RESEARCH ARTICLE

Mis12 controls cyclin B1 stabilization via Cdc14B-mediated APC/C<sup>Cdhl</sup> regulation during meiotic G2/M transition in mouse oocytes

Guang-Yu Bai<sup>1,2</sup>, Min Ho Choe<sup>3</sup>, Jae-Sung Kim<sup>3,*</sup> and Jeong Su Oh<sup>1,2,*</sup>

ABSTRACT
Mammalian oocytes are arrested at G2/prophase of the first meiosis. After a hormone surge, oocytes resume meiosis, undergoing germinal vesicle breakdown (GVBD). This process is regulated by Cdk1/cyclin B1. Here, we report that Mis12 is required for G2/M transition by regulating cyclin B1 accumulation via Cdc14B-mediated APC/C<sup>Cdhl</sup> regulation, but is not essential for spindle and chromosome dynamics during meiotic maturation. Depletion of Mis12 severely compromised GVBD by impairing cyclin B1 accumulation. Importantly, impaired GVBD after Mis12 depletion was rescued not only by overexpressing cyclin B1 but also by depleting Cdc14B or Cdhl. Notably, oocytes rescued by cyclin B1 overexpression exhibited normal spindle and chromosome organization with intact kinetochore-microtubule attachments. In addition, after being rescued by cyclin B1 overexpression, Mis12-depleted oocytes normally extruded polar bodies. Moreover, Mis12-depleted oocytes formed pronuclear structures after fertilization but failed to develop beyond zygotes. Interestingly, Mis12 was localized in the cytoplasm and spindle poles in oocytes, in contrast to kinetochore localization in somatic cells. Therefore, our results demonstrate that Mis12 is required for meiotic G2/M transition but is dispensable for meiotic progression through meiosis I and II.

KEY WORDS: APC/C<sub>Cdhl</sub>, G2/M transition, Mis12, Meiotic resumption, Oocyte maturation

INTRODUCTION
Mammalian oocytes remain arrested at G2/prophase of the first meiosis within antral follicles. During this stage, oocytes are characterized by a large nucleus called the germinal vesicle (GV). Following hormone stimulation, oocytes at the GV stage resume meiosis (known as meiotic resumption or G2/M transition), as indicated by GV breakdown (GVBD). After GVBD, oocytes undergo a further maturational process that is manifested by chromosome congression, bipolar spindle formation, kinetochore-microtubule (kMT) attachment, chromosome segregation, and extrusion of the first polar body. Following a series of these events, oocytes are arrested again at the metaphase of the second meiosis (MII). After fertilization, oocytes are released from MII arrest, resulting in meiotic exit and formation of the pronucleus. Although many factors are involved in the regulation of oocyte maturation, this process is principally governed by maturation-promoting factor (MPF) (Jones, 2004).

MPF is a complex of cyclin B1 and Cdk1 that orchestrates progression through both mitosis and meiosis (Morgan, 1995; Nurse, 1990). During G2/prophase arrest in mammalian oocytes, MPF activity remains suppressed through Wee1 kinase-mediated inhibitory phosphorylation of Cdk1 at the Y15 residue and by the constant degradation of cyclin B1 by the anaphase-promoting complex/cyclosome (APC/C) in association with Cdh1 (APC/C<sub>Cdhl</sub>) (Han et al., 2005; Holt et al., 2011; Marangos et al., 2007; Reis et al., 2006). Indeed, inhibition of Wee1 kinases causes a partial induction of GVBD in mouse oocytes (Han et al., 2005; Oh et al., 2010). Similarly, APC/C<sub>Cdhl</sub> inhibition overrides G2/prophase arrest in mouse oocytes by increasing cyclin B1 levels (Holt et al., 2011; Marangos et al., 2007; Reis et al., 2006). The activity of APC/C<sub>Cdhl</sub> generally parallels the expression level of Cdh1 (Pesin and Orr-Weaver, 2008). However, its activity can also be modulated through inhibitory phosphorylation of Cdh1, which is reversed by Cdc14B phosphatase (Jaspersen et al., 1999; Kramer et al., 2000; Schindler and Schultz, 2009). Although the modulation of Cdh1 through phosphorylation, degradation or direct binding to inhibitory proteins has been demonstrated in mitosis, the regulation of APC/C<sub>Cdhl</sub> activity in mammalian meiosis remains poorly understood.

The Mis12 complex, composed of the Mis12, Pmf1, Nsl1 and Dsn1 subunits, is a highly conserved kinetochore protein that interacts with the Ndc80 and Knl1 complexes, forming the KMN network (Cheeseman et al., 2006; Petrovic et al., 2016, 2010). The KMN network is primarily responsible for stable kMT attachment and the recruitment of spindle assembly checkpoint (SAC) components in unattached kinetochores (Cheeseman et al., 2006). However, a recent study revealed that Hecl (also known as Ndc80), a subunit of the Ndc80 complex, is required for the stabilization of cyclin B2 through APC/C<sub>Cdhl</sub> regulation during G2/M transition in mouse oocytes (Gui and Homer, 2013). This result suggests that kinetochore proteins might exert noncanonical functions beyond either the chromosome segregation process or M phase. Consistent with this, a component of inner kinetochore protein Cenph has been reported to protect cyclin B1 from destruction by competing with the action of APC/C<sub>Cdhl</sub> during G2/M transition in mouse oocytes (Zhang et al., 2017). Similarly, a SAC protein, BubR1, has been shown to regulate meiotic G2/M transition by maintaining elevated levels of Cdh1 in mouse oocytes (Homer et al., 2009). These findings open the possibility that other kinetochore proteins might play an additional role in the regulation of meiotic arrest and resumption in mouse oocytes. Through initial knockdown screening of kinetochore proteins, we found that Mis12 is required for meiotic resumption in mouse oocytes.
Here, we describe a noncanonical function of Mis12 in the regulation of the meiotic G2/M transition in mouse oocytes. Mis12 depletion compromised GVBD by impairing cyclin B1 accumulation. However, this arrest induced by Mis12 depletion was rescued not only by overexpressing cyclin B1 but also by depleting Cdh1 or Cdc14B. Moreover, oocytes rescued by cyclin B1 overexpression exhibited normal spindle and chromosome organization, extruded a polar body, and were fertilized. Therefore, our results demonstrate that Mis12 is required for meiotic G2/M transition but is dispensable for progression through meiosis I and meiosis II.

RESULTS

Mis12 depletion compromises meiotic resumption by impairing cyclin B accumulation

To investigate the function of Mis12 in mouse oocytes, we injected siRNAs that specifically targeted Mis12, or a nonspecific siRNA as a control. After injection, the oocytes were maintained at the GV stage for 12 h with 3-isobutyl-1-methylxanthine (IBMX). Immunoblot analysis showed that Mis12 was efficiently knocked down by siRNA injection (Fig. 1A). To explore the effect of Mis12 depletion on meiotic maturation, oocytes were released from IBMX-mediated GV arrest. Although ~80% of control oocytes underwent GVBD after 3 h culture, most Mis12-depleted oocytes remained arrested at the GV stage (siCtrl and siMis12+hMis12 mRNA groups versus siMis12 group: 82.03±5.75 and 63.94±2.04 versus 19.02±11.22; P<0.001) (Fig. 1B). Moreover, the GV arrest in Mis12-depleted oocytes was not rescued by longer culture up to 24 h (siCtrl group versus siMis12 group: 95.0±5.00 versus 29.84±11.75; P<0.0001) (Fig. 1C), suggesting that GVBD was blocked in Mis12-depleted oocytes. This phenotype was rescued by overexpressing human Mis12, thus excluding the possibility of off-target effects (Fig. 1B). Therefore, our results demonstrate that Mis12 is required for GVBD in mouse oocytes.

Because GVBD in mouse oocytes is driven by the activity of Cdk1 in association with cyclin B1, we determined Cdk1 activity and cyclin B1 levels. Immunoblot analyses showed that levels of inhibitory phosphorylation of Cdk1 at Y15 were not different between control and Mis12-depleted oocytes (Fig. 1D). In contrast, cyclin B1 levels were significantly decreased after Mis12 depletion (Fig. 1E). Consistent with decreased cyclin B1 levels, MPF activity was decreased following Mis12 depletion (Fig. 1F). Therefore, our results suggest that Mis12 is required for cyclin B1 accumulation, which is necessary for GVBD in mouse oocytes.

Mis12 regulates APC/C<sup>Cdh1</sup> activity through Cdc14B regulation

Because cyclin B1 levels are regulated by APC/C<sup>Cdh1</sup> activity during meiotic resumption in mouse oocytes, we investigated Cdh1 levels in Mis12-depleted oocytes. Interestingly, Cdh1 levels were not changed after Mis12 depletion (Fig. 2A). Nevertheless, the impaired GVBD after Mis12 depletion was rescued by Cdh1 depletion (siCtrl and siMis12+Cdh1 morpholino (MO) groups versus siMis12 group: 92.10±5.46 and 83.86±5.65 versus 25.26±2.84; P<0.0001) (Fig. 2B). Along with decreased levels of cyclin B1 after Mis12 depletion, this result implies that APC/C<sup>Cdh1</sup> activity was increased without a change in the Cdh1 level in Mis12-depleted oocytes. Because Cdc14B is required to maintain the APC/C<sup>Cdh1</sup> activity by removing inhibitory phosphorylation of Cdh1 (Jaspersen et al., 1999; Schindler and Schultz, 2009), we examined...
whether knockdown of Cdc14B rescued impaired GVBD in Mis12-depleted oocytes. Notably, Cdc14B levels were increased after Mis12 depletion and impaired GVBD by Mis12 depletion was rescued by Cdc14B depletion (siCtrl and siMis12+dsCdc14B groups versus siMis12 group: 94.32±6.33 and 73.76±8.06 versus 33.81±3.54; P<0.001) (Fig. 2C,D; Fig. S2). Consistent with this, reduced levels of cyclin B1 after Mis12 depletion were recovered by depleting Cdc14B, as well as Cdh1 (Fig. 2E). Therefore, our results suggest that Mis12 is required for cyclin B1 accumulation by suppressing Cdc14B-mediated APC/CCdh1 activity during G2/M transition in mouse oocytes.

Recently, Hec1-dependent cyclin B2 stabilization was shown to be required for meiotic resumption in mouse oocytes (Gui and Homer, 2013). Given that Mis12 is complexed with Hec1 at the kinetochores in mitotic cells, it was of interest to determine cyclin B1 and Hec1 levels after Mis12 depletion. Neither cyclin B2 nor Hec1 was changed after Mis12 depletion (Fig. 2F). Moreover, unlike cyclin B1, cyclin B2 levels were not changed by Cdh1 or Cdc14B depletion in Mis12-depleted oocytes (Fig. S3). Therefore, our results suggest that Mis12 regulates cyclin B1 levels independent of Hec1 and cyclin B2 pathways in mouse oocytes.

Mis12 depletion is rescued by cyclin B1 overexpression

Given that cyclin B1 did not accumulate in Mis12-depleted oocytes, we next sought to investigate whether cyclin B1 overexpression would rescue the impaired GVBD in Mis12-depleted oocytes. After 12 h knockdown with Mis12 siRNAs, oocytes arrested at the GV stage were injected with cyclin B1-GFP mRNAs and cultured for 3 h to allow protein expression. After confirming the GFP signal, oocytes were released from IBMX and scored for GVBD. Interestingly, impaired GVBD after Mis12 depletion was fully restored within 3 h by overexpressing cyclin B1-GFP with a comparable time course of cyclin B1 nuclear entry (siCtrl and siMis12+cyclin B1 mRNA groups versus siMis12 group: 81.67±5.40 and 76.61±10.09 versus 16.15±11.62; P<0.001) (Fig. 3A-C). In contrast to the rescue of GVBD by cyclin B1-GFP overexpression, pharmacological activation of Cdk1 using a Wee1 kinase inhibitor did not lead to GVBD in Mis12-depleted oocytes (control group versus MK-1775 group: 26.71±4.27 versus 43.75±12.68; P>0.05) (Fig. 3D). Therefore, our results further support the notion that impaired GVBD in Mis12-depleted oocytes is due to the failure of cyclin B1 accumulation.

Mis12 depletion does not affect kinetochore-microtubule attachment

Because Mis12 is known to be required for kinetochore assembly during mitosis, we next examined the spindle organization and kMT attachments in Mis12-depleted oocytes. For this experiment, the oocytes rescued by overexpressing cyclin B1-GFP were collected at the metaphase of meiosis I (MI) and spindle organization was examined. Remarkably, no discernible differences were detected in either the size of the spindles or the alignment of the chromosomes between control and rescued oocytes (siCtrl versus siMis12+cyclin B1 groups, spindle length: 44.90±6.21 versus 48.37±7.99; spindle width: 25.15±2.72 versus 27.39±4.67; and metaphase plate width: 13.99±3.73 versus 15.58±4.44; P>0.05) (Fig. 4A-D). To further explore the kMT attachment, MI oocytes were cold treated and
labeled with anticentromere antibody (ACA). The result revealed that kinetochore pairs in Mis12-depleted oocytes showed correct polar MT attachment, comparable with control oocytes (siCtrl group versus siMis12+cyclin B1 group: 6.59±2.57 versus 6.36±3.60; P >0.05) (Fig. 4E,F). These findings suggest that Mis12 depletion has little effect on spindle formation and kMT attachment during meiotic progression in MI.

**Mis12 is dispensable for progression through meiosis I and meiosis II**

Given that cyclin B1 overexpression recovered impaired GVBD in Mis12-depleted oocytes, we investigated whether these rescued oocytes could progress through meiosis I and extrude the polar body. Surprisingly, after cyclin B1-GFP overexpression, ∼72% of Mis12-depleted oocytes completed meiosis I and extruded the polar body at a time comparable with that observed in control oocytes (siCtrl group versus siMis12+cyclin B1 group, polar body extrusion (PBE): 82.19±3.09 versus 72.22±7.86; and time between GVBD and PBE: 9.58±1.25 versus 9.00±1.59; P >0.05) (Fig. 5A,B). We further examined spindle and chromosome organization of the Mis12-depleted MII oocytes rescued by cyclin B1-GFP overexpression. Immunofluorescence and confocal microscopy analyses showed that spindle and chromosome organization in these oocytes was comparable with that of normal MII oocytes.

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**Fig. 3. Overexpression of cyclin B1 rescues the impaired GVBD caused by Mis12 depletion.** (A) The rate of GVBD in oocytes injected with siCtrl, siMis12, and siMis12+cyclin B1-GFP mRNA. Data are mean+s.e.m. **P<0.001.** (B) Live cell imaging of Mis12-depleted oocytes overexpressing cyclin B1-GFP during the onset of GVBD. Representative DIC (top) and GFP (bottom) images are shown. (C) The ratio of nuclear versus cytoplasmic cyclin B1-GFP fluorescence in oocytes. (D) The rate of GVBD in Mis12-depleted oocytes after treating with 50 μM MK-1775. Scale bar: 50 μm.

**Fig. 4. Spindle formation and kMT attachment are normal in Mis12-depleted oocytes.** (A) Immunostaining of oocytes injected with either control siRNA or Mis12 siRNA+cyclin B1-GFP mRNA. Spindle microtubule and chromosomes were stained with anti-α-tubulin and DAPI, respectively. (B-D) Quantification of spindle length, spindle width and metaphase plate width. Numbers in parentheses indicate the total number of oocytes assayed in each group. (E) Following cold treatment for 10 min, MI oocytes were stained for DNA, spindle and kinetochore with DAPI, anti-α-tubulin, and anti-centromere antibody (ACA), respectively. Representative images of bi-oriented kMT attachments are shown with the magnified images. (F) The kMT attachments were quantified. The number of kinetochores analyzed is specified above the bars. Data are mean+s.e.m. Scale bars: 20 μm (A); 10 μm (E).
siMis12+cyclin B1 groups, spindle length: 30.97±2.78 versus 31.64±3.25; spindle width: 18.50±2.80 versus 16.83±1.61; and metaphase plate width: 9.08±1.46 versus 9.87±2.09; P>0.05) (Fig. 5C-F), suggesting that Mis12 is dispensable for meiotic progression after GVBD. The normality of MII oocytes depleted of Mis12 was further supported by the observation that the frequency of aneuploidy in Mis12-depleted oocytes was comparable with the control oocytes (siCtrl group versus siMis12+cyclin B1 group: 18.84±4.09 versus 19.87±4.53; P>0.05) (Fig. 5G).

Because Mis12 was dispensable for meiotic progression after GVBD, we further investigated whether Mis12 is required for fertilization. For this purpose, Mis12-depleted MII oocytes were subjected to intracytoplasmic sperm injection (ICSI), and pronuclear formation was examined. Interestingly, most Mis12-depleted oocytes had formed pronuclei 6 h after ICSI onwards, comparable with the control oocytes (siCtrl group versus siMis12+cyclin B1 group: 98.86±2.27 versus 96.73±2.30; P>0.05) (Fig. 6A), suggesting that Mis12 is dispensable for the completion of meiosis II as well as meiosis I. However, after pronuclear formation, fertilized zygotes depleted of Mis12 were arrested and failed to develop to two-cell embryos (siCtrl group versus siMis12 group: 98.72±2.22 versus 23.91±7.29; P<0.0001) (Fig. 6B). Consistent with this, nuclear envelope breakdown (NEBD) did not occur when zygotes were injected with Mis12 siRNA (Fig. S4A). However, impaired NEBD in Mis12-depleted zygotes was not rescued by ectopic overexpression of cyclin B1-GFP, suggesting that Mis12 is required for mitotic entry during the first mitotic cell cycle in zygotes, but the downstream target of Mis12 might be different between mitosis and meiosis (Fig. 4B,C). Taken together, our results demonstrate that Mis12 is required for meiotic resumption but dispensable for progression through meiosis I and II.

Unexpected cytoplasmic and spindle pole localization of Mis12 in mouse oocytes
To understand the functional difference of Mis12 between meiosis and mitosis, we examined the localization of Mis12 in oocytes and somatic cells. To this end, we had to ectopically overexpress Mis12 in mouse oocytes because of the lack of sensitive and selective antibodies capable of recognizing endogenous Mis12. Surprisingly, we found that Mis12 did not colocalize with chromosomes or with kinetochore proteins in mouse oocytes. Instead, Mis12 was widely distributed in the cytoplasm with punctate foci at the GV stage. After GVBD, Mis12 was localized at the spindle pole but not the kinetochore in mouse oocytes (Fig. 7A). Moreover, Mis12 was mostly colocalized with Cdc14B external to the GV (Fig. 7B), supporting the notion that Mis12 regulates Cdc14B during the onset of meiotic resumption. In striking contrast to the cytoplasmic and spindle pole localization in oocytes, Mis12 was observed in the kinetochores in somatic cells (Fig. S5). Therefore, our results suggest that the different functions of Mis12 in mouse oocytes are probably due to its unique localization in these cells.

DISCUSSION
A well-known function of Mis12 in the context of the KMN network is kinetochore assembly, ensuring kMT attachments for accurate chromosome segregation. In this study, we discovered an unexpected novel function of Mis12 in the regulation of cyclin B1 levels through the Cdc14B-mediated APC/Cdc14B pathway in mouse oocytes. Depletion of Mis12 severely suppressed meiotic resumption by impairing cyclin B1 accumulation. This aborted meiotic resumption could be restored by cyclin B1 overexpression, and rescued oocytes progressed through meiosis I and II without any...
discernible defects. Thus, our results show that Mis12 is required for meiotic resumption, but not for kinetochore assembly and chromosome segregation in mouse oocytes.

The APC/C\(^{\text{Cdh1}}\) is inactivated by multiple phosphorylations of Cdh1 mediated by Cdks, as well as Cdh1 degradation (Pesin and Orr-Weaver, 2008). Because Cdh1 levels were unchanged after Mis12 depletion, it is likely that Mis12 regulates APC/C\(^{\text{Cdh1}}\) activity by modulating Cdh1 phosphorylation status in mouse oocytes. It is known that Cdh1 dephosphorylation depends not only on the inactivation of Cdks, but also on the activation of counteracting phosphatases (Wurzenberger and Gerlich, 2011). Although Cdc14 has been identified as the main phosphatase responsible for Cdh1 dephosphorylation in budding yeast (Jaspersen et al., 1999), its relevance in vertebrates remains unclear. However, recent evidence showed that Cdc14B dephosphorylates Cdh1, hence promoting APC/C\(^{\text{Cdh1}}\) activity in response to genotoxic stress in the G2 phase in somatic cells (Bassermann et al., 2008). Consistent with this, overexpression of Cdc14B has been shown to inhibit GVBD with reduced cyclin B1 levels in mouse oocytes (Schindler and Schultz, 2009). Notably, the effects of Cdc14B overexpression were attenuated by depleting Cdh1, indicating that Cdc14B is an upstream regulator of APC/C\(^{\text{Cdh1}}\) in mouse oocytes (Schindler and Schultz, 2009). In this study, we observed that Cdc14B levels were increased after Mis12 depletion, and depletion of Cdc14B rescued the impaired GVBD in Mis12-depleted oocytes. Therefore, we propose that Mis12 regulates cyclin B1 levels through Cdc14B-mediated APC/C\(^{\text{Cdh1}}\) regulation during the G2/M transition in mouse oocytes (Fig. 8).

Because of the well-conserved function of kinetochore proteins in spindle dynamics and chromosome segregation, we assumed that Mis12-depleted oocytes would fail to progress through meiosis I, regardless of the rescue of impaired GVBD by overexpressing cyclin B1-GFP. However, we found that the Mis12-depleted oocytes entered meiosis I with normal timing and efficiency and...
progressed through meiosis I and II. Moreover, the Mis12-depleted oocytes were fertilized as usual and formed pronuclei. Therefore, our results suggest that in mouse oocytes Mis12 is required for meiotic resumption but dispensable for subsequent progression through meiosis I and II. The reason why Mis12 plays a different function during meiotic maturation is unclear, but it is probably because of the unique localization of Mis12 in mouse oocytes. In somatic cells, Mis12 is associated with chromosome centromeres and is recruited to the kinetochores during mitosis (Gascoigne and Cheeseman, 2013). Consistently, we observed that ectopically expressed Mis12 was localized in the nucleus and kinetochores in somatic cells (Fig. S5). In contrast, in mouse oocytes, Mis12 was localized in the cytoplasm at the GV stage and enriched at the spindle poles instead of the kinetochores at the MI and MII stages (Fig. 7). Similar to our results, the kinetochore protein Hec1 was found to localize in the cytoplasm without associating with chromosomes in GV oocytes, and to regulate meiotic resumption by regulating cyclin B1 levels through the APC/CCdh1 pathway in mouse oocytes (Gui and Homer, 2013). In line with these results, it is also noteworthy that Cdc14B exhibited a different localization in mouse oocytes. In somatic cells, Cdc14B localizes in the nucleolus during interphase and at the spindle poles during mitosis (Bassermann et al., 2008; Cho et al., 2005; Schindler and Schultz, 2009). However, Cdc14B has been shown to colocalize with γ-tubulin at the microtubule organizing center and with the cytoplasmic microtubule network in GV oocytes (Schindler and Schultz, 2009). Given that Cdc14 is sequestered in the nucleolus by associating with its inhibitor Cfi1 and Net1 in yeast (Shou et al., 1999; Visintin et al., 1999), it is likely that in mouse oocytes Mis12 mimics some aspects of yeast inhibitors of Cdc14, thereby sequestering and inhibiting Cdc14B in the cytoplasm of oocytes. Therefore, we suggest that Mis12 inhibits Cdc14B in the cytoplasm in response to the signals triggering meiotic resumption, which thereby decreases APC/CCdh1 activity. This in turn increases cyclin B1 levels in the cytoplasm, which enables the nuclear entry of cyclin B1. In addition to Mis12 and Hec1, the kinetochore proteins BubR1 and Cenph have been shown to regulate meiotic resumption by regulating Cdh1 levels in mouse oocytes (Homer et al., 2009; Wei et al., 2010; Zhang et al., 2017). Therefore, it would be interesting to investigate the function and localization of other kinetochore proteins in mouse oocytes. In this regard, it is tempting to speculate that oocytes might utilize the kinetochore proteins in the regulation of meiotic resumption by spatially sequestering them into specialized localization. However, we could not exclude the possibility that these noncanonical functions of kinetochore proteins are species specific.

Although APC/CCdh1 activity is required for meiotic progression from G2/prophase I to prometaphase I in mouse oocytes (Holt et al., 2011; Marangos et al., 2007; Reis et al., 2006), it is generally accepted that APC/CCdh1 remains inactive during the G2 phase and early mitosis in somatic cells. Instead, the APC/CCdh1-associated with Cdc20 (APC/Cdh1) is activated during early mitosis (Kramer et al., 2000; Pesin and Orr-Weaver, 2008). In this regard, it is likely that a failure to rescue NEBD in Mis12-depleted zygotes by cyclin B1 overexpression is due to the difference in APC/C species in the regulation of G2/M transition between oocytes and early embryos. This is supported by findings that Cdh1 is absent during the early embryonic stage in a number of species, such as Xenopus, Caenorhabditis elegans, and Drosophila (Lorca et al., 1998; Raff et al., 2002; Sigrist and Lehner, 1997). Nevertheless, the Mis12-mediated Cdc14B regulatory pathway appears to be present in zygotes, because overexpression of Cdc14B in zygotes causes a G2 arrest of the first or second mitotic division in mouse (Buffone et al., 2009), which is a phenocopy of Mis12 depletion in zygotes. Given that the principle target for Cdc14B-mediated APC/CCdh1 in somatic cells is Plk1 and not cyclin B1 (Bassermann et al., 2008), it is also possible that the Mis12-Cdc14B-APC/CCdh1 pathway works in zygotes but targets different substrates. However, further studies are needed to clarify the downstream pathway of Mis12 in early embryonic divisions.
In conclusion, our data demonstrate that Mis12 is required to suppress Cdc14B and APC/C\(^{Cdh1}\) activity, thereby promoting cyclin B1 accumulation during the onset of meiotic resumption. However, after GVBD, Mis12 is no longer required for subsequent meiotic maturation, including kMT attachment and chromosome segregation. This unique function of Mis12 during meiosis is probably due to the different localization of Mis12 in mouse oocytes. Given that the molecular mechanisms underlying meiotic maturation are not entirely clear, our findings provide new insights and a deeper understanding of meiotic maturation, adding another layer of APC/C\(^{Cdh1}\) regulation in mammalian oocytes.

**MATERIALS AND METHODS**

**Oocyte collection, culture and microinjection**

All procedures for mouse care and use were conducted in accordance with the guidelines and approved by the Institutional Animal Care and Use Committees of Sungkyunkwan University (approval ID: SKKUIACUC2018-04-01-3). Mice were purchased from a local company (Koatech, Korea). Ovaries were isolated from 3- to 6-week-old CD-1 female mice 46-48 h after intraperitoneal injection of 10 IU of pregnant mare’s serum gonadotropin (PMSG). Oocytes were collected from follicles and recovered in M2 medium supplemented with 200 μM IBMX to prevent meiotic resumption. For in vitro maturation, oocytes were washed and cultured in IBMX-free M2 medium in a 5% CO\(_2\) atmosphere at 37°C. All reagents and media were purchased from Sigma-Aldrich, unless otherwise stated.

For microinjection, 5-10 μl of solution containing 20 μM siRNA, 300 ng/μl mRNA, 1 μg/μl dsRNA or 1 mM MO, was injected into the cytoplasm of fully recovered MII oocytes. For Cdc14B knockdown, double-stranded RNA targeting Cdc14B has been described previously (Reis et al., 2006). The full-length cDNA encoding human Mis12 was obtained by PCR from a 293T cell cDNA library and inserted into pRNA3-mCherry or pcDNA3.1-GFP/V5 (Thermo Fisher Scientific). The primers used were: Cdc14B, 5′-ATTAATACGACTAACTATAGGGAGAATGGTGAGCAAGGGCGAG-3′ and 5′-ATTAATACGACTACCTATGATGGGAGAGGAACATGGTGACAGAAGGGGAG-3′.

**Plasmid construct, morpholino and RNA preparation**

The full-length cDNA encoding human Mis12 was obtained by PCR from a 293T cell cDNA library and inserted into pRNA3-mCherry or pcDNA3.1-GFP/V5 (Thermo Fisher Scientific). The pRNA3-cyclin B1-GFP has been described previously (Reis et al., 2006). The fidelity of the constructions was confirmed by DNA sequencing. The mRNAs for microinjection were prepared with a mMESSAGE mMACHINE Kit (Ambion). The mRNA synthesis was performed by reverse transcription and digestion, followed by polyadenylation, purification and dissolved in nuclease-free water.

**Zygote collection, culture and microinjection**

To obtain zygotes, BDF1 mice were superovulated by the administration of 5 IU PMSG and, 48 h later, with 5 IU hCG. In vivo-fertilized zygotes were collected 20 h post-hCG from the oviduct ampullae of superovulated BDF1 females that had been mated with the BDF1 males. After removing cumulus cells, zygotes were cultured in KSOM medium at 37°C in a 5% CO\(_2\) atmosphere. For injection, zygotes were transferred to M2 medium and siRNA was microinjected into the cytoplasm of zygotes.

**MPF activity assay**

MPF activity was measured using the CDK1 Assay Kit (BPS Bioscience) according to the manufacturer’s instructions. Each assay was performed using 50 oocytes.

**Immunoblotting analysis**

Oocytes were lysed in SDS sample buffer and subjected to SDS-PAGE. After transfer, the membranes were blocked in 5% nonfat dry milk at room temperature for 1 h, and then incubated with primary antibodies overnight at 4°C. After washing three times in Tris-buffered saline with 0.05% Tween 20 [100 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween 20], membranes were incubated with secondary antibodies for 1 h. The blots were developed with the ECL Plus Western Blotting Detection Kit (GE Healthcare). Cropped sections of blots were shown in the main figures with the full length blot included in Fig. S6. The primary antibodies used for immunoblotting were: anti-Mis12 (Abcam, ab70843; 1:1000), anti-cyclin B1 (Cell Signaling Technology, 4138; 1:1000), anti-cyclin B2 (R&D Systems, AF6004; 1:1000), anti-Heclin (Abcam, ab3163; 1:1000), anti-Cdh1 (Abcam, ab3242; 1:1000), anti-pY15-Cdk1 (Cell Signaling Technology, 9111; 1:1000), anti-Cdk1 (Santa Cruz Biotechnology, sc-954; 1:1000), anti-Cdc14B (Abcam, ab203625; 1:5000) and anti-β-actin (Cell Signaling Technology, 4967; 1:2500). The secondary antibodies were HRP-labeled mouse (Jackson ImmunoResearch, 711-005-152; 1:5000) and goat (Sigma-Aldrich, A5420; 715-005-150; 1:10000), rabbit (Jackson ImmunoResearch, 711-005-152; 1:5000) and goat (Sigma-Aldrich, A5420; 1:5000) antibodies.

**Immunofluorescence analysis**

Oocytes at specific stages were fixed in 4% paraformaldehyde for 30 min, permeabilized in PBS with 0.05% Triton X-100 for 40 min, and blocked in PBS with 1% bovine serum albumin for 1 h. Oocytes were then incubated with primary antibodies followed by Alexa Fluor-conjugated 488 (Jackson ImmunoResearch, 715-546-150; 1:500 or 711-545-152; 1:100) and 594 secondary antibodies (Jackson ImmunoResearch, 115-585-044; 1:100). DAPI was used for DNA counterstaining. At least 20 oocytes were examined for each group, unless otherwise stated. The antibodies used in this study were: anti-V5 (Cell Signaling Technology, 13202; 1:100; or Invitrogen, R960-25; 1:100), anti-Cdc14B (Abcam, ab203625; 1:100), anti-acetylated-α-tubulin (Sigma-Aldrich, T7451; 1:500) and anti-centromere (Antibodies Incorporated, 15-234-0001; 1:50). Images were acquired using an LSM 700 confocal laser-scanning microscope (Zeiss) equipped with a C-Apochromat 63 × 1.2 water immersion objective. All microscope settings were set to collect images below saturation and were kept constant for all images. Optical sections were obtained at 1 μm intervals and converted into maximum intensity projections. The spindle intensity was measured and normalized to mean DAPI intensities using ZEN 2010 LSM software.
(Zeiss), and values were expressed as mean values in arbitrary fluorescence units.

**Cold treatment and chromosome spreading**
For analysis of kMT attachment, oocytes at the MI stage were incubated in ice-cold M2 medium for 10 min. After cold treatment, oocytes were fixed and subjected to the immunofluorescence analysis.

For chromosome spreading, MI oocytes were exposed to acidic Tyrode’s solution for 1 min to remove the zona pellucida. After a brief recovery in the M2 medium, the oocytes were fixed in 1% paraformaldehyde in distilled water (pH 9.2) containing 0.15% Triton X-100 and 3 mM dithiothreitol. The slides were dried slowly in a humid chamber for several hours and subjected to the immunofluorescence analysis.

**Time-lapse imaging**
Oocytes injected with cyclin B1-GFP mRNA were placed in a heated solution of M2 medium, the oocytes were fixed in 1% paraformaldehyde in distilled water (pH 9.2) containing 0.15% Triton X-100 and 3 mM dithiothreitol.

**Statistical analysis**
Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software). Data are representative of at least three independent experiments, unless otherwise specified. The significance of differences between groups was analyzed by a paired two-tailed Student’s t-test, and P<0.05 was considered statistically significant.

**Author contributions**

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**Supplementary information**
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**Competing interests**
The authors declare no competing or financial interests.

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Fig. S1. Knockdown of Cdh1. GV oocytes injected with control (Ctrl) or Cdh1 morpholino (MO) were cultured for 12 h in the presence of IBMX. (A) Knockdown of Cdh1 was confirmed by immunoblot analysis. β-actin was used as a loading control. Each lane contains 50 oocytes. (B) The normalized level of Cdh1 was quantified from three independent experiments. Data are mean+SEM. p**<0.001.
**Fig. S2. Knockdown of Cdc14B.** GV oocytes injected with Cdc14B dsRNA (dsCdc14B) were cultured for 12 h in the presence of IBMX. As a control, oocytes were injected with EGFP dsRNA. (A) Knockdown of Cdc14B was confirmed by immunoblot analysis. β-actin was used as a loading control. Each lane contains 100 oocytes. (B) The normalized level of Cdh1 was quantified from three independent experiments. (C) The rate of GVBD was determined after incubating with 50 μM IBMX for 24 h. Data are mean±SEM from three independent experiments. p***<0.0001.
**Figure S3**

**A**

Fig. S3. Cyclin B2 is not associated with Mis12 depletion. (A) Immunoblot analysis of cyclin B2 in oocytes injected with siCtrl, siMis12, siMis12+Cdh1 MO, and siMis12+dsCdc14B. β-actin was used as a loading control. Each lane contains 50 oocytes. (B) Normalized level of cyclin B2 was quantified from three independent experiments. Data are mean+SEM.
Fig. S4. Knockdown of Mis12 in zygotes. (A-C) Zygotes were injected with siCtrl, siMis12, or siMis12+cyclin B1-GFP. After 24 h culture, rate of 2-cell development was determined. The percentage of 2-cell embryos was shown with the representative images. Arrowheads indicate nuclei in zygotes. Scale bar, 100 μm. Data are mean±SEM from three independent experiments. p***<0.0001. (C) The expression and cytoplasmic localization of cyclin B1-GFP was confirmed by fluorescence signal.
Fig. S5. Subcellular localization of Mis12 in somatic cells. (A, B) HEK293 (A) and H1299 (B) cells were transfected with hMis12-GFP/V5 vector for 48 h. The cells were stained with anti-centromere antibody (ACA) with DAPI. Scale bar, 10 μm.
Fig. S6. Full blots of figures.