OLA1 is responsible for normal spindle assembly and SAC activation in mouse oocytes

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Background. OLA1 is a member of the GTPase protein family, unlike other members, it can bind and hydrolyze ATP more efficiently than GTP. OLA1 participates in cell proliferation, oxidative response and tumorigenesis. However, whether OLA1 is also required for oocyte meiosis is still unknown.

Methods. In this study, the localization, expression, and functions of OLA1 in the mouse oocyte meiosis were examined. Immunofluorescent and confocal microscopy were used to explore the location pattern of OLA1 in the mouse oocyte. Moreover, nocodazole treatment was used to confirm the spindle-like location of OLA1 during mouse meiosis. Western blot was used to explore the expression pattern of OLA1 in the mouse oocyte. Microinjection of siRNA was used to explore the OLA1 functions in the mouse oocyte meiosis. In addition, chromosome spreading was used to investigate the spindle assembly checkpoint (SAC) activity.

Results. Immunofluorescent staining showed that OLA1 evenly distributed in the cytoplasm at germinal vesicle (GV) stage. After meiosis resumption (GVBD), OLA1 co-localized with spindles, which was further identified by nocodazole treatment experiments. Knockdown of OLA1 impaired the germinal vesicle breakdown progression and finally resulted in a lower polar body extrusion rate. Immunofluorescence analysis indicated that knockdown of OLA1 led to abnormal spindle assembly, which was evidenced by multipolar spindles in OLA1-RNAi-oocytes. After 6 h post-GVBD in culture, an increased proportion of oocyte which has precociously entered into anaphase/telephase I (A/TI) was observed in OLA1-knockdown oocytes, suggesting that loss of OLA1 resulted in the premature segregation of homologous chromosomes. In addition, the chromosome spread analysis suggested that OLA1 knockdown induced premature anaphase onset was due to the precocious inactivation of SAC. Taken together, we concluded that OLA1 plays important role in GVBD, spindle assembly and SAC activation maintenance in oocyte meiosis.

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ABSTRACT

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induced premature anaphase onset was due to the precocious inactivation of SAC. Taken together, we concluded that OLA1 plays important role in GVBD, spindle assembly and SAC activation maintenance in oocyte meiosis.

INTRODUCTION

Mammalian gametes are yield through an event named meiosis where two consecutive divisions are conducted to halve the chromosomes without an intervening replicative process (*Verlhac et al., 2016*). Spindle assembly and chromosome segregation are both vital courses to keep genomic stability during oocyte meiosis. Errors in these two processes can lead to the failure of meiosis or the generation of aneuploidy (*Ma et al., 2014*; *Jiao et al., 2017; Han et al., 2015*). Moreover, human pregnancy loss has strong correlation with fetal aneuploidy which can be induced by defective spindle structure and abnormal chromosome segregation (*Webster et al., 2017*). To guarantee the normal progress of oocyte meiosis, spindle assembly and chromosome segregation must be correctly commanded.

After GVBD, as absent of centrosome at the mitosis, the meiotic spindle is emanated and nucleated from microtubule organizing centers (MTOCs) (*Schuh et al., 2007*; *Clift et al., 2015; Bennabi et al., 2016*). During pre-metaphase I stage (pre-MI stage), the chromosomes showing unstable attachments to microtubules are under the supervision of SAC to avoid the premature of chromosome segregation (*Vogt et al., 2008*; *Touati et al., 2016; Sanders et al., 2018*). Once the incorrect connection of chromosome with microtubule is amended, all chromosomes are correctly aligned at the metaphase I plate (MI stage) then SAC proteins will dissociate from kinetochore to trigger the segregation of chromosomes (anaphase onset), which finally bring out the polar body extrusion and oocyte will arrest at metaphase II (MII).

OLA1 is a member of the GTPase protein family, unlike other members, it can bind and hydrolyze ATP more efficiently than GTP (*Koller-Eichhorn et al., 2007*). OLA1 can act as a

negative regulator of the antioxidative response via nontranscriptional mechanisms (*Zhang et al., 2009*). During mitosis, OLA1 localizes to centrosomes in interphase and then to the spindle pole after nuclear envelop breakdown (NEBD). OLA1 can directly interact with BRCA1 and γ-tubulin by bounding to the amino-terminal of these two proteins, and this interaction is very important for the centrosomal regulation (*Matsuzawa et al., 2014*). And knockdown of OLA1 results in the centrosome amplification and the activation of microtubule aster formation (*Matsuzawa et al., 2014*; *Yoshino et al., 2018*). It is noteworthy that, BRCA1 has been shown to exert functions in oocyte meiosis, and knockdown of BRCA1 in mouse oocyte causes abnormal spindle assembly and the dysfunction of SAC (*Xiong et al., 2008*). As an interactive factor, whether OLA1 also participates in meiotic progression remains elusive. Thus, we attempt to explore the possible roles of OLA1 in oocyte meiosis.

Oocyte maturation is a complicated process, which can be affected by many factors including functional cellular proteins, and whether OLA1 involve in meiosis is totally unknown. In this study, we have found that OLA1 participates in oocyte meiotic maturation, especially in the progress of germinal vesicle breakdown, spindle assembly and SAC activation.

MATERIALS AND METHODS

Animals

Three-four weeks-old female KM mice were used in this experiment. Animal experiments were approved by Hubei Research Center of Laboratory Animal (Approval ID: SYXK (Hubei) 2014-0082SCXK). Animal care and handling were conformed to regulations of Animal Care and Use Committee of General hospital of the Central Theater Command.

Antibodies and chemicals

All chemicals and culture media were purchased from Sigma (St Louis, MO) unless those specifcally mentioned. Rabbit polyclonal anti-OLA1 antibody (Cat# A4673) was purchased from

ABclonal (Wuhan, China). Mouse monoclonal anti-α-tubulin-FITC antibody (Cat# F2168) was obtained from Sigma (St Louis, MO); Sheep polyclonal anti-BubR1 antibody (Cat# 28193) was obtained from Abcam (Cambridge, UK). FITC-conjugated donkey anti-sheep IgG (H + L) was purchased from Jackson ImmunoResearch Laboratory.

Oocyte collection and culture

To harvest fully grown GV oocyte, 3-4 weeks-old female KM mice firstly intraperitoneally injected with 5 IU pregnant mares serum gonadotropin, after 46-48h, mice were sacrificed by cervical dislocation and ovaries were isolated. Enclosed cumulus oocytes were removed by repeatedly pipetting, and then oocytes were cultured in pre-warmed M16 medium under paraffin oil at 37°C in a 5% CO2 atmosphere. At appropriate time points, oocytes were selected for different experiments. In order to inhibit the spontaneous meiotic resume in vitro culture, M2 medium with 50 μM IBMX was used to maintain the GV stage.

Nocodazole treatment and recovery

To destroy the spindle apparatus, wide-type MI and MII stage oocytes were cultured in M16 medium with 20 μg/ml of nocodazole for 20 min. To re-assembly spindle microtubule, oocytes were then thoroughly washed out of nocodazole and cultured in fresh M16 medium for 30 min.

Microinjection of OLA1 siRNA

OLA1 siRNA (sc-145833; Santa Cruz, CA) was used to knockdown OLA1. Control siRNA (sc-37007; Santa Cruz, CA) was used as negative control. For microinjection, 5 pL of 30 μM control siRNA and OLA1 siRNA were injected into fully grown GV oocytes, to completely degrade the targeted mRNA, oocytes were cultured in M2 medium with 50 μM IBMX for 24 h. After that, oocytes were directly collected for western blotting or culture in fresh M16 medium for meiotic maturation.

Immunofluorescence analysis

Oocytes were fixed and permeabilized in PBS containing 4% paraformaldehyde, 0.5% Triton X-100 (pH=7.4) for 40-50 min. Then, oocytes were blocked in PBS containing 2% BSA 1 h at room temperature and incubated overnight at 4°C with anti-OLA1 antibody (ABclonal, 1:100), anti-α-tubulin-FITC antibody (Sigma, 1:100). After washing 3 times in PBS containing 0.05% Tween-20, oocytes were incubated with appropriate secondary antibodies for 1 h at 37°C. Chromosomes were visualized by staining with 1 μg/ml of DAPI for 5-10 min at room temperature. Finally, oocytes were mounted on slides and immunofluorescent images were observed by a confocal laser scanning microscope (Zeiss LSM 800, Germany). Non-immunized rabbit or mouse IgG was used as negative control.

Western blot

Oocytes were placed in 2X SDS loading buffer and boiled for 5 min at 95°C. The proteins were separated by SDS-PAGE and then electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. After transfer, the PVDF membranes were blocked in TBST containing 5% non-fat milk for 1 h, followed by incubation with OLA1 antibody (ABclonal, 1:1000) overnight at 4°C. After three times washes in TBST buffer, the membranes were incubated with HRP conjugates secondary antibodies at room temperature for 1 h. After three times washes, bands were visualized using ECL kit. GAPDH antibody was served as a loading control.

Chromosome spreading and BubR1 staining

Chromosome spreading was done as described previously (*Chen et al., 2018*). Oocytes were treated with Tyrode's buffer (pH 2.5) for about 30 s at 37°C to remove zona pellucidae. Then oocytes were fixed in drops of speading solution (1% PFA, 0.15% Triton X-100, 3mM DTT in ddH_2O, pH=9.2) on a glass slide. After undisturbed air drying, slides were washed and blocked

149 with PBS containing 1% BSA. Finally, samples were labeled with BubR1 antibody (Abcame,
150 1:200) and FITC-conjugated donkey anti-sheep secondary antibody (1:200). Chromosomes were
151 stained with DAPI.

152 **Statistical analysis**

153 Data are presented as mean ± SEM from at least three independent replicates. Statistical analyses
154 were made with GraphPad. For statistical comparison, Student's t test was used. A value of $P <$
155 0.05 was considered significant.

156 ## RESULTS

157 **Cellular localization and expression pattern of OLA1 during mouse oocyte meiosis**

158 Firstly, we investigated the dynamic distribution of OLA1 during mouse oocyte meiosis by
159 immunofluorescent labeling and confocal microscopy. As shown in Figure 1A, OLA1 was
160 concentrated on the whole cytoplasm at GV stage, after meiotic resume, OLA1 distributed
161 around the chromosomes and co-localized with α-tubulin. At MI and MII stages, OLA1
162 displayed a spindle-like localization, suggesting that OLA1 may have some connections with
163 spindle assembly. To validate the OLA1connection with spindle, MI and MII oocytes were
164 treated with nocodazole. After treatment, the spindles were depolymerized and microtubules
165 were evenly dispersed into the cytoplasm in oocytes. Meanwhile, OLA1 signal also dispersed
166 into the cytoplasm instead of around the chromosomes. Furthermore, as the oocytes were
167 thoroughly washed out of nocodazole and cultured in pre-warmed M16 medium, coupled with
168 spindle re-assembly, OLA1 renewed its spindle-like localization, hinting that OLA1 did co-
169 localized with spindle in mouse oocyte (Figure 1C). Western blot results showed that OLA1
170 consistently expressed during meiotic progression (Figure 1B).

Knockdown of OLA1 impairs the germinal vesicle breakdown (GVBD) leading to a decrease in polar body extrusion (PBE).

To further explore the function of OLA1 in oocyte meiosis, OLA1 specific siRNA were injected into GV oocytes. As shown in Figure 2A and B, western blot analysis revealed that expression of OLA1 was significantly decreased when compared with control ($p < 0.05$), suggesting that OLA1-RNAi achieved good knockdown efficiency for the further study of its function in oocytes. GVBD and PBE are two hallmark events in the meiotic progression. We then checked the GVBD and PBE rate after OLA1 knockdown, as shown in Figure 2C, knockdown of OLA1 significantly inhibited the GVBD progression and the average rate was 86.70 ± 6.12% in control groups, but decreased to 62.83 ± 1.49% in OLA1-RNAi groups ($p < 0.05$). After 12 h in culture, most of the control oocytes extruded the first polar bodies (60.38 ± 6.52%), while only 40.58 ± 2.05% of oocytes extruded the polar body in OLA1-RNAi groups (Figure 2D, $p < 0.05$). To further confirm whether the decline of PBE was due to the block of GVBD in OLA1-RNAi oocytes, we then analyzed the PBE rate in meiosis-resumed oocytes. As shown in Figure 2E, once the oocytes underwent GVBD, knockdown of OLA1 had no effect on PBE in meiosis resumed-oocytes (69.54 ± 3.45%, control vs 64.58 ± 2.34%, $P > 0.05$).

Knockdown of OLA1 induces abnormal spindle assembly and chromosome alignment in mouse oocyte

BRCA1 knockdown causes abnormal spindle assembly in mouse oocytes, as a direct interactive protein, OLA1 may modulate the spindle assembly. To verify this speculation, we then checked spindle morphology at metaphase stage by immunofluorescence after OLA1 knockdown. As shown in Figure 3A, confocal microscopy and quantitative analysis revealed that most control oocytes presented a normal barrel-shape spindle at metaphase stage, while OLA1-RNAi led to

abnormal multipolar and small spindles. The proportion of abnormal spindles in OLA1-RNAi group was significantly higher than the control group (30.06 ± 0.68% vs. 11.34 ± 1.46%, $p < 0.05$; Figure 3B). Meanwhile, chromosome alignment was also disturbed in OLA1-RNAi oocyte. As shown in Figure 3A, chromosomes were well-aligned at the metaphase I plate in control oocytes, while OLA1-RNAi oocytes displayed irregularly scattered chromosomes (red arrow). The proportion of misaligned chromosomes in OLA1-RNAi group was also significantly higher than the control group (13.19 ± 1.59% vs. 7.92 ± 1.57%, $p < 0.05$; Figure 3C).

OLA1 knockdown accelerates the anaphase onset in mouse oocytes

After 6 h post-GVBD in vitro culture , most of meiosis resumed-oocytes in control group have reached at MI, while we found some oocytes were extruding the polar bodies in OLA1-knockdown oocytes (Figure 4A). We speculate that OLA1 knockdown could result in premature anaphase onset. To verify this hypothesis, we then did the cycle analysis by α-tubulin staining and confocal microscope. As shown in Figure 4B, almost all of the control oocytes reached at MI, but we observed a quite number of oocytes have reached at anaphase I or telephase I in OLA1-knockdown oocytes. We also recorded the proportion of oocytes that have reached at MI or A/TI. Results showed that, compared with control, a lower rate of oocytes reached at MI stage in OLA1-RNAi oocytes (88.00 ± 3.09% vs. 74.73 ± 1.27%, $p < 0.05$; Figure 4C), while a significant higher proportion of oocytes has reached A/TI stage (16.73 ± 2.19% vs. 2.62 ± 2.28%, $p < 0.05$; Figure 4C), suggesting that knockdown of OLA1 led to premature of chromosome segregation and accelerated anaphase onset in mouse oocytes.

OLA1 knockdown causes premature inactivation of SAC

SAC will not abrogate its activity until all the chromosomes are correctly aligned at metaphase I plate , and its previous inactivation can lead to premature of chromosome segregation which

could finally cause aneuploidy in oocytes. We have found that knockdown of OLA1 induced premature of anaphase onset, thus we speculated OLA1 knockdown could give rise to the inactivation of SAC, thus we used chromosome spreading and BubR1 staining to check SAC activity at pre-MI stage (6.5 h) after OLA1-RNAi. As shown in Figure 5, BubR1 localized to kinetochores at pre-MI stage in all control oocytes, while we could find BubR1 failed to be loaded onto kinetochores from kinetochores in OLA1-depletion oocytes, indicating that depletion of OLA1 caused the previous inactivation of SAC at pre-MI stage.

DISCUSSION

Spindle assembly and chromosome segregation are two indispensable events during the progress of meiosis. Defective morphology of spindles and abnormal chromosome alignments could result in aneuploidy gametes or the failure of meiosis (*Ma et al., 2014*; *Jiao et al., 2017; Han et al., 2015*). There are many factors including cellular protein function to modulate these two progresses to be orderly. Currently, the function OLA1 mouse oocyte meiosis is still unknown. In this study, we have discovered OLA1 is a novel participator in oocyte meiosis: the participation in GVBD, spindle assembly and SAC activation.

GVBD and PBE are marked as important cell cycle symbols. When immature oocytes begin to mature by exogenous and endogenous factors stimulation both in vivo and in vitro, GVBD occurs and then oocyte resumes meiosis which could finally bring out the polar body extrusion (*Sánchez et al., 2012*). Defects in GVBD can result in the block of meiosis and oocyte will lose their ability for further development. To detect the function of OLA1, we first examined GVBD and PBE rates after OLA1 knockdown. We found that OLA1 knockdown significantly inhibited GVBD and a decline in PBE was also found in the loss of OLA1. However, once the oocyte underwent GVBD then it could directly extrude the polar body even in the loss of OLA1. And

the decline of PBE in OLA1 knockdown oocytes was only due to the block of GVBD. Thus we conclude that OLA1 is critical for the germinal vesicle breakdown but not polar body extrusion.

Spindle assembly is indispensable for meiotic progression, it supports the segregation of chromosomes in oocytes. Abnormal spindle assembly could result in PBE failure or production of aneuploid gametes (*Schuh et al., 2007*; *Clift et al., 2015; Bennabi et al., 2016*). During the time spindle assembly, chromosomes show unstable connections with microtubules until hours post GVBD when a tight link between kinetochores and microtubules is established. To avoid the segregation of chromosomes with incorrect connection with microtubules, SAC persist at kinetochore (SAC activation) to prevent the premature of chromosome segregation (*Vogt et al., 2008*; *Touati et al., 2016; Sanders et al., 2018*). Once all chromosomes align correctly at the metaphase I plate, SAC will dissociated from kinetochores (SAC inactivation) then activates the APC/C activity to accelerate SECURIN degradation and anaphase I onset (*Marston et al., 2017*). Defects in SAC function can result in incorrect attachments of chromosome with microtubules, and leads to missegregation (*Miao et al., 2017*; *Lu et al., 2017; Li et al., 2009*). Study has shown that knockdown of BRCA1 causes abnormal spindles in mouse oocytes (*Xiong et al., 2008*). As an interactive protein in mitosis, we speculated OLA1 may also function in spindle assembly in oocyte. Results showed that multipolar and small spindles occurred in OLA1- knockdown oocytes, indicating that OLA1 also participate in spindle assembly in meiosis. What's more, we suppose that OLA1 could bind to BRCA1 in oocyte exerting its regulatory function in meiotic spindle assembly, but still needs further study to verify that. In addition, a higher proportion of oocytes have reached A/TI stage after 8 h in culture, indicating the premature of anaphase onset in OLA1-knockdown oocytes. Further study identified the inactivation of SAC at pre-MI stage in RNAi oocytes, thus knockdown of OLA1 results in the dysfunction of SAC leading to the

263 premature of chromosome segregation. Importantly, knockdown of BRCA1 in muse oocyte also
264 leads to the inactivation of SAC (*Xiong et al., 2008*). Token all these together, it seems that OLA1
265 may function in meiosis in an OLA1-BRCA1 mediated pathway. In addition, OLA1 knockdown
266 induced spindle defects did not lead to metaphase I arrest, we speculate this might be the bypass
267 of SAC supervision.

268 CONCLUSION

269 In brief, our study highlights that OLA1 exhibits significant function in meiotic progression,
270 especially in GVBD regulation. OLA1 also functions in spindle assembly and SAC activation.

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Figure Legends

Figure 1 **OLA1 localization and expression during mouse oocyte meiosis.** (A) Subcellular localization pattern of OLA1 in mouse oocyte meiosis was explored by immunofluorescent analysis. Samples were harvested at GV, GVBD, MI, and MII stage and then immunolabeled. OLA1, red; α-tubulin, green and Chromosome, blue. Scale bar, 10 μm. (B) Subcelluar expression pattern of OLA1 in mouse oocyte meiosis was investigated by western blot analysis. Samples at indicated stages were collected. (C) Confocal images of OLA1 signal in MI/MII oocytes after treatment with nocodazole and recovery. OLA1, red; α-tubulin, green and Chromosome, blue. Scale bar, 10 μm.

Figure 2 **Knockdown of OLA1 impairs GVBD leading to a decrease in PBE.** Fully grown oocytes injected with OLA1 siRNA or control siRNA were arrested in M2 medium with IBMX for 24 hours, then cultured in vitro for the continuous experiments. (A, B) Knockdown efficiency of OLA1 after OLA1 siRNA was verified by western blot; (C, D) Oocytes after microinjection were released into M16 medium for further culture. The GVBD rate in control group and OLA1 knockdown group were recorded at 2 h; The PBE rate in control group and OLA1 knockdown group were recorded at 12 h; *$p < 0.05$. (E) PBE rate was characterized in meiosis resumed oocytes in control and RNAi groups. 162 control oocytes and 182 OLA1-RNAi oocytes were calculated.

Figure 3 **OLA1 knockdown leads to defects of spindle assembly and chromosome alignment.** (A) Spindle morphology in control and OLA1-depletion oocytes after 6 h post-

GVBD culture. α-tubulin, green; Chromosome, blue. Scale bar, 10 μm; (B) Quantification of aberrant spindles rate in control and OLA1-RNAi oocytes; (C) Quantification of misaligned chromosomes rate in control and OLA1-RNAi oocytes. 88 control oocytes and 90 OLA1-RNAi oocytes were calculated in abnormal spindles and misaligned chromosomes. *$p < 0.05$.

Figure 4 **OLA1 knockdown accelerates the anaphase onset in mouse oocytes.** (A) Images exhibited different status of the first polar extraction in control and OLA1-depletion groups; (B) The spindle and chromosome morphologies in control and OLA1-knockdown oocytes after 6 h post-GVBD culture. α-tubulin, green; Chromosome, blue. Scale bar, 10 μm; (C) Cell cycle analysis in control oocytes (n = 97) and OLA1-RNAi oocytes (n = 92) after 6 h post-GVBD culture. *$p < 0.05$.

Figure 5 **OLA1 knockdown causes precocious inactivation of SAC**. The status of BubR1 at pre-MI stage from control and OLA1-knockdown oocytes. After knockdown, GV oocytes in control and OLA1-knockdown groups were released and cultured in pre-warmed M16 medium for 6.5 h, normally corresponding to Pre-MI stage. Then oocytes were collected for chromosome spreading and stained with BubR1. BubR1, green; Chromosome, Blue. Scale bar, 20 μm.

Figure 1

Cellular localization and expression pattern of OLA1 in mouse oocyte meiosis

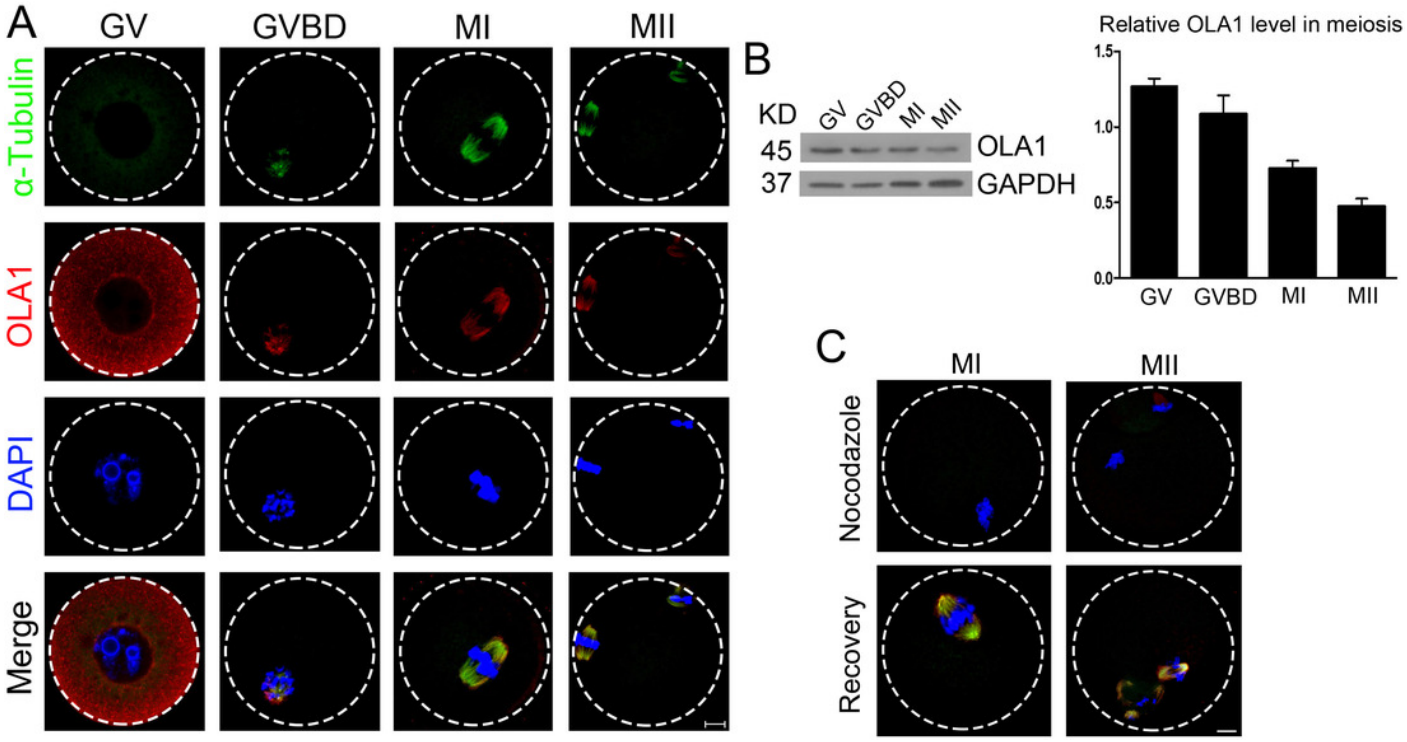

Figure 2

Knockdown of OLA1 impairs GVBD leading to a decrease in PBE

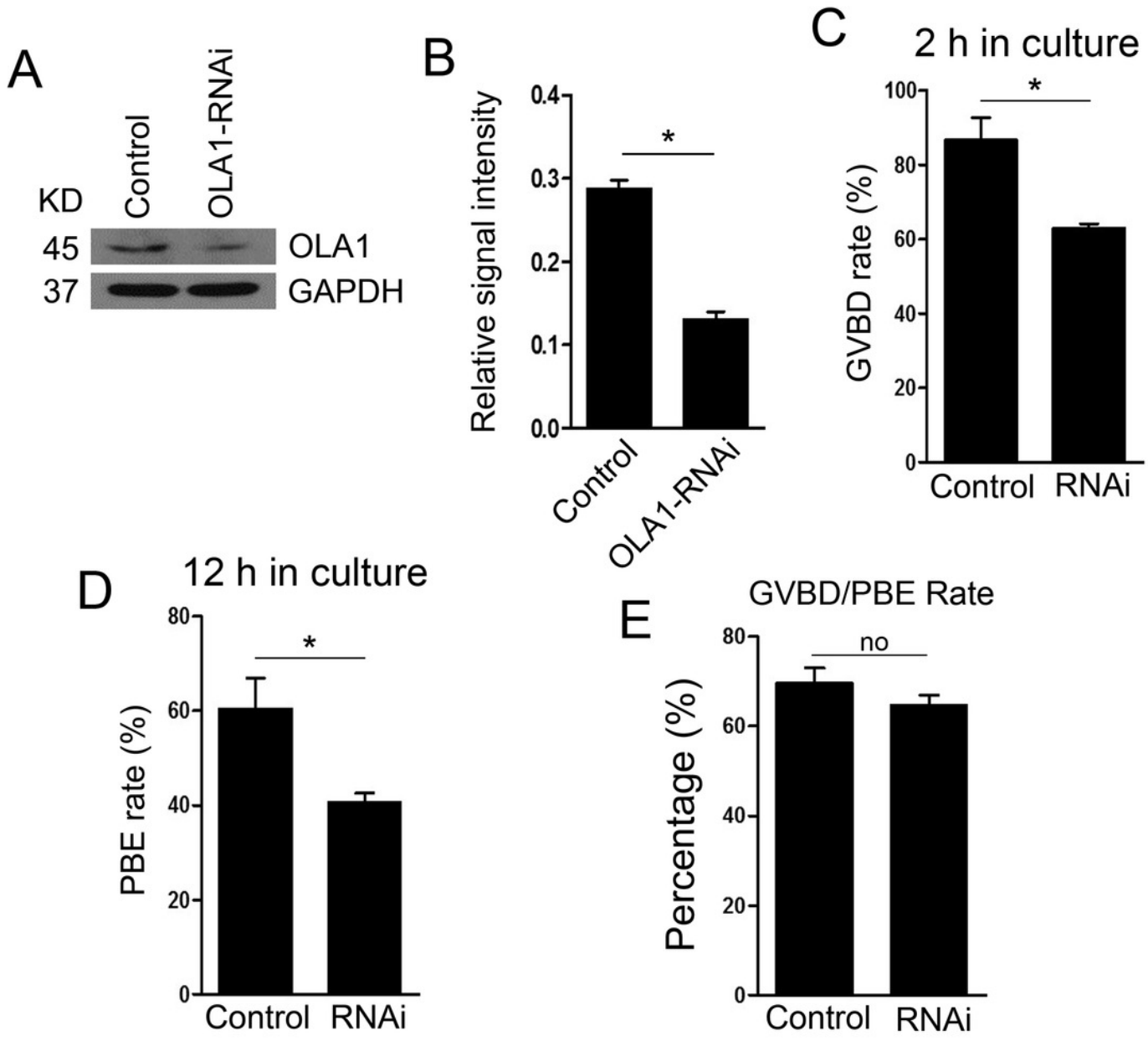

Figure 3

OLA1 is required for spindle assembly and chromosome alignment in mouse oocytes

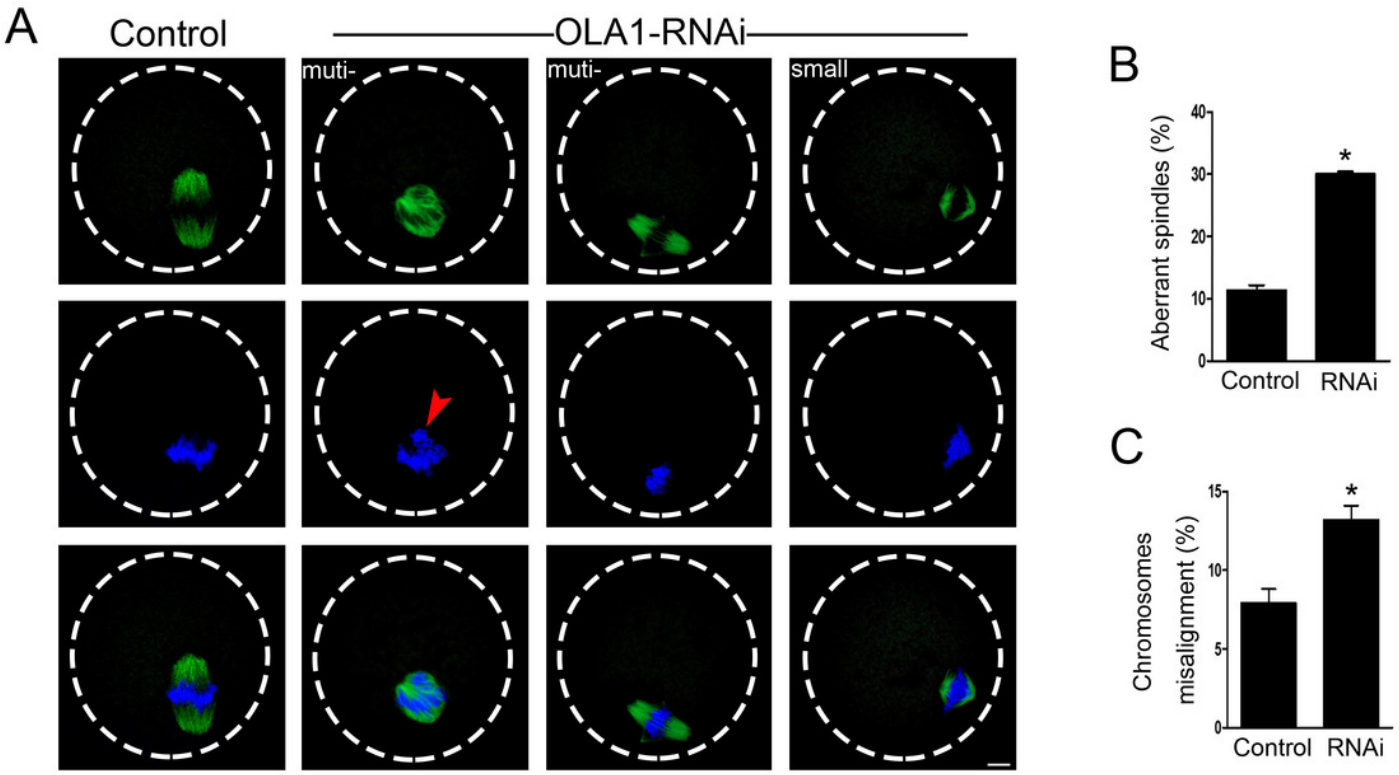

Figure 4

OLA1 knockdown accelerates the anaphase onset in mouse oocytes

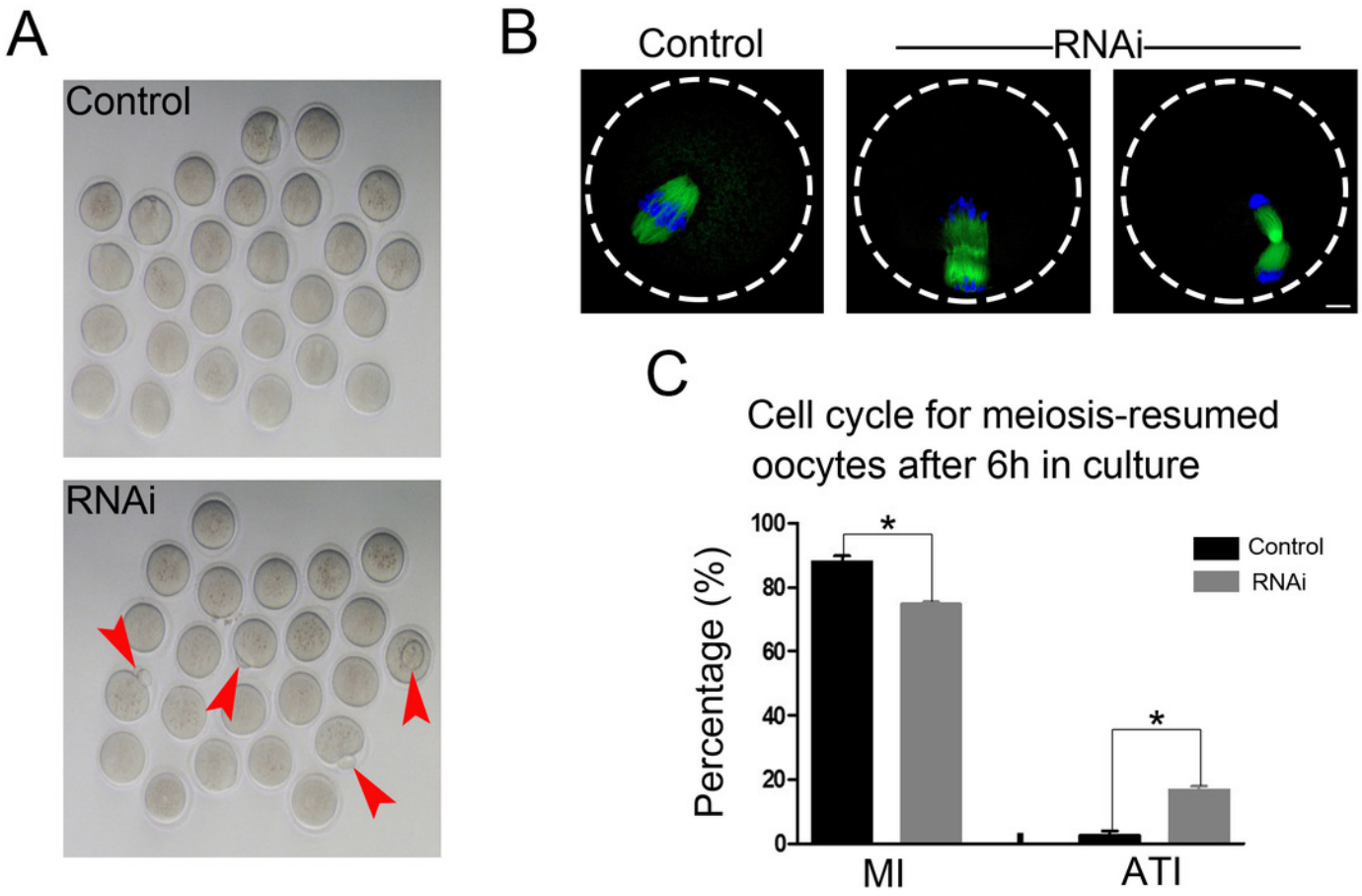

Figure 5

OLA1 knockdown causes inactivation of spindle assembly checkpoint