Cdk1, but not Cdk2, is the sole Cdk that is essential and sufficient to drive resumption of meiosis in mouse oocytes

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Mammalian oocytes are arrested at the prophase of meiosis I during fetal or postnatal development, and the meiosis is resumed by the preovulatory surge of luteinizing hormone. The in vivo functional roles of cyclin-dependent kinases (Cdks) during the resumption of meiosis in mammalian oocytes are largely unknown. Previous studies have shown that deletions of Cdk3, Cdk4 or Cdk6 in mice result in viable animals with normal oocyte maturation, indicating that these Cdks are not essential for the meiotic maturation of oocytes. In addition, conventional knockout of Cdk1 and Cdk2 leads to embryonic lethality and postnatal follicular depletion, respectively, making it impossible to study the functions of Cdk1 and Cdk2 in oocyte meiosis. In this study, we generated conditional knockout mice with oocyte-specific deletions of Cdk1 and Cdk2. We showed that the lack of Cdk1, but not of Cdk2, leads to female infertility due to a failure of the resumption of meiosis in the oocyte. Re-introduction of Cdk1 mRNA into Cdk1-null oocytes largely resumed meiosis. Thus, Cdk1 is the sole Cdk that is essential and sufficient to drive resumption of meiosis in mouse oocytes. We also found that Cdk1 maintains the phosphorylation status of protein phosphatase 1 and lamin A/C in oocytes in order for meiosis resumption to occur.

INTRODUCTION

In contrast to yeast where a single cyclin-dependent kinase (Cdk) mediates cell division (1), cell-cycle progression in mammals is controlled by multiple Cdks (2,3). Mice lacking Cdk2, Cdk3, Cdk4 or Cdk6 are viable, as Cdk1 can compensate for their loss by forming active complexes with A-, B-, E- and D-type cyclins, indicating that these Cdks are not essential for completing the mitotic cell cycle (2–5). At the same time, deletion of Cdk1 per se leads to early embryonic death, suggesting that Cdk1 is the only Cdk that is essential for driving the mitotic cell cycle in mammals (5,6).

This raises the question of whether there is a single Cdk that is sufficient for driving oocyte meiosis in mammals. In mammals, such as humans, pigs, cows and mice, all oocytes are arrested at the prophase of meiosis I (MI) when they are enclosed in individual ovarian follicles. The resumption of meiosis occurs as a result of a preovulatory surge of luteinizing hormone, with a hallmark of germinal vesicle (GV) breakdown (GVBD) followed by chromosome condensation and spindle formation and the completion of MI (7,8). Fully grown mouse oocytes also resume meiosis spontaneously when released from the follicles and cultured in vitro, and then become arrested at the metaphase of meiosis II (MII) until fertilization (7).

Since much of the mitotic cell-cycle machinery is shared by meiosis, quite often findings from biochemical studies of mitosis are generalized to meiosis (9,10). However, several peculiarities have recently been found in meiosis (3). For
example, although Cdk2−/− mice are viable, deletion of Cdk2 in mice leads to a postnatal loss of all oocytes, indicating that Cdk2 is important for oocyte survival, and probably oocyte meiosis also (11,12). Previous studies showed that in porcine oocytes, blocking of Cdk2 activities by anti-Cdk2 antibody resulted in failure of entry into the second meiosis (13). In addition, Cdk2−/− spermatocytes exhibit abnormal homologous pairing, recombination and sex-body formation (14). Cdk2−/− oocytes die during transition from pachytene to diplotene of prophase I, indicating that Cdk2 is important for the early stages of oocyte development and meiosis.

In the current study, we generated mouse models with oocyte-specific deletion of Cdk1 or Cdk2 and studied the specific requirements of Cdk1 and Cdk2 during the resumption of oocyte meiosis. We found that deletion of Cdk1 from oocytes results in a permanent arrest of the oocytes at the GV stage, which is caused by an inability to phosphorylate and suppress the downstream protein phosphatase 1 (PP1), and a subsequent failure to phosphorylate lamin A/C which is required for the disassembly of the nuclear envelope. On the other hand, mutant female mice lacking Cdk2 in their oocytes show normal oocyte maturation and MII-stage arrest, and they are fertile. Our study, therefore, provides direct in vivo evidence that Cdk1 is the only Cdk that is essential and sufficient for driving resumption of meiosis in mouse oocytes.

**RESULTS**

**Infertility caused by deletion of Cdk1, but not Cdk2, in oocytes**

We generated mutant mouse models where the Cdk1 or the Cdk2 gene was deleted in oocytes from primordial and further developed follicles (referred to as OoCdk1−/− or OoCdk2−/− mice). By western blot, we confirmed that the expression of Cdk1 and Cdk2 proteins was completely absent in GV-stage OoCdk1−/− oocytes (Fig. 1Aa) and OoCdk2−/− oocytes (Fig. 1Ab), respectively.

We found that the OoCdk1−/− females were completely infertile (Fig. 1Ac). However, oocytes from OoCdk1−/− mice did not show any morphologic difference from control OoCdk1+/+ oocytes, as shown in Figure 1Ad. Comparable numbers of ovulated oocytes were recovered from oviducts of OoCdk1−/− (8.4 ± 1.5) and OoCdk1+/+ (8.0 ± 2.6) females after natural mating with stud males (Fig. 1Ae), and corpora lutea (CL) were observed in their ovaries (Fig. 1Ad).

In comparison, during a testing period from 5 to 27 weeks of age, the OoCdk2−/− mice were completely fertile, with normal litter sizes (Fig. 1Ba). Moreover, oocytes from OoCdk2−/− mice did not show any morphologic difference from control OoCdk2+/+ oocytes, as shown in Figure 1Bb. Comparable numbers of ovulated oocytes were recovered from oviducts of OoCdk2−/− (8.4 ± 1.1) and OoCdk2+/+ (8.6 ± 0.5) females after natural mating with stud males (Fig. 1Bc).

**Normal resumption of meiosis, maturation and MII arrest of OoCdk2−/− oocytes**

We determined whether Cdk2 is essential for the resumption of meiosis in mouse oocytes. As shown in Figure 1Bd, the GVBD rates in OoCdk2−/− oocytes (76.8 ± 5.8%) were similar to those in control OoCdk2+/+ oocytes (76.2 ± 0.2%), indicating that Cdk2 does not have an indispensable role in triggering resumption of oocyte meiosis.

Normally, ovulated oocytes in the oviducts have completed the MI stage and contain a polar body, and remain arrested at the MII stage until fertilization. Cdk2 has been believed to be essential for MII arrest in Xenopus oocytes (15). This role of Cdk2 in MII arrest of mouse oocytes has not been shown in vivo, however. As shown in Figure 1Be, 96.3 ± 4.7% of oocytes recovered from oviducts of OoCdk2−/− mice were arrested at the MII stage and contained a polar body, which was similar to the rate in control OoCdk2+/+ oocytes (94.9 ± 8.7%). Moreover, Cdk2-null oocytes do not undergo spontaneous parthenogenesis. This result is in accordance with the normal fertility of the OoCdk2−/− female mice (Fig. 1Ba), and indicates that Cdk2 is not essential for MII arrest in mouse oocytes.

Thus, our genetic evidence shows that Cdk2 is neither essential for the resumption of meiosis nor for MII arrest in mouse oocytes.

**Meiotic arrest of OoCdk1−/− oocytes at the GV stage**

We found that all the ovulated OoCdk1−/− oocytes (n = 198) were arrested at the GV stage (Fig. 2Ab, arrows). As a comparison, the control-ovulated OoCdk1+/+ oocytes (n = 40) had emitted the first polar body (Fig. 2Aa, arrows), indicating entry into the MII stage.

Moreover, instead of two-cell embryos, GV-stage oocytes were recovered from oviducts of OoCdk1−/− females at embryonic day (E) 1.5 after mating with stud males. These oocytes failed to develop further during a 24 h culture period (Fig. 2Ba and b). Control two-cell embryos were recovered from oviducts of OoCdk1+/+ females at E1.5 (Fig. 2Bc), which subsequently developed into four- to eight-cell embryos over a 24 h culture period (Fig. 2Bd).

We also investigated whether OoCdk1−/− oocytes can undergo meiotic maturation in vitro. In mice, GVBD usually takes place <90 min when oocytes are removed from follicles and cultured in vitro; this is accompanied by condensation of chromosomes and spindle formation (7,16). We found that OoCdk1−/− oocytes that were cultured in vitro remained at the GV stage even after 8 h of culture (Fig. 2Cd and e, arrowhead), and no spindle formation and chromosome condensation occurred (Fig. 2Cf, arrowhead). In comparison, the first meiotic spindle formation and alignment of chromosomes along the metaphase plate could be seen in OoCdk1+/+ oocytes that were cultured for 8 h (Fig. 2Cc).

These results indicate that OoCdk1−/− oocytes are permanently arrested at the GV stage and are not able to resume meiosis.

**Microinjection of Cdk1 mRNA led to meiotic resumption of OoCdk1−/− oocytes**

To ascertain that the GV-stage arrest of OoCdk1−/− oocytes was indeed caused by the lack of Cdk1, we injected in vitro-transcribed Cdk1 mRNA into OoCdk1−/− oocytes. Water
was injected into OoCdk1+/- and OoCdk1-/- oocytes as controls.

As shown in Figure 2D, after the introduction of Cdk1 mRNA, 78.2 ± 16.1% of the injected OoCdk1-/- oocytes underwent GVBD, a rate comparable with the GVBD rate for OoCdk1+/- oocytes injected with water (88.5 ± 3.4%). None of the OoCdk1-/- oocytes injected with water underwent GVBD (Fig. 2D). Moreover, the OoCdk1-/- oocytes that were injected with Cdk1 mRNA did not erode the first polar body and remained at the metaphase I stage even after 18 h, the longest observation time (data not shown); this is probably due to sustained Cdk1 activity as a result of the high expression of Cdk1 during GVBD.

The above results suggest that the GV-stage arrest as observed in OoCdk1-/- oocyte was solely caused by the lack of Cdk1.

Phosphorylation of lamin A/C was abolished in OoCdk1-/- oocytes

Two major classes of lamins in mammalian cells have been reported: A-type lamins (lamin A/C) and B-type lamins (lamin B) (17,18). A major function of the lamins is to maintain the structural integrity of the nuclear envelope (19). During mitosis, Cdk1 has been reported to promote the disassembly of nuclear lamina by phosphorylating specific sites on lamins (17,18,20).

In this study, we found that in control OoCdk1+/- oocytes, along with oocyte maturation, phosphorylation of lamin A/C (Ser22 and Ser392) was elevated 1 h after the in vitro incubation (Fig. 3A), was downregulated at 10 h and was again increased at 14 h of the in vitro culture (Fig. 3A). Nevertheless, in OoCdk1-/- oocytes, no apparent phosphorylation of lamin A/C was observed during the culture period of 0–14 h (Fig. 3A). This result suggests that the phosphorylation and disassembly of nuclear lamina are downstream of Cdk1 during GVBD.

To confirm that Cdk1 mediates resumption of oocyte meiosis through the phosphorylation of lamin A/C, we re-introduced Cdk1 mRNA into OoCdk1-/- oocytes. As shown in Figure 3B, phosphorylation of lamin A/C was recovered in OoCdk1-/- oocytes that had been supplied with Cdk1 mRNA.

Phosphorylation of PP1 was abolished in OoCdk1-/- oocytes

PP1 is a serine/threonine phosphatase that regulates a number of cellular processes through the dephosphorylation of its substrates (21). In mitosis, Cdk1 suppresses the phosphatase activity of PP1 through an inhibitory phosphorylation at its threonine (Thr) 320 (22,23).

In the current study, we found that in OoCdk1+/- oocytes, phosphorylation of PP1 at Thr320 was elevated along with the progress of oocyte meiotic maturation (Fig. 4A, 1–6 h) and was reduced during the MI–MII transition (Fig. 4A, 10 h). In OoCdk1-/- oocytes, however, phosphorylation of PP1 (Thr320) was completely absent during a culture period of 14 h (Fig. 4A), indicating that Cdk1 is the upstream kinase that regulates PP1 activity during the resumption of oocyte meiosis.

To confirm that Cdk1 mediates resumption of oocyte meiosis through the phosphorylation of PP1, we re-introduced Cdk1 mRNA into OoCdk1-/- oocytes. As shown in Figure 4B, phosphorylation of PP1 was recovered in OoCdk1-/- oocytes that were supplied with Cdk1 mRNA.

The above results suggest that the phosphorylation and suppression of PP1 and subsequent phosphorylation of lamin A/C are key downstream events, following the activity of Cdk1 during the resumption of meiosis in mouse oocytes.

PP inhibitor okadaic acid resumed GVBD of OoCdk1-/- oocytes

To investigate whether chemical inhibition of PP1 could mimic the phosphorylation and suppression of PP1 by Cdk1 and resume GVBD in OoCdk1-/- oocytes, we cultured OoCdk1-/- oocytes with the PP inhibitor okadaic acid (OA, 2 µM). OA inhibits both PP1 and PP2A, and inhibits PP1 activity at a concentration of ≥2 µM (23,24).

As shown in Figure 5, after treatment with OA, GVBD was resumed in OoCdk1-/- oocytes, with rates of 0.5, 1.3, 5.5, 42 and 79% after 2, 4, 8, 12 and 16 h of treatment, respectively. The eventual GVBD rate of OoCdk1-/- oocytes after OA treatment (79.5 ± 9.0%) was comparable with that in OoCdk1+/- oocytes cultured with OA (90.6%).

Notably, lamin A/C phosphorylation in OoCdk1-/- oocytes was also elevated 2 h after OA treatment (Fig. 5), a time point when most of the oocytes were still at the GV stage. These results indicate that the inhibition of PP1 is required for GVBD, and that a major role of Cdk1 in oocyte GVBD is to inactivate PP1. The suppressed PP1 activity in oocytes then facilitates to maintain the phosphorylated status of lamin A/C during oocyte GVBD, which is required for disassembly of the nuclear envelope.

DISCUSSION

In Xenopus and starfish, resumption of meiosis in oocytes has been linked to the activation of maturation-promoting factor (MPF) (7). Based on studies using an anti-PSTAIRE antibody (which should recognize Cdk1 only) (25), and the fission yeast protein p13null (which was thought to bind to Cdk1 only) (26), MPF has been presumed to be a complex of Cdk1 and cyclin B1 in Xenopus and starfish eggs (25–27). However, due to the fact that there are homologous sequences in different Cdks, the anti-PSTAIRE antibody and p13null reagents recognize not only Cdk1, but also Cdk2 and Cdk3 (28–31).

In mouse oocytes, it was found by biochemical experiments that histone H1 kinase activity, presumably addressed as p34cdc2, rises shortly before GVBD and reaches a plateau at the metaphase of MI (7,32). Nevertheless, histone H1 can be phosphorylated not only by Cdk1 but also by at least three other Cdks (33,34). Likewise, although roscovitine prevents GVBD of mouse oocytes (24), it is a non-specific inhibitor of Cdk1, Cdk2 and Cdk5 (35). Thus, there has been no functional evidence in vivo to show whether it is Cdk1 or Cdk2 that is indispensable for the triggering of GVBD in mouse oocytes.

In the present study, we deleted Cdk1 and Cdk2 from mouse oocytes, and have provided genetic evidence that Cdk1 is the
Figure 1. Infertility in OoCdk1<sup>−/−</sup> mice but normal fertility in OoCdk2<sup>−/−</sup> mice. (A) (a) Oocyte-specific deletion of Cdk1 in mice. Western blots demonstrating the absence of Cdk1 protein expression in oocytes of OoCdk1<sup>−/−</sup> mice. GV-stage oocytes were isolated from ovaries of postnatal day (PD) 25 OoCdk1<sup>+/+</sup> and OoCdk1<sup>−/−</sup> mice as described in Materials and Methods. For each experiment, material from 3–5 mice was used per lane and 300 oocytes were loaded. The level of β-actin was used as an internal control. The experiment was repeated three times and a representative image is shown. (b) Oocyte-specific deletion of Cdk2 in mice. Western blots demonstrating the absence of Cdk2 protein expression in oocytes of OoCdk2<sup>−/−</sup> mice. GV-stage oocytes were isolated from ovaries of PD25 OoCdk2<sup>+/+</sup> and OoCdk2<sup>−/−</sup> mice as described in Materials and Methods. For each experiment, material from three to five mice was used per lane. For each lane, 300 oocytes were loaded. The level of β-actin was used as an internal control. The experiment was repeated three times and a representative image is shown. (c) Comparison of the cumulative number of pups per OoCdk1<sup>−/−</sup> female (n = 6, red line) and per OoCdk1<sup>+/+</sup> female (n = 6, blue line). (d) Normal ovary morphology and ovulation of OoCdk1<sup>−/−</sup> mice. Morphologies of ovaries from 3-month-old OoCdk1<sup>−/−</sup> and OoCdk1<sup>+/+</sup> mice are shown. OoCdk1<sup>−/−</sup> females had been ovulating, as indicated by the presence of CL in their ovaries and their normal fertility. (e) Comparison of the number of ovulated eggs (mean ± SEM) per OoCdk1<sup>−/−</sup> female (n = 5) and per OoCdk1<sup>+/+</sup> female (n = 5). Female mice that were 5–6 weeks old were housed with stud males, and vaginal plugs were checked every morning. The mated female mice were sacrificed and ovulated oocytes were recovered from their oviducts and counted. (B) (a) Normal fertility, ovary morphology, GVBD and MII arrest in OoCdk2<sup>−/−</sup> mice. (b) Normal ovary morphology of OoCdk2<sup>−/−</sup> mice.

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only Cdk1 that is indispensable for the triggering of the resumption of meiosis in oocytes. In the absence of Cdk1, fully grown oocytes are permanently arrested at the GV stage, resulting in infertility in the females. We also found that Cdk1 in mouse oocytes triggers GVBD through phosphorylation and inhibition of the phosphatase activity of PP1, thereby maintaining the phosphorylation status of lamin A/C and possibly other proteins needed for the resumption of meiosis. Similar results have been obtained when Cdk1 was deleted from oocytes, using transgenic mice expressing a Zona pellucida 3 (Zp3) promoter-mediated Cre recombinase (36) (data not shown).

We also showed that the injection of in vitro-transcribed Cdk1 mRNA into OoCdk1−/− oocytes causes GVBD. However, Cdk1 mRNA-injected OoCdk1−/− oocytes do not extrude their first polar body; rather, they remain arrested at the metaphase I. It is likely that the overexpressed Cdk1 mRNA has generated several fold more Cdk1–cyclin B1 complexes than what is generated under physiologic conditions. It is known that a 7-fold higher amount of cyclin B1 molecules than Cdk1 are generally present in mouse oocytes (37), and the anaphase-promoting complex may not be able to degrade the excessive amount of cyclin B1 bound to Cdk1.

In mitosis, phosphorylation of lamin A/C is needed to trigger the disassembly of the nuclear envelope (20). Our results in the current study show that in the absence of Cdk1, PP1 in mouse oocytes remains unphosphorylated, and active, thus maintaining lamin A/C in an unphosphorylated state. This suppresses the occurrence of GVBD in Cdk1-null oocytes. On the other hand, when the PP inhibitor OA was applied to Cdk1-null oocytes, GVBD was largely resumed, due to the inhibition of the phosphatase activity of OA-sensitive PP, thereby making it possible for lamin A/C to be phosphorylated. In our experiment, lamin was phosphorylated over 8–12 h of the culture with OA, and oocyte GVBD occurred mostly at 8–16 h of culture, suggesting that lamin has to be phosphorylated first in order for OA-induced GVBD to occur. Further experiments are required to determine whether it is PP1, or PP2A, that plays an in vivo role in mediating GVBD of mouse oocytes.

Interestingly, our unpublished data showed that OA treatment also induced the phosphorylation (indicating activation) of MEK (Ser217/221) and MAPK (Thr202/Tyr204) in OoCdk1−/− oocytes (not shown). Inhibition of MEK by its specific inhibitor PD98059 can largely inhibit OA-induced GVBD and phosphorylation of lamin A/C in OoCdk1−/− oocytes (data not shown). Thus, MEK/MAPK may be a side pathway of kinases that phosphorylates lamin A/C in maturing mouse oocytes.

Based on our results, we propose that a low Cdk1 activity in GV-stage oocytes is important in facilitating a low phosphorylation level of PP1, thus keeping PP1 active, which in turn maintains meiotic phosphoproteins in hypophosphorylated states, and thereby preventing GVBD. When Cdk1 activity rises, PP1 becomes phosphorylated and its phosphatase activity is suppressed, which facilitates the phosphorylation of key meiotic phosphoproteins, leading to GVBD.

Earlier in vitro studies have shown that Cdk2 is essential for MII arrest in Xenopus oocytes (15). However, our results here conclusively show that Cdk2 is not required for MII arrest in mouse oocytes.

In addition, although conventional knockout of Cdk2 in mice leads to the demise of all oocytes by 2 weeks after birth (11,12), oocyte-specific deletion of Cdk2 from the primordial follicle stage does not lead to any abnormality in oocyte maturation or female fertility. These findings suggest that Cdk2 is not required for the resumption of meiosis in mouse oocytes. In conventional Cdk2−/− mice, oocytes fail to undergo transition from pachytene to diplotene of prophase I, indicating that Cdk2 in oocytes is probably important for early stages of meiosis, which is an interesting question for further studies.

In summary, using mutant mouse models with oocyte-specific deletions of Cdk1 and Cdk2, we have shown that Cdk1 is indispensable for the triggering of the resumption of meiosis in mammalian oocytes. This is achieved through phosphorylation and suppression of PP1, and subsequent phosphorylation of lamin A/C and possibly other key proteins required for GVBD. On the other hand, Cdk2-null oocytes resume meiosis and are fertilized normally, proving that Cdk2 is not essential for oocyte maturation in mice. Our findings implicate Cdk1 as the only Cdk that is essential and sufficient for driving resumption of meiosis in mammalian oocytes. The information obtained will also provide useful knowledge for the treatment of female infertility that is caused by meiosis-arrested oocytes, which are often seen in in vitro fertilization clinics.

MATERIALS AND METHODS

Mice
Cdk1loxP/loxP and Cdk2loxP/loxP mice (6) with a mixed genomic background of 129S4/SvJae and C57BL/6J were crossed with transgenic mice carrying growth differentiation factor 9 (Gdf-9) promoter-mediated Cre recombinase (38). After multiple rounds of crossing, we obtained homozygous mutant female mice lacking Cdk1 (OoCdk1−/− mice) and Cdk2 (OoCdk2−/− mice) in oocytes. Control mice that do not carry the Cre transgene are referred to as OoCdk1+/+ and OoCdk2+/+ mice. The mice were housed under controlled environmental conditions, with free access to water and food. Illumination was on between 0600 and 1800 hours.

4-month-old OoCdk2−/− and OoCdk2−/− mice were embedded in paraffin, and sections of 8 μm thickness were prepared and stained with hematoxylin.

(c) Comparison of the number of ovulated eggs (mean ± SEM) per OoCdk2−/− female (n = 5) and per OoCdk2−/+ female (n = 5). Female mice that were 5–7 weeks old were housed with stud males, and vaginal plugs were checked every morning. The mated female mice were sacrificed, and ovulated oocytes were recovered from their oviducts and counted. (d) Comparison of GVBD percentage between OoCdk2−/− and OoCdk2−/− oocytes. Oocytes from 3- to 4-week-old PMSG primed OoCdk2−/− and OoCdk2−/− mice were collected in M2 medium. They were cultured further in M16 medium for GVBD analysis. GVBD rate was scored after a culture period of 3 h. The GVBD rates were comparable between OoCdk2−/− oocytes and control oocytes. (c) Normal MII arrest of OoCdk2−/− oocytes in vitro. Three- to 4-week-old female mice were superovulated with PMSG-ICG as described in Materials and Methods. Ovulated oocytes were collected from the oviducts, and cumulus cells were removed by treatment with 0.1% hyaluronidase in M2 medium. Numbers of polar bodies in each oocyte were counted. The numbers of oocytes used (n) are shown.
Lysate from 180 oocytes was loaded in each lane. Cdk1 and DAPI (blue), respectively, as described in Materials and Methods. All experiments were repeated at least three times. Lysate from 180 oocytes was loaded in each lane. (B) Lack of lamin A/C phosphorylation in OoCdk1−/− oocytes after 18 h of incubation, whereas phosphorylation of lamin A/C (Ser22 and Ser392) was below the detection limit in OoCdk1−/+ oocytes. Ovulated oocytes were collected from the oviducts of female mice as described in Materials and Methods. OoCdk1−/− oocytes were already at the two-cell stage (c) and developed to the four- to eight-cell stage by E2.5 (d). (C) Failure of OoCdk1−/− and OoCdk1−/+ oocytes to be fertilized. OoCdk1−/− and OoCdk1−/+ female mice were mated with stud males, and oocytes/zygotes were recovered from the oviducts at E1.5 as described in Materials and Methods. OoCdk1−/− oocytes were still at the one-cell stage (a) and failed to develop further upon culture in vitro up to E2.5 (b). In contrast, E1.5 OoCdk1−/+ embryos were already at the two-cell stage (c) and developed to the four- to eight-cell stage by E2.5 (d). (C) Failure of meiotic maturation in OoCdk1−/− and OoCdk1+/− oocytes in vitro. GV-stage OoCdk1−/+ and OoCdk1−/− oocytes were collected and cultured in vitro for 8 h. Spindle and chromosomes were labeled with α-tubulin (green) and DAPI (blue), respectively, as described in Materials and Methods. OoCdk1−/− oocytes underwent GVBD (b), formed first meiotic spindle and chromosomes were aligned along the metaphase plate (c). On the other hand, OoCdk1−/+ oocytes were still at the GV stage (e), and there was no chromosome condensation and spindle formation (f). Scale bar corresponds to 25 μm. (D) GVBD rate (compared with OoCdk1−/+ oocytes) in OoCdk1−/− oocytes after microinjection of Cdk1 mRNA. In vitro-transcribed Cdk1 mRNA was microinjected into OoCdk1−/− oocytes (OoCdk1−/+ + mRNA). As controls, distilled water was injected into OoCdk1−/− (OoCdk1−/− + water) and OoCdk1−/+ (OoCdk1−/+ + water) oocytes as described in Materials and Methods. 78.2 ± 16.1% of the OoCdk1−/+ + mRNA oocytes underwent GVBD when compared with the OoCdk1−/+ + water negative controls (0%). This GVBD rate in OoCdk1−/− + mRNA was only slightly lower than in the OoCdk1−/+ + water controls (88.5 ± 3.4%).

Figure 2. Permanent arrest of OoCdk1−/− oocytes at the GV stage. (A) Failure of meiotic maturation in OoCdk1−/− oocytes in vivo. Ovulated oocytes were collected from the oviducts of OoCdk1−/+ and OoCdk1−/− mice as described in Materials and Methods. Ovulated OoCdk1−/+ oocytes had already emitted their first polar body (a), whereas ovulated OoCdk1−/− oocytes were still at the GV stage (b). (B) Failure of OoCdk1−/− oocytes to be fertilized. OoCdk1−/+ and OoCdk1−/− female mice were mated with stud males, and oocytes/zygotes were recovered from the oviducts at E1.5 as described in Materials and Methods. OoCdk1−/− oocytes were still at the one-cell stage (a) and failed to develop further upon culture in vitro up to E2.5 (b). In contrast, E1.5 OoCdk1−/+ embryos were already at the two-cell stage (c) and developed to the four- to eight-cell stage by E2.5 (d). (C) Failure of meiotic maturation in OoCdk1−/− oocytes in vitro. GV-stage OoCdk1−/+ and OoCdk1−/− oocytes were collected and cultured in vitro for 8 h. Spindle and chromosomes were labeled with α-tubulin (green) and DAPI (blue), respectively, as described in Materials and Methods. OoCdk1−/− oocytes underwent GVBD (b), formed first meiotic spindle and chromosomes were aligned along the metaphase plate (c). On the other hand, OoCdk1−/+ oocytes were still at the GV stage (e), and there was no chromosome condensation and spindle formation (f). Scale bar corresponds to 25 μm. (D) GVBD rate (compared with OoCdk1−/+ oocytes) in OoCdk1−/− oocytes after microinjection of Cdk1 mRNA. In vitro-transcribed Cdk1 mRNA was microinjected into OoCdk1−/− oocytes (OoCdk1−/+ + mRNA). As controls, distilled water was injected into OoCdk1−/− (OoCdk1−/− + water) and OoCdk1−/+ (OoCdk1−/+ + water) oocytes as described in Materials and Methods. 78.2 ± 16.1% of the OoCdk1−/+ + mRNA oocytes underwent GVBD when compared with the OoCdk1−/+ + water negative controls (0%). This GVBD rate in OoCdk1−/− + mRNA was only slightly lower than in the OoCdk1−/+ + water controls (88.5 ± 3.4%).

Figure 3. Lack of lamin A/C phosphorylation in OoCdk1−/− oocytes. (A) GV-stage OoCdk1−/+ and OoCdk1−/− oocytes were collected and cultured in vitro for 0, 1, 3, 6, 10 and 14 h, and western blots were performed as described in Materials and Methods. In vitro culture of OoCdk1−/− oocytes was found to lead to the phosphorylation of lamin A/C after 1 h of incubation, which was then downregulated at 10 h, and was again increased at 14 h of in vitro culture. On the contrary, in OoCdk1−/+ oocytes, no apparent phosphorylation of lamin A/C was observed during 14 h of in vitro culture. The level of β-actin was used as internal control. All experiments were repeated at least three times. Lysate from 180 oocytes was loaded in each lane. (B) Restoration of lamin A/C phosphorylation in OoCdk1−/+ oocytes after microinjection of Cdk1 mRNA. In vitro-transcribed Cdk1 mRNA was microinjected into OoCdk1−−/− oocytes. As a control, distilled water was injected into OoCdk1−−/− oocytes as described in Materials and Methods. Microinjection of Cdk1 mRNA was found to restore phosphorylation of lamin A/C (Ser22 and Ser392) after 18 h of incubation, whereas phosphorylation of lamin A/C (Ser22 and Ser392) was below the detection limit in OoCdk1−−/− oocytes injected with distilled water. The level of lamin A/C was used as internal control. All experiments were repeated at least three times. Lysate from 180 oocytes was loaded in each lane.

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Reagents, antibodies and immunologic detection methods

Mouse monoclonal antibodies to Cdk1, lamin A/C and rabbit polyclonal antibody to Cdk2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies to PP1α, phospho-PP1α (Thr320) and phospho-lamin A/C (Ser22) were obtained from Cell Signaling Technologies (Beverly, MA, USA). Rabbit polyclonal antibody to phospho-lamin (Ser392) was purchased from Abcam (Cambridge, UK). Mouse monoclonal antibody to β-actin, M2 and M16 media, Hank’s balanced salt solution (HBSS), dibutyryl cyclic AMP (dbcAMP), pregnant mare serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), hyaluronidase, 4,6-diamidino-2-phenylindole (DAPI), mouse monoclonal anti-α-tubulin-FITC and mineral oil were purchased from Sigma. OA was obtained from Tocris Biosciences (Ellisville, MO, USA). Western blots were carried out according to the instructions of the suppliers of the different antibodies, and visualized using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Uppsala, Sweden).

Histologic analysis of ovarian tissues

Histologic analysis of ovary was performed as previously described (39). Briefly, ovaries were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Paraffin-embedded ovaries were serially sectioned at 8 μm thickness and stained with hematoxylin for morphologic observation.

Gonadotropin-induced ovulation

To obtain ovulated oocytes for the observation of GVBD and polar body extrusion in vivo, 3- to 4-week-old female mice were injected with 7.5 IU PMSG, and 48 h later with 5 IU hCG intraperitoneally. Ovulated oocytes were collected from the oviducts 16 h after hCG injection. Cumulus cells were removed by treatment with 0.1% hyaluronidase in M2 medium.

Oocyte collection and analysis

Three- to 4-week-old female mice were injected with 7.5 IU PMSG intraperitoneally, and 42–44 h later the mice were sacrificed and their ovaries were collected in 100 μg/ml dbcAMP-containing M2 medium. Fully grown GV-stage oocytes surrounded by cumulus cells were released by puncturing the ovaries in M2 medium supplemented with 100 μg/ml dbcAMP to maintain meiotic arrest during in vitro operation. Oocytes were freed from attached cumulus cells by repetitive pipetting through a narrow-bore glass
pipette. If GV-stage oocytes were required for western blots, the denuded oocytes were rinsed in HBSS and lysed in lysis buffer as previously described (39).

To observe GVBD, the denuded oocytes were washed twice in dbcAMP-free M2 medium, followed by once in M16 medium and then cultured in M16 medium (or M16 medium containing different inhibitors as mentioned in the appropriate figure legend) at 37°C in a humidified atmosphere of 5% CO₂ under mineral oil. GVBD rates were recorded at the end of the incubation, and the oocytes were rinsed in HBSS before lysing in lysis buffer. About 20 oocytes were cultured in a 50 μL drop of M16 medium. For western blots, unless otherwise stated specifically, 180 oocytes were used per lane.

For inhibitor studies, the PP inhibitor OA (2 μM) was used in M16 medium. Oocytes were cultured for different lengths of time as indicated in the figure legends. At the end of incubation, GVBD rates were recorded and the oocytes were rinsed in HBSS before being lysed in lysis buffer for western blot analysis.

**Observation of embryonic development**

Oocdk1−/− and Oocdk1+/+ female mice that were 5–6 weeks old were housed with stud males, and vaginal plugs were checked every morning. E0.5 refers to the day that a vaginal plug was found. The mated female mice were sacrificed at E1.5 and zygotes/oocytes were recovered from their vaginal plug. The mated female mice were sacrificed at E1.5 and zygotes/oocytes were recovered from their vaginal plug. The developmental stages of the zygotes/oocytes were recorded.

**In vitro transcription and microinjection**

To generate Cdk1 mRNA for microinjection, pBluescript II KS vector containing full-length mouse Cdk1 (pBS-CDK1) (GenBank accession no. NM_007659.3) was linearized with HindIII. A mMESSAGE mMACHINE T7 ULTRA kit (Ambion) was used to synthesize mRNAs containing 5’ cap and 3’ poly A by *in vitro* transcription. RNA was purified using the RNeasy Mini Kit (Qiagen) and was quantified before microinjection.

About 5 pl of mRNA (500 ng/μl) was microinjected per oocyte, using a Leitz inverted microscope equipped with Narishige MNO-202N hydraulic three-dimensional micromanipulators (Narishige). As a control, oocytes were microinjected with the same amount of water. The oocytes were kept in M2 medium supplemented with 100 μg/ml dbcAMP to prevent GVBD during *in vitro* manipulation. After microinjection, the oocytes were washed three times in M16 medium to eliminate dbcAMP, and then cultured in M16 medium at 37°C in a humidified atmosphere of 5% CO₂ under mineral oil.

**Immunofluorescence and confocal microscopy**

Oocdk1−/− and Oocdk1+/+ oocytes that were cultured *in vitro* for 8 h in M16 medium were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 30 min at room temperature (RT). After permeabilization with 0.5% Triton X-100 at RT for 20 min, they were blocked with 1% bovine serum albumin-supplemented PBS for 1 h and incubated with 1:200 anti-α-tubulin-FITC antibody for 1 h at RT. After three washes in PBS containing 0.1% Tween 20 and 0.01% Triton X-100, the oocytes were co-stained with DAPI (1 μg/ml in PBS). Finally, they were mounted on glass slides and examined by laser scanning confocal microscopy (Leica TSP-2; Leica Heidelberg, Germany).

**Conflict of Interest statement.** None declared.

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