Epithelial Cell Transforming Protein 2 (ECT2) Depletion Blocks Polar Body Extrusion and Generates Mouse Oocytes Containing Two Metaphase II Spindles

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Completion of the first meiosis in oocytes is achieved by the extrusion of the first polar body (PBI), a particular example of cell division. In mitosis, the small GTPase RhoA, which is activated by epithelial cell transforming protein 2 (ECT2), orchestrates contractile ring constriction, thus enabling cytokinesis. However, the involvement of this pathway in mammalian oocytes has not been established. To characterize the role of ECT2 in PBI emission in mouse oocytes, the small interfering RNA approach was employed. We found that ECT2 depletion significantly reduces PBI emission, induces first metaphase arrest, and generates oocytes containing two properly formed spindles of the second metaphase. Moreover, we describe, for the first time, that before PBI emission, RhoA forms a ring that is preceded by a dome-like accumulation at the oocyte cortex, next to the spindle. This unique mode of RhoA translocation failed to occur in the absence of ECT2. We further found that the Rho-dependent kinase, a main RhoA effector, is essential for PBI emission. In addition, we demonstrate herein that ECT2 is subjected to phosphorylation/dephosphorylation throughout meiosis in oocytes and further reveal that PBI emission is temporally associated with ECT2 dephosphorylation. Our data provide the first demonstration that an active cyclin-dependent kinase 1, the catalytic subunit of the maturation-promoting factor, phosphorylates ECT2 during the first meiotic metaphase and that cyclin-dependent kinase 1 inactivation at anaphase allows ECT2 dephosphorylation. In conclusion, our study demonstrates the indispensable role of the maturation-promoting factor/ECT2/RhoA pathway in PBI extrusion in mouse oocytes. (Endocrinology 151: 755–765, 2010)
PBI emission is a particular case of cytokinesis, a process executed by the constriction of the actomyosin-based contractile ring (10–12). This ring enables furrow ingression, and subsequent abscission, leading to complete separation of daughter cells (reviewed in Ref. 13). RhoA small GTPase induces constriction of the contractile ring via three main effectors as follows: it promotes the polymerization of actin necessary for contractile ring formation through the formin family (14); it phosphorylates myosin II light chain, which generates the force required for the contractile ring ingestion through the Rho-dependent kinase (ROCK) (15, 16); and it regulates late steps of abscission through citron kinase (12) (reviewed in Ref. 17). In somatic cells, the presence of RhoA during mitosis is absolutely essential for both furrow formation and ingression (10, 18, 19). Furthermore, induction of actomyosin constriction requires local accumulation and activation of RhoA at the equatorial cortex (20–23).

Like most small G proteins, RhoA is a molecular switch that cycles between resting GDP-bound and active GTP-bound states. The epithelial cell transforming protein 2 (ECT2) is the guanine nucleotide exchange factor that cycles between resting GDP-bound and active GTP-bound states. The epithelial cell transforming protein 2 (ECT2) is the guanine nucleotide exchange factor that activates RhoA in dividing epithelial cells. Specifically, ECT2 directs the precise localization of RhoA at the equatorial cortex by its accumulation at the midzone, a structure formed between segregated chromatids at the onset of anaphase. Upon ECT2 inhibition in somatic cells, RhoA fails to accumulate at the cell cortex, cytokinesis does not occur, and multinucleated cells are formed (21, 22, 24–28). ECT2, which is known to be regulated by phosphorylation, was reported to be phosphorylated during G2 and M phases in HeLa cells (28). Later studies showed that ECT2 is dephosphorylated during cytokinesis (23, 29).

It was recently shown in Xenopus oocytes that ECT2 controls RhoA localization and PBI emission (30). However, the signaling cascade of ECT2, as well as the phenotype of ECT2-depleted oocytes, was not examined. In the present study, we report that ECT2 is essential for the ring-shaped accumulation of RhoA, without which PBI does not form. Some oocytes that failed to extrude PBI contained two distinct spindles of the second meiotic metaphase (MII), a phenomenon that is actually analogous to binucleation reported in somatic cells. Finally, we show for the first time that MPF inactivation at the metaphase-to-anaphase transition brings about ECT2 dephosphorylation.

Materials and Methods

Reagents
Leibovitz’s L-15 tissue culture medium and fetal bovine serum (FBS) were purchased from Biological Industries (Kibbutz Beit Hemeek, Israel). Antibiotics were purchased from Bio-Lab Ltd. (Jerusalem, Israel), pregnant mare’s serum gonadotropin (PMSG) from Chronogest Intervet (Boxmeer, The Netherlands) and ovine LH (o-LH-26) from National Hormone and Pituitary Program (Harbor-UCLA Medical Center, Torrance, CA). The ROCK inhibitor Y27632 was purchased from Calbiochem (San Diego, CA), MG132, and cilostamide from Alexis Biochemicals (San Diego, CA) and roscovitine from LC Laboratories (Woburn, MA). Protease inhibitor cocktail, phenylmethylsulfonyl fluoride, leupeptin, and pepstatin as well as anti-β-tubulin and fluorescein isothiocyanate-conjugated anti-α-tubulin monoclonal antibodies were from Sigma-Aldrich Corp. (St Louis, MO). Anti-RhoA, anti-ECT2 and anti-general ERK1/2 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA; sc-418, sc-1005, and sc-135900, respectively) and Cy3-conjugated goat antimouse F(ab)2 IgG antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA). 4’,6-Diamidino-2-phenylindole (DAPI) stain was purchased from Molecular Probes (Eugene, OR).

Animals
Sexually immature C57BL/6 female mice (25 d old) were purchased from Harlan Laboratories (Rehovot, Israel) and handled according to the guidelines of the National Institutes of Health and of the Weizmann Institute for management of laboratory animals. This study was approved by the institutional animal care and use committee. The mice were housed in a light- and temperature-controlled room, with food and water provided ad libitum.

Oocytes collection and culture
The aforementioned mice, sc injected with 5 IU PMSG for induction of follicular development and sacrificed 48 h later, were used. The ovaries were removed and placed in L-15 medium, supplemented with 5% FBS, penicillin (100 IU/ml), and streptomycin (100 mg/ml). To maintain the oocytes in meiotic arrest, the phosphodiesterase 3A (PDE3A)-specific inhibitor cilostamide (7.5 mM) was included in the medium of incubation (31). The large antral follicles were punctured under a stereoscopic microscope to release the cumulus-oocyte complexes. Cumulus cells were removed by repetitive pipetting, and the denuded fully grown oocytes were incubated in a 37°C humidified incubator. Oocytes were examined for maturation and PBI emission using a stereoscopic microscope (SMZ 1500; Nikon, Tokyo, Japan).

Small interfering RNA (siRNA) microinjection
Oocytes collected as mentioned above were denuded and incubated in a cilostamide-containing medium. They were transferred to 10-μl drops, under 3.5 ml of paraffin oil in a 35-mm Falcon petri dish. Dishes were placed on a heated stage (28°C) of an inverted microscope equipped with differential interference contrast optics (Axiovert 35; Zeiss, Oberkochen, Germany). Oocytes were microinjected with ECT2 siRNA as well as nontargeting scrambled siRNA (approximately 10 pl) using a three-dimensional motor coarse control micromanipulator (Eppendorf, Hamburg, Germany). The siRNA pools were purchased from Dharmacon (Lafayette, CO): ECT2 siGENOME SMARTpool, catalog no. M-045026-00 NM_007900) and nontargeting siGENOME (pool number 2). Noninjected oocytes served as a control for the effect of the microinjection.
RNA extraction and cDNA preparation

Liquid nitrogen-frozen oocytes were homogenized in 500 μl Tri-Reagent (Sigma-Aldrich) through a 21-gauge syringe. Glycogen (10 μl) (Roche Applied Science, Mannheim, Germany) was added to allow better precipitation of RNA. After adding 100 μl chloroform to allow phase separation by centrifugation (at 4 °C), 250 μl isopropanol was added to the aqueous phase. After several hours in −20 °C, the RNA was precipitated by centrifugation. The pellet was washed in cold 70% ethanol and then dried. The RNA pellet was resuspended, and deoxyribonuclease treatment was performed according to the manufacturer’s instructions (Ambion, Austin, TX). Quality and quantity of total RNA extracted were assessed using a Nanodrop spectrophotometer. RNA samples (200 ng) were reverse transcribed using recombinant reverse transcriptase (Moloney murine leukemia virus; Promega, Madison, WI) and oligo-deoxythymidine primer, as indicated in the manufacturer’s protocol. The cDNA was then diluted two times.

Semiquantitative PCR

The reverse-transcribed diluted cDNA was amplified by PCR. The nucleotide sequences are as follows: RhoA (NM_016802), CCAGAAAGCCCAAGTCCA and TGAGAAAGGCTATGCCACCT; RhoB (NM_007483), CGAACCTTGTGCTGTGCTTAC and CCGTGTTGCTTCTTTTACCA; RhoC (NM_007484), TCCTCAACCTCCACCTT and GCTACTACCCCAAGGCAAACC; PDE3A (NM_018779), ATGGGTAGAGCGAGCTGTGT and ACCTTGTTGAGTTCGCCCAC; PDE4D (NM_011056), CATGTGAAGCACTGATAAACC and ACTACGATTTGCTTCACCA. The primers were designed using the Primer3 program and validated for their specificity by the NCBI-BLAST program. PCR was performed in 25-μl reaction volumes containing 2 μl cDNA, 5 pmol each primer, and 5 μl ReddyMix PCR Master Mix (ABgenehouse, Epsom, Surrey, UK).

Protein extraction and Western blot analysis

Oocytes were lysed in RIPA buffer supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml pepstatin, protease inhibitor (according to manufacturer’s instructions), and 400 μM NaVO₄. After pipetting, the lysates were kept on ice for 30 min and then centrifuged for 20 min, after which the supernatants were collected. The samples were dissolved in protein sample buffer [2% β-mercaptoethanol, 2% sodium dodecyl sulfate, 50 mm Tris, HCl (pH 6.8), 10% glycerol, and 0.01% bromophenol blue], boiled, and loaded onto 10% SDS-PAGE. For better resolution of the ECT2 phosphorylation shift, 8% SDS-PAGE was used, and the bisacrylamide in the monomer mixture was reduced from 8 to 0.12%. After electrophoretic separation, the proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA), which were washed for 1 h with a blocking solution (5% milk, 0.05% Tween in PBS) and then incubated with primary antibodies (overnight, 4 °C) and antirabbit horseradish peroxidase-conjugated antibodies (1: 4000 for 1 h at room temperature). Chemiluminescent signals were generated by incubation with the enhanced chemiluminescence reagent (Amersham, Buckinghamshire, UK). For quantification, intensity values of bands were measured from three different repeats using image J (National Institutes of Health, Bethesda, MD).

Immunofluorescence

The oocytes were fixed in ice-cold 10% trichloroacetic acid in distilled water for 15 min (32) followed by extensive washing with GB-PBS (PBS containing 10 mg/ml BSA and 10 mM glycine, pH 7.4), permeabilization in Triton X-100 (1% in PBS) for 4 min, and transfer to blocking solution (GB-PBS containing 10% PBS) for 1 h. The oocytes were then incubated overnight with RhoA primary antibodies, extensively washed, and further incubated with the secondary antibodies, cy3 goat antimesum F(ab’)2 (1:200) for 2 h. They were then incubated in fluorescein isothiocyanate-conjugated tubulin antibodies (1:100) and DAPI for 2 h, washed, and mounted in elvanol on silicone-coated glass slides and covered by coverslips resting on a silicone ring containing 100-mm glass beads that served as spacers. The oocytes were visualized using an LSM710 confocal microscope (Zeiss). In some oocytes, serial images were acquired at different planes along the z-axis (Z-stack acquisition). From these serial images, a three-dimensional picture was generated using Zeiss’s ZEN 2008 software.

Results

ECT2 depletion blocks PBI extrusion

To examine the necessity of ECT2 for PBI extrusion, denuded oocytes, which were incubated with ciliostamide to keep them meiotically arrested, were microinjected with either ECT2 or nontargeting scrambled siRNA. Noninjected oocytes served as a control for the effect of this manipulation. Meiotic arrest was maintained for 24 h, after which ciliostamide was washed, and the oocytes were allowed to undergo spontaneous maturation. The oocytes were monitored for the presence of PBI after an additional 24 h. Semiquantitative RT-PCR and Western blot analysis revealed that ECT2 levels were substantially down-regulated, demonstrating high efficiency of the siRNA treatment (Fig. 1, A and B). Microscopic examination revealed that the fraction of oocytes extruding PBI after ECT2 depletion was reduced by 57% compared with scrambled siRNA-injected oocytes (24.1%, n = 421; 55.7%, n = 373, respectively; two way ANOVA P = 0.0032) (Fig. 1C). Interestingly, some of the ECT2 siRNA-injected oocytes that failed to extrude PBI exhibited a cytoplasmic elongated protrusion (18.9% vs. 4.5% in scrambled siRNA-injected oocytes, Fig. 1, D and Ec). The formation of these protrusions suggests that these oocytes may have initiated, but failed to complete, the process of cytokinesis. The microinjection itself did not affect the experimental outcome (Fisher’s least significant difference).

ECT2 depletion induces MI arrest and generates oocytes containing two MI spindles

To examine their chromosomal configuration, ECT2 siRNA-injected oocytes were fixed 24 h after the initiation of spontaneous maturation and stained with antitubulin antibodies as well as with the DAPI fluorescent DNA.
Interestingly, the progression to MII still occurred and each set of chromosomes arranged at the equator of the newly formed MII spindle. Each of the two spindles was aligned in parallel to the cortex, resembling the orientation of a single MII spindle during normal second metaphase arrest. Other oocytes seem to be arrested at MI (Fig. 2A, b1–b3), some of which contained partially or totally segregated chromosomes attached to the spindle poles (Fig. 2A, c1–c3 and d1–d3). Quantification of these configurations revealed that 23.5% of the ECT2-depleted oocytes contained two well-formed MII spindles, whereas this phenotype was not observed in control oocytes (n = 210 and n = 192, respectively; P = 0.02) (Fig. 2B). About half (48.2%) of the ECT2-depleted oocytes failed to complete homologous chromosome segregation, a phenotype observed in only 18.6% of the scrambled siRNA-injected oocytes (P = 0.02). Some of the ECT2-depleted oocytes that contained a metaphase-like spindle displayed partially segregated chromosomes (37.8%); no partial DNA segregation was observed in control oocytes (P = 0.01) (Fig. 2A, c1–c3). About 25% of the ECT2-depleted oocytes contained a single typical MII spindle, which is consistent with the fraction of these oocytes that extruded PBI (24.1%, Fig. 1C).

### RhoA expression and localization in mouse oocytes

Mammalian cells express three main isoforms of Rho proteins: RhoA, RhoB, and RhoC. To characterize their expression, RNA was extracted from mouse oocytes and cDNA was synthesized and subjected to PCR. For comparison, PCRs were also performed on cDNA from whole ovaries. A possible cross-contamination was ruled out by the use of two control genes: PDE3A, an oocyte-specific gene, and PDE4D, a granulosa-specific PDE isoform. We found that RhoA and RhoB are expressed in mouse oocytes, whereas RhoC mRNA is absent (Fig. 3A). Intact ovaries, however, expressed all three isoforms. Because RhoA is the isoform that plays a major role in cytokinesis in somatic cells, its localization during PBI emission was next determined. Mouse oocytes were continuously monitored between 10 and 14 h of their spon-
taneous maturation and fixed at PBI emission. They were subsequently stained using anti-RhoA and antitubulin antibodies as well as DAPI. We show herein, for the first time in mammalian oocytes, a distinct pattern of RhoA accumulation at the cortical region, adjacent to the spindle (Fig. 3B). Specifically, during meiotic arrest, RhoA is distributed uniformly on the cortex of the oocyte (Fig. 3B, a1–a3). After the organization of the spindle, RhoA translocates to a relatively large area of the membrane surface, forming a dome-like structure in the vicinity of the spindle (Fig. 3B, b1–b3, and supplemental Fig. 2). This structure is later remodeled into a ring at the shoulders of the protrusion, marking the cortical region for subsequent meiotic furrow formation (Fig. 3B, c–e, and supplemental Fig. 3). The RhoA ring then shrinks around the spindle center, enabling separation of the homologous chromosomes and PBI emission (Fig. 3B, f1–f3, and supplemental Fig. 4). It finally appears as a dot at the region of the polar body and the oocyte membrane apposition. Unexpectedly, most oocytes exhibited a spindle oriented parallel, and not perpendicular, to the cortex. At initial stages of outpocketing, the entire spindle was localized within the protrusion, between the forming contractile ring and the oocyte cortex (Fig. 3B, e1–e3; supplemental Fig. 5 and Fig. 3). It is only after membrane constriction that the spindle becomes perpendicular to the cortex (Fig. 3B, f1–f3).

ECT2 is essential for RhoA translocation

The requirement of ECT2 for RhoA translocation was next assessed. For this purpose, the previously described
ECT2 siRNA-microinjected oocytes, which were allowed to undergo spontaneous maturation, were fixed during PBI emission and stained with anti-RhoA and antitubulin antibodies as well as DAPI. A considerable difference in RhoA localization was observed between the control and the ECT2 siRNA-treated groups. In scrambled siRNA-injected oocytes, RhoA localization was similar to that observed in nontreated oocytes (n = 64, Fig. 4, a and b). Interestingly, even though the cortex had begun to constrict in some oocytes, RhoA still failed to accumulate at this precise site (n = 12, Fig. 4, b1–b3). A minor fraction of oocytes presented aberrant RhoA accumulation (n = 5, Fig. 4, c and d). RhoA localized normally in one fourth of the oocytes, in high correlation with the fraction of oocytes that extruded PBI.

**ROCK is required for PBI extrusion**

We next examined the role of ROCK, the downstream effector of Rho, during PBI emission. Oocytes were incubated in the presence of Y27632, a specific ROCK inhibitor. Y27632 reduced PBI extrusion in a dose-dependent manner (Fig. 5A). Moreover, similar to ECT2-depleted oocytes, some of the Y27632-treated oocytes that failed to extrude PBI exhibited an elongated protrusion (13.8% in the 100 μM-treated group, n = 222, Fig. 5B). The earlier stages of oocyte maturation were unaffected by the ROCK inhibitor, with all oocytes completing GVB within less than 6 h (Fig. 5C).

**CDK1 phosphorylates ECT2 during oocyte maturation**

Our next experiments were directed at understanding the mode of regulation of ECT2. These experiments re-
revealed no significant change in ECT2 expression level during meiotic maturation (Fig. 6, A and B). However, a shift in ECT2 electrophoretic mobility that was previously attributed to its phosphorylation (28) was observed upon reinitiation of meiosis (Fig. 6C). This shift was partially abrogated at the onset of PBI extrusion, which occurs after approximately 12 h incubation. ECT2 electrophoretic mobility was retarded again after 24 h incubation, when the oocytes reached MII. Strikingly, the ECT2 pattern of phosphorylation described herein is in full accordance with the well-established pattern of CDK1 activity as follows: activation upon resumption of meiosis, inactivation around the time of PBI emission, and further reactivation at the MII stage (4–6). To examine whether CDK1 induces ECT2 phosphorylation at the GVB stage, we employed roscovitine, a selective CDK1 inhibitor. Oocytes were incubated for 5 h to allow the activation of CDK1 associated with their spontaneous reinitiation of meiosis. They were then transferred to a roscovitine-containing medium for 1 h. CDK1 inactivation indeed reduced the mobility shift of ECT2, observed after 6 h isolation in the nontreated oocytes (Fig. 6D). It should be noted that this effect was detected after only 1 h roscovitine treatment, suggesting that a continuous activity of CDK1 is needed to maintain ECT2 in its phosphorylated state during GVB and early MI stages.

To further establish whether CDK1 inactivation induces ECT2 dephosphorylation, CDK1 was maintained experimentally active by incubating spontaneously maturing oocytes with MG132, a potent proteasome inhibitor. We previously showed that MG132-incubated rat oocytes accumulate cyclin B and subsequently maintain a high level of CDK1 activity (9). This treatment brought about inhibition of PBI extrusion and formation of a nose-like protrusion. In the present study, this phenotype was fully reproduced; none of the MG132-treated oocytes extruded PBI, and some of them exhibited an elongated protrusion (Fig. 6F, left, insets). The electrophoretic mobility shift assay revealed that unlike the nontreated oocytes, ECT2 in the MG132-treated oocytes exhibited a retarded mobility, suggesting that CDK1 inactivation is responsible for ECT2 dephosphorylation (Fig. 6E). Because MG132 is not a MPF-specific inhibitor, a control group of oocytes was incubated in a culture medium containing the combination of MG132 and roscovitine. Under these conditions, the MG132-induced activation of CDK1, promoted by accumulation of the MPF regulatory subunit cyclin B1, should be abolished by roscovitine. Indeed, the addition of roscovitine to the MG132-treated oocytes reversed the electrophoretic mobility shift of ECT2 induced by MG132, confirming that the MG132-induced mobility shift occurred due to CDK1 prolonged activation (Fig. 6E). Taken together, these data suggest that CDK1-sustained activation is required for ECT2 phosphorylation dur-
ing meiosis. At the onset of anaphase, CDK1 inactivation is sufficient to induce ECT2 dephosphorylation.

Discussion

We report herein that at the onset of the MI, ECT2 undergoes a CDK1-dependent phosphorylation. This is followed by its dephosphorylation upon transition to anaphase, resulting from CDK1 inactivation. In the absence of ECT2, completion of the first meiotic division is severely impaired. These oocytes, which failed to emit PBI, are either arrested at MI or contain two MII spindles, resembling binucleated somatic cells. In addition, we show that ECT2 is indispensable for RhoA translocation at the vicinity of the spindle. Finally, ROCK, the downstream effector of RhoA, mediates PBI emission.

ECT2 is required for PBI emission and its depletion results in oocytes that are either arrested at MI or contain two MII spindles

Half of the oocytes failed to complete homologous chromosome segregation (MI) in the absence of ECT2 (see model in supplemental Fig. 6B). A similar phenotype was previously described in cytochalasin D-treated oocytes (33) and in formin-2-deficient oocytes (34). In these reports, the homologous chromosomes segregated and initiated two MII spindle assembly, which then retracted and remerged rapidly back into a single spindle. What seems like an MI arrest in the ECT2-depleted oocytes could in fact represent the outcome of the above mentioned process. It should be noted that no similar phenotype was ever described in ECT2-depleted somatic cells, which may suggest that ECT2 activity may affect aspects unique to the meiotic, rather than mitotic, cell cycle.

A third of the oocytes that failed to emit PBI upon ECT2 depletion contained two MII spindles (see model in supplemental Fig. 6B). This phenomenon, which resembles binucleation in ECT2-depleted somatic cells (24, 27), has never been reported in oocytes. It apparently represents the completion of chromosome segregation that was not followed by PBI emission. Remarkably, under the conditions generated in the ooplasm in this experiment, each of the two separated sets of homologous chromosomes has formed its own stable MII spindle. Regardless of cell division failure, each spindle was aligned in parallel to the cortex, resembling the orientation of a single MII spindle during normal MI arrest. It is clear that the two well-formed MII spindles observed in the present study are not transitory structures as previously mentioned (33, 34) because they were detected 20 h after GVB.

The unique RhoA localization during PBI emission

At the onset of anaphase in mitotically dividing cells, RhoA accumulates in a ring-like manner around the spindle, preceding furrow ingress (20–23). Previous reports in somatic cells showed that RhoA promotes actin polymerization and myosin phosphorylation, thus bringing about the formation of the contractile ring (10, 12, 16). Similarly, the ring-shaped RhoA accumulation in oocytes apparently marks the localization of the future contractile ring. Unlike somatic cells, the formation of the RhoA ring in oocytes is preceded by RhoA translocation to the cortex adjacent to the spindle, forming a dome-like structure (see model in supplemental Fig. 6A). Two hypotheses can be raised to explain the unique dome-like accumulation of RhoA in oocytes. In somatic cells, the spindle expands throughout the entire cell, enabling activation of the equatorial cortex by the centralspindlin. However, the oocyte not only is much larger than a somatic cell but also undergoes an asymmetric cell division. Under these conditions, a limited centralspindlin-cortex contact area is available. A multistep process, in which RhoA is first crudely recruited to the cortex in the vicinity of the spindle...
to form a dome-like accumulation and later reorganizes into the functional ring, may be particularly adapted to fit the oocyte properties. The GTPase flux model, which provides evidence for the considerable plasticity of the RhoA zone, easily explains the remodeling of the RhoA shape (35–37). The second explanation for the requirement of a dome-like accumulation in oocytes takes into account the polarity of the cortex of the extruding PBI. The RhoA accumulation at the region of the cortex that is adjacent to the spindle may be a prerequisite for further cortex polarization.

Supporting this hypothesis, CDC42 activation, which controls PBI outpocketing, requires RhoA activation (30). Further research is necessary to examine these hypotheses.

Role of ROCK in PBI emission

The downstream effector of RhoA, ROCK, is one of the kinases that phosphorylates the myosin II regulatory light chain during cytokinesis, inducing constriction of the actomyosin ring. In agreement with a previous publication (38), we show herein that ROCK inhibition blocks PBI extrusion. This finding is somewhat unexpected because depletion of both ROCK-I and ROCK-II isoforms in HeLa cells induced only a minor increase in the multinucleation incidence, suggesting that ROCK is not necessary for cytokinesis in somatic cells (16, 20, 39). This discrepancy can be explained by a redundant mechanism responsible for the activation of myosin II in somatic cells. In addition, an adhesion-mediated cell division pathway that bypasses the myosin-induced cell division, has been suggested to explain the dispensability of ROCK in somatic cells (40, 41). However, because the oocytes do not adhere to the substrate, this adhesion-dependent pathway evidently does not exist. Furthermore, the absolute requisite of ROCK for PBI emission denies the presence of another kinase that would activate myosin II in oocytes.

Unlike previous studies suggesting that ROCK is also involved in an early stage of meiosis maturation, inhibition of this kinase in our hands had no effect on GVB (38, 42). Our finding indicates that the ECT2-RhoA-ROCK pathway is not active during early meiosis, supporting the assumption that up to metaphase, ECT2 is maintained in a phosphorylated inactive state, avoiding premature cytokinesis.

CDK1 regulates the ECT2 phosphorylation state

We demonstrate herein, for the first time, that ECT2 is subjected to phosphorylation/dephosphorylation throughout meiosis in oocytes. We reveal that PBI emission is temporally associated with ECT2 dephosphorylation. Interestingly, the phosphorylation state of ECT2 is tightly correlated with the pattern of CDK1 activity. Furthermore, our data provide the first demonstration that an active CDK1 phosphorylates ECT2 during the first meiotic metaphase and that CDK1 inactivation upon anaphase allows ECT2 dephosphorylation.
a specific phosphatase, and therefore, a sustained kinase activity is necessary to maintain it in a phosphorylated state. This kinetics assessment of the CDK1-induced ECT2 phosphorylation could be analyzed in oocytes due to the substantial longer duration of meiosis compared with mitosis.

The question whether CDK1 inactivation generates the sufficient condition to induce ECT2 dephosphorylation was never assessed in cell cycle studies. In this study, we provide evidence that it is the inactivation of CDK1 that allows ECT2 dephosphorylation. Since ECT2 is known to be regulated by its phosphorylation, we suggest that CDK1 inactivation induces PBI emission via ECT2 dephosphorylation and its subsequent activation.

ECT2 displays an interesting pattern of phosphorylation/dephosphorylation. It is phosphorylated at prometaphase/metaphase, dephosphorylated at the metaphase-to-anaphase transition, and rephosphorylated during MII arrest. A similar pattern of phosphorylation was observed for several other proteins such as protein regulator of cytokinesis 1 (PRC1), mitotic kinesin-like protein-1 (MKLP1), and cell division cycle20 homolog 1 (CDH1), which play a critical role during cytokinesis (43–45).

Their phosphorylation by CDK1 during metaphase inhibits its cytokinesis, whereas their dephosphorylation during anaphase, upon CDK1 inactivation, permits cell division. This mechanism ensures that cytokinesis occurs at the appropriate time point during the cell cycle, avoiding aneuploidy. We therefore suggest that ECT2 is regulated in a similar mode: the CDK1 phosphorylation-mediated inactivation at MI is followed by a dephosphorylation-dependent activation at the first anaphase. According to this model, the balance between the phosphorylated and dephosphorylated states provides a tight regulation on ECT2 activity. This hypothesis assumes that the phosphorylated ECT2 is inactive. Interestingly, subsequent to PBI emission, ECT2 is rephosphorylated. This event is presumably responsible for its inactivation that would apparently prevent premature extrusion of the second PB. Supporting this idea, only dephosphorylated ECT2 was shown to interact with the centralspindlin component Rac GTPase-activating protein 1 RACGAP1, also known as CYK-4, which is required for completion of cytokinesis (23).

However, in disagreement to this theory, phosphorylated and dephosphorylated ECT2 were both reported active and localized properly during cytokinesis (29). Further investigations are needed to elucidate the phosphorylation requirement for ECT2 activity.

In conclusion, our results demonstrate the necessity of the ECT2-RhoA-ROCK pathway for PBI emission in mammalian oocytes. This pathway appears to be turned on at anaphase I, upon CDK1 inactivation. In part of the ECT2-depleted oocytes, the homologous chromosomes have separated, generating oocytes that contain two MII spindles. Those oocytes that did not generate two spindles seemed to be arrested at MI. Furthermore, ECT2 triggers the formation of the RhoA ring. RhoA further activates its effector ROCK, which, by phosphorylating the myosin light chain, induces constriction of the contractile ring and the subsequent PBI emission.

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