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REPORT

Zap70 and downstream *RanBP2* are required for the exact timing of the meiotic cell cycle in oocytes

Hyun-Jung Kim[a,†], Su-Yeon Lee[b,†], Hyun-Seo Lee[c], Eun-Young Kim[b], Jung-Jae Ko[b], and Kyung-Ah Lee[b]

[a]Fertility Center of CHA Gangnam Medical Center, College of Medicine, CHA University, Seoul, Korea; [b]Institute of Reproductive Medicine, Department of Biomedical Science, College of Life Science, CHA University, Pan-Gyo, Korea; [c]Research Center for Cancer Immunotherapy, Chonnam National University Hwasun Hospital, Jeollanam-do, Korea

ABSTRACT

In previous studies, we observed that Zeta-chain-associated protein kinase 70 (Zap70) regulates spindle assembly and chromosome alignment in mouse oocyte and that Ran binding protein 2 (RanBP2) is a highly associated gene with Zap70 based on a microarray analysis. Because RanBP2 is related to nuclear envelope breakdown (NEBD) during mitosis, the aim of the present study was to elucidate the molecular mechanism of Zap70 with respect to RanBP2 in the germinal vesicle breakdown (GVBD) of oocytes. Results indicated that RanBP2 expression was regulated by Zap70 and that depletion of RanBP2 using *RanBP2* RNAi manifested comparable phenotypes to those observed in *Zap70* RNAi-treated oocytes, which presented faster processing of GVBD. Additionally, *Zap70* RNAi-treated oocytes showed faster meiotic resumption with premature activation of maturation-promoting factor (MPF), premature division of chromosomes at approximately 6–8 h and more rapid degradation of securin. In conclusion, we report that Zap70 is a crucial factor for controlling the exact timing of meiotic progression in mouse oocytes.

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chromosome segregation; mouse oocyte; MPF; premature meiotic resumption; RanBP2; securin; Zap70

Introduction

Mammalian oocytes enter the prophase stage of meiosis and remain arrested at the diplotene stage of prophase I, namely, the GV stage in the primordial follicles, and some of these primordial follicles selectively begin to grow after the onset of puberty.[1,2] Meiotic resumption of oocytes in full-grown follicles occurs in response to a surge of luteinizing hormone (LH) from the pituitary gland during the oestrous cycle in animals and menstrual cycle in women before ovulation. The processes by which the oocyte completes the first meiotic division and undergoes cytoplasmic changes for the ultimate goal of fertilization by a male gamete compose oocyte maturation.[3]

Meiotic resumption is initiated by the disassembly of the nuclear membrane of oocytes known as germinal vesicle breakdown (GVBD). This key process in meiotic division ensures accurate chromosome segregation and spindle formation during mammalian meiosis. Correct assembly and segregation of chromosomes in mitotic and meiotic cells requires the formation of a bipolar spindle.[4] During meiosis, oocytes undergo chromosome condensation, spindle formation, microtubule organization, the separation of one set of homologous chromosomes into the first polar body, and the progression of meiosis without DNA replication; afterwards, the oocyte arrests at the metaphase II (MII) stage.[5,6]

Previously, we found that Zap70 is highly expressed in GV oocytes compared with MII oocytes and plays a role in oocyte maturation.[7,8] Zap70 is a cytoplasmic Syk family tyrosine kinase that consists of 2 tandem SH2 domains, which are essential elements in coupling immune receptors to intracellular response cascades, and a kinase domain at the amino terminus.[9] Prior to our discovery, Zap70 expression was exclusively reported in normal T cells and natural killer cells and served as a pivotal regulator of antigen-mediated receptor signaling.[10] After we identified Zap70 expression in the female gamete, its presence and function were also evaluated in stem cells, and it was determined that Zap70 critically regulates the balance between self-renewal and pluripotency in undifferentiated mouse embryonic stem cells (mESCs). Zap70 also plays important roles in modulating the LIF/JAK/STAT3 signaling pathway to balance the pluripotent state of mESCs between maintaining self-renewal and permitting differentiation.[11]

In a series of previous studies, we reported that oocytes arrest at the metaphase I (MI) stage and cannot progress through the meiotic cell cycle without Zap70 expression.[8] *Zap70* RNAi treatment results in MI-arrested oocytes that present elongated spindles with abnormally aggregated chromosomes. We analyzed the genes downstream of Zap70 in oocytes using *Zap70* RNAi followed by microarray analysis .[12] Based on these findings, the present study was conducted to further elucidate the molecular mechanisms involved in Zap70-mediated regulation of meiosis in oocytes. Here, we report that Zap70 regulates the timely processes of oocyte maturation, including meiotic resumption, bipolar spindle formation and chromosome segregation in association with RanBP2, one of its downstream effectors.

CONTACT Kyung-Ah Lee, PhD leeka@cha.ac.kr 335 Pangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do, Korea.
Supplemental data for this article can be accessed on the publisher's website.
[†]Co-first authors: These authors contributed equally.

Results

Zap70 is expressed on the chromosome in mature mouse oocytes

Elevated expression levels of Zap70 mRNA and protein were detected during the GV, GVBD, and MI stages of oocytes, and slightly decreased levels were observed during the MI-MII transition (Fig. 1A and B). Immunofluorescence staining showed that Zap70 was dispersed in the cytoplasm during the GV stage but was concentrated and localized on the chromosomes in mature oocytes after the GVBD stage (Fig. 1C). Based on our previous results that the effects of *Zap70* RNAi were found in disorganization of chromosome and spindle structure,[12,13] chromosomal localization of Zap70 in this study may suggest that Zap70 plays a role in chromosome segregation during the MI-MII transition.

RanBP2, a downstream gene of Zap70, also contributes to rapid meiotic resumption

In our previous study, we found that a total of 1,152 genes in MI-arrested oocytes subjected to *Zap70* RNAi had a greater than 2-fold change in expression compared with those in the control MI oocytes.[12] Among the top 10 genes that were downregulated by *Zap70* RNAi, *RanBP2* was highly downregulated in response to *Zap70* RNAi (Table S1). In mitotic cells, RanBP2 has been known to be involved in nuclear envelope breakdown (NEBD) as the core component of the 100-nm filaments that extend from the cytoplasmic face of the nuclear pore complex (NPC).[14] Using immunofluorescence analysis, we observed that RanBP2 is also localized to the nuclear envelope during the GV stage and that those signals diminished when the oocytes initiated GVBD (Fig. S1). After treatment with *Zap70* RNAi, the RanBP2 mRNA and protein levels in GV oocytes were decreased, and its expression on the nuclear envelope was significantly reduced (Fig. 2A and B). These results show that RanBP2 expression is regulated by Zap70, confirming that RanBP2 is a downstream effector of Zap70 in oocytes. Because RanBP2 plays a role in the process of NEBD during mitosis, we predicted that RanBP2 also plays a role in meiosis. Accordingly, we subjected GV oocytes to *RanBP2* RNAi and measured the *in vitro* maturation rate. After GV oocytes were treated with *RanBP2* RNAi treatment and incubated in IBMX-supplemented medium for 8 h, the *RanBP2* transcript had

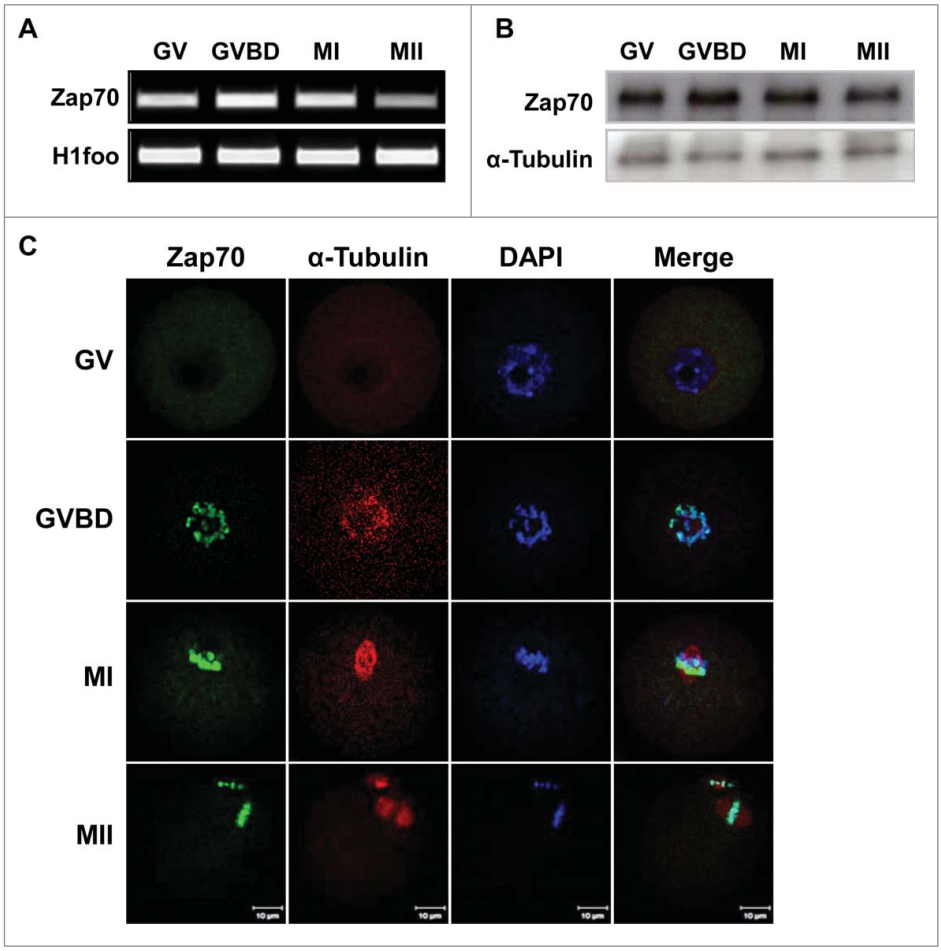

Figure 1. Expression and localization of Zap70 during mouse oocyte maturation. (A) The expression of *Zap70* mRNA in oocytes during *in vitro* oocyte maturation. For semi-quantitative PCR, single-oocyte-equivalent cDNA was used as a template for amplification. GV, GVBD, MI and MII oocytes were harvested at 0, 2, 8 and 16 h during *in vitro* oocyte maturation, respectively. Constitutively expressed oocyte-specific histone (*H1foo*) was used as an internal control. (B) Western blot analysis of Zap70 expression during *in vitro* oocyte maturation. Each lane contained protein extract from 100 oocytes, and α-Tubulin was used as a loading control. (C) Confocal microscopy images showing immunofluorescence for Zap70 (green), Tubulin (red), and DNA (blue) at the GV, GVBD, MI and MII stages. At the GV stage, Zap70 is dispersed in the cytoplasm of the oocytes. When maturation is initiated, the fluorescence from the GVBD stage throughout MI and MII shifts precisely to the chromosomes of the mouse oocytes. Scale bars = 10 μm.

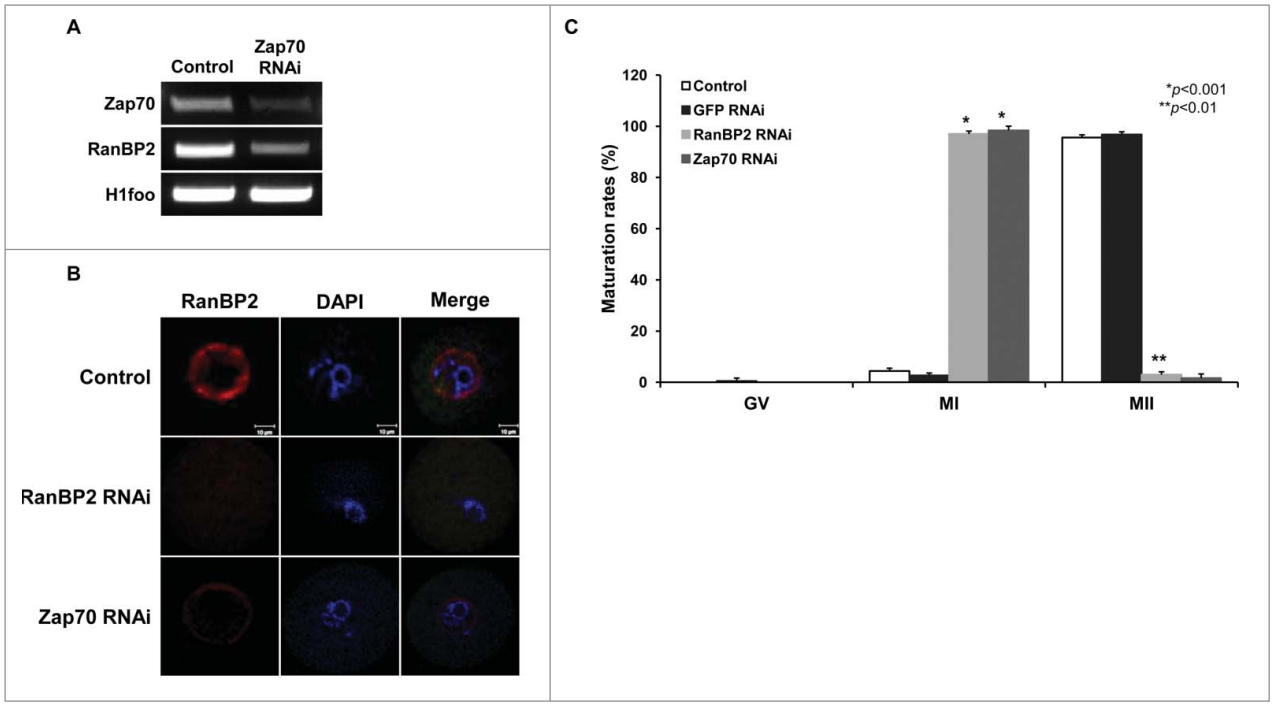

Figure 2. Changes in expression of RanBP2 after *Zap70* RNAi treatment and maturation rates after *RanBP2* or *Zap70* RNAi treatment. (A) *Zap70* RNAi treatment resulted in specific suppression of *Zap70* mRNA expression. In addition, *RanBP2* mRNA was decreased by *Zap70* RNAi. Oocyte-specific *H1foo* was used as an internal control. (B) The RanBP2 signals were decreased in *Zap70* RNAi oocytes and completely absent in *RanBP2* RNAi oocytes compared with those in control GV oocytes, suggesting that RanBP2 expression is regulated by Zap70. Oocytes were cultured in M16 medium containing IBMX for 8 h, and staining was conducted using antibodies against RanBP2 (red), DNA (blue) and α-Tubulin (green). Scale bar = 10 μm. (C) Maturation rates of oocytes after culture with IBMX for 8 h followed by 16 h of culture in plain M16 medium. *RanBP2* or *Zap70* RNAi-treated oocytes were showed abnormal MI-MII transition and oocytes were showed as like MI stage with neither GV nor polar bodies. We categorized it as MI. The asterisk indicates statistical significance at $p < 0.001$ or $p < 0.01$.

completely disappeared, and the RanBP2 protein levels were decreased by 80% (Fig. 3A and B). When the speed of GVBD was examined at 30 min of *in vitro* maturation, the *RanBP2* RNAi-treated oocytes showed a faster resumption of spontaneous maturation (76% GV) compared with control (100% GV) and *GFP* RNAi oocytes (92% GV) (Fig. 3C). *In vitro* oocyte maturation also progressed abnormally, and almost of the oocytes showed atypical MI-MII transition with no GV nor polar bodies were arrested (97%) at the MI stage when oocytes subjected to *RanBP2* RNAi treatment (Fig. 2C, Table S2).

Zap70 RNAi-treated oocytes undergo GVBD faster than untreated oocytes

As mentioned above, Zap70 is upstream of RanBP2, which is regulator of meiotic resumption of mouse oocytes. Based on these results, we observed whether Zap70 could also influence the timing of GVBD. After GV oocytes were injected with Zap70 dsRNA, the corresponding *Zap70* transcripts were completely eliminated after oocytes were cultured in IBMX-supplemented medium for 8 h (Fig. 4A). However, small amounts of Zap70 protein were observed, which may have existed in the oocytes before the *Zap70* RNAi treatment (Fig. 4B). In the presence of IBMX, control, *GFP* RNAi- and *Zap70* RNAi-treated oocytes were all maintained at the GV stage (100%). To measure the timing of GVBD, we counted the number of oocytes showing disappearance of the nuclear envelope at 30 min and 1 h after the transfer of oocytes to plain M16 medium for spontaneous maturation *in vitro*. As a result, *Zap70* RNAi-treated oocytes showed faster resumption of meiosis than either control or *GFP* RNAi-treated oocytes (*Zap70* RNAi = 31% GV, *GFP* RNAi = 92% GV, and control = 100% GV) after 30 min in plain M16 medium (Fig. 4C). After 1 h of culture, most of the oocytes in all the groups eventually completed GVBD. Similarly, we used time-lapse video microscopy to track the speed of oocyte maturation during *in vitro* culture and found that GVBD occurred approximately 20 min earlier in *Zap70* RNAi-treated oocytes. (Fig. 4D).

However, *Zap70* RNAi-treated oocytes failed to complete nuclear maturation. Control and *GFP* RNAi-treated oocytes successfully reached the MII stage (95.6% and 96.8% in control and *GFP* RNAi-treated oocytes, respectively) with extrusion of the first polar body after 16 h of culture. In contrast, 98.4% of the *Zap70* RNAi-treated oocytes were look like as arrested at the MI stage without polar body extrusion (Fig. 2C). This observed phenotypic change of MI arrest after *Zap70* RNAi is consistent with our previous findings.[8]

In oocytes subjected to *Zap70* RNAi, the rate of GVBD at 30 min after spontaneous maturation was higher than that found in oocytes subjected to *RanBP2* RNAi treatment (Fig. 3C and 4C). Because RanBP2 is a gene downstream of Zap70, this suggests that the resumption of oocyte maturation in *in vitro* culture requires Zap70 as well as RanBP2.

The premature activation of MPF by Zap70 RNAi also contributes to the faster meiotic resumption of oocytes

Prophase I arrest of immature oocytes is the result of low maturation-promoting factor (MPF) activity, and hormonal signals induce MPF activation and the resumption of meiosis.[15-17] MPF and other protein kinases form a positive

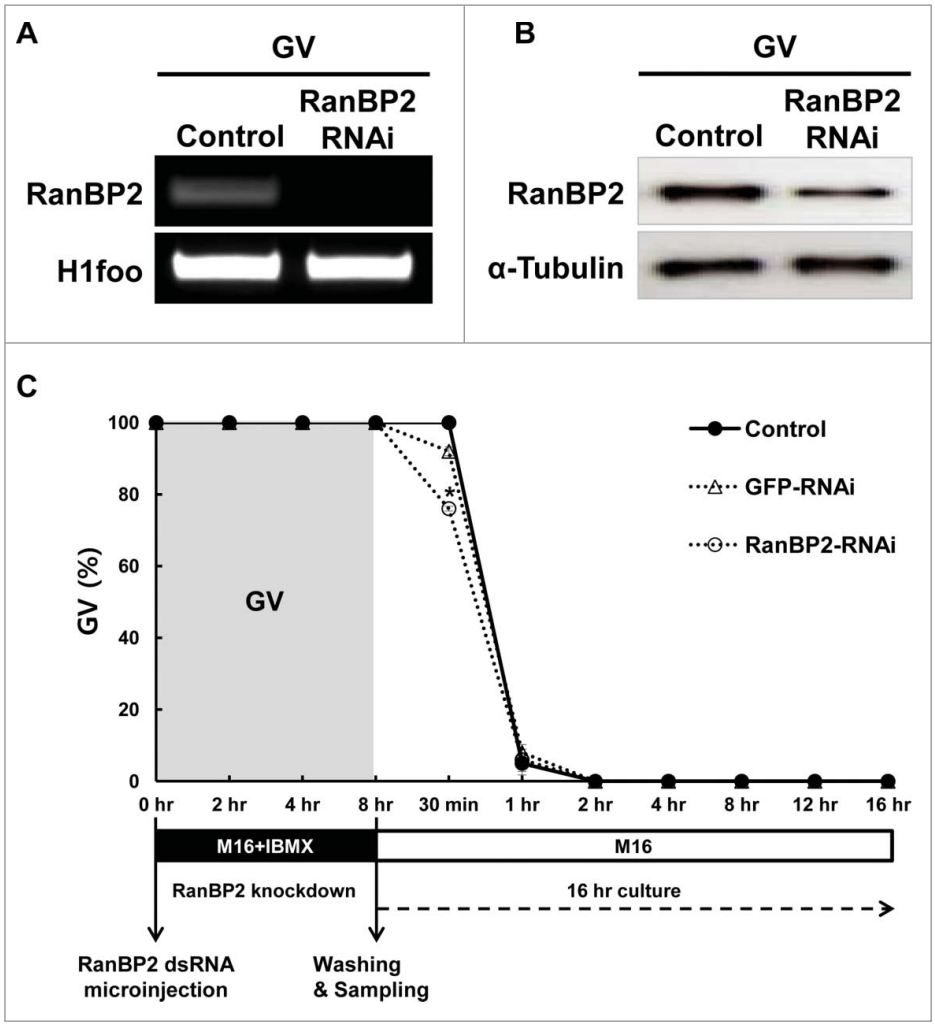

Figure 3. Effects of *RanBP2* RNAi on the timing of the resumption of oocyte nuclear maturation during *in vitro* culture. (A) *RanBP2* RNAi treatment resulted in the specific suppression of *RanBP2* mRNA expression. Oocyte-specific *H1foo* was used as an internal control. (B) Protein knockdown after *RanBP2* RNAi was confirmed using Western blot analysis. Each lane contained proteins extracted from 100 GV oocytes. α-Tubulin was used as an internal control. (C) The maturation rates of oocytes in the control, *GFP* RNAi and *RanBP2* RNAi treatment groups were scored at 30 min intervals during the first 1 h and at 2 h intervals thereafter. *RanBP2* RNAi oocytes, similar to the *Zap70* RNAi oocytes, resumed maturation earlier than the control groups. The asterisk indicates statistical significance at $p < 0.05$.

feedback loop, and these associations induce many nuclear and cytoplasmic changes during meiosis, including chromatin condensation, NEBD, spindle formation, and other cytoplasmic changes.[18] As we observed faster resumption of meiosis in *Zap70* RNAi-treated oocytes, the kinase activity of MPF and MAPK, which are highly required for meiotic resumption, was measured during the first few hours of *in vitro* maturation. The results indicated that MPF was activated more rapidly at 30 min of *in vitro* maturation in *Zap70* RNAi-treated oocytes than MPF in untreated oocytes, which was activated at 1 h and gradually increased until 8 h after maturation (Fig. 5A and B). In this regard, the expression of CDC25C phosphatase, which dephosphorylates cyclin-dependent kinase 1 (Cdk1) on the inhibitory residue Tyrosine 15 (pTyr15-CDK1), was increased, whereas pTyr15-CDK1 was decreased in premature GVBD oocytes subjected to *Zap70* RNAi treatment (Fig. 5C). The activating phosphorylation of Cdk1 on Threonine 161 (pThr161-CDK1) was unchanged. Taken together, these data indicate that faster meiotic resumption upon Zap70 depletion is caused by premature activation of MPF.

In contrast, MAPK activity was activated at 2 h after *in vitro* maturation in both untreated and *Zap70* RNAi-treated oocytes. However, *Zap70* RNAi-treated oocytes showed relatively decreased activity of MAPK compared with untreated oocytes between 4 h and 8 h (Fig. 5A), which is consistent with our previous research.[8]

Zap70 RNAi-treated oocytes showed more rapid chromosome segregation

We showed that Zap70 localized to chromosomes during oocyte maturation and that *Zap70* RNAi-treated oocytes had abnormalities in meiotic spindle formation and chromosome alignment. Accordingly, we observed the structural aspects of chromosome segregation every 2 h during *in vitro* maturation in oocytes treated with *Zap70* RNAi. The spindle and chromosome structures of *Zap70* RNAi-treated oocytes were not significantly different from those in control and *GFP* RNAi-treated oocytes at 2 and 4 h (Fig. 6A-C). Interestingly, at 6 h, the spindles and chromosomes of several *Zap70* RNAi-treated oocytes were already divided

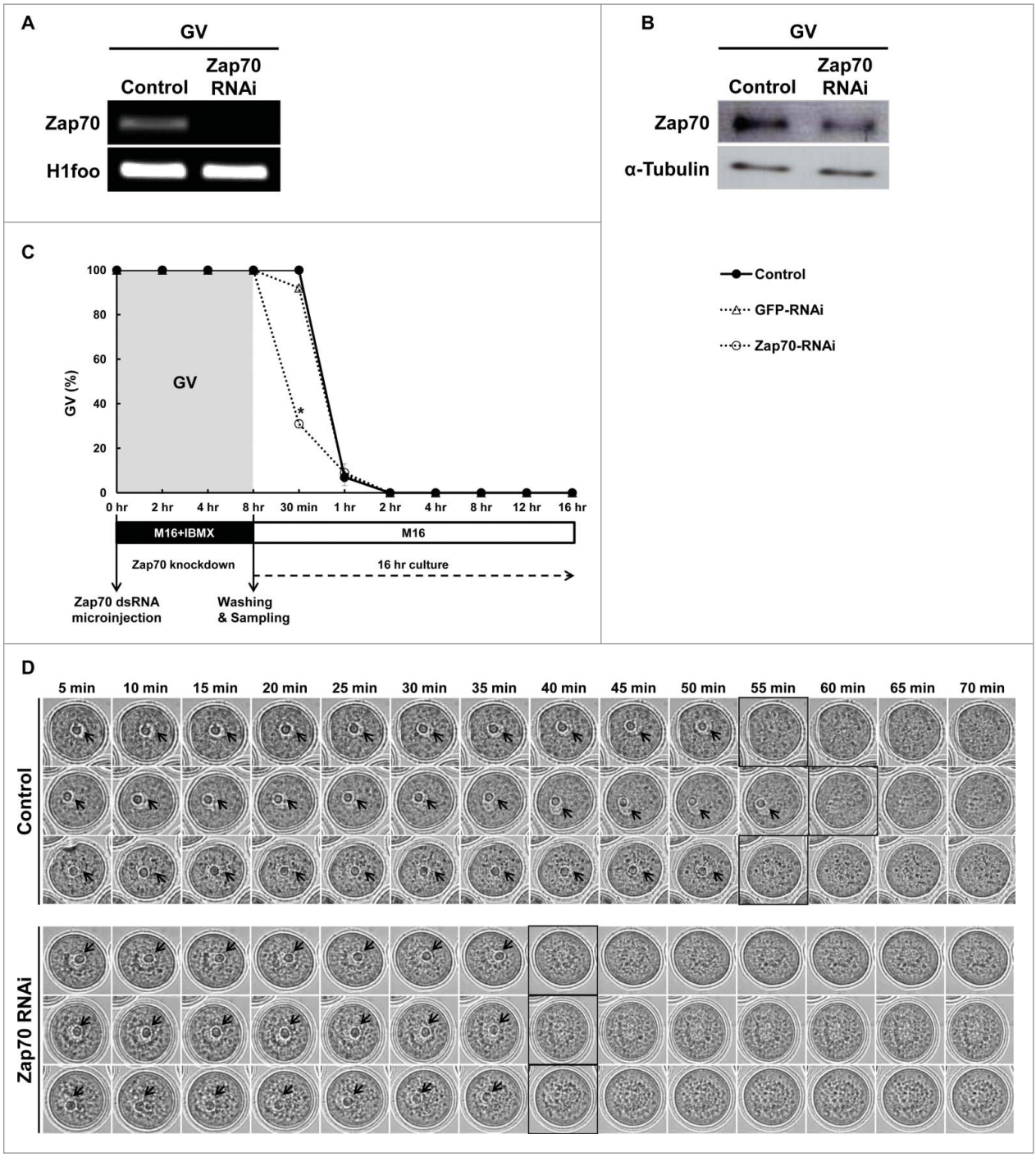

Figure 4. Effects of *Zap70* RNAi on the timing of the resumption of oocyte nuclear maturation during *in vitro* culture. (A) *Zap70* RNAi treatment resulted in the specific suppression of *Zap70* mRNA expression. Oocyte-specific *H1foo* was used as an internal control. (B) Protein knockdown in oocytes treated with *Zap70* RNAi was confirmed by Western blot analysis. Each lane contained proteins extracted from 200 GV oocytes. α-Tubulin was used as an internal control. (C) The maturation rates of oocytes in the control, *GFP* RNAi and *Zap70* RNAi treatment groups were scored at 30 min intervals during the first h and then at 2 h intervals thereafter. All oocytes were incubated in M16 medium containing IBMX for 8 h after microinjection of dsRNA and then transferred to IBMX-free M16 medium for 16 h to measure changes in *in vitro* oocyte maturation. *Zap70* RNAi oocytes resumed maturation earlier than the control groups. Asterisks represent statistical significance at $p < 0.05$. (D) Representative time-lapse images of oocytes from the imaging video from initiation of resumption to the time of anaphase onset. The time stamp of each captured picture is shown on top of the oocytes.

akin to the anaphase I (AI) stage (Fig. 6C). At 8 h, in contrast to the normal barrel shape of spindles in the control and *GFP* RNAi-treated oocytes, most of the *Zap70* RNAi-treated oocytes showed divided chromosomes with abnormally aggregated spindles (Fig. 6D). Taken together with the results that *Zap70* RNAi treatment of GV oocytes leads to faster meiotic resumption, these findings strongly imply that the loss of Zap70 at the GV stage accelerates the progression of the meiotic chromosome separation as well.

Interestingly, when we evaluated the meiotic spindle structure and chromosome organization of oocytes treated with *RanBP2* RNAi, we observed abnormal spindle shapes and chromosome alignments at 8 h, including irregularly divided or scattered chromosomes with elongated spindles similar to the

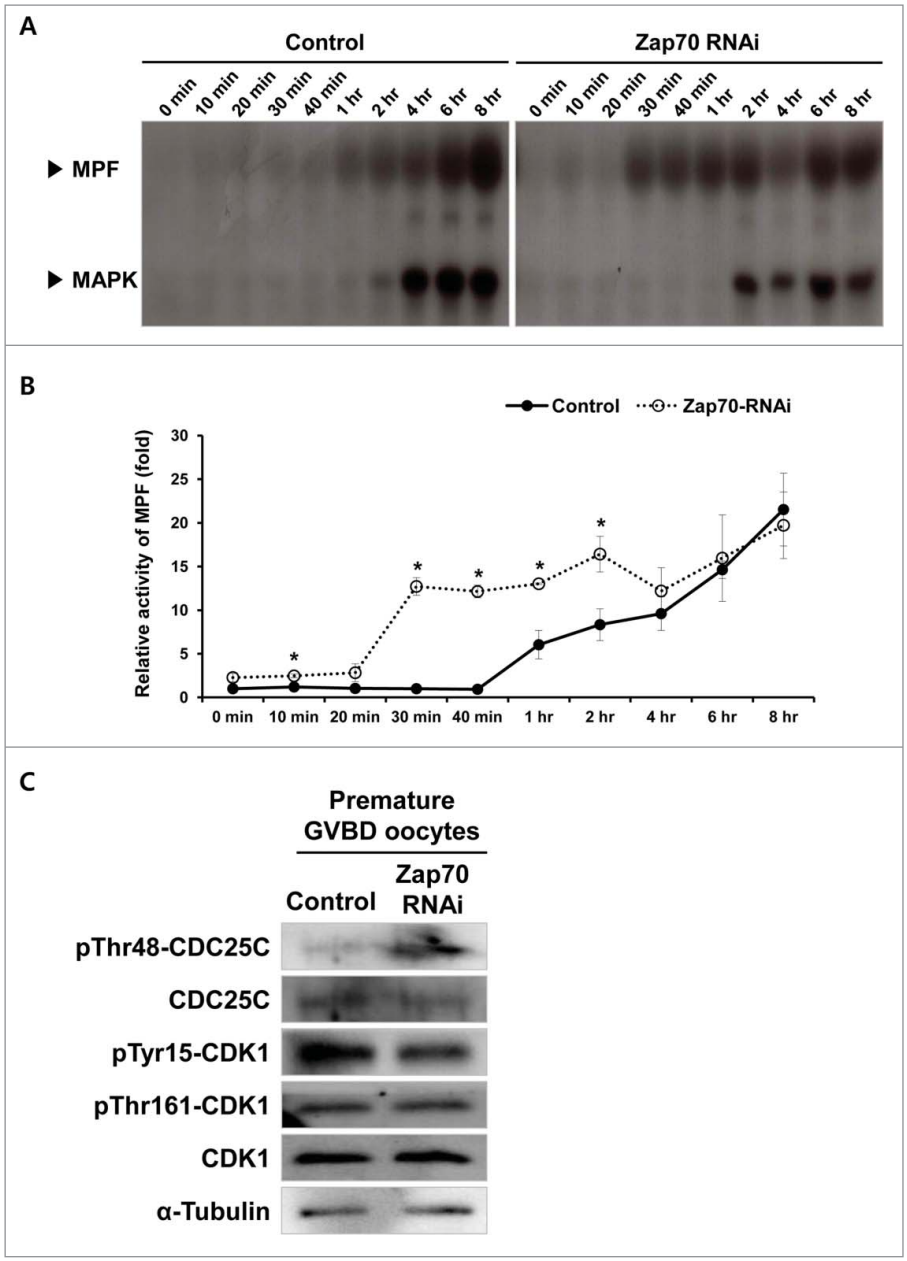

Figure 5. The premature activation of MPF after *Zap70* RNAi treatment in GV oocytes. ((A)and B) Dual kinase activity assay after *Zap70* RNAi treatment. MPF and MAPK activities were analyzed by measuring the phosphorylation levels of histone H1 and myelin basic protein, which are substrates of MPF and MAPK, respectively. Control and *Zap70* RNAi-treated oocytes were collected at specific time points during *in vitro* maturation. The lower panel depicts the relative MPF activity, which was determined by calculating the phosphorylation levels of substrates followed by normalization against the levels in the oocytes at the initial time point. Asterisks indicate statistically significant differences compared with controls ($p < 0.05$). (C) Changes in the MPF-related proteins after *Zap70* RNAi treatment was confirmed by Western blot analysis. Each lane contained proteins extracted from 200 oocytes. α-Tubulin was used as an internal control. Control oocytes, *GFP* RNAi- and *Zap70* RNAi-treated oocytes were collected 1 h after spontaneous oocyte maturation.

morphology and distribution observed in *Zap70* RNAi-treated oocytes (Fig. S2).

Zap70 RNAi-treated oocytes undergo premature degradation of securin

As the substrate of APC/C, securin regulates the timing of anaphase via inhibition of separase activity, which initiates sister chromatid segregation by cleavage.[19] When securin is degraded, separase subsequently activates the breakdown of the cohesin connecting the sister-chromatids during the MI-AI transition during oocyte maturation.[20] In mouse oocytes, separase is inhibited until the Mad2-dependent spindle assembly checkpoint is completed, after which it is activated at the MI-MII transition by either APC/C regulation or securin ubiquitination.[20-24] Hence, we speculated that the earlier separation of chromosomes in oocytes treated with *Zap70* RNAi may occur due to more rapid securin degradation following the premature meiotic progression from the early GV and GVBD stages. Based on our hypothesis, we evaluated the amount of securin protein using Western blot analysis and found that the securin protein levels in oocytes treated with *Zap70* RNAi were already decreased at 1 h

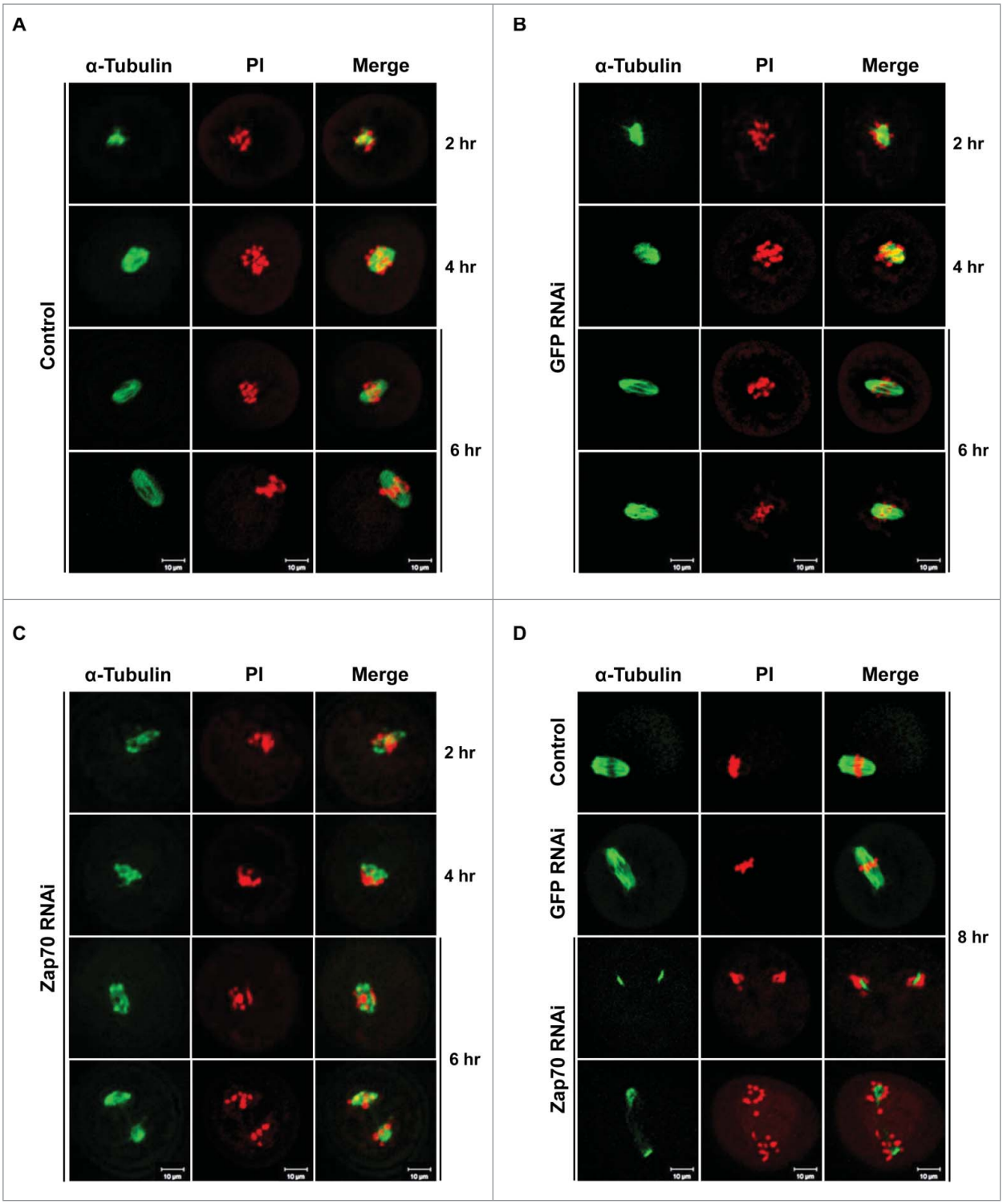

Figure 6. Immunofluorescence staining of the spindles and chromosomes in oocytes treated with *Zap70* RNAi. Immunofluorescence staining of spindles and chromosomes in (A) control, (B) *GFP* RNAi oocytes, and (C) *Zap70* RNAi oocytes after 2, 4, 6 h, and (D) 8 h of culture. *Zap70* RNAi MI oocytes showed elongated spindles with prematurely divided chromosomes at approximately 6 h and 8 h. Oocytes were fixed in 4% paraformaldehyde, stained with an anti-α-Tubulin antibody (green), and counterstained with PI (red) to identify DNA. Scale bars = 10 μm.

after *in vitro* maturation (Fig. 7A and B). Additionally, the cytoplasmic securin levels were diminished in *Zap70* RNAi-treated oocytes, which confirm that proteolysis had already occurred due to loss of Zap70 (Fig. 7C). These results suggest that separase may be prematurely activated by more rapid securin degradation and cause the earlier division of meiotic chromosomes.

Discussion

During oocyte maturation, oocytes undergo 2 successive cellular divisions in a sequence of events that are tightly regulated by the cytoskeleton and microtubules. Microtubules are essential for chromosome movements during the first and second meiotic stages.[25] This process is continuous

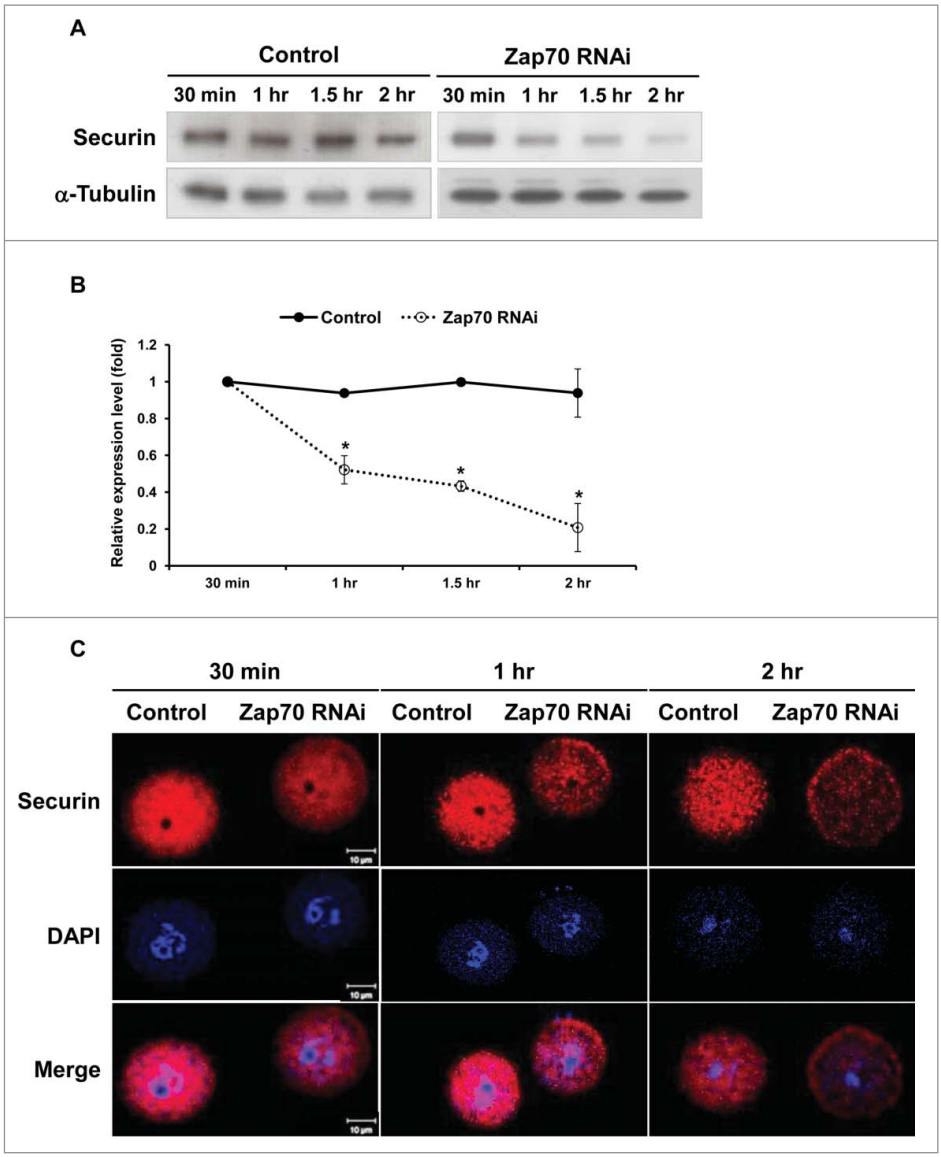

Figure 7. Securin was already degraded at early stages in *Zap70*-RNAi oocytes than in control oocytes. (A) Securin expression decreased more rapidly in *Zap70* RNAi oocytes than in control oocytes. Lysates from 100 oocytes at 30 min, 1 h, 1.5 h and 2 h were analyzed, and α-Tubulin was used as a loading control. (B) The relative protein levels of securin in oocytes treated with *Zap70* RNAi were calculated by measuring the density and area of the protein bands and were normalized to those of the control oocytes. Asterisks indicate statistically significant differences compared with control samples ($p < 0.05$). (C) Immunofluorescence showed distinct changes in the expression of securin at approximately 2 h after *Zap70* RNAi treatment. Securin (red), DAPI (blue). Scale bars = 10 μm.

with the separate but intricately linked events of nuclear and cytoplasmic maturation. Nuclear maturation involves chromosomal arrangement, organization and segregation, whereas cytoplasmic maturation involves the redistribution of intracellular organelles and cytoskeletal systems.[5]

Previously, we reported that Zap70 is necessary for MI-MII transition during mouse oocyte maturation through regulation of chromosome and spindle formation. In this study, we additionally found that Zap70 also regulates the timing of meiotic progression, including GVBD and chromosome segregation, in a similar manner as how RanBP2 regulates the anaphase I stage; interestingly, RanBP2 is downstream of Zap70. Especially, our finding that Zap70 was localized to the chromosomes in mouse oocytes suggests the function of Zap70 in regulating the dynamics of chromosome segregation during meiotic cell division in oocytes.

The results of microarray analysis indicated that many genes involved in various functional pathways were changed by *Zap70* RNAi. Among them, RanBP2 has the closest association with Zap70.[12] RanBP2 is a pleiotropic protein that interacts with multiple partners to exert numerous cellular and molecular activities. RanBP2 is an extremely large protein (358 kDa) and a major element of the cytoplasmic filaments of the nuclear pore complex (NPC). During mitosis in higher eukaryotic cells, NEBD leads to the disassembly of the nuclear membrane and NPC.[26] The spatial connection between the nucleus and cytoplasm, genome organization and stability, and gene expression are all intimately related to NPC proteins.[27,28] Between NEBD and pre-anaphase, when chromosomes align on the metaphase plate, it is essentially free of membranes to allow separation.[29] This NEBD process is facilitated by microtubules in the mitotic spindle. Microtubule dynamics efficiently disassemble the nucleus and clear the peripheral chromatin from the nuclear

envelope membrane network, thereby promoting spindle assembly.[30] The spindle itself plays a dynamic role in nuclear membrane dispersion during the pro-metaphase stage.[14,31] In mitosis, NPC components are required for spindle assembly *in vivo* and *in vitro*, and NEBD is assisted by a microtubule-dependent tearing mechanism in somatic cells.[32]

RanBP2 performs its enzymatic function via an unusual SUMO E3 ligase domain and targets DNA topoisomerase IIα.[33,34] RanBP2 forms a complex with RanGAP1 on kinetochores and microtubules during mitosis.[35] Knockdown of the RanGAP1-RanBP2 complex leads to the formation of a defective bipolar spindle and the accumulation of misarranged chromosomes.[14,36] Depletion of RanBP2 produced severe defects in mitosis, including perturbations in microtubule attachment, mislocalization of kinetochores and spindle assembly checkpoint (SAC) proteins and the formation of multipolar spindles.[36] In our study, RanBP2 was localized on the germinal vesicle (nucleus) of the oocytes, suggesting that RanBP2 also regulates NEBD during meiotic processes. Expectedly, the loss of RanBP2 in GV oocytes resulted in not only a more rapid GVBD but also premature chromosome segregation during *in vitro* oocyte maturation. Interestingly, the phenotypes of the *RanBP2* RNAi-treated oocytes were very similar to the phenotypes of the *Zap70* RNAi-treated oocytes. Therefore, we concluded that RanBP2 acts as a structural protein in the GV membrane and is involved in regulation of GVBD as a downstream effector of Zap70 in oocytes. Further functional studies should address the enzymatic role of RanBP2 during meiosis.

In addition to the decreased RanBP2 expression, premature activation of MPF after *Zap70* RNAi treatment is strongly involved in regulating the positive feedback for premature GVBD and other cellular features, including chromatin condensation and spindle formation, to advance the cell cycle. The present study reveals the molecular mechanism regulating the premature activation of MPF. We concluded that changes in the networking of kinase activities began with the loss of Zap70, a tyrosine kinase.[37]

MPF is a complex comprising the catalytic subunit Cdk1 and the regulatory subunit Cyclin B1.[38] One of the major mechanisms regulating MPF activity involves either an activating phosphorylation event at the Thr161 residue or an inhibitory phosphorylation event at the Thr14 and Tyr15 residues of Cdk1. Thus, the phosphorylation and/or dephosphorylation status of the Thr14 and Tyr15 residues of Cdk1 is a decisive factor for MPF activation at the G2/M phase transition.[39] Myt1 and Wee1 are well known protein kinases that phosphorylate Thr14 and Tyr15 (Wee1 only catalyzes the phosphorylation of Tyr15), whereas Cdc25 phosphatases are required for dephosphorylation.[40] Three isoforms (A, B and C) of Cdc25 have been identified in mammalian cells. Among them, Cdc25A functions during G1/S transition, whereas Cdc25B and Cdc25C regulate G2/M transition. Meiotic resumption, i.e., GVBD entry, is a similar process with the G2/M transition in mitosis, which is associated with MPF activation.[38] In mouse oocytes, the regulatory roles of Cdc25C have been less established than those of Cdc25B in meiotic resumption; [41,42] however, Cdc25C also activates MPF by promoting dephosphorylation of Thr14 and Thy15 on Cdk1.[43,44] In this study, we found that Zap70 induces MPF activation during GVBD by regulating the phosphorylation of Thy15 and subsequently inactivating Cdc25C. It remains unknown whether Zap70 directly phosphorylates Thy15 as a tyrosine kinase or indirectly acts through Cdc25C. However, investigating the involvement of Zap70 in the phosphorylation/dephosphorylation of these well established regulatory mechanisms of MPF is an important task. Zap70 expression was originally considered T cell- and B cell-specific, but the present study clearly shows that Zap70 is also expressed in oocytes and plays important roles in the regulation of meiosis. Therefore, identifying substrates for Zap70 in oocytes should also be completed in the near future.

The untimely segregation of chromosomes at 6–8 h of the meiotic cell cycle in oocytes subjected to *Zap70* RNAi treatment strongly supports the fact that premature anaphase has occurred. In mitosis, separase associates with its inhibitory factor securin and loses its ability to dissociate from cohesin.[45-48] SAC and APC/C control the dissociation of securin from separase. SAC is inactivated and APC/C^{Cdc20} ubiquitinates securin, resulting in the degradation of securin by proteasomes, which in turn allows separase to become active and degrade cohesin.[49] Inhibitory signals from the checkpoint stabilize the interaction between securin and separase, preventing the cleavage of cohesin and the MI-AI transition. During meiosis, mechanisms regulating separase are similar to those in mitosis, as APC/C and securin are also key regulators of meiosis.[20] APC/C is responsible for the degradation of key regulators at the MI-MII transition by destabilizing its substrates cyclin B1 and securin.[50-53] In addition, APC/C-mediated degradation of securin also regulates the MI-AI transition.[20,53] In this study, RNAi-treated oocytes showed different fluctuations in the changes in MPF activity compared with control oocytes, indicating dysfunction of the activation/inactivation system for the MPF components Cdc2 and Cyclin B1. We showed that the loss of Zap70 induced premature activation of MPF and concurrent premature proteolysis of securin, suggesting premature activation of APC/C in oocytes treated with RNAi (Fig. 8).

In summary, Zap70 is an upstream regulator of *RanBP2* in oocytes. Based on the loss-of-function studies, we confirmed that Zap70 and RanBP2 act as inhibitors of premature MPF activation and the untimely degradation of securin in oocyte maturation, thereby preserving the accurate timing of the resumption of maturation and meiotic progression in mouse oocytes. To the best of our knowledge, this is the first report studying the localization and molecular roles of Zap70 during meiosis and the regulation of the timing of meiotic resumption and chromosome segregation in mouse oocytes. Understanding the molecular mechanism regulating the process of spindle formation and chromosome segregation in relation with Zap70 as well as RanBP2 is the goal of future studies.

Materials and methods

Animals

ICR mice were obtained from Koatech (Pyengtaek, Korea) and maintained at the animal facility at the CHA Stem Cell Institute of CHA University. All procedures described here were

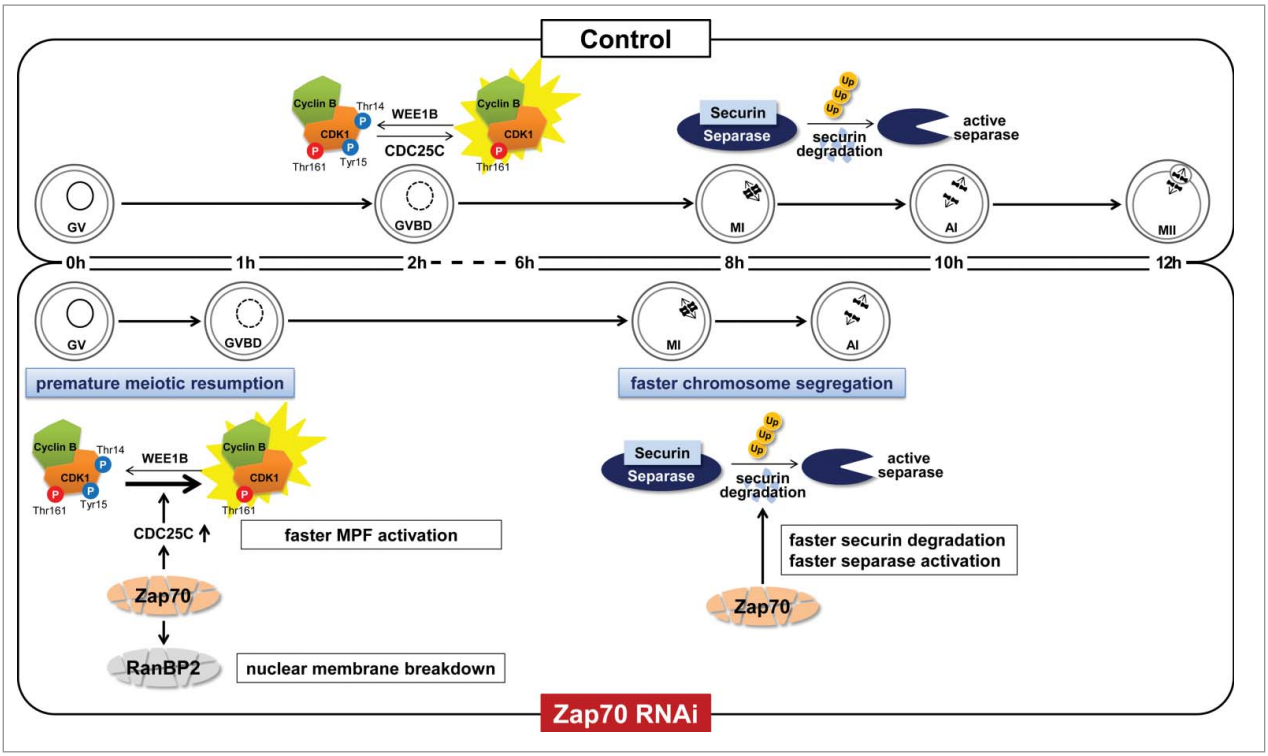

Figure 8. A schematic diagram summarizing the proposed role of Zap70 and/or RanBP2 in the oocyte maturation process. (A) In control oocytes, MPF activity increased from 2–8 h during *in vitro* maturation. Securin induces activation of separase, which induces the cleavage of cohesin followed by chromosome separation. AI occurs at approximately 12 h, and meiosis I is completed with the first polar body extrusion. (B) In *Zap70* RNAi -treated oocytes, MPF is activated prematurely within 30 min of maturation. Consistently, securin degradation is also accelerated, and the RNAi-treated oocytes showed abnormal MI-MII transition with abnormalities in spindle organization and prematurely separated chromosomes, which is similar to AI oocytes.

reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at CHA University and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Isolation of oocytes

For the isolation of GV oocytes from preovulatory follicles, 3-week-old female ICR mice were injected with 5 IU of pregnant mare's serum gonadotropin (PMSG; Sigma-Aldrich, St. Louis, MO, USA) and killed 46 h later. Cumulus-enclosed oocyte complexes (COCs) were recovered from the ovaries by puncturing the preovulatory follicles with 27 gauge needles. M2 medium (Sigma-Aldrich) containing 0.2 mM IBMX (Sigma-Aldrich) was used to inhibit GVBD while handling the oocytes. Isolated oocytes were snap-frozen and stored at -80°C before RNA preparation.

Preparation of dsRNA for Zap70, RanBP2 and GFP

To prepare double-stranded RNA (dsRNA), we used Zap70-A and RanBP2-A primers to amplify the complementary DNA (cDNA) of Zap70 and RanBP2, respectively (Table S3). The pEGFP-C1 vector (Clontech, Palo Alto, CA, USA) was used as a template for amplifying GFP cDNA. The PCR products were gel eluted and cloned into the pGEM T-easy vector (Promega, Madison, WI, USA). Each dsRNA was synthesized using a MEGAscript RNAi Kit (Ambion, Austin, TX, USA) and diluted to a final concentration of 3.5 μg/μl.

Microinjection of dsRNA into oocyte cytoplasm and in vitro *culture*

To elucidate the function of Zap70 during oocyte maturation, *Zap70* mRNA was knocked down by microinjecting *Zap70* dsRNA. GV oocytes were microinjected with *Zap70* dsRNA diluted in M2 medium (Sigma-Aldrich) containing 0.2 mM IBMX. An injection pipette containing the dsRNA solution injected 10 pl into the cytoplasm of oocytes using a constant flow system (Femtojet; Eppendorf, Hamburg, Germany). To functionally study several genes of interest, each dsRNA-treated oocyte was cultured in M16 medium (Sigma-Aldrich) containing 0.2 mM IBMX for 8 h to ensure complete knockdown of each target transcript and then cultured in plain M16 medium (Sigma-Aldrich) for 16 h at 37°C in an atmosphere containing 5% CO_2. Control oocytes were cultured using the same protocol. After the RNAi experiments, the *in vitro* oocyte maturation rates and morphological changes were recorded.

Time-lapse video microscopy

Time-lapse video microscopy was performed to track phenotypic changes and the speed of oocyte maturation during *in vitro* culture. A time-lapse microscope (JuLI^{TM}; Digital Bio, Seoul, Korea) was placed in the incubator at 37°C in an atmosphere containing 5% CO_2, and a culture dish containing the oocytes was placed on the microscope stage. Images were automatically captured every 5 min for 2 h, and the resulting sequential time-lapse images were converted to movie files using JuLI operation software.

mRNA isolation and RT-PCR

Oocyte mRNAs were isolated using a Dynabeads mRNA DIRECT Kit (Invitrogen Dynal AS, Oslo, Norway) according to the manufacturer's instructions. Briefly, the oocytes were resuspended with lysis/binding buffer (100 mM Tris-HCl, 500 mM LiCl, 10 mM EDTA, 10% LiDS (SDS), and 5 mM DTT) and mixed with prewashed Dynabeads with linked oligo dT_{25}. After RNA binding, the beads were washed with buffer A twice followed by a buffer B wash, and RNA was eluted using Tris-HCl (10 mM Tris-HCl, pH 7.5). The isolated mRNA was used as a template for reverse transcription using oligo dT_{25} primers according to the MMLV protocol. PCR was performed with single-oocyte-equivalent amounts of cDNA and primers (Table S3). PCR products were separated on 1.5% agarose gels and quantified using ImageJ software (NIH, Bethesda, MD, USA).

Western blot analysis

Protein extracts (100 oocytes per lane) were separated using 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ, USA). Each membrane was blocked for 1 h in Tris-buffered saline Tween (TBST; 0.2 M NaCl, 0.1% Tween-20, and 10 mM Tris [pH 7.4]) containing 5% non-fat dry milk. The blocked membrane was then incubated with a rabbit polyclonal anti-Zap70 antibody (1:1000; sc-574, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-RanBP2 antibody (1:500; sc-74518, Santa Cruz Biotechnology), mouse monoclonal anti-securin antibody (1:500; ab3305, Abcam, Cambridge, UK), or mouse monoclonal anti-α-Tubulin antibody (1:1000; sc-8035, Santa Cruz Biotechnology) in TBST buffer. After this incubation, the membranes were treated with horseradish peroxidase-conjugated (HRP) anti-rabbit IgG (1:1000; A-1949, Sigma-Aldrich) or anti-mouse IgG (1:1000; A-2554, Sigma-Aldrich) in TBST buffer for 1 h at room temperature. After each step, membranes were washed with TBST buffer, and the bound antibody was detected using an enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology) according to the manufacturer's instructions. The protein levels were quantified by measuring the density and area for each band using ImageJ software. These values were then normalized to those of anti-α-Tubulin and are expressed as a percentage compared with the control oocytes.

Immunofluorescence staining

For immunofluorescence staining, denuded oocytes were placed in phosphate-buffered saline (PBS) containing 0.1% polyvinyl alcohol (PVA), 4% paraformaldehyde and 0.2% Triton X-100 and then fixed for 40 min at room temperature. Fixed oocytes were washed 3 times for 10 min per wash in 1% bovine serum albumin (BSA)-supplemented PBS-PVA and stored overnight in 1% BSA-supplemented PBS-PVA. Oocytes were blocked in 3% BSA-supplemented PBS-PVA for 1 h and incubated overnight at 4°C with anti-Zap70 (1:20; sc-574, Santa Cruz Biotechnology), anti-α-Tubulin (1:100; sc-8035, Santa Cruz Biotechnology), anti-RanBP2 (1:50; sc-74518, Santa Cruz Biotechnology), or anti-securin (1:50; ab3305, Abcam) primary antibodies. After washing, the following secondary antibodies were used for 1 h at room temperature: FITC-anti-rabbit IgG (1:100; A-1949, Sigma-Aldrich), Alexa-anti-mouse IgG (1:100; A-21422, Invitrogen) and Alexa-anti-rabbit IgG (1:100; A-11034, Invitrogen). After 3 washes with 1% BSA-supplemented PBS-PVA, the oocytes were stained with either propidium iodide (Sigma-Aldrich, PI; 1 mg/ml in PBS) or 4′, 6-diamidino-2-phenylindole (Sigma-Aldrich, DAPI; 1 mg/ml in PBS), mounted on slides, and observed using a laser-scanning confocal microscope (Zeiss LSM 510 META, Germany).

Dual kinase activity assay

To measure the activities of the 2 kinases MPF and MAPK, a dual kinase activity assay was performed as described previously.[54] The oocytes were collected at certain time intervals (0, 10, 20, 30, and 40 min, and 1, 2, 4, 6, and 8 h) during *in vitro* culture, after which they were then washed in 0.1% PVA-PBS, lysed with 4 μl of lysis buffer, snap-frozen, and stored at −80°C until further use. After thawing, the oocytes were added to 5 μl of kinase buffer containing 0.3 μCi/μl [γ-^{32}P]-ATP (250 μCi /25 μl; Amersham Bioscience) and 5 μl of substrates and were incubated for 20 min at 37°C. The substrate solution for the MPF and MAPK double-kinase assay contained 4.5 μl of histone H1 and 0.5 μl of myelin basic protein (MBP). The reaction was terminated by the addition of 5 μl of 4x SDS sample buffer and boiling for 5 min. The samples were separated on a 15% gel using SDS-PAGE, and labeled MBP and Histone H1 were analyzed using autoradiography. The kinase activity was quantified by measuring the area of each band using ImageJ software (NIH).

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) and a log linear model. Data derived from at least 3 separate and independent experiments are expressed as the mean ± SEM, with values of $p < 0.05$ considered statistically significant.

Abbreviations

AI	Anaphase I
Cdk1	Cyclin-dependent kinase 1
GVBD	Germinal vesicle breakdown
LH	Luteinizing hormone
mESC	mouse embryonic stem cell
MI	Metaphase I
MII	Metaphase II
MPF	Maturation-promoting factor
NEBD	Nuclear envelope breakdown
NPC	Nuclear pore complex
RanBP2	Ran binding protein 2
Zap70	Zeta-chain-associated protein kinase 70

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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