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RESEARCH ARTICLE

CENP-T regulates both the G2/M transition and anaphase entry by acting through CDH1 in meiotic oocytes

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ABSTRACT

Oocyte meiotic maturation failure is one of the major causes for female infertility. Meiotic resumption (the G2/M transition) and progression through metaphase I (MI) are two critical stages of oocyte meiotic maturation. Here, we report that centromere protein T (CENP-T), an internal kinetochore protein, plays a critical role in meiotic resumption of mouse oocytes. Depletion of CENP-T by siRNA injection increased the CDH1 (also known as FZR1) level, resulting in increased activity of the anaphase-promoting complex (APC)–CDH1 complex, and further leading to decreased levels of the cyclin protein CCNB1, attenuated maturation-promoting factor (MPF) activity, and finally severely compromised meiotic resumption. The impaired meiotic resumption caused by CENP-T depletion could be rescued by overexpression of exogenous CCNB1 or knockdown of endogenous CDH1. Overexpression of exogenous CENP-T resulted in decreased CDH1 levels, which accelerated the progression of G2/M transition, and accelerated meiotic cell cycle progression after germinal vesicle breakdown (GVBD). Unexpectedly, spindle organization after GVBD was not affected by the overexpression, but the distribution of chromosomes was affected. Our findings reveal a novel role for CENP-T in regulating meiotic progression by acting through CDH1.

KEY WORDS: CENP-T, CDH1, FZR1, CCNB1, Oocyte, Meiotic resumption

INTRODUCTION

Oocytes remain arrested at prophase of first meiosis until a gonadotropin surge takes place. Subsequently, the germinal vesicle breakdown (GVBD) takes place, which represents the initial stage of oocyte maturation. After GVBD, oocytes pass through metaphase (MI), the stage during which all chromosomes become well aligned at the equatorial plate of the meiotic spindle, and then enter anaphase, followed by first polar body (PB1) extrusion and arrest at metaphase of the second meiotic division until fertilization takes place.

The prophase I oocyte arrest is the result of low maturation promoting factor (MPF) activity (Choi et al., 1991). Inhibition of MPF activity prevents GVBD (Aritro, 2013); conversely, a high MPF level promotes GVBD. Two components constitute MPF, catalytic CDK1 (also known as Cdc2) and a regulatory cyclin, CCNB1 (Mattioli et al., 1991). CDK1 can be phosphorylated by inhibitory protein kinase, like Wee1 or Myt1 (also known as PKMYT1), at Thr14 and Tyr15, while it can be dephosphorylated by Cdc25 proteins (CDC25A–CDC25C) to keep the heterodimer MPF in an active state (Han et al., 2005; Jones, 2004). The regulatory subunit CCNB1 is continuously degraded by the anaphase-promoting complex/cyclosome (APC/C) during the prophase I arrest (Reis et al., 2006). CDH1 (also called Fzr1) binds to APC/C during prophase I to mediate CCNB1 destruction (Solc et al., 2010). Knockout of *Cdh1* leads to increased CCNB1 levels, and coordinates the timing of meiotic resumption. Meanwhile, *Cdh1* knockout also accelerates the progression of oocytes so that they pass through meiosis I (Holt et al., 2012, 2011). A protein called early mitotic inhibitor 1 (Emi1, also known as FBXO5), which is an inhibitor of the APC, can regulate the CDH1 level. Exogenous Emi1 or inhibition of Emi1 destruction in germinal vesicle (GV) oocytes leads to stabilization of CCNB1 and triggers GVBD, while this also causes arrest at the MI stage. In contrast, the depletion of Emi1 increases CCNB1 destruction, resulting in a delayed meiotic resumption (Marangos et al., 2007). Interestingly, the two critical stages of oocyte maturation, prophase I arrest and progression through metaphase I, can be regulated by the spindle assembly checkpoint protein BubR1 (also known as BUB1B). Depletion of BubR1 in oocytes causes spontaneous GVBD in the presence of the phosphodiesterase inhibitor IBMX. Surprisingly, depletion of BubR1 decreases the expression of CDH1 levels, but does not affect CCNB1 levels (Homer et al., 2009).

CENP-T is widely known as a kinetochore protein. It is a component of the constitutive centromere associated network (CCAN), and it is required to recruit the Knl1–Mis12–Ndc80 (KMN) network, which, in turn, is necessary to assemble a functional kinetochore (Hori et al., 2008; Gascoigne et al., 2011; Suzuki et al., 2011). One of the most important regions of CENP-T, the C-terminal, was thought to directly bind to CENP-A; however, recent findings have shown that the CENP-T C-terminal is localized next to histone H3.1, suggesting that the CCAN bridges a CENP-A- and a H3-containing nucleosome (Abendroth et al., 2015). The C-terminal region of CENP-T is also required for the formation of the CENP-T–CENP-W–CENP-S–CENP-X complex, and a nucleosome-like structure is next formed to bind to centromeric DNA (Hori et al., 2008; Nishino et al., 2012). In addition, the N-terminal region of CENP-T, which is associated with the outer kinetochore, has been reported to be necessary for outer kinetochore assembly via interacting with the 'RWD' domain in the Spc24–Spc25 portion of the Ndc80 complex (Gascoigne et al., 2011; Nishino et al., 2013). Phosphorylation at Thr11 or Thr85 is needed for CENP-T to become active, which is accomplished by a cyclin-dependent kinase (CDK), thus, deletion of CENP-T or prevention of CDK phosphorylation all lead to improper recruitment to the kinetochore (Kim and Yu, 2015;

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Rago et al., 2015; Veld et al., 2016). In addition, CENP-T directly interacts with CSN5, the fifth component of the COP9 signalosome and a key modulator of the cell cycle and cancer (Chun et al., 2013). Results show that depletion of CENP-C results in decreased CENP-T level at the centromere in mitosis (Krizaic el al., 2015). Direct depletion of CENPs-T by siRNA in HeLa cells results in defects in spindle assembly (Prendergast et al., 2011), and knockdown of CENP-T in zebrafish causes aberrations in cell division with abnormal chromosomal segregation (Hung et al., 2017). Based on the above findings, CENP-T has an essential role in mitosis; however, the function of CENP-T in meiosis is still unknown.

Here, we investigated the function of CENP-T in regulating the meiotic progression in mouse oocytes. CENP-T, working as a kinetochore protein, was first thought to affect the chromosome segregation. However, unexpectedly, we found that depletion of CENP-T inhibits the occurrence of GVBD (G2/M transition) by increasing APC–CDH1 activity, resulting in degradation of CCNB1. The prophase I arrest induced by CENP-T depletion could be rescued by exogenous *Ccnb1* mRNA injection or CDH1 knockdown induced by morpholino injection. Moreover, overexpression of CENP-T inversely promoted GVBD. Finally, we showed that overexpression of CENP-T also accelerated the passage through meiosis I, resulting in abnormal chromosome segregation.

RESULTS

Expression and subcellular localization of CENP-T during oocyte meiotic progression

We first examined the expression and subcellular localization of CENP-T. Mouse oocytes were collected and cultured for 0, 4, 8 and 12 h after release into M2 medium, corresponding to stages of the GV, GVBD, MI and metaphase II (MII), respectively. Immunoblotting analysis showed that CENP-T was present from the GV to MII stages, and the level at the GVBD stage was significantly higher than in other stages (Fig. 1A,B). Immunofluorescence staining of CENP-T in the collected oocytes was next examined, and clear staining of CENP-T was seen concentrated in the nucleus of the GV oocyte and could be observed at kinetochores from the GVBD stage to MII stage, as shown in Fig. 1C.

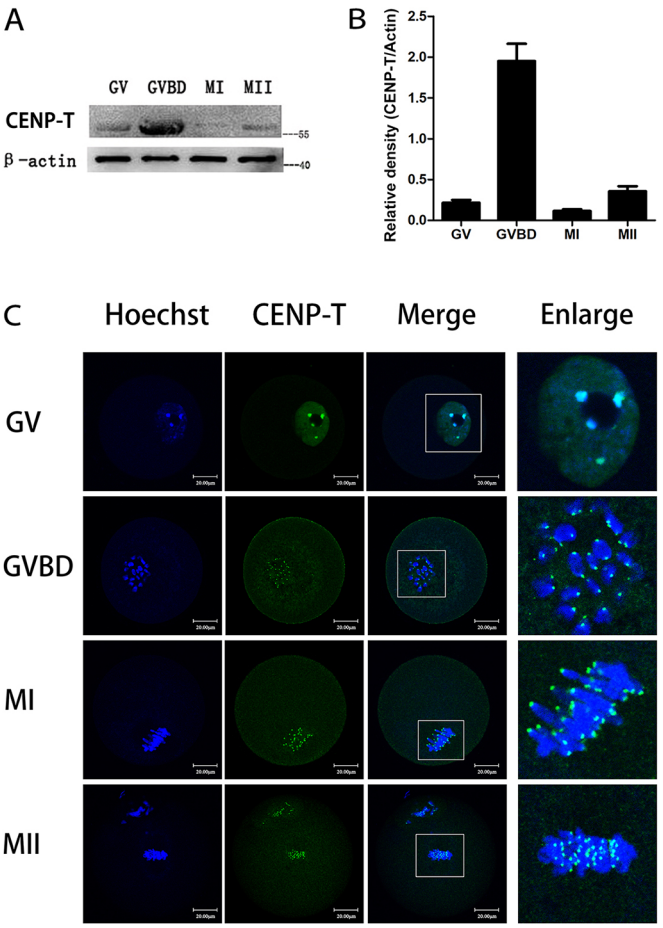

Fig. 1. Expression and subcellular localization of CENP-T during mouse oocyte meiotic maturation. (A) Expression of CENP-T protein as revealed by western blot analysis. Samples of 200 oocytes were collected after culture for 0, 4, 8 and 12 h, the time points when most oocytes had reached the GV, GVBD, MI and MII stages, respectively. β-actin is shown as a loading control. (B) The relative staining intensity of CENP-T was assessed through densitometry (mean±s.e.m., *n*=3). (C) Confocal microscopy images showing the subcellular localization of CENP-T (green) in mouse oocytes at the GV, GVBD, MI and MII stages. DNA (blue) was counterstained with Hoechst 33342. Scale bars: 20 µm.

Depletion of CENP-T results in low MPF activity and prophase I arrest of oocytes

For effectively depleting CENP-T in oocytes *in vitro*, siRNA was used. Oocytes at the GV stage were collected from female mice at 6 weeks of age, and next CENP-T siRNA was microinjected into oocytes followed by a 24 h incubation in IBMX to deplete the protein. We found that up to 90% CENP-T was effectively depleted by CENP-T siRNA injection compared with the control group (Fig. 2A). Unexpectedly, only 30% of CENP-T-depleted oocytes underwent GVBD 3 h after release from IBMX. The percentage of oocytes completing meiotic resumption was significantly lower in the CENP-T-depleted group than in the control group (29.91± 9.85% versus 92.04±10.09%, mean±s.e.m., $P<0.05$) (Fig. 2B). These results indicate that CENP-T was effectively depleted in our experiments, and that CENP-T-depleted oocytes maintained an arrest at the GV stage.

To further investigate the underlying reasons for the prophase I arrest, we next explored MPF activity. As shown in Fig. 2C, the MPF activity as determined with a histone H1 kinase assay in the CENP-T-depleted oocytes was reduced compared with that in the control group, which is responsible for the prophase I arrest in CENP-T-siRNA-injected group. We further examined the two critical components of MPF, CDK1 and CCNB1. As CDK1 has to be dephosphorylated at site Tyr15 to become active, we first tested CDK1 activity by examining the phosphorylation level of the state of Tyr15 and found that the level of phosphorylation in CENP-T-depleted oocytes was much higher than that of control oocytes by 1 h following release from IBMX, indicating that the CDK1 activity is inhibited in siRNA-injected oocytes (Fig. 2D). We next examined the expression level of CCNB1 and showed that the CCNB1 level was significantly reduced in CENP-T-depleted oocytes (Fig. 2E). We also tested the expression level of securin, and found no significant difference (Fig. 2F). Thus, CENP-T depletion leads to oocyte arrest in prophase I, which is caused by loss of CCNB1 and lack of CDK1 dephosphorylation, further leading to decreased MPF activity.

To further reveal whether low level of CCNB1 caused GVBD inhibition in CENP-T-depleted oocytes, *Ccnb1-GFP* mRNA was microinjected to show the location of CCNB1. As shown in Fig. 2G, besides the reduced fluorescence intensity (Fig. 2H), the nuclear entry of CCNB1, which could be easily found in control oocytes, disappeared in CENP-T-depleted oocytes. We also constructed a vector carrying mutant CDK1 gene in which the phosphorylation

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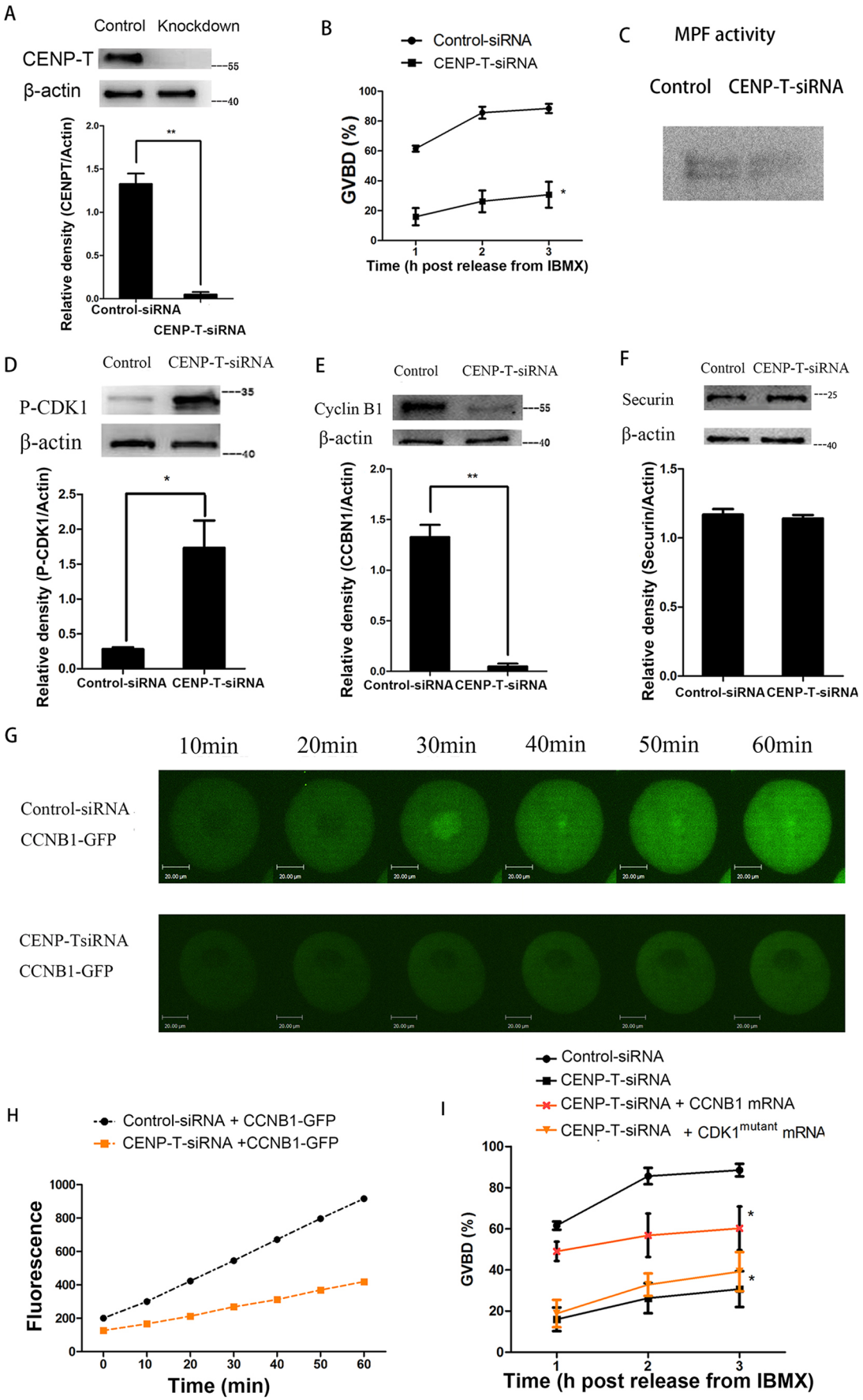

Fig. 2. See next page for legend.

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Fig. 2. Depletion of CENP-T impairs GVBD and MPF activity, while prophase I arrest can be rescued by exogenous cyclin B. (A) Western blotting results for CENP-T and β-actin in the CENP-T-siRNA injected oocytes and control oocytes by 1 h following release from IBMX (200 oocytes per sample). The relative staining intensity of CENP-T was assessed by densitometry. (B) Percentage of oocytes that had undergone GVBD by 1, 2 and 3 h following release from IBMX for control-siRNA- and CENP-T-siRNA-injected oocytes. (C) MPF activity level of CENP-T-siRNA-injected oocytes and control oocytes as revealed by a histone H1 kinase assay. (D) The phosphorylation level of Tyr15 of CDK1 in CENP-T-siRNA-injected oocytes and control oocytes. The relative staining intensity of phosphorylated CDK1 (P-CDK1) was assessed by densitometry. (E) The expression levels of CCNB1 in control oocytes and CENP-T-siRNA-injected oocytes at 1 h following release from IBMX (200 oocytes per sample). The relative staining intensity was assessed by densitometry. (F) The expression level of securin in control oocytes and CENP-T-siRNA-injected oocytes at 1 h following release from IBMX (200 oocytes per sample). The relative staining intensity of securin was assessed by densitometry. (G) After injection with CENP-T-siRNA or control-siRNA into the GV oocytes followed by 20 h incubation in 200 µM IBMX, CCNB1–GFP mRNA was injected into CENP-T-siRNA- or control-injected oocytes. Following microinjection of CCNB1–GFP mRNA, oocytes were maintained for 1 h in 200 µM IBMX. Live-cell imaging shows the dynamics of CCNB1–GFP at 10 min intervals. Scale bars: 20 µm. (H) The relative fluorescence intensity (arbitrary units) of CCNB1–GFP in oocytes as in G. (I) Oocytes injected with CENP-T-siRNA or control-siRNA were then injected with CCNB1 mRNA, mutant CDK1 mRNA or control mRNA followed by 20 h incubation in 200 µM IBMX. Oocytes were maintained for 2 h in 200 µM IBMX followed by release from IBMX, and the percentage of oocytes undergoing GVBD were recorded. All quantitative data are presented as means±s.e.m. of three independent experiments (*P<0.05; **P<0.01).

domain was destroyed, and hence that produces a CDK1 form that cannot be phosphorylated and is constitutively active. We injected the mutant CDK1 mRNA and *Ccnb1-GFP* mRNA separately in different groups of oocytes, and the impaired GVBD was only rescued by exogenous CCNB1, and not by active CDK1 (Fig. 2I). These results suggest that the main reason for the prophase I arrest in CENP-T-depleted oocytes is the decrease in CCNB1 levels, and that CENP-T may be required for CCNB1 stabilization, which in turn plays an indispensable role in the meiotic resumption.

CENP-T depletion-induced MPF activity reduction and prophase I arrest are caused by enhanced CDH1 expression and are independent of BubR1

As the kinetochore protein BubR1 has been reported to be required for meiotic resumption in mouse oocytes (Homer et al., 2009), we next asked whether CENP-T depletion caused meiotic prophase I arrest through affecting BubR1. Oocytes at the GV stage were microinjected with CENP-T siRNA and cultured for 24 h in IBMX before being collected for western blotting. No obvious difference between the CENP-T knockdown group and the control group (Fig. 3A) was observed, indicating that CENP-T and BubR1 do not function in the same pathway to mediate meiotic resumption.

As it is widely known that CCNB1 destruction is mainly mediated by APC–CDH1 during G2/M transition, we hypothesized that the low level of CCNB1 was mediated by increased APC–CDH1 activity; therefore, we tested the expression of CDH1 in oocytes. Results showed significantly increased CDH1 levels in CENP-T-knockdown oocytes compared to that in the control ones (Fig. 3B). To further test our hypothesis that an increased CDH1 level caused CCNB1 destruction, we overexpressed CDH1 to determine whether it could cause prophase I arrest. After microinjection with CDH1 mRNA following 2 h incubation in IBMX, we released the injected oocytes for IBMX. Then we counted the numbers of the GV and GVBD oocytes in each group at 3 h after release, and found that prophase I arrest did take place (Fig. 3D). Based on these results, we can say that the low level of CCNB1 in CENP-T depleted oocytes is caused by the increased CDH1 level. We next examined our hypothesis further. To do this, morpholino (MO) antisense was used for microinjection to knockdown CDH1 in oocytes. Before performing the experiment, we first tested whether MO worked well in oocytes. In line with this, oocytes were incubated for 24 h following MO microinjection, and results showed that CDH1 was knocked down effectively (Fig. 3C). Then we injected siRNA and MO separately in each group and incubated the oocytes for 24 h in medium containing 30 µM IBMX. The oocytes that were injected with both CENP-T siRNA and CDH1 MO underwent spontaneous GVBD (54.32±7.42%; and 86.61±8.10%; mean±s.e.m.); however, the oocytes injected with CENP-T siRNA combined with control MO and the control oocytes treated with control siRNA and control MO were still arrested at prophase I (Fig. 3E). In addition, we set out to determine whether knocking down CDH1 could rescue the prophase I arrest. As expected, the prophase I arrest caused by CENP-T depletion could be partly rescued by CDH1 knockdown (Fig. 3F). The above data suggest that a high expression of CDH1 is the main reason for failed meiotic resumption caused by CENP-T depletion. We further tested whether CENP-T could directly interact with CDH1 by co-immunoprecipitation analysis, and results showed that CDH1 did directly bind to CENP-T (Fig. 3G).

CENP-T overexpression promotes G2/M transition by reducing CDH1 expression

To further prove the function of CDH1 in CENP-T-regulated G2/M transition in meiotic oocytes, we performed CENP-T overexpression. We added a Myc tag on the CENP-T gene, and we achieved the CENP-T overexpression by microinjection of its mRNA followed by 2 h incubation in IBMX. We then performed western blotting with anti-Myc antibody to show the overexpression of CENP-T (Fig. 4B). As expected, the CDH1 level was significantly reduced in the CENP-T-overexpressing oocytes (Fig. 4A). These data suggest that CENP-T is necessary to mediate CDH1 levels in oocytes.

As the CENP-T-depleted oocytes arrested at prophase I, we expected that overexpression of CENP-T would stimulate GVBD. We released CENP-T-overexpressing oocytes after 2 h incubation in IBMX, and found that these oocytes underwent GVBD 15 min earlier than the control oocytes (Fig. 4C). In addition, if we continuously incubated the CENP-T-overexpressing oocytes in 30 µM IBMX, oocytes underwent spontaneous GVBD, while most of the control oocytes were still arrested at the GV stage. In conclusion, CENP-T depletion arrests oocytes at the GV stage; in contrast, CENP-T overexpression stimulates GVBD, suggesting that CENP-T acts through CDH1 to regulate meiotic resumption in meiotic oocytes.

CENP-T overexpression accelerates progression through meiosis I by reducing CDH1 levels

After the CENP-T-overexpressing oocytes underwent GVBD, we incubated these oocytes for an additional 10 h, and no significant difference was found for the percentage of oocytes that extruded PB1 compared with the control group (Fig. 5A). However, the CENP-T-overexpressing oocytes extruded PB1 ∼2 h earlier than control oocytes (Fig. 5B,C). We also found that oocytes at the MII stage showed increased percentages of abnormal chromosome distribution in CENP-T-mRNA-injected oocytes. Chromosomes showed a scattered distribution in CENP-T-overexpressing oocytes, although the spindle structure showed no obvious abnormality (Fig. 5F), while chromosomes were neatly arranged on the equatorial plate of control

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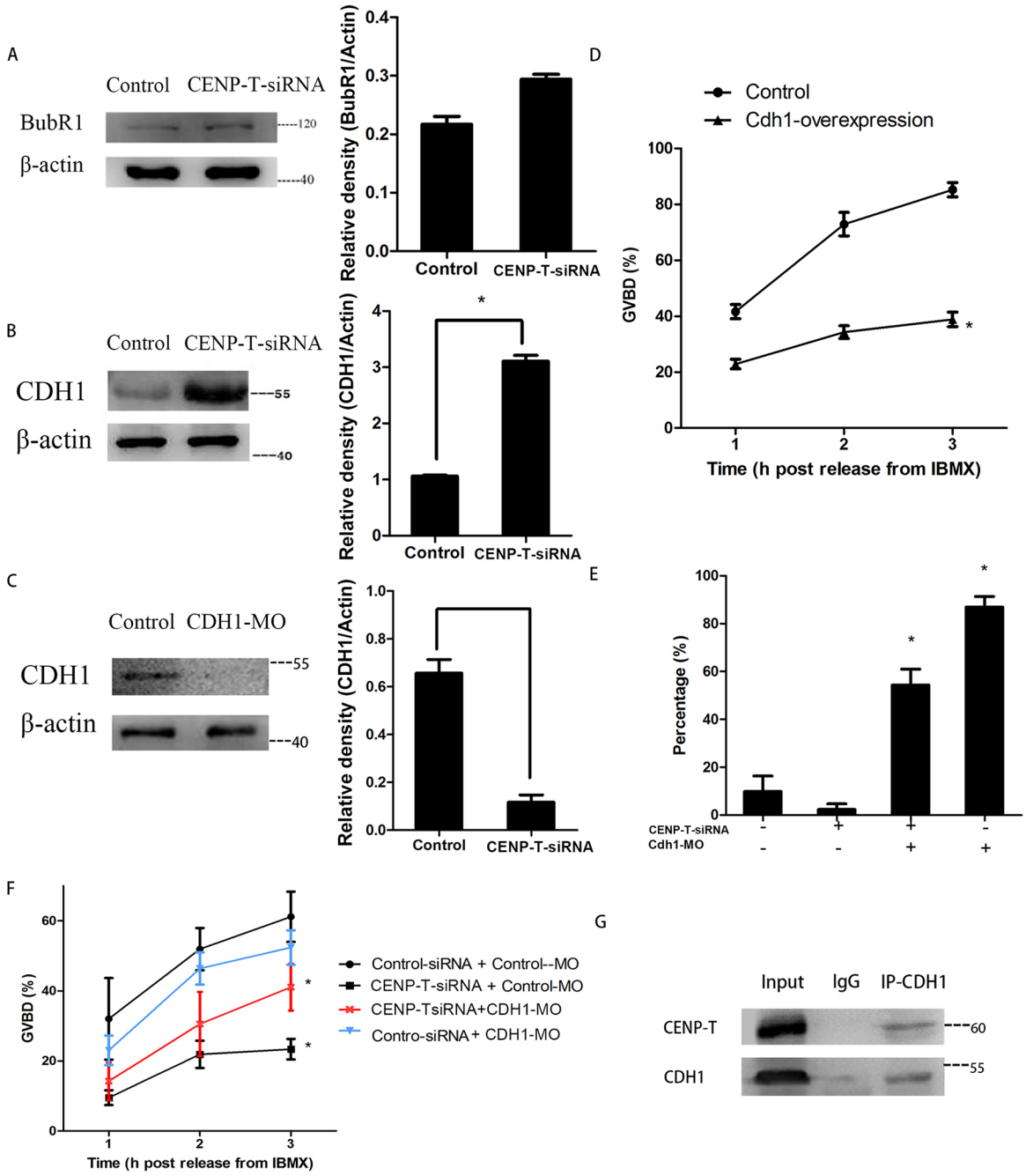

Fig. 3. CENP-T regulates G2/M transition by acting through CDH1. (A–C) Western blotting results for BubR1, CDH1 and β-actin in CENP-T-siRNA- oocytes and control-injected oocytes at 1 h following release from IBMX (200 oocytes per sample). The relative staining intensity was assessed by densitometry. (D) CDH1 mRNA was injected in control GV oocytes followed by 2 h incubation in 200 µM IBMX. The percentage of oocytes that had undergone GVBD by 1, 2 and 3 h following release from IBMX of oocytes was recorded. (E) Oocytes were injected with CENP-T-siRNA or control-siRNA mixed with CDH1-MO or control-MO following 24 h incubation in 30 µM IBMX. Then the percentage of spontaneous GVBD was assessed. (F) Oocytes were injected with CENP-T-siRNA or control-siRNA mixed with CDH1-MO or control-MO following 24 h incubation in 200 µM IBMX. Percentage of oocytes that had undergone GVBD 1, 2 and 3 h following release from IBMX of oocytes were assessed. All quantitative data are presented as means±s.e.m. of three independent experiments (*P<0.05). (G) Two ovaries were lysed for co-immunoprecipitation of CDH1 and CENP-T, and specific anti-CDH1 antibody was used in this test. After incubation with antibody and resin overnight at 4°C, the resultant immune complexes were subjected to western blotting. A representative final western blot for CDH1 and CENP-T, with appropriate controls, is shown. This experiment was repeated at least three times.

oocytes (Fig. 5D,E), indicating that CENP-T also regulates the meiotic cell cycle progression following GVBD.

Further experiments were conducted to understand why CENP-T-overexpressing oocytes progressed faster through the first meiosis. As degradation of CCNB1 is needed for oocytes to pass through metaphase I, we first traced CCNB1 after GVBD, and we also examined the timing of CCNB1 degradation. It was shown that CCNB1 was degraded earlier in CENP-T-overexpressing oocytes compared to in the control group (Fig. 6A,B). Next we examined whether the spindle assembly checkpoint (SAC) is prematurely inactivated after GVBD in CENP-T-mRNA-injected oocytes, and found that SAC inactivation in CENP-T-overexpressing oocytes

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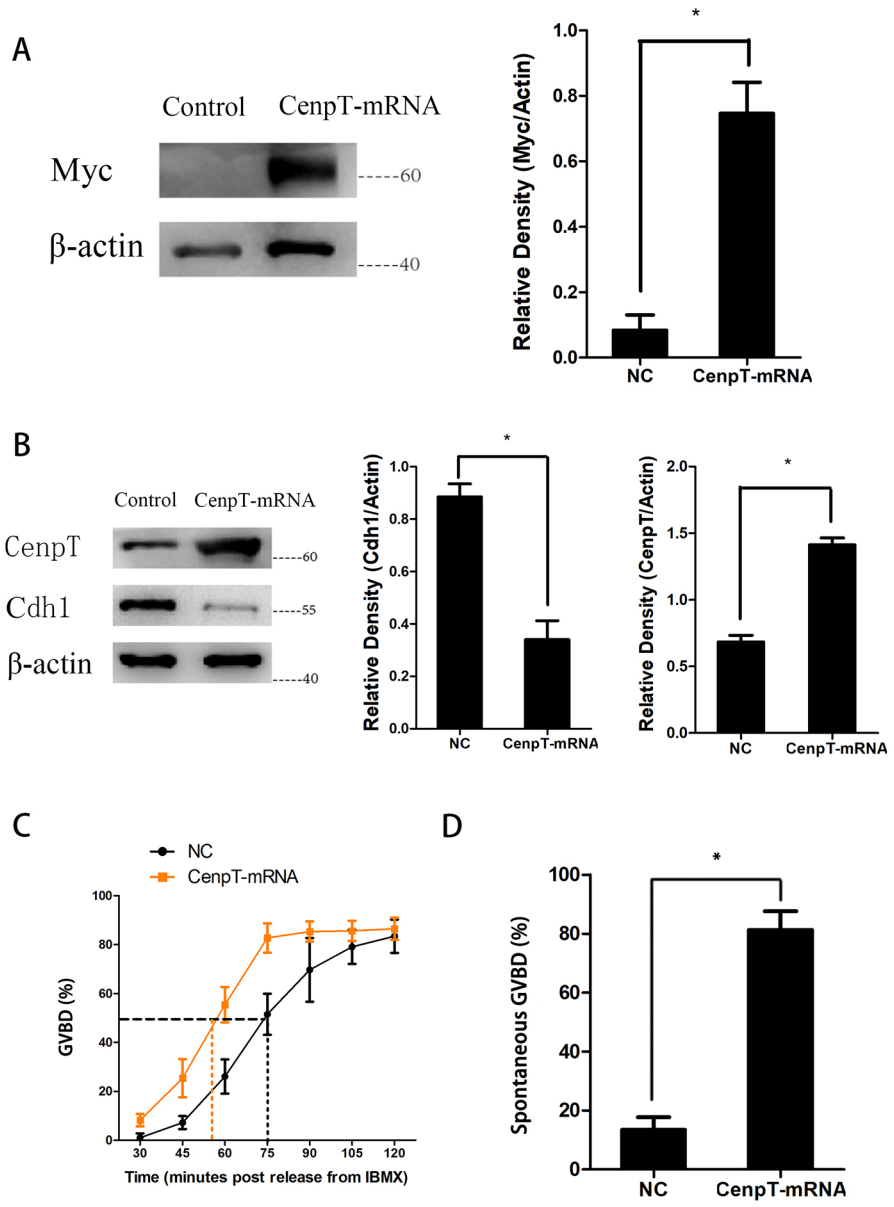

Fig. 4. Overexpression of CENP-T promotes GVBD. (A,B) Western blotting results for Myc, CENP-T, CDH1 and β-actin in the CENP-T-Myc mRNA-injected oocytes and control (NC, negative control) oocytes (200 oocytes per sample). The relative staining intensity was assessed by densitometry. (C) The percentage of oocytes CENP-T-mRNA-injected oocytes and control oocytes that had undergone GVBD were recorded from 30 min to 120 min after release from IBMX. (D) After injection with CENP-T mRNA, the percentages of oocytes that underwent GVBD were assessed after 3 h incubation in 30 µM IBMX. Data are presented as means±s.e.m. of three independent experiments (*P<0.05).

occurred earlier than in the control group. Bub3, a mitotic checkpoint protein, signals were observed at kinetochores at 4.5 h and 5.5 h post GVBD, but disappeared at 6.5 h post GVBD in CENP-T-overexpressing oocytes, while SAC signal could still be found in control oocytes at the same time point (Fig. 6C). Based on these data, we conclude that CENP-T is also involved in meiotic cell cycle regulation: overexpression of CENP-T causes premature SAC inactivation and CCNB1 degradation and thus accelerated meiosis I completion. We further analyzed the expression level of CDH1, which works as an activator of the APC/C from the GV stage to prometaphase I, at 1.5 h, 2.5 h, 3.5 h, 4.5 h, 5.5 h and 6.5 h post GVBD. Results showed that the CDH1 level was relatively high from 1.5 h to 5.5 h post GVBD, and obviously decreased at 6.5 h post GVBD in the control group. However, a significantly lower expression level of CDH1 was detected in CENP-T-overexpressing oocytes from 1.5 h to 6.5 h, and almost no signal could be found after 3.5 h post GVBD (Fig. 6D,E). We also tested the expression level of another APC/C activator protein, CDC20, which functions with APC/C after CDH1 degradation, at the same time points post GVBD. Results showed that CDC20 could almost not be detected at 1.5 h and 2.5 h post GVBD, and increased expression was found at 6.5 h post GVBD in control oocytes. Comparatively, expression level of CDC20 was significantly higher than that of control group at 1.5 h and 2.5 h post GVBD, and also increased at 6.5 h post GVBD (Fig. 6F,G). These results suggest that CENP-T also has critical functions after GBVD during meiotic progression by acting through CDH1, and the decreased CDH1 level caused by CENP-T overexpression may cause accelerated progression through meiosis I in CENP-T-overexpressing oocytes.

DISCUSSION

CENP-T, an important member of the kinetochore protein family, works along with CENP-W to form a complex of the inner kinetochore, and mediates duplication and elongation of kinetochores. CENP-T has been reported to play a critical role in regulating the mitotic cell cycle (Giunta and Funabiki, 2017; Prendergast el al, 2017). Here, we first provide evidence for a novel role of CENP-T in regulating the meiotic cell cycle by regulating the

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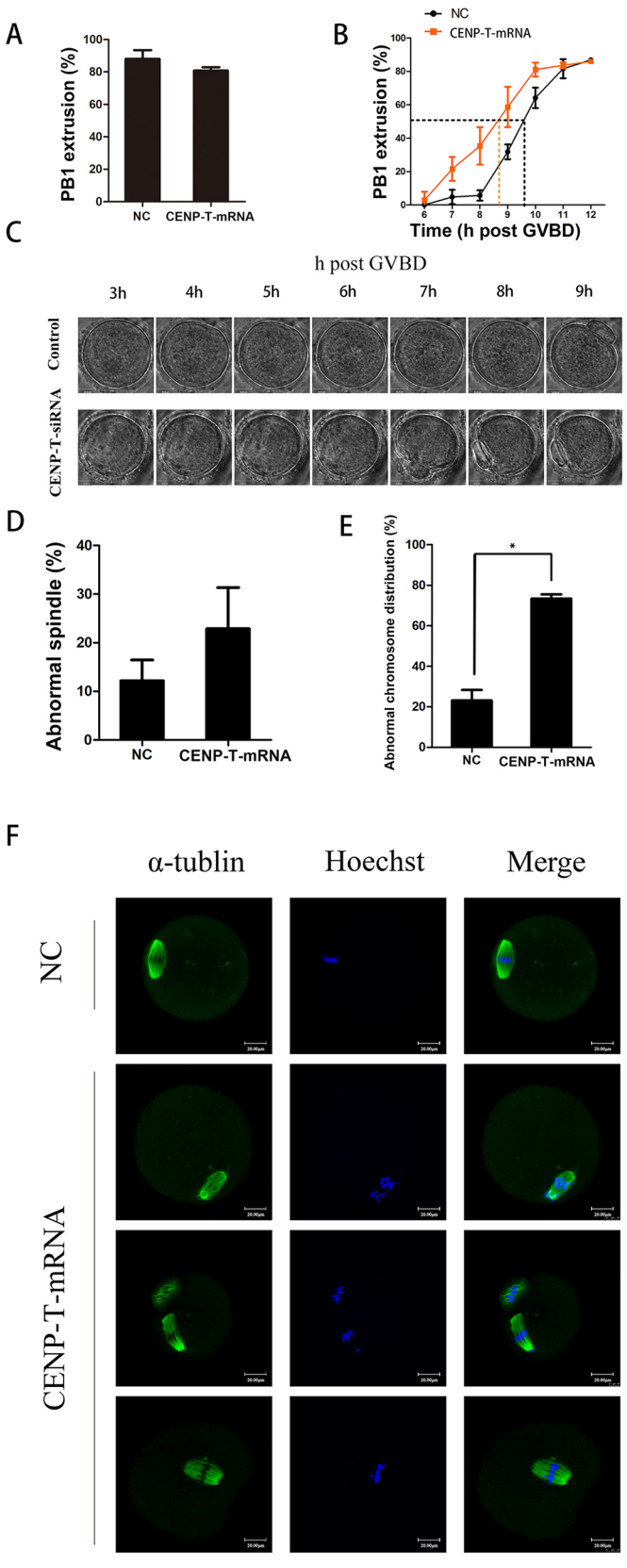

Fig. 5. CENP-T overexpression accelerates progression through meiosis I. (A) The percentages of oocytes that had extruded PB1 extrusion for control (NC) oocytes and CENP-T-overexpressing oocytes were recorded at 14 h after release from IBMX. (B) CENP-T-overexpression oocytes and control oocytes that underwent progression through meiosis were counted from 6 h to 12 h after release from IBMX. The progression of PB1 extrusion was obviously earlier in CENP-T mRNA-injected oocytes than in control oocytes. (C) Live-cell imaging showing the time of PB1 extrusion in both CENP-T-mRNA-injected oocytes and control oocytes. See also Movies 1 and 2. (D) The percentage of abnormal spindle structures seen in CENP-T-overexpressing oocytes and control oocytes. (E) The percentage of abnormal chromosome distribution seen in CENP-T-overexpressing oocytes and control oocytes. (F) Confocal microscopy showing the distribution of chromosomes in CENP-T-overexpressing oocytes and control oocytes. α-tubulin (green) was stained with anti-α-tubulin–FITC antibody, and DNA (blue) was stained with Hoechst 33342. Scale bars: 20 µm. All quantitative data are presented as means± s.e.m. of three independent experiments (*P<0.05).

CDH1 expression level, which sharply differs from the role of CENP-T in mitosis. In meiosis, CENP-T was localized in the nucleus at the GV stage and showed the same subcellular localization pattern at kinetochores after GVBD as that in mitosis. CENP-T was expressed throughout meiotic maturation, and it was highly expressed at the stage of GVBD, which indicated that CENP-T might be involved in the progression of GVBD. Based on the expression pattern of CENP-T, we focused our observation on the progression of GVBD and found that CENP-T plays a critical role in G2/M transition, as we hypothesized. We depleted CENP-T in the GV oocytes by siRNA microinjection, and found that only 30% of CENP-T-injected oocytes underwent GVBD 3 h following release from IBMX, providing evidence showing that CENP-T, as a kinetochore protein, not only takes part in the assembly of kinetochore but also has a role in mediating meiotic resumption in mouse oocytes.

We next set out to find the reason for meiosis I arrest in CENP-T-depleted oocytes. As prophase I arrest is mainly maintained through a low activity of MPF, which is formed by CCNB1 and CDK1 (Aritro, 2013; Kishimoto, 2018), we tested the MPF activity of CENP-T-depleted oocytes, and results showed that the MPF activity of CENP-T-depleted oocytes was obviously lower than that of control oocytes, as indicated by reduced CCNB1 level and an increased CDK1 phosphorylation level as well as decreased H1 histone kinase activity. We further asked whether CCNB1 failed to enter the nucleus, which is required for MPF activation. Although, we did find failure of CCNB1 entry into the nucleus in CENP-T-depleted oocytes, it was hard for us to determinate whether this was caused by CCNB1 dysfunction or its low level expression, which should be further tested in future experiments. To further understand what actually caused the prophase I arrest, we injected exogenous CCNB1 and active CDK1 to perform rescue experiments, and found that only exogenous CCNB1 rescued the G2/M transition in CENP-T-depleted oocytes, so we believe that the CCNB1 accumulation failure was the actual reason for prophase I arrest, and the explanation for high phosphorylation of CDK1 might be due to the low expression of CCNB1 in oocytes, as CDK1 activity can be influenced by the CCNB1 expression level (Holt et al., 2011; Marangos and Carroll, 2008). In a previous study, BubR1 was reported to play a role in mediating meiosis resumption (Homer et al., 2009); we doubted whether CENP-T would interact with BubR1 to mediate the G2/M transition, and we indeed found that BubR1 was not affected after knockdown of CENP-T in oocytes, which excluded the possibility that CENP-T affected GVBD through the BubR1 pathway. Next, we hypothesized that the reduced CCNB1 was caused by accelerated destruction. In line with this, we first considered the function of APC/C, which could mediate destruction of CCNB1 during oocyte maturation (Aritro, 2013). As expected, the expression of CDH1 was significantly increased, indicating that APC/C activity was very high in CENP-T-depleted oocytes, which might cause accelerated CCNB1

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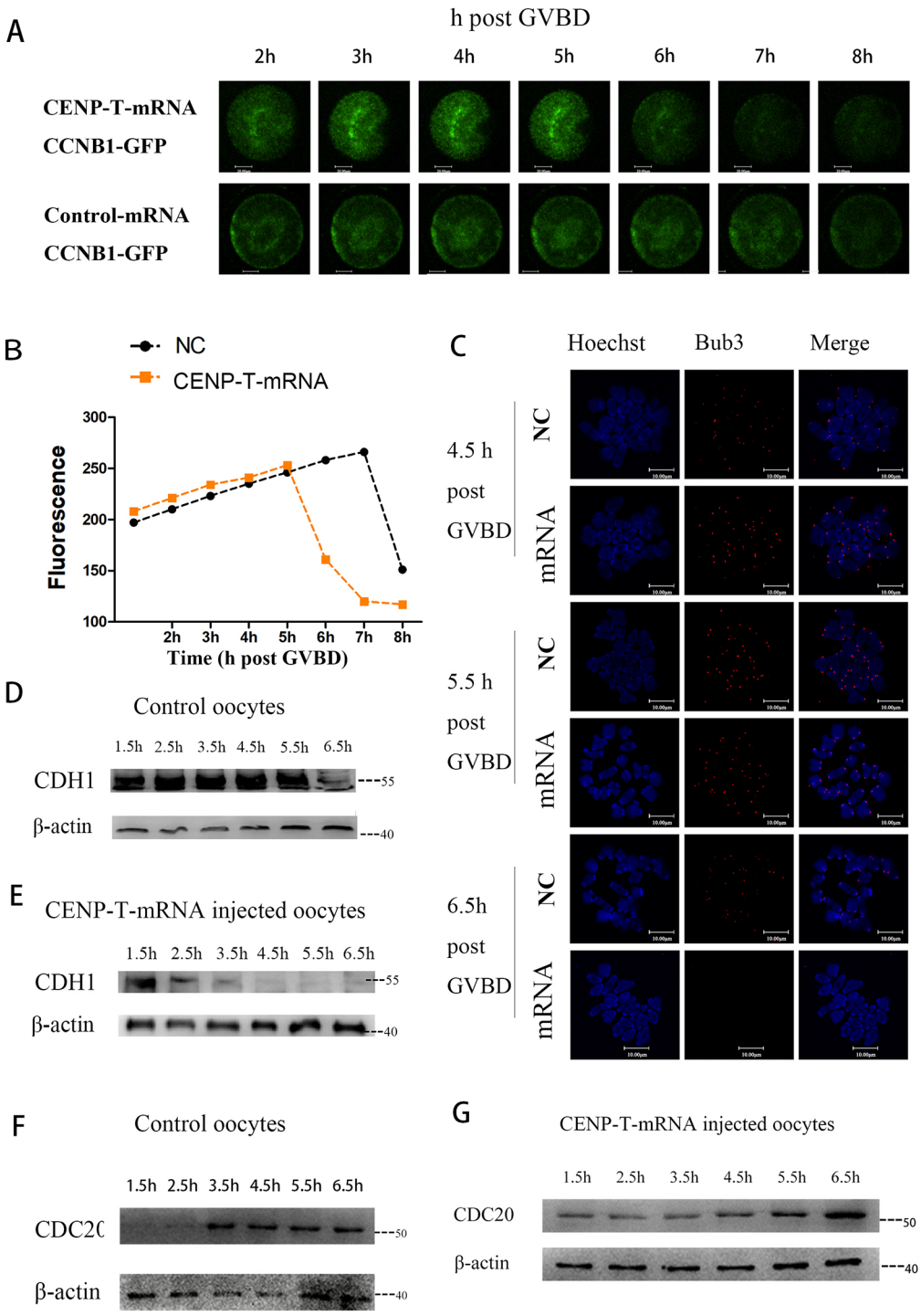

Fig. 6. CENP-T overexpression regulates anaphase entry by acting through CDH1. (A) After injection with CENP-T mRNA or control mRNA (NC) followed by 2 h incubation in 200 µM IBMX, 10 ng/µl CCNB1 mRNA was microinjected into oocytes. Oocytes were then maintained for 15 min in 200 µM IBMX for protein expression. Live-cell imaging showed the dynamics of CCNB1–GFP at 1 h intervals. (B) The relative fluorescence intensity (arbitrary units) of CCNB1–GFP was assessed in oocytes as in A. (C) Oocytes injected with CENP-T mRNA or control mRNA were collected at 4.5 h, 5.5 h and 6.5 h post GVBD. Bub3 was stained with rabbit polyclonal anti-Bub3 antibody (red), and chromosomes were stained with Hoechst 33342 (blue). (D) Control oocytes were collected at 1.5 h, 2.5 h, 3.5 h, 4.5 h, 5.5 h and 6.5 h post GVBD (200 oocytes per sample), and western blotting results for CDH1 and β-actin are shown. (E) Western blotting results for CDH1 and β-actin in the CENP-T-mRNA injected oocytes collected at 1.5 h, 2.5 h, 3.5 h, 4.5 h, 5.5 h and 6.5 h post GVBD (200 oocytes per sample). (F) Control oocytes were collected at 1.5 h, 2.5 h, 3.5 h, 4.5 h, 5.5 h and 6.5 h post GVBD (200 oocytes per sample), and the expression level of CDC20 and β-actin were determined by western blotting. (G) CENP-T-mRNA-injected oocytes were collected at 1.5 h, 2.5 h, 3.5 h, 4.5 h, 5.5 h and 6.5 h post GVBD (200 oocytes per sample), and western blotting of CDH1 and β-actin was performed. Scale bars: 20 µm (A); 10 µm (C).

destruction. To prove the hypothesis that it was the increased CDH1 expression level that caused prophase I arrest in CENP-T-depleted oocytes, we knocked down or overexpressed CDH1 in oocytes, and we also overexpressed CENP-T to inversely test the possibility. All the results indicate that CENP-T function in meiotic resumption is indeed mediated by CDH1. Whether CENP-T, working mainly as a kinetochore protein, interacts with CDH1 was unknown. We used anti-CDH1 antibody to conduct co-immunoprecipitation, and found that CENP-T can directly interact with CDH1, and hence, these roles might be mediated via a direct interaction.

To further understand the function of CENP-T in meiotic progression after GVBD, we again used culture of CENP-T-overexpressing oocytes, and found that CENP-T-overexpressing oocytes extruded PB1 earlier than control oocytes. We also found that CENP-T-overexpressing oocytes had a normal spindle structure and abnormal chromosome distribution. Because SAC inactivation and CCNB1 destruction are actually needed for oocytes to pass through the MI stage (Touati and Wassmann, 2015; Homer, 2006), we examined both the SAC and CCNB1 in CENP-T-overexpressing oocytes, and found that the signals for both the SAC and CCNB1 disappeared earlier than in control oocytes, which might be the

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cause of the earlier metaphase-to-anaphase transition and PB1 extrusion. We found that CENP-T-overexpressing oocytes had similar phenotypes to those in CDH1-knockout oocytes; they both spontaneously underwent GVBD in 30 µM IBMX and both extruded the PB1 earlier than control oocytes. The abnormal chromosome distribution was also found in both CDH1-depleted oocytes and CENP-T-overexpressing oocytes. In addition, degradation of CDC20 in CENP-T-overexpressing oocytes could not be found at 1.5 h post GVBD, which has also been previously shown to be the case in CDH1-depleted oocytes (Holt et al., 2011, 2012; Fukushima et al., 2013; Reis et al., 2007). Therefore, we hypothesize that overexpression of CENP-T in oocytes caused reduced CDH1 and thus led to similar phenotypes to those seen in CDH1-knockout oocytes. To prove our hypothesis, we examined CDH1 after GVBD in CENP-T-overexpressing oocytes, and a low level of CDH1 was detected in CENP-T-overexpressing oocytes, as expected. Taken together, we conclude that CENP-T has a critical role in meiotic anaphase entry by regulating the CDH1 expression level.

In summary, this study has provided evidence showing a novel function of kinetochore protein CENP-T in the G2/M transition of meiotic oocytes via regulating CDH1 level, which further affects MPF activity. We also demonstrated that there was no interaction between CENP-T and BubR1. CENP-T also plays a critical role in meiotic anaphase entry by regulating the CDH1 expression (Fig. 7). More experiments need to be performed to further understand the pathway of CENP-T-mediated meiotic cell cycle regulation.

MATERIALS AND METHODS

Oocyte collection and culture

Care and handing of 6–8-week-old ICR mice was conducted in accordance with policies promulgated by the Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences. Oocytes were cultured in M2 medium (Sigma) supplemented with 200 µM 3-isobutyl-1-methylxanthine (IBMX) to maintain them at the GV stage. After different culture times in IBMX, oocytes were released and washed thoroughly three times, followed by culture in M2 medium without IBMX to specific stages. All oocytes were cultured in medium under liquid paraffin oil at 37°C in an atmosphere of 5% CO_2 in air.

Antibodies

Mouse polyclonal anti-CENP-T antibody (1:1000 for western blotting, and 1:50 for immunofluorescence analysis) was purchased from Genetex (GTX51767); mouse monoclonal anti-α-tubulin–FITC antibody (1:4000) was obtained from Sigma-Aldrich (F2168); mouse monoclonal anti-CCNB1 antibody (1:1000) was purchased from Santa Cruz Biotechnology (sc-245); rabbit polyclonal anti-CDK1 p34 (Tyr 15) antibody (1:1000) was purchased from Santa Cruz Biotechnology (sc-12340-R); rabbit monoclonal anti-Myc antibody (1:4000) for western blotting, and 1:200 for immunofluorescence analysis) was purchased from Abcam (ab32072); rabbit polyclonal anti-Bub3 (1:200) antibody was obtained from Santa Cruz Biotechnology (sc-28258); sheep polyclonal anti-BubR1 antibody (1:2000) was purchased from Abcam (ab28193).

CENP-T, CDH1, CCNB1–GFP and mutant CDK1 expression plasmid construction

Plasmid construction was performed as described previously (Xu et al., 2015). Briefly, total RNA was extracted from 150 mouse GV oocytes by

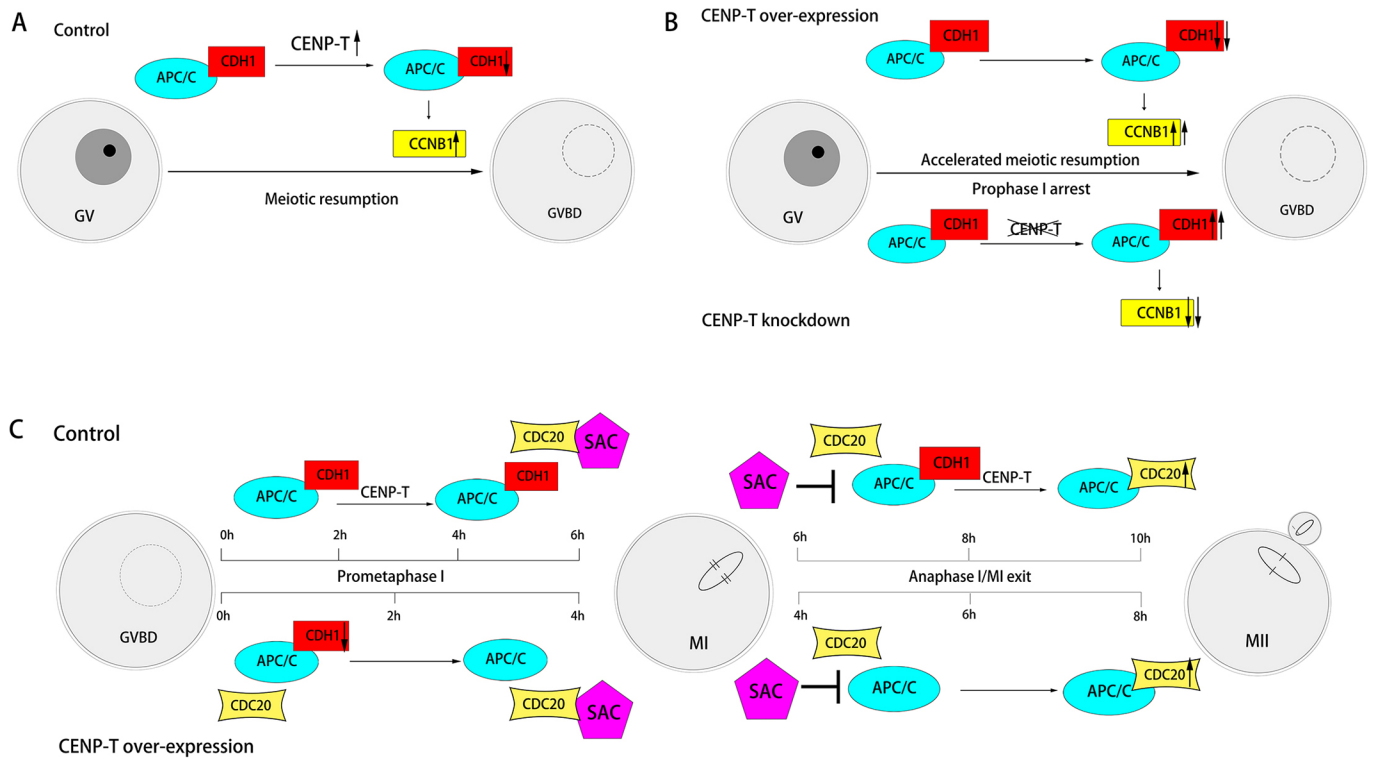

Fig. 7. Effects of CENP-T during meiosis in oocytes. (A) Mechanism for meiotic resumption in normal oocytes. (B) Depletion of CENP-T in mouse oocytes leads to upregulation of CDH1, causing instability of CCNB1 and prophase I arrest. Conversely, CENP-T overexpression causes CDH1 degradation, in turn leading to increased CCNB1 activity, accelerating meiotic resumption. (C) Mechanism for MI–anaphase transition for both control oocytes and CENP-T-overexpressing oocytes. CDH1 binds to APC complex at prometaphase, and overexpression of CENP-T caused downregulation of CDH1 as well as upregulation of CDC20, which accelerates PB1 extrusion. The SAC, working as a checkpoint for meiosis, becomes inactivated earlier in CENP-T-overexpressing oocytes compared with that of control oocytes.

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using the RNeasy micro purification kit (Qiagen) and the first-strand cDNA was generated with a cDNA synthesis kit (Takara) using poly(dT) primers. Nested PCR was used to amplify the full-length specific cDNA for each gene (5′-GGCCGGCCCGGATCAATGCACAAAGAAG-3′ and 5′-CCG-CGCGGCATATTTCAGAAATTAAGGA-3′ for CENPT; 5′- GGCCGG-CCGCTCCTGCTGCAGGTCTCCT-3′ and 5′-CCGCGCGGAATTGTC-AAATTGTTTTGAG-3′ for CDH1; 5′-GGCCGGCCCTAGGAACACG-AAAATTAAC-3′ and 5′-CCGCGCGGTTTCGAAAGGTGGTTATTTA-AAA-3′ for CCNB-GFP; 5′-GGCCGGCCATTGGAGAAGGTACTTA-CGG-3′ and 5′-CCGCGCGGAAGACAAACACTTATTAGGAA-3′ for mutant CDK1). PCR products were digested using FseI and AscI (New England Biolabs, Inc.) and linked with GFP or Myc plasmid. The fusion plasmid was transfected into T1 competent cells (Transgen Biotech). The modified plasmid was then extracted (Transgen) and linearized. Capped mRNA was produced by the mMESSAGE mMACHINE T3 (Ambion) from the linearized plasmids (linearized by Sfi, New England Biolabs), and a poly(A) tail was added with a poly(A) polymerase tailing kit (Epicenter, AP-31220). Finally, an RNeasy cleanup kit (Qiagen) was used to purify the mRNA.

Microinjection of siRNA, MO or mRNA

Microinjections were performed using a Narishige microinjector and completed within 30 min. For CENP-T knockdown in mouse oocytes, CENP-T siRNA (sc93326, Santa Cruz Biotechnology) was diluted with nuclease-free water (Sigma) to give a 2 mM stock concentration. The same amount of negative control siRNA was used as control. After microinjection, oocytes were kept at the GV stage for 24 h in IBMX-containing medium. A volume of 2 mM CDH1 MO 5′-CCTTCGCTCATAGTCCTGGTCCATG-3′ (Gene Tools, Philomath, OR) was microinjected to knockdown CDH1 in oocytes, and the same volume of negative control MO 5′-CCTCTTACCT-CAGTTACAATTTATA-3′ (Gene Tools, Philomath, OR) was used as control. For CCNB1–GFP overexpression, 1 µg/µl *Ccnb1* mRNA solution was injected into the cytoplasm of the GV oocytes followed by 2 h incubation in IBMX-containing medium. For CCNB1–GFP dynamics analysis, 10 ng/µl *Ccnb1* mRNA solution was injected into the cytoplasm of the GV oocytes followed by 2 h incubation in IBMX-containing medium. The same amount of GFP mRNA was injected as control. For CENP-T and CDH1 overexpression, 1 µg/µl mRNA was microinjected in oocytes, and oocytes were arrested at the GV stage in IBMX-containing medium for 2 h.

Immunofluorescence analysis

Immunofluorescence was performed as described previously (Wang et al., 2019). Briefly, oocytes were first fixed in 4% paraformaldehyde in PBS buffer for 30 min at room temperature, and permeabilized with 0.5% Triton X-100 for 20 min. Then they were blocked in 1% BSA-supplemented PBS for 1 h at room temperature. For staining of α-tubulin, oocytes were incubated overnight at 4°C with 1:800 anti-α-tubulin–FITC antibody. For other staining of CENP-T, Myc or BUB3, oocytes were incubated with primary antibody overnight at 4°C, and with secondary antibody for 1 h at room temperature. DNA was stained for 15 min with Hoechst 33342. Finally, oocytes were mounted on glass slides and viewed under a confocal laser-scanning microscope (Zeiss LSM 780).

Chromosome spreading

Oocytes were placed in Tyrode's solution (Sigma, T1788) to remove the zona pellucida. A 1 cm×1 cm frame was drawn on a glass slide with a hydrophobic pen and 100 µl methanol:glacial acetic acid (3:1) was added in the frame area. Oocytes were dropped onto the slide for breaking and fixing. Then Bub3 antibody was added, followed by incubation overnight at 4°C, and then secondary antibody was added for 1 h at room temperature. Finally, 10 µg/ml Hoechst 33342 was added to stain the chromosomes.

Western blotting

Immunoblotting was performed as described previously (Zhang et al., 2016). Briefly, a total of 150 mouse oocytes were collected in a 7 µl 2× SDS buffer and heated for 5 min at 100°C. The proteins were separated by SDS-PAGE and then transferred onto PVDF membranes. Next, the membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% BSA for 2 h at room temperature, followed by incubation overnight at 4°C with primary antibody, then incubation with specific secondary antibody at room temperature for 1 h, after three washes in TBST buffer, for 10 min each time. Finally, the membranes were processed using the enhanced chemiluminescence detection system (Bio-Rad, CA).

Time-lapse live imaging experiments

CCNB1–GFP dynamics were filmed on a PerkinElmer Ultra VIEW VoX confocal imaging system. A narrow band-pass EGFP filter set and a 30% cut neutral density filter from Chroma was used. Exposure time was set ranging from 300–800 ms depending on the CCNB1–GFP fluorescence levels. The acquisition of digital time-lapse images was controlled by IP Lab (Scanalytics) or AQM6 (Andor/Kinetic-imaging) software packages. Confocal images of CCNB1 in live oocytes were acquired with a 20× oil objective on a spinning-disc confocal microscope (PerkinElmer).

Histone H1 kinase assay

The MPF activity assay was performed as described previously (Kubiak, 2011). Briefly, 20 oocytes were collected in 1 µl 20 mg/ml BSA with a 1.5 ml Eppendorf tube after washing five times in solution with the same concentration of BSA. Then the samples were repeatedly frozen and thawed at least three times for collecting oocytes for lysis. 2 µl of HK buffer (15 mM $Mgcl_2$, 20 mM EGTA and 1 mM DTT) was next added to bring the total volume to 3 µl, and the samples were gently spun down using a table centrifuge (12,000 ***g*** for 1 min). The samples were placed back on crushed ice and 1 µl of the radioactive reaction solution (3.3 mg/ml histone H1, 1 mM ATP, 0.25 mCi/µl [^{32}P]ATP) was added to the inside wall of the Eppendorf tubes without shaking. Following centrifugation at a low speed (5000 ***g*** for 1 min), the tubes were transferred immediately to a 37°C incubation bath for 50 min, then placed on crushed ice and 20 µl of Laemmli buffer was immediately added to obtain a final volume of 24 µl. The tubes were heated for 5 min at 90°C and then cooled on crushed ice. Then samples were subjected to SDS-PAGE. Next, SDS-PAGE gels were removed and fixed using 70% ethanol and 10% acetic acid, and gels were stained with Coomassie Brilliant Blue solution in 70% ethanol and 10% acetic acid. Finally, the gels were exposed to X-ray film in a dark room or to a phosphoimager screen.

Co-immunoprecipitation

The co-immunoprecipitation assay was performed as described previously (Takahashi, 2015). Briefly, ovary was lysed in an appropriate lysis buffer for 1 h on ice, followed by centrifugation (12,000 ***g*** for 10 min), and supernatant liquid was transferred to another tube. CDH1 in the lysate was captured using a specific antibody and precipitated along with its binding proteins using beads overnight at 4°C. Next, five washes with lysis buffer were performed to remove non-bound proteins in the lysate. The resultant immune complexes were subjected to western blotting to determine the interaction of CDH1 and CENP-T. 10 µl of lysate without any antibody added was used as Input, and rabbit IgG was used as control antibody in control group.

Image analysis

Images were acquired using a confocal laser-scanning microscope (LSM 780; Zeiss) equipped with a C-Apochromat 40 ×water immersion objective. Data analysis was performed using ZEN 2012 LSM software (Zeiss) and ImageJ software.

Statistical analysis

Data (mean±s.e.m.) were generated from at least three replicates per experiment and analyzed by one-way ANOVA using SPSS software (SPSS Inc., Chicago, IL) followed by Student–Newman–Keuls test. A difference at $P<0.05$ was considered to be statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Y.W.; Methodology: Y.W., Y.-C.O., Z.W.; Software: Y.W., W.Y.; Validation: Y.W., Y.H.; Formal analysis: Y.W., F.D.; Investigation: Y.W.; Resources: Y.W., J.L.; Data curation: Y.W.; Writing - original draft: Y.W.; Writing - review & editing: Y.W., H.S., Q.-Y.S.; Visualization: Y.W., Q.-Y.S.; Supervision: Q.-Y.S.; Project administration: Q.-Y.S.; Funding acquisition: Q.-Y.S.

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Supplementary information

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.238105.supplemental

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