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Degradation of Ccnb3 is essential for maintenance of MII arrest in oocyte

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ABSTRACT

Before fertilization, ovulated mammalian oocytes are arrested at the metaphase of second meiosis (MII), which is maintained by the so-called cytostatic factor (CSF). It is well known that the continuous synthesis and accumulation of cyclin B is critical for maintaining the CSF-mediated MII arrest. Recent studies by us and others have shown that Ccnb3 is required for the metaphase-to-anaphase transition during the first meiosis of mouse oocytes, but whether Ccnb3 plays a role in MII arrest and exit remains unknown. Here, we showed that the protein level of Ccnb3 gradually decreased during oocyte meiotic maturation, and exogenous expression of Ccnb3 led to release of MII arrest, degradation of securin, separation of sister chromatids, extrusion of the second polar body (PB2), and finally entry into interphase. These phenotypes could be rescued by inhibition of Wee1B or CDK2. Our results indicate that Ccnb3 plays a critical regulatory role in MII arrest and exit in mouse oocytes.

1. Introduction

Meiosis is a cell division pattern unique to the formation of haploid gametes in sexually reproducing animals. During meiosis, the chromosomes are replicated once, and then the cell divides twice in succession. During the first meiosis, homologous chromosomes are separated and sister chromatids are still linked together; sister chromatids are separated during the second meiosis, resulting in a halving of the number of chromosomes in the gametes. In mice, ovulated mature oocytes are arrested at the metaphase of second meiosis (MII) until fertilization by a cytostatic factor (CSF) [1,2]. CSF is a multi-subunit E3 ubiquitin ligase3, which inhibits the anaphase-promoting complex/cyclosome (APC/C) and thereby preventing the degradation of cyclin B and thus exit from MII stage [3] by regulating the kinase activity of maturation promoting factor (MPF), which consists of cyclin B (Ccnb) and cdc2 (also known as CDK1) [4]. The kinase activity of MPF is regulated by the phosphorylation of cdc2 residues, and Wee1B and Cdc25 play antagonistic roles in regulating the activity of cdc2. Wee1B is a protein kinase that phosphorylates $Cdc2^{Y15}$ and inhibits Cdc2 kinase activity [5], while Cdc25 is a protein phosphatase that dephosphorylates p-$Cdc2^{Y15}$and activates Cdc2 kinase activity [6].

Cyclin synthesis and degradation cooperate with cyclin-dependent kinases (CDKs) to control the progression of both meiosis and mitosis. B-type cyclins represent essential components regulating meiotic progression. Three mammalian B-type cyclins have been identified, B1, B2 and B3 [7]. Our latest study reveals a compensatory mechanism between cyclinB2 and cyclin B1 in MPF activation in meiosis I, updating our understanding of MPF regulation [8]. Cyclin B3 (Ccnb3) is conserved across higher eukaryote evolution. Compared to other cyclic proteins, Ccnb3 protein has a higher molecular weight. Prolonged Ccnb3 expression perturbed spermatogenesis and cyclin B3 interacted with CDK2 [9,10]. In our previous report, RNAi-mediated knockdown of *ccnb3* in oocytes resulted in meiotic MI arrest [11]. Recently, *ccnb3* knockout has further demonstrated that Ccnb3 participates in the separation of homologous chromosomes during the first meiotic process by forming a complex with CDK1 [12,13]. However, because both RNAi and $ccnb3^{-/-}$ cause metaphase I arrest of the meiotic cell cycle, whether ccnb3 is involved in the regulation of MII arrest and release remains unclear.

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To gain insights into the potential roles of Ccnb3 in meiosis II in oocyte, we exogenously expressed Ccnb3 by microinjection of *ccnb3* mRNA in MII oocytes. Strikingly, we found that exogenous expression of Ccnb3 induced MII exit and separation of sister chromatids. Furthermore, both Wee1B inhibitor and CDK2 inhibitor can antagonize the effects of exogenously expressed Ccnb3 on MII exit in mouse oocytes. Our data have updated our understanding on the regulation of oocyte MII arrest and exit.

2. Materials and methods

2.1. Mouse oocytes collection and culture

The GV oocytes were collected from ovaries of 6–8 week-old ICR female mice in M2 medium (Sigma) supplemented with or without 200 μM IBMX.

To collect MII oocytes, each 6–8-week-old ICR female mouse was injected with 10 IU PMSG, followed by 10 IU hCG after 48 h to promote oocyte maturation and ovulation. The oviducts were dissected and cumulus–oocyte complexes were collected at 14h of hCG treatment. After a 2min treatment with 0.5 mg/ml hyaluronidase (Sigma, St. Louis, MO, USA) in the M2 medium, oocytes were collected. Oocytes were cultured in the M2 medium (Sigma) under paraffin oil at 37 °C with 5% CO_2 in air.

2.2. Western blot analysis

Western blot was performed as described previously (???). All the related primary antibodies used as follows: mouse anti-cyclin B3 (C500, 1:500), rabbit polyclonal anti-phospho-CDC2 (CDK1)-Y15(ABclonal, AP0016, 1:1000), rabbit monoclonal anti-C-Myc antibody (Sigma, M4439, 1:2000) or mouse polyclonal anti-β-actin antibody (Zhongshanjinqiao, TA-09, 1:2000).

2.3. Chromosome spreading

Chromosome spreads were performed as described previously [14]. Briefly, the oocytes were placed in acid Tyrode's solution (Sigma) for 1 min at 37 °C to remove the zona pellucida. After three washes with M2 medium, the oocytes were placed onto glass slides and fixed in a solution of 1% paraformaldehyde in distilled H_2O (pH 9.2) containing 0.15% Triton X-100 and 3 mM dithiothreitol. The slides were allowed to dry slowly in a humid chamber for several hours, and then blocked with 1% BSA in PBS for 1 h at room temperature. The slides were then incubated with Hec1 (1:100), a component of the essential kinetochore-associated NDC80 complex, overnight at 4 °C. After brief washes with washing buffer, chromosomes on the slides were stained with the corresponding secondary antibodies and 5 μg/ml DAPI, and the specimens were mounted for immunofluorescence microscopy observation.

2.4. Immunofluorescence and confocal imaging

Oocytes and embryos were washed in M2 medium and fixed in 4% paraformaldehyde in PBS for 30 min, permeabilized for 20 min in 0.1% Triton X-100 in PBS, and then blocked in PBS containing 1 mg/ml BSA(PBS/BSA) for 1h at room temperature. After blocking, the cells were stained with α-tubulin overnight at 4 °C. After washing three times with PBS/BSA, the cells were incubated for 1 h with specific fluorescent secondary antibodies at room

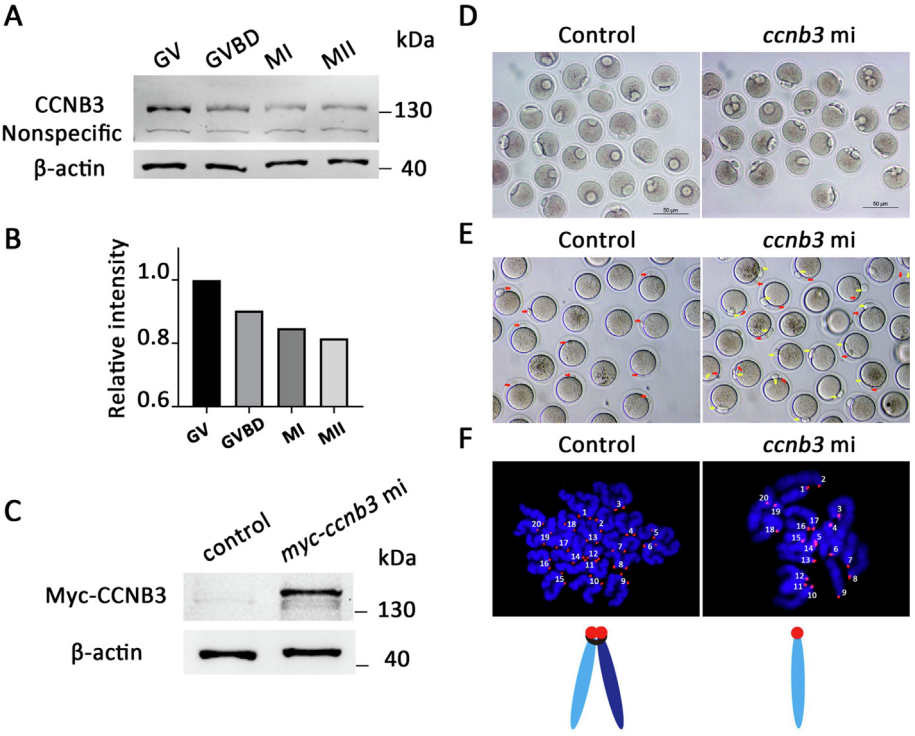

Fig. 1. Exogenous expression of Ccnb3 leads to MII exit. (A) Oocytes were collected after culturing for 0, 4, 8 and 12 h, corresponding to the GV, GVBD, MI and MII stages, respectively. Level of β-actin was used as an internal control. A total of 150 oocytes were used for each lane; (B) The relative level staining intensity of Ccnb3 was accessed by gray-scale analysis carried out by software Quantity One (Bio-Rad); (C) Expression of Myc-Ccnb3 in the GV oocytes microinjected with *myc-ccnb3* mRNA, and the control GV oocytes were microinjected with nuclease-free water. The GV oocytes were cultured to 8 h for the expression of mRNA in M2 medium containing 2.5 μM Milrinone, and then collected for western blot. A total of 100 oocytes were used per sample. The exogenous Myc-Ccnb3 was detected with anti-Myc antibody; (D) The GV oocytes microinjected with *ccnb3* mRNA showed failure of MII arrest and extrusion of second polar body when cultured for18 h; (E) MII oocytes microinjected with *ccnb3* mRNA showed MII exit and extrusion of secondary polar body (red arrows indicate PB1, yellow arrows indicate PB2); (F) Chromosome spreads of MII oocytes in control and oocytes$^{exo-Ccnb3}$ groups.

temperature, followed by incubation with Hoechst 33342 for 20 min. These cells were mounted on glass slides and examined with a Zeiss LSM 780 confocal laser-scanning microscope.

2.5. Time-lapse confocal microscopy of live oocytes

To analysis whether the MII exit was due to the activation of APC/C activity in MII oocytes, we tracked the dynamic changes of the APC/C substrate, securin, after exogenous expression of Ccnb3. MII oocytes were injected with 10 pl of 200ng/ul mRNA econding securin-mCherry in M2 medium. Following mRNA injection, oocytes were cultured for 2h at 37 °C to allow SECURIN-mCherry protein expression. Then half of these treated oocytes were injected with 10 pl of 200ng/ul mRNA encoding myc-ccnb3 in M2 medium. Then oocytes were transferred and cultured in M2 medium placed in a European Molecular Biology Laboratory environmental microscope incubator (GP106), allowing cells to be maintained in a 5% CO2 atmosphere at 37 °C with humidity control during imaging. Time-lapse image acquisitions were performed using a customized Zeiss LSM510 META confocal microscope equipped with a 532-nm excitation laser, a long-pass 545-nm emission filter, a 403 C-Apochromat 1.2 NA water immersion objective lens, and an in-house developed 3D tracking macro (Rabut and Ellenberg, 2004).

2.6. Inhibitor treatment

Inhibitors were prepared as 50 mM stock solutions in DMSO and stored at −80 °C. MII oocytes were collected and transferred into M2 medium containing Wee1 inhibitor (50 μM, Selleckchem, MK-1775), type II CDK2 inhibitor (20 μM, TargetMol, K03861). MII oocytes cultured in M2 medium containing equivalent DMSO were used as control.

2.7. Statistical analysis

All data presented were collected in at least three independent experiments and analyzed using GraphPad Prism 8. Data were expressed as mean ± S.E.M. and significance of differences was evaluated with Student's t-test.

3. Results

3.1. Exogenous expression of Ccnb3 leads to MII exit

Given that both RNAi-mediated depletion of *ccnb3* and *ccnb3* knockout perturbed oocyte meiosis I progression, so we asked whether ccnb3 has a potential role in meiosis II in oocyte. We first examined the expression profile of CCNB3 during oocyte meiotic maturation by western blot, and found that the protein level of Ccnb3 gradually decreased (Fig. 1A and B). Then mRNA encoding Myc-Ccnb3 was injected into wild-type GV oocytes, and oocytes were cultured in M2 medium (Fig. 1C). Exogenously expressed Ccnb3 did not affect meiotic resumption and first polar body extrusion in oocytes (data was not shown). However, these oocytes microinjected with *myc-ccnb3* mRNA showed failure in MII arrest and extruded the second polar body when cultured to 18h (Fig. 1D). Hence, our results suggested that exogenously expressed ccnb3 may cause MII arrest release. To test this, *myc-ccnb3* mRNA was directly injected into wild-type MII oocytes. As we expected, almost all oocytes discharged the second polar body (Fig. 1E). We further observed that sister chromatids were segregated by chromosome spreading (Fig. 1F).

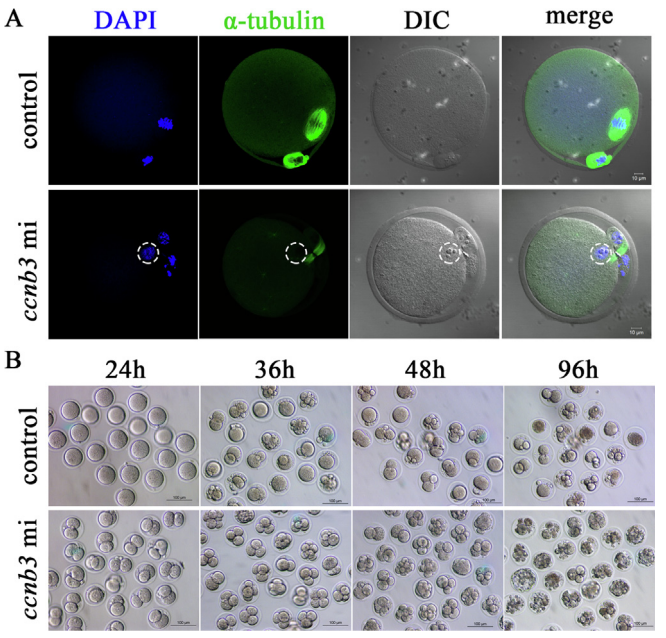

Fig. 2. Formation of pronuclei after *ccnb3* mRNA microinjection in MII oocyte. (A) Representative immunofluorescence images of formation of pronuclei in MII oocytes$^{exo-Ccnb3}$; (B) Representative images of oocytes$^{exo-Ccnb3}$ were cultured in KSOM at different time points. Scale bars: 20 μm.

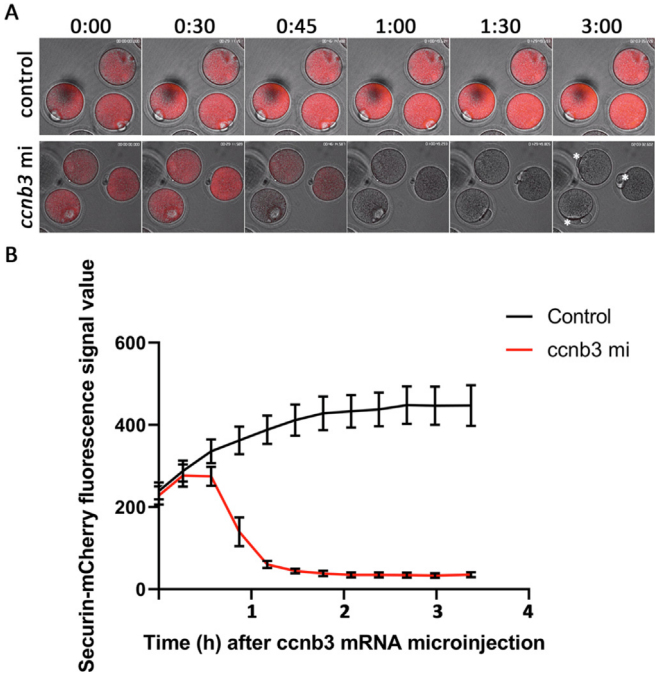

Fig. 3. MII arrest disruption is associated with complete APC/C activation in MII oocytes$^{exo-Ccnb3}$. (A) Time-lapse fluorescence measurement of Securin-mCherry expression of indicated mRNA injection in MII oocytes; (B) The integrated intensities of mCherry were measured, background corrected, and normalized to the initial-intensity value obtained per oocyte. At least 20 oocytes for Securin-mCherry expression were analyzed. Measurements were aligned after Securin-mCherry mRNA microinjected for 1 h as the starting time. Statistical analyses for differential Securin-mCherry fluorescence intensity changes. Error bars represent mean ± SEM.

3.2. Formation of pronuclei after ccnb3 mRNA microinjection in MII oocyte

Generally, fertilization triggers the MII exit and completion of meiosis II with segregation of sister chromatids, then paternal and maternal pronuclei are formed. To further define the MII exit caused by exogenously expressed ccnb3, the oocytes were fixed and stained with anti-α-tubulin-FITC and DAPI. As shown in Fig. 2A, the pronuclei were detected adjacent to cell membrane. In order to observe the development potential of the oocytes exogenously expressed Ccnb3 after MII exit, then these oocytes were cultured in KSOM medium. Similar with normal fertilization, majority of these oocytes could develop to 2 cell-like embryos, 4 cell-like embryos and 8 cell-like embryos. However, very few oocytes could develop into blastocysts (Fig. 2B).

MII arrest disruption is associated with complete APC/C activation in oocytes with exogenously expressed Ccnb3.

To test whether the failure of MII arrest was due to the insufficiency of APC/C activity in exogenously expressed Ccnb3 MII oocytes (MII oocytes$^{exo-Ccnb3}$), we traced dynamic changes of the APC/C substrates, securin, by microinjecting both *securin-mcherry* mRNA and *myc-ccnb3* mRNA into MII oocytes. The red fluorescence intensity remained almost unchanged in control oocytes, while the oocytes microinjected with *myc-ccnb3* mRNA experienced a significant decline in PB2 extrusion (Fig. 3). Our results suggested that there was a significant increase of APC/C activity for Securin degradation in MII oocytes$^{exo-Ccnb3}$, and exogenously expressed Ccnb3 could cause full activation of APC/C, thus disrupting the MII arrest in oocytes.

3.3. Wee1B inhibitor and CDK2 inhibitor can antagonize the effects of exogenously expressed Ccnb3 on MII exit in mouse oocytes

It is well known that Emi2 is involved in maintenance of CSF arrest in an egg. Cdc2/Cyclin B-mediated Emi2 phosphorylation could inhibit Emi2 bounding to the core APC/C. Upon fertilization or activation, meiosis resumes, and Cdc2/Cyclin B activity declines. We measured Cdc2 activity by examining its Tyr15 phosphorylation level, which suggested the inhibitory state of Cdc2 activity. As shown in Fig. 4A, there was a significant increase of Tyr15 phosphorylation level of Cdc2 (Cdc2^{Y15}). Given that Wee1B could phosphorylate Cdc2^{Y15} and inhibits Cdc2 kinase activity, we speculated that inhibition of Wee1B activity may rescue the disruption of MII arrest. Then we found that there was no extrusion of second polar body in MII oocytes$^{exo-Ccnb3}$ treated with MK-1775, an inhibitor of Wee1B (Fig. 4B and C). In a previous study, it was found that Ccnb3 interacted with CDK2 [9]. MII oocytes$^{exo-Ccnb3}$ were cultured in M2 medium with K03861, a type II CDK2 inhibitor.

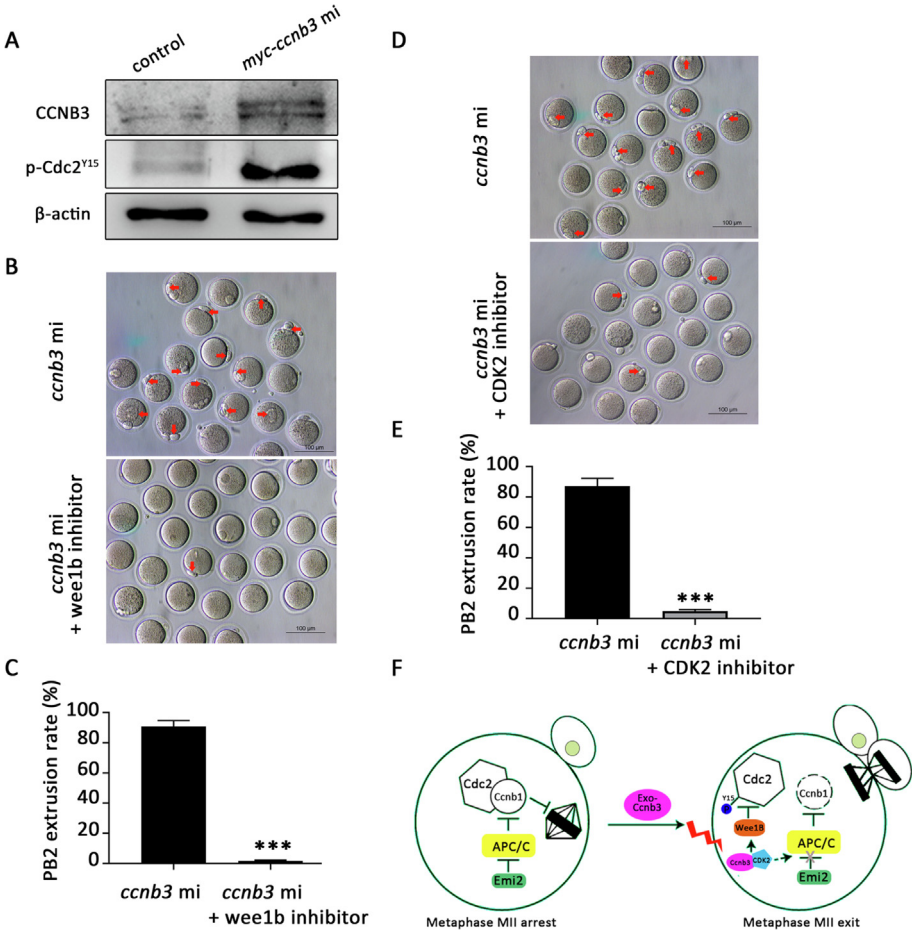

Fig. 4. Wee1B inhibitor and CDK2 inhibitor can antagonize the failure of MII oocytes$^{exo-Ccnb3}$ arrest. (A) Western blotting of p-Cdc2^{Y15} and Ccnb3 in MII oocytes injected with *myc-ccnb3* mRNA and nuclease-free water, respectively. Level of β-actin was used as an internal control. A total of 150 oocytes were used for each lane. The endogenous and exogenous Ccnb3 was detected with anti-Ccnb3 antibody (C500); (B) Representative images of MII oocytes$^{exo-Ccnb3}$ cultured in M2 medium containing Wee1B inhibitor. (red arrows indicate PB2); (C) Percentage of PB2 extrusion after Wee1B inhibitor treatment in MII oocytes$^{exo-Ccnb3}$. Data are presented as means ± SEM; (D) Representative images of MII oocytes$^{exo-Ccnb3}$ were cultured in M2 medium containing CDK2 inhibitor. (red arrows indicate PB2); (E) Percentage of PB2 extrusion after CDK2 inhibitor treatment in MII oocytes$^{exo-Ccnb3}$. Data are presented as means ± SEM; (F) Schematic figure showing possible mechanism of MII arrest release in MII oocytes$^{exo-Ccnb3}$.

Likewise, as treated with MK-1775, majority of MII oocytes$^{exo-Ccnb3}$ no longer discharged the second polar body (Fig. 4D and E).

4. Discussion

Oocytes arrest at MII stage with high MPF activity as the result of existence of CSF. Although MPF is activated in both mitotic and meiotic cell cycles, the sustaining high MPF activity is only found in MII oocytes because the APC/C will become active and cyclin B will be degraded once the cell reaches metaphase of mitosis and the first meiosis. Furthermore, Emi2 could competitively inhibits APC/C–Ube2S binding before fertilization, while its degradation in fertilization relieves the inhibition for APC/C activation [15].

Various cyclic proteins share a common molecular structural feature, which contains a 9-residue destruction motif, the destruction box (D-box) [16]. B-type cyclins represent essential regulating components in both mitosis and meiosis. Three mammalian B-type cyclins have been reported [7]. Unlike Cdc2 protein, the content of which in the cell cycle is relatively stable, the content of cyclin B is cyclical. Cyclin synthesis and degradation cooperate with cyclin-dependent kinases (CDKs) to control the progression of meiosis [3].

Ccnb3 is much larger than other cyclins as the result of extension of a single exon. Our previous reports and the latest reports show that Ccnb3 controls anaphase onset during meiosis I in oocytes, which is independent of the spindle assembly checkpoint. But it is unclear that whether Ccnb3 is involved in meiosis II maintenance and resumption. By immunoblotting, we showed that Ccnb3 protein levels gradually decreased during meiotic maturation, so we considered maintaining Ccnb3 protein level in eggs by exogenous expression of Ccnb3. Strikingly, we found that MII arrest was released.

Our further studies have found that the activity of Cdc2 is significantly decreased. Wee1B is a protein kinase that phosphorylates Cdc2^{Y15} and inhibits Cdc2 kinase activity. Naturally, we tried to use Wee1B inhibitor treatment to analyze the upstream and downstream relationship between Wee1B and Ccnb3. Wee1B inhibitor could antagonize the effects of exogenously expressed Ccnb3 on MII exit in mouse oocytes, which indicates that Ccnb3 is located upstream of the Wee1B signaling pathway.

Li and Karasu et al. reported that Ccnb3 mainly functions as a complex with cdc2 during meiosis [12,13]. What's more, prolonged cyclin B3 expression perturbed spermatogenesis and cyclin B3 interacted with CDK2 [9,10]. In MII oocytes$^{exo-Ccnb3}$, there was a significant increase of Cdc2^{Y15}, which suggests that Cdc2 is unlikely to be the functional partner of CCNB3. We hypothesize that whether CDK2 is the potential functional partner of Ccnb3 in MII oocytes$^{exo-Ccnb3}$. Then we found that CDK2 inhibitor could rescue the failure of MII arrest in MII oocytes$^{exo-Ccnb3}$, which indicated that exogenously expressed Ccnb3 could cause MII exit by interact with CDK2.

In summary, our study suggests that a low protein level of Ccnb3 is essential for maintaining the MII arrest in oocyte. Our results may provide an important in vitro model (Fig. 4F) for further understanding the mechanism of MII arrest and exit of mammalian oocytes.

Declaration of competing interest

All the authors declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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