (0.5 μg/ml) before placing them in the imaging chamber. Approximately 12 min later we observed synchronous chromosome separation (Fig. 4A, 1:00). Within a few minutes of anaphase initiation, the homologues destined to the polar body were seen protruded above the plasma membrane (Fig. 4A, 5:00). However, the protruding polar body chromosomes returned inside the oocytes (Fig. 4A, 12:00), indicating the failure of cytokinesis completion (polar body retraction). Of the nine oocytes examined, only two successfully emitted the second polar body, making this method unsuitable for studying cytokinesis. We reasoned that the widespread calcium influx caused by A23187 may be responsible for the failure of cytokinesis. In an attempt to more closely simulate fertilization, we employed pricking metaphase II oocytes with a fine glass needle (~10 μm tip diameter; Parmenter, 1933). Indeed, we observed consistent polar body emission when metaphase II oocytes were pricked with a glass needle (Fig. 4B, 17:00; representative of more than 50 oocytes).

Upon establishing a reliable method for triggering second polar body emission, we wished to determine whether Cdc42 and RhoA were similarly activated temporally and spatially during egg activation, as we have shown during oocyte maturation (Ma et al., 2006; Zhang et al., 2008). GV oocytes injected with eGFP-rGBD and RFP-wGBD mRNAs were treated with progesterone overnight to trigger first polar body emission (Fig. 5, top panel depicting the first polar body) and metaphase II arrest. The metaphase II oocytes were pricked and followed by time-lapse imaging. Twelve (12) minutes after pricking, an active Cdc42 cap could be seen as a slight bulge, which was circumvented by an active RhoA contractile ring (Fig. 5, 00:12). This was followed by the rapid constriction of the RhoA ring (Fig. 5, bottom panels), accompanied by further elevation of the protruding polar body containing active Cdc42 (00:14). Within a few minutes, the RhoA contractile ring had closed and polar body emission appeared complete (00:20). These observations mirrored those during first polar body emission (Ma et al., 2006; Zhang et al., 2008).

Over-expression of Cdc42N17 diminishes Cdc42 activation, prevents membrane protrusion and inhibits first polar body emission (Ma et al., 2006; Zhang et al., 2008). To determine if Cdc42 is similarly required for second polar body emission at fertilization, we decided on a strategy that allowed us to examine both first polar body and second polar body. GV oocytes were injected with eGFP-wGBD and RFP-H2B, labeling active Cdc42 and chromosomes, respectively. Half of the oocytes were further injected with Cdc42N17 mRNA (10 ng per oocyte). Both groups were incubated overnight with progesterone. Control oocytes exhibited a metaphase II spindle with roughly twice as many chromosomes (Fig. 6B, 00:00), indicating that Cdc42N17 did not interfere with homolog separation but blocked cytokinesis, as we have reported previously (Ma et al., 2006; Zhang et al., 2008). When these oocytes were pricked, we also observed synchronous chromosome separation 12 min later (Fig. 6B, 00:03, arrows). However, the chromosomes destined for the polar body never protruded (Fig. 6B and C) and fell back into

**Figure 3** APR2 is localized at the polar body cap. Oocytes were treated with progesterone and fixed between 120 and 140 min after GVBD. The fixed oocytes were stained with anti-ARP2 antibodies (sc-15389, Santa Cruz Biotechnology) and DRAQ1® DNA dye (Biostatus; 1000 dilution). Images were obtained by confocal microscopy and volume rendered. Shown are two representative oocytes of a total of 19, all of which exhibited ARP2 signals at the polar body cap.

**Cdc42 is required for second polar body emission at parthenogenetic activation**

All of the above results (Figs 2 and 3) depict first polar body emission during oocyte maturation, as do those in our previous publications (Ma et al., 2006; Zhang et al., 2008). We wanted to know if Cdc42 was similarly required for second polar body emission, which occurs at fertilization in the frogs. To do this, we needed first to develop a protocol capable of allowing live cell imaging during second polar body emission. We injected eGFP-H2B mRNA into GV oocytes followed by overnight incubation in the presence of 1 μM progesterone. Examining these oocytes confirmed that they were properly matured and 140 min following GVBD. These experiments demonstrated that ARP2 was indeed found at the cortex of protruding polar body (Fig. 3; 2 representative images of 19 total), very similar to the pattern of active Cdc42 at these stages of polar body emission. Therefore, Cdc42 appears to promote membrane protrusion via activating ARP2/3 nucleated F-actin polymerization during polar body emission.
the oocyte (Fig. 6B, 00:18 and 00:24). Therefore, second polar body emission at fertilization requires Cdc42 activation and Cdc42-mediated membrane protrusion, similar to first polar body emission during oocyte maturation.

Discussion

As this article is part of the special issue on polar body, we take this opportunity to discuss our work in the broader context of cytokinesis of this special form of cell division, and to speculate on both the regulatory mechanism that controls Cdc42 activity, as well the mechanism by which Cdc42 promotes membrane protrusion during polar body emission.

Cdc42 promotes membrane protrusion during polar body emission

Unlike RhoA which is universally required to build the contractile ring during cytokinesis in animal cells (Bement et al., 2005), the role of Cdc42 described here appears to be uniquely required for cytokinesis during polar body emission. The unique requirement for Cdc42 in polar body emission appears to stem from the need for membrane protrusion during cytokinesis. Indeed, over-expression of Cdc42N17 diminishes Cdc42 activation and prevents membrane protrusion. However, these oocytes still exhibit robust, albeit broadened, ring-shaped RhoA activation at the membrane and the RhoA-GTP ring still constricts, albeit futilely over a flat membrane (Zhang et al., 2008). These results suggest that, in addition to promoting membrane protrusion, Cdc42 may also contribute to building a normal contractile ring by preventing RhoA activity zone from broadening. Similarly, over-expression of Cdc42V12 (constitutively active Cdc42) or Cdc42N17 in Xenopus embryos causes cleavage furrow broadening during the first and second cleavage (Drechsel et al., 1997).

In addition, Cdc42 is found to be associated with meiotic spindles in mouse oocytes (Bielak-Zmijewska et al., 2008). Over-expression of Cdc42N17 in mouse oocytes similarly prevents polar body emission (Na and Zernicka-Goetz, 2006). However, the mouse oocytes exhibit a more complex phenotype. The most notable is distortion of the meiotic spindles and misalignment of chromosomes. These earlier defects presumably preclude analyses of cytokinetic defects (Na and Zernicka-Goetz, 2006). A similar role for Cdc42 in chromosome alignment has been reported in HeLa cells (Yasuda et al., 2004). It is intriguing that Cdc42, which is a membrane protein by virtue of its prenylation (Ziman et al., 1993), is required for spindle assembly, a process requiring mainly microtubules (and associated proteins) and chromosomes. However, earlier electron microscopic studies have clearly indicated the presence of membrane vesicles within the boundary of mitotic spindles (Harris, 1975). Therefore, it remains possible that residual Cdc42 activity persists in frog oocytes injected with Cdc42N17 (Fig. 6B), which was sufficient to support spindle assembly but which was insufficient to promote polar body membrane protrusion.

A critical step in polar body emission is the asymmetric positioning of the meiotic spindle prior to anaphase, with one pole attached to the animal pole cortex and the other pole projecting into the cytoplasm (Fig. 1L). This unique positioning of the meiotic spindle is dependent on cortical F-actin network and its interaction with the spindle, through both chromosome and microtubule components. In Xenopus oocytes, the giant nucleus (~400 μm in diameter) is located at the animal hemisphere. Within 30 min of GVBD, the condensing chromosomes reach the animal pole cortex in a process that is dependent on F-actin but not microtubules (Gard...
et al., 1995). Spindle assembly occurs at the cortical region of the animal pole, achieving the asymmetric positioning at least 1 h before anaphase or Cdc42 activation (Zhang et al., 2008). Furthermore, this asymmetric spindle positioning is not affected by Cdc42N17 over-expression (Zhang et al., 2008). In mouse oocytes, meiosis I spindle assembles at the center of the oocyte and then moves towards the cortex, apparently through an actomyosin contraction mechanism: spindle pole-associated myosin appears to pull the spindle towards the cortex through F-actin whose plus ends are anchored at the cortex (Schuh and Ellenberg, 2008). Intriguingly though, in the absence of microtubules, the chromosomes can also move to the cortex, with slightly increased velocity than that of the intact spindle (Verlhac et al., 2000; Li et al., 2008). The cortically localized chromosomes can also induce a membrane bulge rich in F-actin (Deng et al., 2007; Halet and Carroll, 2007), apparently through activation of Rac1 GTPase (Halet and Carroll, 2007). However, chromatin-induced membrane bulge does not lead to cytokinesis (Deng and Li, 2009). The exact relationship between the chromosome induced membrane bulge in mouse oocytes and membrane protrusion during polar body emission in Xenopus oocytes therefore remains unknown. It would be of great interest to analyze Cdc42 and Rac1 simultaneously during polar body emission in frog oocytes and/or mouse oocytes.

Mechanisms that control Cdc42 activation during polar body emission

Like other members of the Rho family GTPases, Cdc42 is prenylated (Ziman et al., 1993) and is associated with the inner leaflet of the plasma membrane and the cytoplasmic side of the Golgi apparatus (Erickson et al., 1996). However, our live cell imaging experiments have indicated that the active form of Cdc42 is restricted to the cortex of the protruding polar body following anaphase in X. laevis oocytes (Ma et al., 2006; Zhang et al., 2008). These results clearly indicate temporal (during polar body emission) as well as spatial (in relationship to the spindle apparatus) elements that regulate Cdc42 activation.

Spatial regulation: requirement of microtubules

Clearly the spindle apparatus plays a dominant role in restricting Cdc42 activation during polar body emission. In addition, the unique position and orientation of the meiotic spindle—perpendicular to the oocyte cortex and with one pole anchored—is essential. If the orientation of the spindle is changed, by disrupting the cortical F-actin network, such that the spindle becomes parallel to the cortex, Cdc42 activation is abolished. Under this condition, anaphase proceeds normally but no membrane protrusion occurs and cytokinesis completely fails (Zhang et al., 2008).
Further supporting the role of microtubules in Cdc42 activation during polar body emission, we demonstrated here a close relationship between the size of the spindle and the size of the active Cdc42 ‘cap’. Specifically, Cdc42 activation is limited to the spindle–cortex contact site, either in the presence of microtubule-destabilizing nocodazole or microtubule-stabilizing taxol (Fig. 2E). Our results further indicate that Cdc42 activation requires a spindle structure, not mere presence of microtubules or chromatin (Fig. 2D).

**Temporal regulation: linking to anaphase**

Simple attachment of the meiosis spindle to the oocyte cortex alone does not lead to Cdc42 activation. Instead, Cdc42 activation is closely linked to initiation of anaphase. Anaphase initiation in mitosis, and meiosis, is thought to be brought about by the activation of the E3 ligase, APC/C, as a result of the spindle-based inactivation of the spindle assembly checkpoint. However, since Cdc42-mediated

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**Figure 6** Over-expression of Cdc42N17 inhibits both first and second polar body emission. GV oocytes were injected with mRNAs for RFP-H2B and eGFP-wGBD. Half of the oocytes were used as control (A) and the half were further injected with mRNA for Cdc42N17 (10 ng per oocyte) (B). Six hours after mRNA injection, both groups of oocytes were incubated overnight with 1 μM progesterone to reach metaphase II arrest. (A) Time series of a typical control metaphase II oocyte following pricking activation, depicting Cdc42 activation during second polar body emission. Note chromosome separation at anaphase II (00:08, arrows) and the emission of the second polar body. The haploid egg chromosomes typically moved quickly inside the egg and became invisible (00:21). (B) Time series of a typical Cdc42N17 oocyte following pricking activation. The presence of roughly twice as many chromosomes in the single metaphase array (00:00) indicated cytokinesis failure during first polar body emission. Note the synchronous segregation of all chromosomes (00:03, arrows), indicating anaphase II and the failure of polar body protrusion. Eventually, all chromosomes moved inside the cytoplasm and became too deep to be detected (00:24). 00:00 represents 10 min after pricking in this oocyte. (C) Single plane top (XY) and side (XZ or YZ) views of the control oocyte (00:12 in A) depicting active Cdc42 at the cortex of the protruding second polar body (arrows). In contrast, no active Cdc42 or membrane protrusion at similar time in Cdc42N17 oocytes (B, 00:13).
membrane protrusion is unique for polar body emission, it is not at all clear whether activation of Cdc42 is also regulated by APC/C or it requires a separate signaling pathway.

Given that Cdc42 activation only occurs at the spindle pole–cortex contact site and at the beginning of anaphase, one possibility might be that a microtubule-associated guanine nucleotide exchange factor (GEF) is activated at the onset of anaphase and this GEF in turn activates Cdc42. An interesting candidate might be GEF-H1 (also known as XLfc), a microtubule-binding GEF for the Rho family GTPases, that becomes activated when microtubules are depolymerized, either as a result of inherent instability or after treatment with microtubule-depolymerizing drugs (Krendel et al., 2002; Kwan and Kirschner, 2005; Chang et al., 2008). Although these studies have implicated a function of GEF-H1 as an activator of RhoA, it remains possible that the rapid depolymerization of microtubules at anaphase (as a driving force of chromosome poleward movement) may activate this GEF, leading to cortical Cdc42 activation and polar body membrane protrusion. Such a possibility is currently being investigated.

That Cdc42 is similarly activated during second polar body emission suggests the possible involvement of calcium signaling, since pricking is thought to mimic sperm-induced intracellular calcium signaling (Parmenter, 1933). In this regard, it is interesting that an earlier study (Sun and Machaca, 2004) has demonstrated that, contrary to previous belief, Ca$^{2+}$ signaling during oocyte maturation plays an essential role in first polar body emission. Specifically, inhibition of intracellular calcium signaling does not block initiation of meiosis I, suggesting a role for localized calcium release in spindle function and/or cytokinesis (Fitz-Harris et al., 2007). Similarly, ER enrichment has been noticed around metaphase II spindle of oocytes in several species (Mehlmann et al., 1995; Terasaki et al., 2001).

**The roles of Cdc42 during polar body emission: promoting membrane protrusion via branched F-actin meshwork**

We propose that the precise spatial/temporal activation of Cdc42 serves to promote polar body membrane protrusion during cytokinesis (Fig. 7). Cdc42 does so by promoting dynamic F-actin polymerization at the protruding polar body cortex. This notion is supported by our previously published evidence that active Cdc42 precisely localizes at the protruding membrane (Zhang et al., 2008). We have substantiated this conclusion by demonstrating here that ARP2/3 complex had similar/identical spatial–temporal localization at the protruding polar body (Fig. 3). As such, the function of Cdc42 during polar body emission is reminiscent of its role in promoting other membrane protrusions, such as filopodia and lamellipodia, in somatic cells (Mattila and Lappalainen, 2008). Therefore, shortly after anaphase initiation, an active Cdc42 cap promotes polymerization of branching F-actin network through the classical Cdc42-GTP/N-WASP/ARP2/3 pathway. This dynamic F-actin zone is circumvented by the active RhoA ring, which patterns an actomyosin contractile ring. The contractile ring is made of F-actin poorly labeled with fluorescent G-actin probe, consistent with the notion that RhoA promotes formin-
nucleated unbranched F-actin (Kovar et al., 2006). The interplay between Cdc42 and RhoA thus accomplishes this extreme form of asymmetric cytokinesis. Not unlike polar body emission, other processes, such as single cell wound healing (Benink and Bement, 2005) and cortical granule membrane retrieval (Sokac et al., 2003), also require Cdc42 and RhoA in similarly complementary but mutually exclusive activity zones. Furthermore, in all cases, the process involves actomyosin-mediated constriction and is dependent on intracellular calcium signals. A recent study has identified a multi-functional regulator of the Rho family GTPases Abr (Vaughan et al., 2011) as a key player in maintaining the spatial separation of the Cdc42 and RhoA activity zones during wound healing (Benink and Bement, 2005). It is clearly an intriguing possibility that Abr is similarly involved in maintaining the dynamic and spatially distinct activity zones of the two GTPases during polar body emission.

Authors’ roles


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