© 2019. Published by The Company of Biologists Ltd | Development (2019) 146, dev182519. doi:10.1242/dev.182519

RESEARCH ARTICLE

The cyclin B2/CDK1 complex inhibits separase activity in mouse oocyte meiosis I

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ABSTRACT

Chromosome segregation is driven by separase, activity of which is inhibited by binding to securin and cyclin B1/CDK1. In meiosis, premature separase activity will induce aneuploidy or abolish chromosome segregation owing to the untimely destruction of cohesin. Recently, we have proved that cyclin B2 can compensate for cyclin B1 in CDK1 activation for the oocyte meiosis G2/M transition. In the present study, we identify an interaction between cyclin B2/CDK1 and separase in mouse oocytes. We find that cyclin B2 degradation is required for separase activation during the metaphase I-anaphase I transition because the presence of stable cyclin B2 leads to failure of homologous chromosome separation and to metaphase I arrest, especially in the simultaneous absence of securin and cyclin B1. Moreover, non-phosphorylatable separase rescues the separation of homologous chromosomes in stable cyclin B2-arrested cyclin B1-null oocytes. Our results indicate that cyclin B2/CDK1 is also responsible for separase inhibition via inhibitory phosphorylation to regulate chromosome separation in oocyte meiosis, which may not occur in other cell types.

KEY WORDS: Cyclin B2, Separase, Meiosis, Oocyte

INTRODUCTION

In order to produce a haploid egg, mammalian oocytes undergo two continuous cell divisions, with separation of homologous chromosomes first and separation of sister chromatids second (Petronczki et al., 2003). The two rounds of chromosome segregation are executed by separase-dependent cleavage of cohesin, which is under precise regulation. In meiosis I, only the cohesin on chromosome arms is removed by separase to resolve the chiasmata to allow the separation of homologous chromosomes. In meiosis II, the centromeric cohesin is then removed by separase to achieve the separation of sister chromatids. Notably, the centromeric cohesin is protected from cleavage in meiosis I through Sgo2-dependent PP2A recruitment to centromeres. PP2A is responsible for dephosphorylation of the cohesin subunit Rec8, preventing it from being cleaved by separase in meiosis I (Kitajima et al., 2006; Riedel et al., 2006).

Importantly, separase activity needs to be controlled properly to prevent the damage of cohesin until all chromosomes are correctly attached and aligned at the metaphase plate. It is now accepted that separase is inhibited through mutual binding to securin (also known as PTTG1) (Lee and Orr-Weaver, 2001; Petronczki et al., 2003; Zou et al., 1999) and maturation-promoting factor (MPF), a heterodimer of cyclin B1 and CDK1 (Gorr et al., 2005; Holland and Taylor, 2006; Stemmann et al., 2001). Different from the direct inhibition of securin binding, cyclin B1/CDK1 inhibits separase through inhibitory phosphorylation of separase, and the binding of cyclin B1 to separase enhances this phosphorylation (Gorr et al., 2005); in turn, separase is an inhibitor of cyclin B1/CDK1 (Gorr et al., 2005, 2006). Degradation of securin and cyclin B1 is dependent on the anaphase-promoting complex or cyclosome (APC/C) activity (Herbert et al., 2003; Reis et al., 2007), which is activated after the spindle assembly checkpoint (SAC) (Wassmann et al., 2003).

However, although deletion of securin results in lethal phenotypes in yeast (Ciosk et al., 1998) and *Drosophila* (Stratmann and Lehner, 1996), securin-mutant mice are viable and fertile (Mei et al., 2001; Wang et al., 2001), suggesting that securin is not the only regulator required for separase inhibition in mammalian cells. The cyclin B1/CDK1 complex seems to take on the responsibility for separase inhibition in the absence of securin in oocyte meiosis because securin loss induced by morpholino in germinal vesicle (GV) oocytes does not lead to premature separation of homologues during meiosis I (Chiang et al., 2011; Nabti et al., 2008). Nevertheless, we previously found that cyclin B1-null oocytes unexpectedly resumed and went through meiosis I because of compensation by cyclin B2 (Li et al., 2018), which potentially activated CDK1 for MPF formation. We raised the hypothesis that cyclin B2 may be involved in the regulation of separase activity in oocyte meiosis.

In this study, we focused on the role of cyclin B2 in separase inhibition in cyclin B1-null mouse oocytes. We show that cyclin B2 degradation is required for anaphase onset and separation of homologous chromosomes, and that the cyclin B2/CDK1 complex controls separase activity through inhibition of phosphorylation. Remarkably, cyclin B2/CDK1 interacts with separase by binding of cyclin B2 with separase. Our findings reveal an unexpected role for cyclin B2 in restraining separase activity, providing new evidence for the crucial function of cyclin B2 in oocyte meiosis progression.

RESULTS

Homologous chromosomes separate normally during meiosis I in cyclin B1-null oocytes

We recently reported that cyclin B1-null oocytes did not arrest at the metaphase of meiosis II (MII) after the first polar body extrusion (PBE) but entered interphase with nucleus reformation (Li et al., 2018). Notably, the chromatin underwent decondensation, which prevented the analysis of chromosome separation in meiosis I. This phenotype appeared to be the result of inactivation of CDK1. Because both CDK1 and protein phosphatase 2A (PP2A) are involved in MII arrest (Adhikari et al., 2014), and a major role of

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Received 11 July 2019; Accepted 4 November 2019

Development (2019) 146, dev182519. doi:10.1242/dev.182519

CDK1 is to inactivate PP2A through the Greatwall kinase (MASTL) (Hara et al., 2012), we wondered whether inhibition of PP2A could break the interphase arrest in *GDF9-Ccnb1*$^{-/-}$ oocytes. To test this hypothesis, mature *GDF9-Ccnb1*$^{-/-}$ oocytes were collected from oviducts after superovulation and treated with the protein phosphatase inhibitor okadaic acid (OA; 50 nM) (Adhikari and Liu, 2014; Chang et al., 2011; Tay et al., 2012). As expected, we found that OA treatment led to breakdown of the nuclear membrane and chromatin condensation in *GDF9-Ccnb1*$^{-/-}$ oocytes, but no metaphase II spindle was formed (Fig. 1A). This implies that cyclin B1 may be required for spindle assembly during meiosis II in mouse oocytes.

Because OA treatment could restore the condensation of chromosomes, we checked whether homologous chromosomes separated normally in the absence of cyclin B1. After PBE, *GDF9-Ccnb1*$^{-/-}$ oocytes were transferred into OA-containing M2 medium and chromosomes were spread as soon as possible (Fig. 1B). To identify each chromatid clearly, we stained the centromeres before DNA staining. We found that the number of centromeres in the *GDF9-Ccnb1*$^{-/-}$ oocytes was the same as that of control oocytes (Fig. 1C), indicating that homologous chromosomes separated equally in the absence of cyclin B1. Moreover, the univalents were maintained well after PBE in the *GDF9-Ccnb1*$^{-/-}$ oocytes because the centromeric cohesin was intact (Fig. 1D).

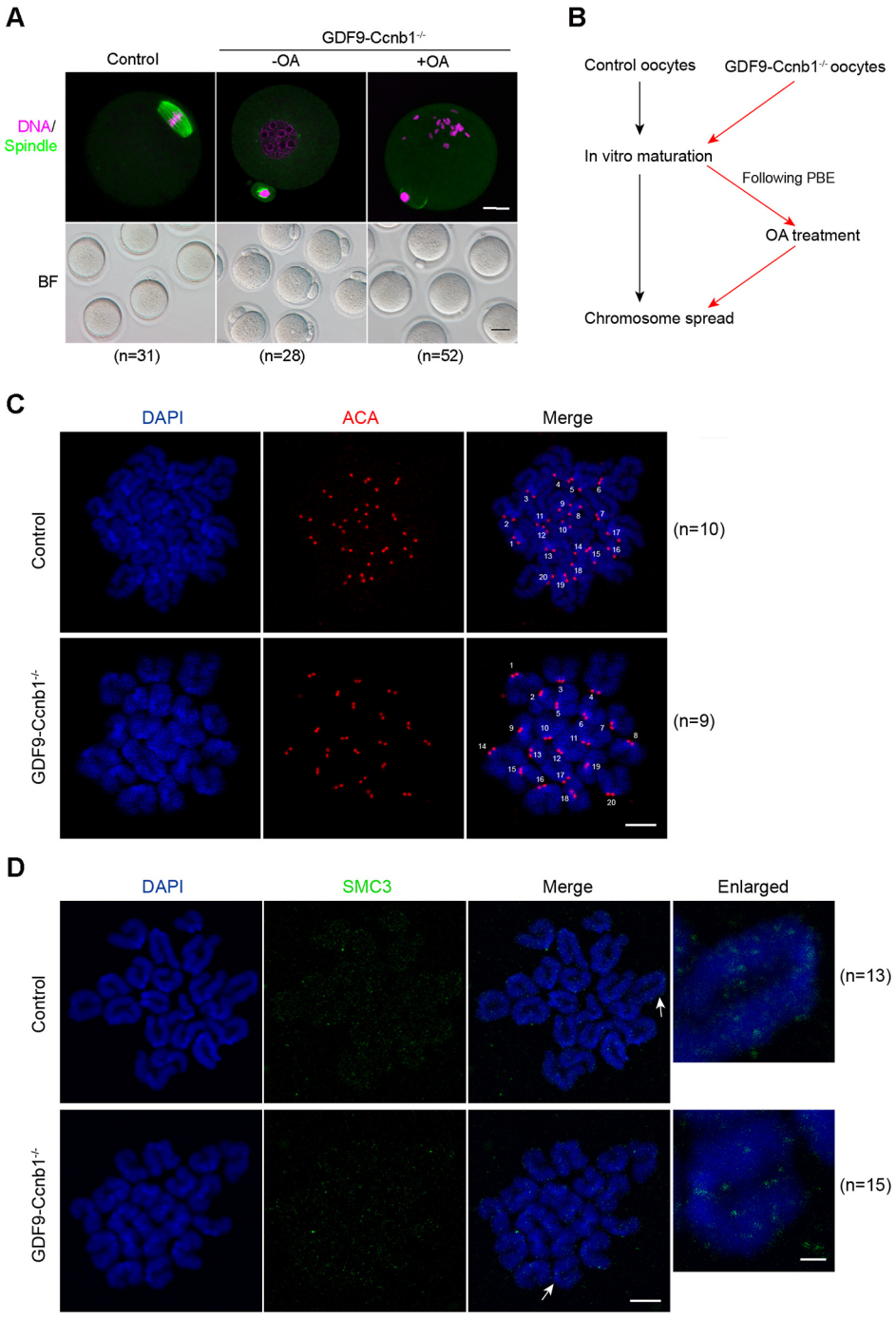

Fig. 1. Normal separation of homologous chromosomes in cyclin B1-null oocytes. (A) OA treatment induced breakdown of the nuclear membrane and rescued chromatin condensation in ovulated *GDF9-Ccnb1*$^{-/-}$ oocytes. The ovulated *GDF9-Ccnb1*$^{-/-}$ oocytes were treated with OA and then fixed for immunofluorescence (IF); spindle and DNA were stained with α-tubulin-FITC antibody and DAPI, respectively. BF, brightfield; Scale bars: 20 µm (IF); 40 µm (BF). (B) Method used for chromosome analysis in control and *GDF9-Ccnb1*$^{-/-}$ oocytes. (C) Chromosome spreads for control and *GDF9-Ccnb1*$^{-/-}$ oocytes post-PBE. Centromeres and chromosomes were stained with anti-centromeric antibodies (ACA) and DAPI, respectively. Merge panel shows counting of sister centromere pairs. Scale bar: 5 µm. (D) Chromosome spread and centromeric cohesin detection following PBE in control and *GDF9-Ccnb1*$^{-/-}$ oocytes. Cohesin and chromosomes were stained with SMC3 and DAPI, respectively. Arrows indicate the dyads enlarged. Scale bars: 5 µm (main panels); 1 µm (enlarged panels). The numbers of oocytes used (*n*) are shown.

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Separase functions normally during meiosis I in cyclin B1-null oocytes

As mentioned above, separase directly promotes the separation of chromosomes and, importantly, appropriate supervision of separase activity is crucial for the correct segregation of homologous chromosomes. Generally, the arm cohesin can be cleaved by separase and centromeric cohesin is protected in meiosis I. The separase should be inhibited before metaphase I and released at the correct time during the metaphase I-anaphase I transition. The release of separase activity is achieved by APC/C-dependent degradation of securin and cyclin B1. Premature separase activity may induce the damage of cohesin before chromosomes are well aligned, leading to disjunction error and aneuploidy. Given that cyclin B1/CDK1 inhibits separase but chromosome segregation occurs normally in the cyclin B1-null oocytes, we proposed that the separase should exert proper control during meiosis I in the cyclin B1-null oocytes. To test the separase activity and integrity of cohesin, we determined the state of chromosomes and the integrity of cohesin during metaphase I in cyclin B1-null oocytes. We found that the bivalents were maintained well around the metaphase I plate in the *GDF9-Ccnb1*$^{-/-}$ oocytes (Fig. 2A); in addition, the expression of Smc3, a subunit of cohesin, in the *GDF9-Ccnb1*$^{-/-}$ oocytes was comparable to that of the control oocytes (Fig. 2B,C). These results suggested that the separase activity was well controlled during meiosis I in cyclin B1-null oocytes.

Cyclin B2 is degraded during the metaphase I-anaphase I transition in oocytes

Considering that cyclin B2 can compensate for cyclin B1 in meiosis I progression, cyclin B2/CDK1 should control separase activity in

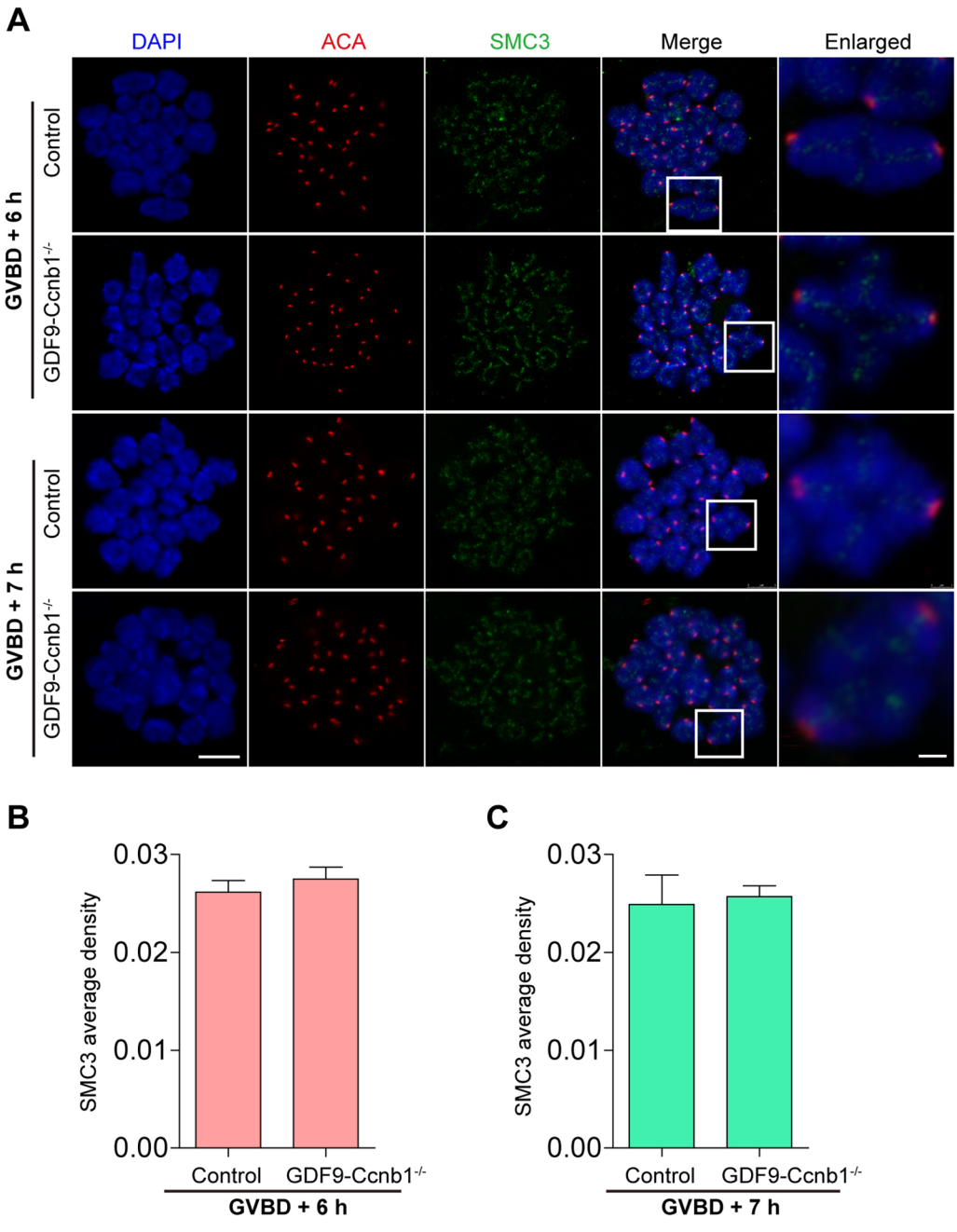

Fig. 2. The integrity of cohesin during meiosis I in cyclin B1-null oocytes. (A) Chromosome spread and SMC3 detection around metaphase I in control and *GDF9-Ccnb1*$^{-/-}$ oocytes. Oocytes were examined at 6 and 7 h after GVBD. Enlarged panels show high-magnification views of the boxed areas. ACA, anti-centromeric antibodies. Scale bars: 5 µm (main panels); 1 µm (enlarged panels). (B,C) Analysis of SMC3 density in oocytes at 6 h (B) and 7 h (C) after GVBD. Data are presented as mean+s.e.m.

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cyclin B1-null oocytes. To address this point, we firstly wanted to explore the expression pattern of cyclin B2 in the cyclin B1-null oocytes. To trace the temporal and spatial expression of cyclin B2, we performed confocal live-cell imaging for 18 h using the fusion protein cyclin B2-Venus (Fig. 3A). We found that cyclin B2-Venus increased from the GV stage to metaphase I and reached a peak at metaphase I, then it dropped sharply before PBE both in the control and in *GDF9-Ccnb1*$^{-/-}$ oocytes (Fig. 3B; Movies 1 and 2).We therefore suggest that cyclin B2 degradation was coordinated with subsequent anaphase onset and chromosome segregation in a chronological order.

Expression of stable cyclin B2 prevents the metaphase I-anaphase I transition and chromosome separation

To confirm that cyclin B2 degradation is important for anaphase I onset in the cyclin B1-null oocytes, we designed experiments to stabilize the expression of cyclin B2 by destroying its D-box (destruction box). The N-terminal 50 amino acids of cyclin B2 were deleted to construct a Δ50cyclin B2-Venus fusion protein that is undegradable by APC/C. Then, *Δ50cyclin B2-Venus* mRNA was injected into control and *GDF9-Ccnb1*$^{-/-}$ oocytes, and the oocytes were incubated in IBMX-containing M2 medium for 3 h before live-cell imaging by confocal microscopy (Fig. 4A). The injected oocytes underwent germinal vesicle breakdown (GVBD) in the presence of IBMX, indicating that expression of Δ50cyclin B2-Venus could break the GV arrest induced by CDK1 inhibition; moreover, Δ50cyclin B2-Venus increased and was maintained at a high level without degradation (Fig. 4B; Movies 3 and 4). None of the *Δ50cyclin B2-Venus* mRNA injected-oocytes extruded the first polar body (PB1) (Fig. 4C) but they instead arrested at metaphase I (Fig. 4D). This result strongly demonstrates that cyclin B2 degradation is required for the metaphase I-anaphase I transition in oocyte meiosis.

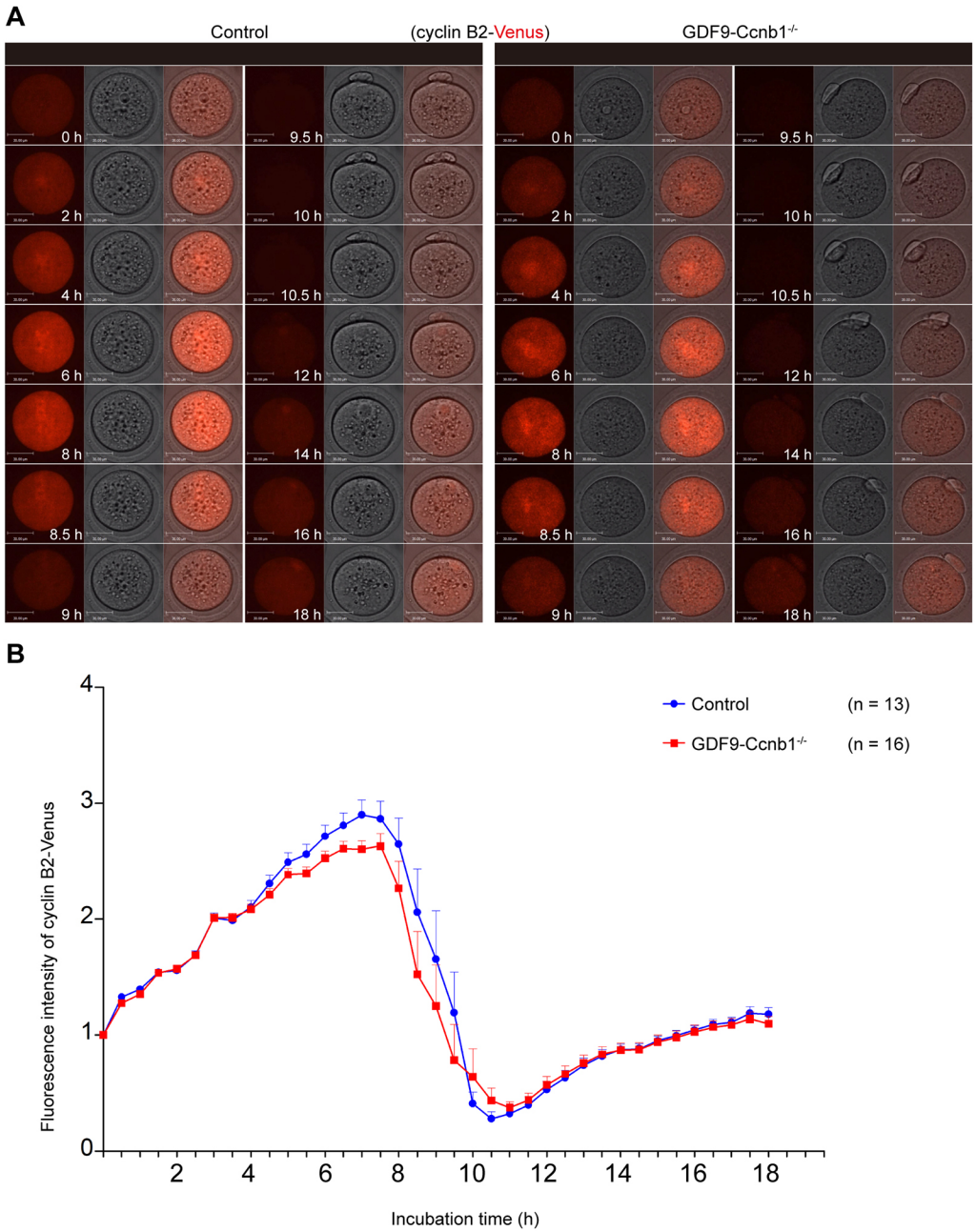

Fig. 3. Cyclin B2 is degraded during the metaphase I-anaphase I transition in oocytes. (A) Representative time-lapse confocal images for variation of cyclin B2-Venus during meiosis in control and *GDF9-Ccnb1*$^{-/-}$ oocytes. The concentration of *cyclin B2-Venus* mRNA used was 200 ng/µl. Scale bars: 30 µm. (B) Analysis of cyclin B2-Venus fluorescence intensity in control and *GDF9-Ccnb1*$^{-/-}$ oocytes. The cytoplasmic cyclin B2-Venus fluorescence intensities were measured every 30 min for up to 18 h in control and *GDF9-Ccnb1*$^{-/-}$ oocytes. The numbers of oocytes used (*n*) are shown.

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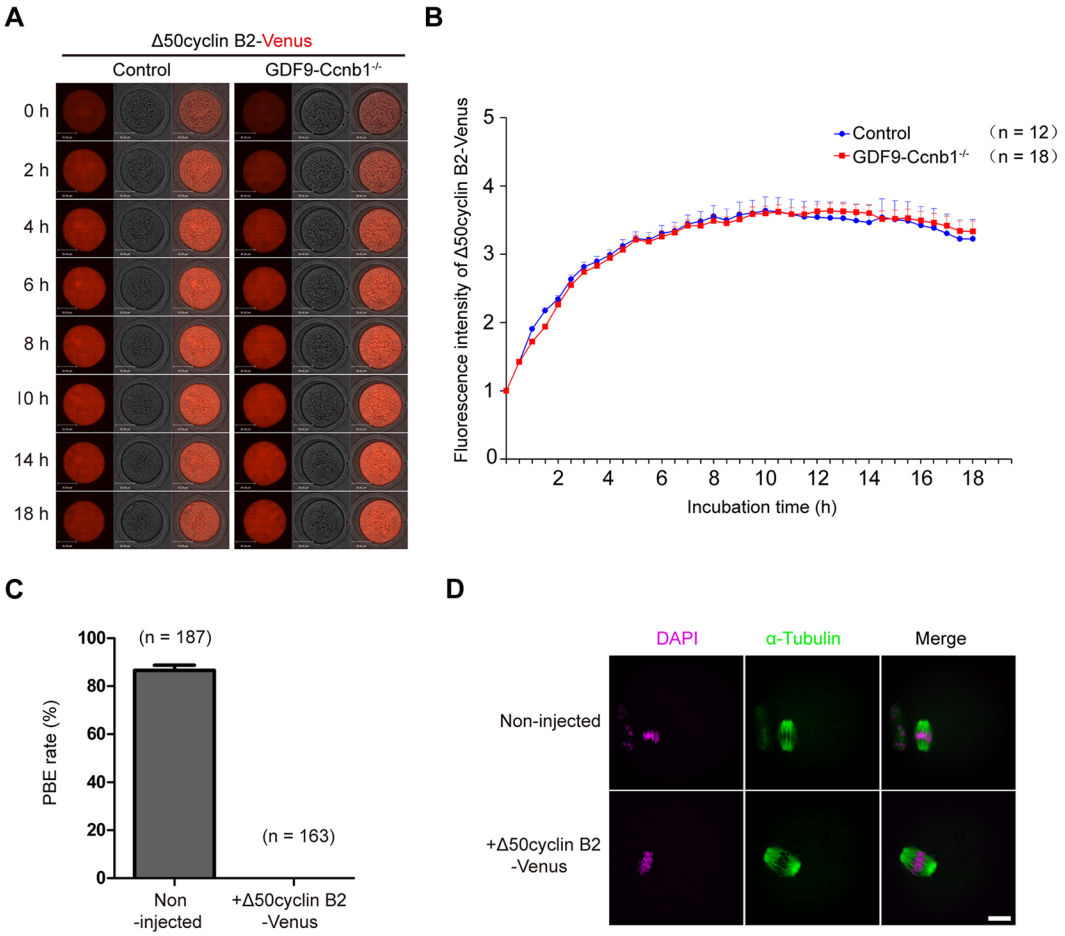

Fig. 4. Stable cyclin B2 prevents the metaphase I-anaphase I transition. (A) Representative time-lapse confocal images of the variation of Δ50cyclin B2-Venus in control and *GDF9-Ccnb1*$^{-/-}$ oocytes. Scale bars: 30 µm. (B) Analysis of Δ50cyclin B2-Venus fluorescence intensity in control and *GDF9-Ccnb1*$^{-/-}$ oocytes. The cytoplasmic Δ50cyclin B2-Venus fluorescence intensities were measured every 30 min for 18 h in control and *GDF9-Ccnb1*$^{-/-}$ oocytes. The numbers of oocytes used (*n*) are shown. (C) PBE rates of non-injected (86.67±2.0%) and *Δ50cyclin B2-Venus*-injected (0%) oocytes. The PBE rates were scored after an 18 h incubation period. The numbers of oocytes used (*n*) are shown. Data are presented as mean+s.e.m. (D) The *Δ50cyclin B2-Venus*-injected oocytes arrested at metaphase I. Spindle and DNA were stained with α-tubulin-FITC antibody and DAPI, respectively. Scale bar: 20 µm.

APC/C-dependent degradation of securin takes place in a timely manner in stable cyclin B2-arrested oocytes

Homologous chromosomes failed to separate in stable cyclin B2-arrested oocytes, which should have resulted from the separase inhibition by cyclin B2, especially in the *GDF9-Ccnb1*$^{-/-}$ oocytes. However, given that securin, which is degraded by APC/C before chromosome separation, also inhibits separase activity, we asked whether securin was degraded in stable cyclin B2-arrested oocytes. To address this question, mCherry-tagged securin was co-expressed with Δ50Cyclin B2-Venus in oocytes. The *mCherry-securin* mRNA and *Δ50Cyclin B2-Venus* mRNA were injected into the control and the *GDF9-Ccnb1*$^{-/-}$ oocytes, followed by confocal live-cell imaging for 18 h (Fig. 5A,B). We found that the degradation of securin occurred at the same time as the increase in stable cyclin B2 expression in both control (Fig. 5A,C; Movie 5) and *GDF9-Ccnb1*$^{-/-}$ oocytes (Fig. 5B,D; Movie 6). These results indicated that it was the stable cyclin B2 itself that resulted in metaphase I arrest and failure of chromosome separation, along with APC/C-dependent degradation.

Cyclin B2 inhibits separase through its associated CDK1 activity

To illustrate the effect of stable cyclin B2 on separase activity, we determined chromosome separation in live *GDF9-Ccnb1*$^{-/-}$ oocytes using a separase sensor (Fig. 6A), which is similar to a sensor reported previously (Nikalayevich et al., 2018). Notably, we replaced the YFP with an EGFP tag, to facilitate clear imaging. H2B localizes the sensor to chromosomes and can be visualized by both mCherry and EGFP signals, and cleavage of the RAD21 fragment by separase will discern the EGFP signal from chromosomes. In control oocytes, the GFP signal on chromosomes disappeared suddenly during chromosome separation in meiosis I (Fig. 6B; Movie 7). By contrast, the GFP signal was maintained on chromosomes in stable cyclin B2-expressed oocytes (Fig. 6B; Movie 8), indicating that separase was not activated in these oocytes. This result clearly demonstrated the inhibitory role of cyclin B2 on separase activity in oocyte meiosis I.

Next, we wondered whether the above observed phenotype (metaphase I arrest) depends on cyclin B2-associated CDK1 activity. To answer this question, the 'cyclin box' (Cbox) of stable cyclin B2, which is required for interaction with CDK1, was mutated and Δ50cyclin B2$^{\Delta Cbox}$-Venus was expressed in wild-type oocytes. As expected, the stable and kinase-disabled cyclin B2 did not affect the metaphase I-anaphase I transition, or exit from meiosis I (Fig. 6C) and homologous chromosome separation (Fig. 6D). Hence, the function of stable cyclin B2 relies on CDK1 activity. Based on this result, we tried to rescue chromosome separation in stable cyclin

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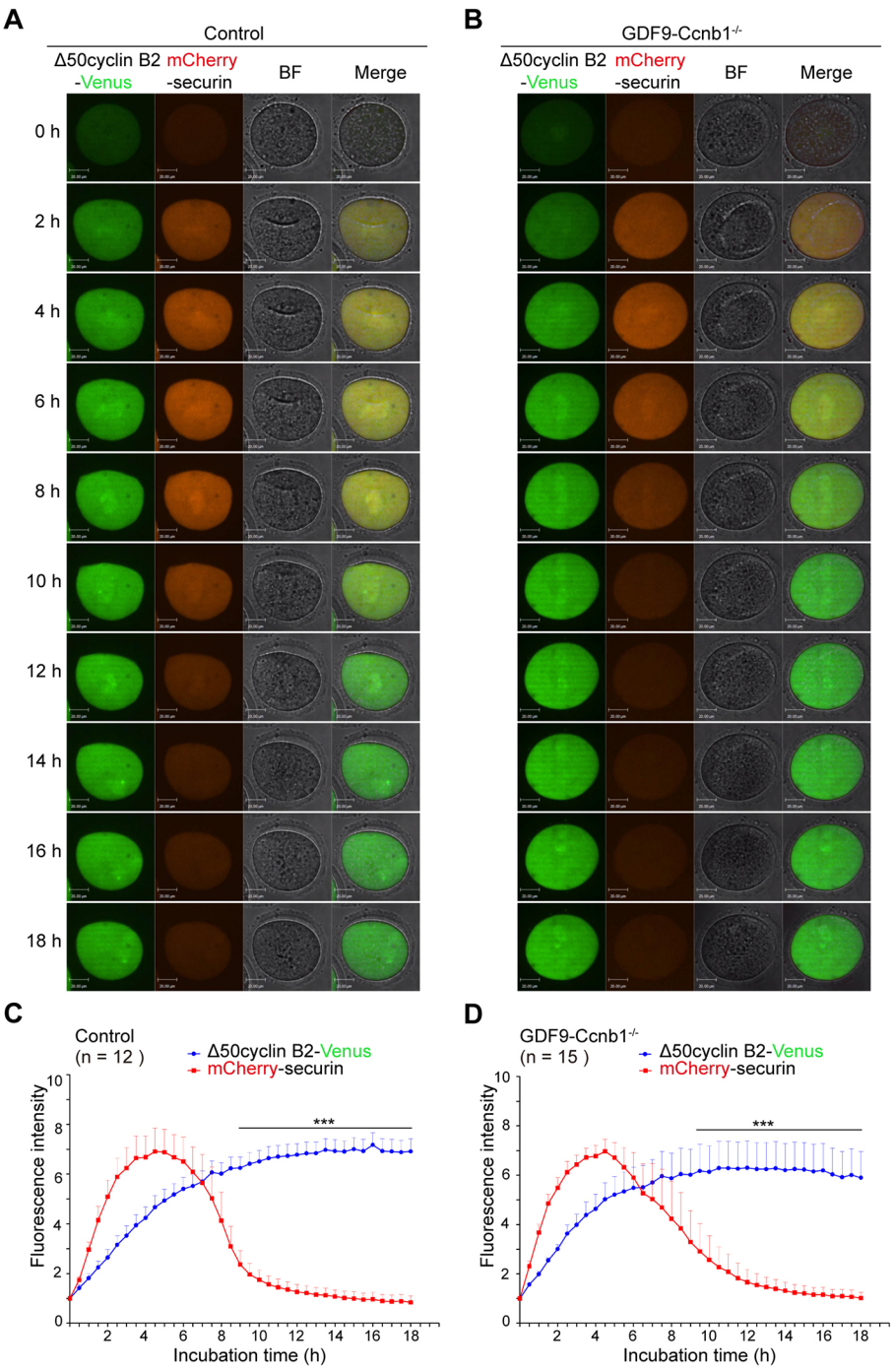

Fig. 5. Securin degradation occurs in a timely manner in stable cyclin B2-arrested oocytes. (A,B) Representative time-lapse confocal images for Δ50cyclin B2-Venus and mCherry-securin in control (A) and *GDF9-Ccnb1*$^{-/-}$ (B) oocytes. Scale bars: 20 µm. (C) Analysis of Δ50cyclin B2-Venus and mCherry-securin fluorescence intensities in control (C) and *GDF9-Ccnb1*$^{-/-}$ (D) oocytes. Data are presented as mean+s.e.m. ***P<0.0001 by Student's *t*-test.

B2-arrested oocytes by inhibiting CDK1 activity with its inhibitor roscovitine (Fig. 6E). Roscovitine treatment allowed the separation of homologous chromosomes in stable cyclin B2-arrested oocytes without PBE (Fig. 6F). However, a few roscovitine-treated oocytes extruded PB1 but entered an interphase-like stage (Fig. 6G), similar to the phenotype displayed by *GDF9-Ccnb1*$^{-/-}$ oocytes. To summarize, these results confirmed that cyclin B2 exerts its role on the inhibition of separase through CDK1 kinase activity.

Cyclin B2 interacts with separase directly

Next, we asked how cyclin B2/CDK1 interacts with separase in oocytes. In mitosis, it was previously demonstrated that CDK1-dependent phosphorylation was not sufficient for separase inhibition; notably, the binding of its regulatory subunit, cyclin B1, with separase is important for inhibition of separase activity. Based on these data, it is very possible that cyclin B2/CDK1 regulates separase activity through binding of cyclin B2 with separase and subsequently plays an inhibitory role in phosphorylation. To test this hypothesis, myc-tagged Δ50cyclin B2 and EGFP-tagged separase were expressed in 293T cells for immunoprecipitation (IP) experiments. As expected, Δ50cyclin B2 clearly immunoprecipitated with separase (Fig. 7). This confirms that cyclin B2/CDK1 interacts with separase by direct binding of cyclin B2 and separase.

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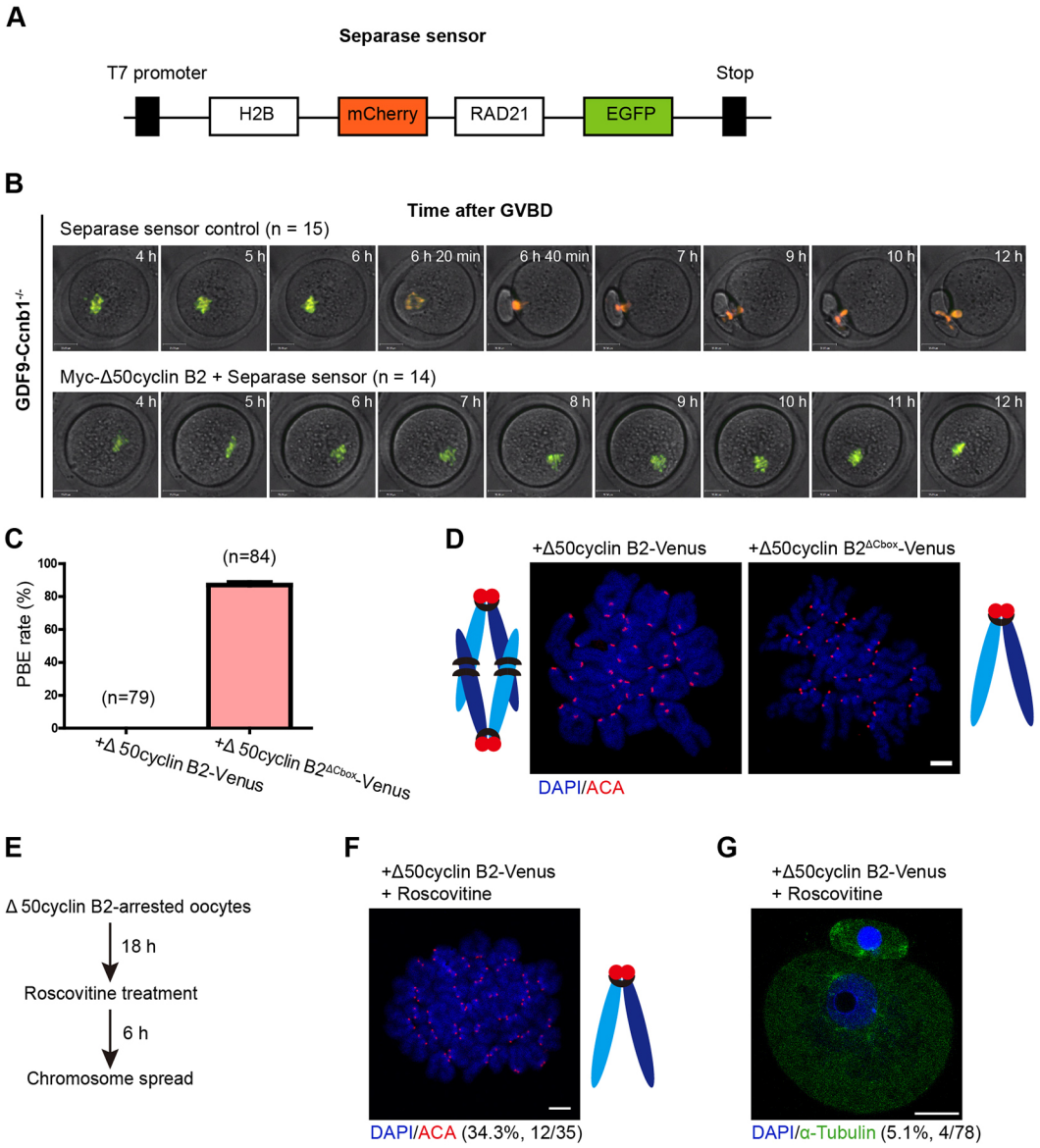

Fig. 6. Cyclin B2 inhibits separase through its associated CDK1 activity. (A) Schematic of the separase sensor. (B) Representative time-lapse confocal images of the separase sensor in stable cyclin B2-expressing *GDF9-Ccnb1*$^{-/-}$ oocytes. The concentration of separase sensor mRNA used was 200 ng/µl. Scale bars: 20 µm. (C) PBE rates in oocytes injected with *Δ50cyclin B2-Venus* (0%) or *Δ50cyclin B2*$^{\Delta Cbox}$*-Venus* (87.00±1.5%) mRNAs. Data are presented as mean+s.e.m., and the numbers of oocytes used (*n*) are shown. (D) Chromosome spreads for oocytes expressing Δ50cyclin B2-Venus or Δ50cyclin B2$^{\Delta Cbox}$-Venus. Scale bar: 5 µm. (E) Roscovitine treatment regime in stable cyclin B2-arrested oocytes. (F) Roscovitine treatment rescued the separation of homologous chromosomes in stable cyclin B2-arrested oocytes. Scale bar: 5 µm. ACA, anti-centromeric antibodies. (G) Immunofluorescence of stable cyclin B2-arrested oocytes with PBE after roscovitine treatment. Spindle and DNA were stained with α-tubulin-FITC antibody and DAPI, respectively. Scale bar: 20 µm. Diagrams in D and F illustrate that cyclin B2 plays its role on inhibition of separase via associated CDK1 kinase activity; red represents the centromere, black represents cohesin, and light blue and dark blue represent sister chromatids, respectively.

Non-phosphorylatable separase induces chromosome disjunction in stable cyclin B2-arrested oocytes

To clarify the inhibitory phosphorylation of separase by the cyclin B2/CDK1 complex, we asked whether the non-phosphorylatable separase could break the metaphase I arrest and induce chromosome separation in stable cyclin B2-arrested oocytes. To address this issue, a known CDK1-resistant phosphorylation site-mutated (S1121A) murine separase (PM-separase) was expressed (Huang et al., 2005; Touati et al., 2012); as a comparison, the wild-type separase (WT-separase) was expressed as well. In both control and *GDF9-Ccnb1*$^{-/-}$ oocytes, co-expression of WT-separase with stable cyclin B2 did not affect the metaphase I arrest (Fig. 8A), or the separation of homologous chromosomes (Fig. 8B). In contrast, co-expression of PM-separase with stable cyclin B2 destroyed the metaphase I arrest (Fig. 8C), and allowed the homologous chromosomes to separate in the control and *GDF9-Ccnb1*$^{-/-}$ oocytes (Fig. 8D), although without the extrusion of PB1, which might be due to impaired spindle morphology and dynamics (Fig. 8C). Overall, we conclude that cyclin B2/CDK1 inhibits separase via inhibitory phosphorylation in oocyte meiosis I.

DISCUSSION

Separase has been shown to be negatively regulated by securin binding and cyclin B1/CDK1-dependent phosphorylation (Stemmann et al., 2001; Touati et al., 2012; Zou et al., 1999); overexpression of

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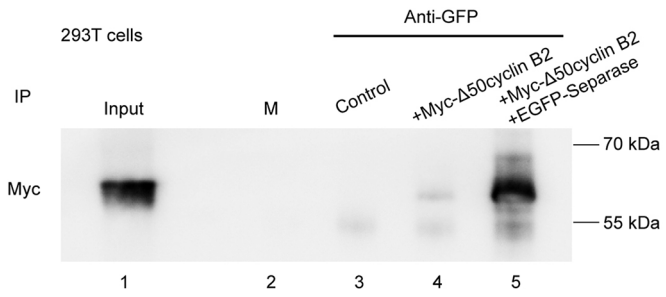

Fig. 7. IP of the interaction between cyclin B2 and separase. Myc-Δ50cyclin B2 and EGFP-separase were co-expressed in 293T cells (lane 5). IP of EGFP-separase was performed with anti-GFP antibody, and precipitates were analyzed by western blot using an anti-Myc antibody. Wild-type 293T cells (lane 3) and 293T cells expressing Myc-Δ50cyclin B2 alone (lane 4) were used as controls. The protein of cells co-expressing Myc-Δ50cyclin B2 and EGFP-separase was loaded as input (lane 1). Lane 2 is for protein marker (M).

either non-degradable securin or cyclin B1 has the capacity to prevent homologous chromosome separation (Touati et al., 2012). In this study, we demonstrated that stable cyclin B2 expression led to metaphase I arrest and non-disjunction of homologous chromosomes as a result of separase inhibition by cyclin B2-associated CDK1 kinase activity, which furthers our understanding of oocyte meiotic regulation. It should be noted that this mechanism for separase regulation by cyclin B2/CDK1 may be specific to mammalian oocytes (Fig. 9).

We have previously shown that cyclin B2 can compensate for cyclin B1 in meiosis I progression (Li et al., 2018). In this study, we describe a similar compensatory ability of cyclin B2/CDK1 to interact with separase, further elucidating the role of cyclin B2 in oocyte meiosis. This cyclin compensation may ensure the appropriate regulation of separase activity for correct chromosome separation. Therefore, we suggest that cyclin B2/CDK1 and cyclin B1/CDK1 complexes likely function together in wild-type oocytes, with some potential overlap. Given that the loss of cyclin B2, not cyclin B1, affected meiosis I progression (Li et al., 2018), we infer that cyclin B2/CDK1 is important for separase inhibition in mammalian meiotic cells. Interestingly, a recent study showed that increased errors in chromosome segregation were observed in cyclin B2-null oocytes (Daldello et al., 2019). As expected, deletion of cyclin B2 led to defective activation of CDK1 during meiosis I, which released the separase activity early, resulting in increased aneuploidy in oocytes. In addition, deletion of cyclin B2 compromised the metaphase I-anaphase I transition because the defective CDK1 activity delayed activation of the APC/C complex, which is responsible for the degradation of securin and cyclin B. However, we cannot exclude the possibility that the loss of cyclin B2-associated CDK1 may cause some other negative effects on meiotic events during GVBD and before the metaphase I-anaphase I transition, such as chromosome condensation and modification, spindle assembly, and modifications of kinetochore-associated factors. It is possible that the commitment to later events depends on the correct completion of early events (Hartwell and Weinert, 1989). However, we found that expression of stable cyclin B2 completely blocked the metaphase I-anaphase I transition, even though APC/C-dependent degradation occurred normally. Therefore, we suggest that degradation of cyclin B2 is required for anaphase onset in meiosis.

Aside from cyclin B1 and cyclin B2, another B-type cyclin, cyclin B3 has been reported to interact with CDK1 to form an active kinase complex (Karasu et al., 2019; Li et al., 2019b) and cyclin B3-associated kinase activity has been shown to be required for anaphase I onset, which may ensure efficient APC/C-dependent degradation. Nevertheless, how cyclin B3/CDK1 regulates APC/C activity remains unclear. Expression of stable cyclin B3 in wild-type oocytes did not prevent PBE, indicating that cyclin B3 degradation is dispensable for anaphase I. As expected, cyclin B3 expression had no evident contribution to MPF activity (Karasu et al., 2019). In contrast, stable cyclin B1 or cyclin B2 lead to metaphase I arrest because cyclin B1 and cyclin B2-associated CDK1 activate MPF directly, which is essential for meiotic resumption and progression (Li et al., 2019a). We propose that the cyclin B3/CDK1 complex may have substrates that are specific and distinct from those of the cyclin B1/CDK1 and cyclin B2/CDK1 complexes in oocyte meiosis.

In a previous study, it was demonstrated that pharmacological inhibition of CDK1 activity allowed chromosome segregation and PB1 extrusion even under conditions of suppressed proteasomal degradation, which maintains securin intact in mouse oocytes (Pomerantz et al., 2012); CDK1-dependent phosphorylation is sufficient for separase inhibition, and securin binding seems to be dispensable. Consistently, knockdown of securin alone in mouse GV oocytes did not induce premature chromosome separation in meiosis I (Chiang et al., 2011; Nabti et al., 2008). In the present study, using non-phosphorylatable separase, we identified the biochemical interaction between cyclin B2-CDK1 and separase, contributing new information on the regulatory role of CDK1 over separase in oocytes.

In addition, we also found that some sister chromatids separated completely in the oocytes with co-expression of stable cyclin B2 and PM-separase (Fig. S1), indicating that the expression of PM-separase abolished the protection of centromeric cohesin mediated by the Sgo2-dependent PP2A recruitment mechanism. It implies that, apart from the protection of centromeric cohesin by PP2A, cyclin B/CDK1-mediated inhibitory phosphorylation of separase is of significant importance for the protection of centromeric cohesin. It will be interesting to investigate whether stable cyclin B2 can prevent separation of sister chromatids in meiosis II.

Taken together, our results demonstrate that cyclin B2/CDK1 appears to inhibit separase activity through inhibitory phosphorylation during oocyte meiosis I. Importantly, we demonstrated an interaction between murine cyclin B2 and separase, revealing the underlying mechanism for separase regulation.

MATERIALS AND METHODS

Mice

Ccnb1$^{Flox/Flox}$ mice (on a C57BL/6 background) were used and crossed with *GDF9-Cre* transgenic mice to generate *Ccnb1*$^{Flox/Flox}$*;GDF9-Cre* (*GDF9-Ccnb1*$^{-/-}$) female mice as previously reported (Li et al., 2018; Tang et al., 2017). Littermates genotyped as *Ccnb1*$^{Flox/Flox}$ were used as control animals.

All experimental protocols and animal handling procedures were conducted in accordance with the guidelines and procedures approved by the Institutional Animal Care Committee of Institute of Zoology (IOZ), University of Chinese Academy of Sciences (UCAS).

Oocyte collection and manipulation

Six- to eight-week-old female mice were used. Mice were injected intraperitoneally with 10 U pregnant mare serum gonadotrophin (PMSG) before oocyte collection. After 44-48 h, GV-intact oocytes were collected from the ovary and the surrounding cumulus cells were removed mechanically with a pipette. The denuded oocytes were incubated in M2 medium (M7167, Sigma-Aldrich) containing 50 µm IBMX under mineral oil (Sigma-Aldrich) for microinjection, or cultured in IBMX-free M2 medium for *in vitro* maturation at 37°C in a humidified atmosphere of 5% CO_2 in air. For OA (okadaic acid) treatment, the *GDF9-Ccnb1*$^{-/-}$ oocytes that extruded the first polar body were immediately transferred into the M2 medium containing 50 nM OA (Sigma-Aldrich) for treatment. For roscovitine treatment, stable cyclin B2-arrested oocytes incubated for 18 h in M2 medium were transferred into M2 medium containing 0.2 mM roscovitine (Selleck).

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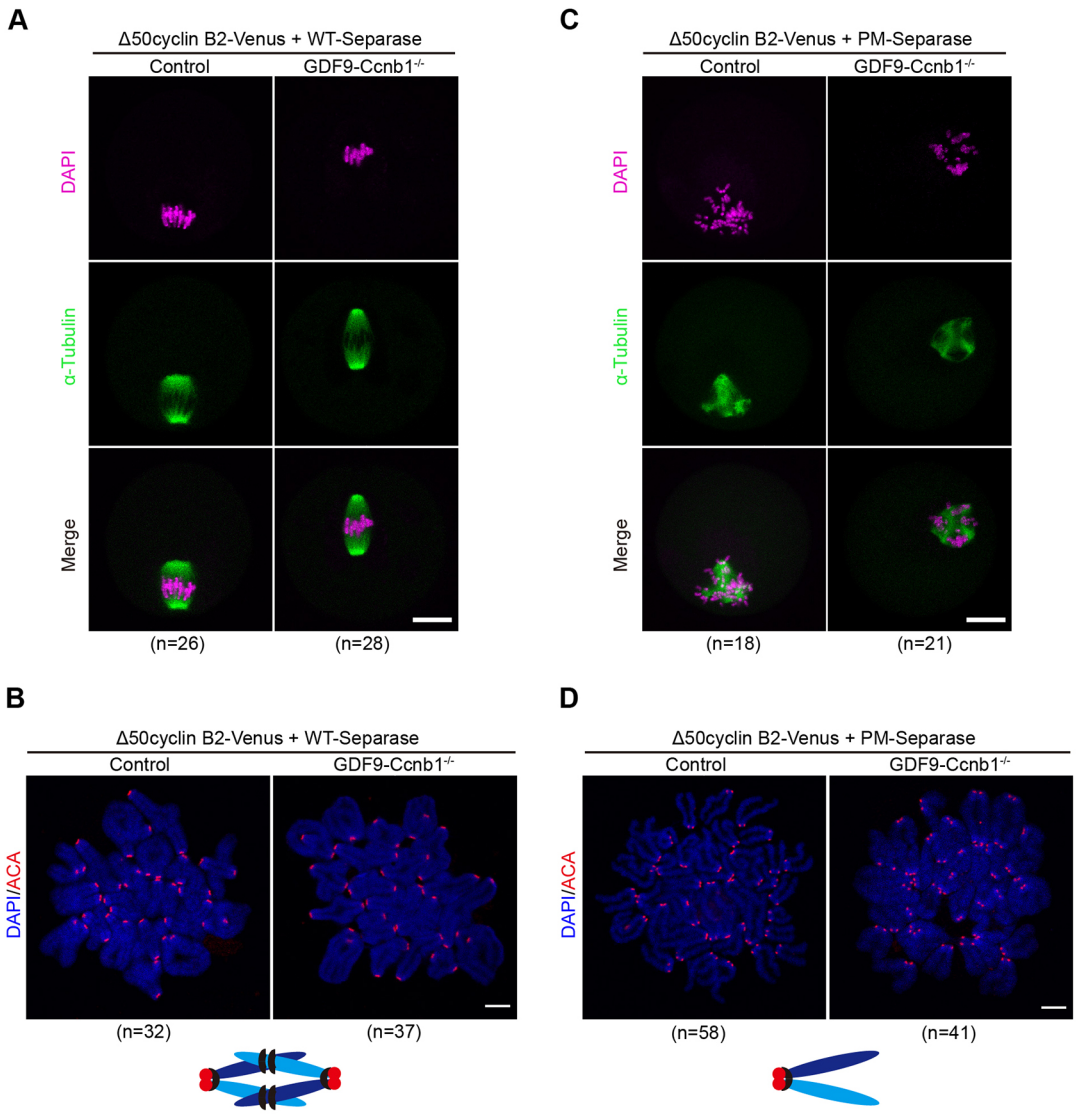

Fig. 8. Non-phosphorylatable separase induces chromosome separation in stable cyclin B2-arrested oocytes. (A) Co-expression of Δ50cyclin B2-Venus and WT-separase did not affect metaphase I arrest in both either control or *GDF9-Ccnb1*$^{-/-}$ oocytes. (B) Chromosome spreads for control and *GDF9-Ccnb1*$^{-/-}$ oocytes co-expressing Δ50cyclin B2-Venus and WT-separase. (C) Co-expression of Δ50cyclin B2-Venus and PM-separase broke the metaphase I arrest in both control and *GDF9-Ccnb1*$^{-/-}$ oocytes. (D) Chromosome spreads for control and *GDF9-Ccnb1*$^{-/-}$ oocytes co-expressing Δ50cyclin B2-Venus and PM-separase. Spindle and DNA were stained with α-tubulin-FITC antibody and DAPI, respectively. ACA, anti-centromeric antibodies. Diagrams in B and D illustrate that PM-separase, not WT-separase, induced separation of homologous chromosomes in stable cyclin B2-expressing oocytes; red represents the centromere, black represents cohesin, and light blue and dark blue represent sister chromatids, respectively. Scale bars: 20 µm (A,C); 5 µm (B,D).

Chromosome spreads

First, oocytes were exposed to Acid Tyrode's solution (Sigma-Aldrich) to remove the zona pellucida properly at room temperature (RT), avoiding over-digestion. Then, the oocytes were transferred into pre-warmed M2 medium for a brief recovery, and, subsequently, the oocytes were transferred onto a clean glass slide and exposed to a solution of 1% paraformaldehyde (PFA) in distilled H_2O (pH 9.2) containing 0.15% Triton X-100 and 3 mM dithiothreitol as previously reported (Hodges and Hunt, 2002). The slides were placed in a half-open humidified chamber to dry slowly for several hours, and the fixed oocytes were blocked with 2% bovine serum albumin (BSA) in PBS for 1 h at RT. The oocytes were then incubated with primary antibodies overnight at 4°C. After three washes (10 min each wash) with washing buffer (PBS containing 0.1% Tween-20 and 0.01% Triton X-100), the slides were then incubated with corresponding secondary antibodies for 2 h at RT. Primary human anti-ACA (anti-centromere antibody) antibody (1:50; 15-234, Antibodies Incorporated) was used for detecting centromeres with a corresponding secondary antibody conjugated with Alexa Fluor Cy5 (709-175-149, Jackson ImmunoResearch). Primary rabbit anti-SMC3 antibody (1:20; ab128919, Abcam) and a corresponding secondary antibody conjugated with Alexa Fluor 488 (A-11008, Thermo Fisher Scientific) were also used.

Immunofluorescence analysis

For spindle staining, oocytes were fixed in 4% PFA in PBS buffer for at least 30 min at RT before permeabilization for 20 min with 0.5% Triton X-100 at RT. The oocytes were then blocked in PBS containing 1% BSA for 1 h at RT. The oocytes were incubated with a FITC-conjugated anti-α-tubulin antibody (322588, Thermo Fisher Scientific) overnight at 4°C. The following morning, oocytes were washed three times in washing buffer (PBS containing 0.1% Tween-20 and 0.01% Triton X-100) and then co-stained with DAPI (1 µg/ml in PBS; Sigma-Aldrich) for 15 min. Finally, the oocytes were mounted on glass slides and imaged with a confocal laser-scanning microscope (TCS SP8, Leica Microsystems) equipped with a high-contrast Plan Apochromat 40×1.10 water-immersion objective lens (Leica Microsystems) at RT, and Application Suite X software (2.0.0.14332, Leica Microsystems) was used for image acquisition.

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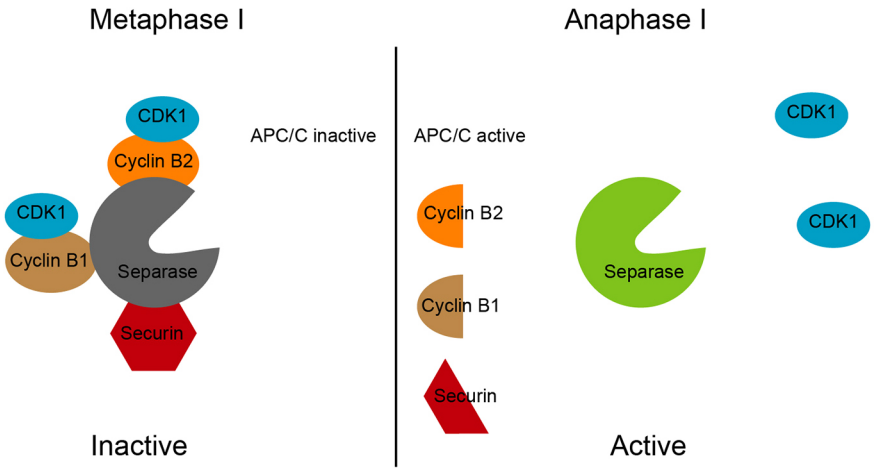

Fig. 9. Model for separase regulation in oocyte meiosis I. Separase is negatively controlled by securin and the cyclin B1/CDK1 and cyclin B2/CDK1 complexes before anaphase onset when APC/C is inactive. Upon APC/C activation during the metaphase I-anaphase I transition, securin, cyclin B1 and cyclin B2 are degraded, thereby releasing active separase for chromosome separation.

Plasmid construction, cRNA preparation and microinjection

The mouse *Ccnb2* gene (NM_007630.2) was cloned into a pcDNA3.1-Venus vector as described (Li et al., 2018). To make the non-degradable cyclin B2, the N-terminal 50 amino acids (Destruction-box) of murine cyclin B2 were deleted to construct Δ50cyclin B2, which was cloned into a pcDNA3.1-Venus vector and a pCS2(+)-Myc vector. To block the binding of Δ50cyclin B2 with CDK1, amino acids 165-200 (Cyclin-box) of murine cyclin B2 were deleted and cloned into a pcDNA3.1-Venus vector (Δ50cyclin B2$^{\Delta Cbox}$–Venus). mCherry-securin (human) was cloned into a pCS2(+) vector. EGFP-WT-separase (murine) and EGFP-PM-separase (murine, S1121A) constructs have been reported previously (Touati et al., 2012). cRNAs were prepared using T7 or SP6 mMessage mMachine (Ambion) correspondingly and purified with RNeasy Mini kits (Qiagen), dissolved in nuclease-free water, and stored at −80°C. A concentration of 500 ng/µl was used for microinjection unless indicated otherwise. Microinjection was performed with a Nikon operating system.

Time-lapse confocal live-cell imaging

Confocal live-cell imaging was performed using a PerkinElmer Ultra VIEW-VoX confocal imaging system equipped with an a CO_2 incubator chamber (5% CO_2 at 37°C) filled with M2 medium covered by mineral oil. Digital time-lapse images (30 *z*-slices with 2-µm spacing) were acquired using a 20×0.75 objective lens, and Volocity 6.0 software was used for image acquisition. Injected oocytes were incubated in M2 medium supplemented with 50 µm IBMX (Sigma-Aldrich) for 3 h at 37°C and 5% CO_2. Then, the oocytes were released into M2 medium and prepared for time-lapse imaging. To track and record the expression changes for cyclin B2-Venus, Δ50cyclin B2-Venus and mCherry-securin, images were taken every 30 min for 18 h. To track the change of separase sensor, images were taken every 20 min for 18 h.

Immunoprecipitation

First, the Myc-Δ50cyclin B2 and EGFP-separase plasmids were transfected into 293T cells using Lipofectamine 2000 (11668-019, Invitrogen) according to the reagent protocol, and Myc-Δ50cyclin B2 was transfected alone as a control. After 24-48 h, the cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% NP-40) supplemented with protease and phosphatase inhibitors (Roche, cOmplete Mini tablets) and incubated on ice for 30 min to extract the proteins. Next, the lysates were centrifuged at 14,000 ***g*** for 10 min at 4°C, the supernatant was collected and 100 µl was removed for incubation with 2 µg primary antibody overnight with rotation at 4°C. The following morning, 20 µl Magnetic Beads Protein A/G (B23201, Bimake) was added and incubation was continued for 4-6 h with rotation at 4°C. After washing with IP lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 0.5 mM DTT and 1 mM PMSF/cocktail) at least five times, 40 µl 1× SDS buffer was added and the supernatant was used for western blotting. Western blotting was performed as described previously (Li et al., 2018). The antibodies used were anti-GFP (1:4000; ab290, Abcam) and anti-c-Myc (1:2000; M4439, Sigma-Aldrich).

Statistical analysis

Images were analyzed with ImageJ software (National Institutes of Health) and Photoshop CS5 (Adobe), composed by Illustrator CC5 (Adobe). Quantitative data (mean+s.e.m.) were processed by Student's *t*-test using Prism 5 (GraphPad Software) with $P<0.05$ considered significant.

Acknowledgements

We thank Professor Heide Schatten at the University of Missouri-Columbia for editing this manuscript. We thank Professor Olaf Stemmann at the Department of Genetics, University of Bayreuth for help with the EGFP-WT-Separase and EGFP-PM-Separase constructs. We thank Lijuan Wang for assistance with live cell imaging; Shiwen Li, Hua Qin and Xili Zhu for assistance with confocal imaging. We thank Yuanyuan Li for the culture of 293T cells.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Methodology: J.L., Y.-C.O.; Formal analysis: C.-H.Z.; Investigation: J.L.; Resources: Y.-C.O.; Writing - original draft: J.L.; Writing - review & editing: Q.-Y.S.; Supervision: W.-P.Q., Q.-Y.S.; Project administration: J.L.; Funding acquisition: W.-P.Q., Q.-Y.S.

Funding

This study was supported by the National Natural Science Foundation of China (31900593) and project funded by the China Postdoctoral Science Foundation (2019M652992), the Project for Research Team of Female Reproductive Health and Fertility Preservation (SZSM201612065) funded by the Science, Technology and Innovation Commission of Shenzhen Municipality, the Project for Medical Discipline Advancement of Health and Family Planning commission of Shenzhen Municipality (SZXJ2017003) funded by the Health Commission of Shenzhen Municipality, and the Project for Exploration of New method of Noninvasive Fertility Evaluation and Establishment of National Standard (2018YFC1002104) funded by the Ministry of Science and Technology of the People's Republic of China.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.182519.supplemental

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