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Research Article

Maternal RNA regulates Aurora C kinase during mouse oocyte maturation in a translation-independent fashion†

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Abstract

During oocyte meiotic maturation, Aurora kinase C (AURKC) is required to accomplish many critical functions including destabilizing erroneous kinetochore–microtubule (K-MT)attachments and regulating bipolar spindle assembly. How localized activity of AURKC is regulated in mammalian oocytes, however, is not fully understood. Female gametes from many species, including mouse, contain stores of maternal transcripts that are required for downstream developmental events. We show here that depletion of maternal RNA in mouse oocytes resulted in impaired meiotic progression, increased incidence of chromosome misalignment and abnormal spindle formation at metaphase I (Met I), and cytokinesis defects. Importantly, depletion of maternal RNA perturbed the localization and activity of AURKC within the chromosomal passenger complex (CPC). These perturbations were not observed when translation was inhibited by cycloheximide (CHX) treatment. These results demonstrate a translation-independent function of maternal RNA to regulate AURKC-CPC function in mouse oocytes.

Summary Sentence

Maternal RNA contained in mouse oocytes regulates localized AURKC-CPC activity independent of its role in translation to support meiotic maturation.

Key words: maternal RNA, Aurora kinase C, mouse oocyte, meiosis, chromosomal passenger complex.

Introduction

In mitotic cells, the fidelity of chromosome segregation requires a functional chromosomal passenger complex (CPC) that is driven by its catalytic subunit, aurora kinase (AURK) B. AURKB carries out its regulatory duties by controlling many cellular processes including triggering destabilization of improper kinetochore microtubule (K-MT) attachments, signaling to the spindle assembly checkpoint (SAC) to appropriately delay anaphase onset, controlling bipolar spindle assembly, and regulating cytokinesis [1–4]. In contrast to mitotic cells, mammalian oocytes utilize an AURKB homolog, AURKC, as the primary kinase in the CPC to regulate meiosis I (MI) [5–9]. AURKC perturbations in mouse oocytes either in vivo or in vitro resulted in higher rates of aneuploidy [6, 7, 10], the leading genetic abnormality causing miscarriage and congenital anomalies [11]. Expression of a dominant-negative mutant of AURKC revealed that AURKC functions in oocytes include regulation of meiotic progression, destabilization of improper K-MT attachments, and regulation of microtubule organizing center (MTOC) clustering to assemble bipolar spindles [7, 12]. However, how AURKC is regulated to control all of these cellular events is not fully elucidated.

Given the important roles played by AURKC to govern MI, it is anticipated that mammalian oocytes have multiple regulatory pathways to ensure the fidelity of AURKC-CPC function. We previously demonstrated that histone modifications such as deacetylation and phosphorylation are critical to regulate AURKC-CPC localized function during MI. Inhibition of the maturation-associated decrease of histone deacetylation resulted in loss of AURKC function [13, 14]. Moreover, inhibition of H3T3 phosphorylation mediated by haspin kinase perturbed AURKC localization and activity along chromosome arms during metaphase of MI (Met I)[10]. Whether AURKC function is governed by other regulatory mechanisms in mammalian oocytes, as AURKB is in mitosis, is still unknown.

During the growth phase of oogenesis, mammalian oocytes accumulate large cytoplasmic pools of RNAs, including messenger RNAs (mRNA) [15]. These stores of maternal mRNA are critical to support the subsequent transcriptionally silent stages of meiotic maturation, fertilization, and early embryonic development prior to zygotic genome activation [16–18]. Therefore, the most widely accepted function of maternal RNA is to assure the translation and synthesis of required proteins at a particular place and time during development [19]. However, recent studies demonstrated that mRNAs in *Drosophila* and *Xenopus* eggs have translation-independent functions. For example, in *Drosophila*, there are alleles of the maternal effect gene, oskar, that produce no detectable RNA and these mutant flies fail to produce viable eggs. This phenotype can be rescued by expression of the 3′ untranslated region of the gene, indicating that mRNA itself, and not the protein, can have functions in oocyte development [20]. Consistent with roles in regulating cellular function, RNAs can be found either tightly associated with the MT cytoskeleton [21] or bound to proteins thereby forming ribonucleoprotein (RNP) complexes [22, 23]. The association of RNA to the cytoskeleton is synergistic. Microtubules are essential for mRNA transport and trafficking to the location where translation is required [24], and the RNAs are required for MT organization and stability. The latter example is supported by depletion experiments that induced a collapsed cytoskeleton, and rescue experiments where this phenotype was reversed by injection of synthetic mRNAs [25, 26]. In mitotic cells, AURKB coprecipitates with minor satellite RNA in RNA pull-down assays [27]. Importantly, the RNP complex is prerequisite to stabilize and activate AURKB-CPC at centromeres, and this regulation is essential for MT polymerization and bipolar spindle assembly [3, 27].

Because oocytes contain abundant stores of maternal RNAs, we hypothesized that AURKC-CPC is also regulated by RNA. Here we demonstrate that maternal RNAs regulate AURKC localization and activity, and spindle assembly in mouse oocytes in a manner independent from translation during MI.

Material and methods

Ethics

All animals were maintained and experiments were conducted in accordance with Rutgers Institutional Animal Use and Care Committee (#11–032) and the National Institutes of Health guidelines.

Cloning and in vitro cRNA synthesis

DNA linearization of *Gfp*-containing plasmid was carried out using Nde I restriction enzyme (New England BioLabs). After DNA purification, in vitro transcription was carried out using an mMessage mMachine T7 kit (Ambion) according to the manufacturer's protocol. The synthesized cRNA was then purified using an RNAEasy kit (Qiagen).

Oocyte collection, microinjection, and culture

Prophase I-arrested oocytes were obtained from 6-week-old CF-1 female mice previously primed (44–48 h) with pregnant mare serum gonadotropin (PMSG) (Calbiochem #367 222) and mechanically stripped of cumulus cells [28]. The collection and injection medium for oocytes was bicarbonate-free minimal essential medium (MEM) containing 25 mM Hepes (pH 7.3), 3 mg/ml polyvinylpyrollidone (MEM/PVP), and 2.5 μM milrinone (Sigma #M4659) to prevent nuclear envelope breakdown and meiotic resumption [29].

Each denuded oocyte was microinjected with ~10 pl of 10, 50, or 100 μg/μl of RNase A (Sigma, R4642). This RNase A is molecular biology grade and has no detected endonuclease, exonucleases, or DNase activities. The microinjected oocytes were then incubated in Chatot, Ziomek, and Bavister (CZB) medium containing 2.5 μM milrinone for 1–2 h followed by in vitro maturation in milrinone-free CZB medium for 6 h (Met I) or 16 h (Met II) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cycloheximide (Sigma, C7698) was dissolved in phosphate-buffered saline (PBS) and then added to CZB culture medium to a final concentration of 10 μg/ml.

Quantitative reverse transcription polymerase chain reaction

Twenty oocytes were sampled and frozen at –80°C prior to processing. After thawing on ice, 2 ng of *Gfp* cRNA was added to each sample. RNA extraction and cDNA synthesis was performed using Cell lysis and reverse transcription (RT) kit for quantitative polymerase chain reaction (qPCR) (Toyobo) following the manufacturer's instructions. All cDNA samples were stored at –80°C. The expression level of *GAPDH* relative to *Gfp* was assessed using KAPA SYBR FAST qPCR kit optimized for LightCycler 480 (KAPA Biosystem), and data were acquired using LightCycler Nano (Roche). The comparative Ct method was employed to determine differences in the expression level between groups.

Immunocytochemistry and confocal microscopy

For detection of survivin, oocytes were fixed in freshly prepared 4% paraformaldehyde containing 0.1% Triton X-100 in PBS for 30 min at room temperature. In all other experiments, 2% paraformaldehyde in PBS was used as fixative for 20 min at room temperature. After fixation, the oocytes were permeabilized in PBS with 0.1% Triton X-100 for 15 min and placed in blocking buffer (PBS + 0.3% BSA+0.01% Tween-20) for 15 min. After 1 h incubation with primary antibody, oocytes were washed three times in blocking solution, 10 min each. The oocytes were then incubated in secondary antibodies for 1 h. Oocytes were washed again in blocking solutions three times, 10 min each followed by mounting and DNA staining with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Life Technologies #D1306; 1:170) diluted in VectaShield (Vector Laboratories). Fluorescence signals were detected on Zeiss 510 meta laser-scanning confocal microscope under a ×63 objective.

All oocytes in the same experiment were processed simultaneously. The laser power was carefully adjusted to induce signal intensity just below saturation for the group that displayed the highest intensity, and all oocytes were then scanned at the same laser power to allow for intensity quantification. The intensity of fluorescence was quantified using NIH image J software under the same

processing parameters when experimental analysis required intensity measurements.

Cytotoxicity assays

A TUNEL assay kit was used to assess the presence of apoptotic cells (In Situ Cell Death Detection Kit; Roche Applied Science) according to the manufacturer's instructions. The cells were fixed in 4% (w/v) paraformaldehyde solution (pH 7.4) for 1 h followed by washing twice in PBS containing 0.3% BSA and 0.01% Tween-20 for 10 min each. After washing, fixed oocytes were permeabilized in PBS with 0.1% Triton X-100 for 20 min. The fragmented DNA ends of the cells were labeled with fluorescein-dUTP for 60 min at 37°C. After incubation, the cells were washed in PBS containing 0.3% BSA and 0.01% Tween-20 three times for 5 min each, followed by mounting onto glass slides using Vectashield mounting solution containing DAPI (Vector Laboratories). Pretreatment of oocytes with DNase I recombinant served as a positive control, while omitting fluorescein-dUTP served as a negative control. The fluorescence of fragmented DNA ends was detected by fluorescence microscopy (Leica).

To evaluate cell viability, oocytes were incubated with a double staining of 10 μg/ml of Hoechst 33 342 (Life Technologies #C10337) and 10 μg/ml of propidium iodide (PI) (Invitrogen #P3566) in MEM/PVP for 30 min at room temperature. Nuclear PI staining is specific for cells with a damaged membrane, indicative of cytotoxicity, while nuclear Hoechst staining is universal for live and dead cells. Control oocytes were treated with 1 mM hydrogen peroxide for 2 h. Oocytes were examined under a fluorescent microscope EVOS FL Auto Imaging System (Life Technologies) with a ×20 objective.

Live cell imaging

Oocytes were transferred into a 96-well optical-bottom dish containing CZB medium covered with mineral oil (Greiner Bio One, # 655 892). Bright-field image acquisition was started at prometaphase I using an EVOS FL Auto Imaging System (Life Technologies) with a ×20 objective. The imaging system was equipped with EVOS Onstage environmental chamber to maintain humidity, temperature (37°C) and 5% CO_2. Images were then captured every 20 min and processed using NIH image J software.

Antibodies

The following primary antibodies were used in immunofluorescence: AURKC (Bethyl #A400–023A- BL1217; 1:30), phospho-specific Ser893/Ser894 INCENP (gift from M. Lampson, U. of Pennsylvania [30]; 1:1000), Survivin (Cell Signaling Technology #2808S; 1:500), α-tubulin-Alexa Fluor 488 conjugate (Life Technologies #322 588; 1:100), phospho-specific H3S10 (Millipore; #05–806; 1:100).

Statistical analysis

One-way ANOVA and Student *t*-test were used to evaluate the differences between groups using GraphPad Prism. ANOVA test was followed by the Tukey post hoc test. The differences of $P < 0.05$ were considered significant. Each experiment was replicated at least two times. The data were expressed as means ± SEM.

Results

Maternal RNA is essential for meiosis I in mouse oocytes

Mammalian oocytes contain an abundance of maternal RNAs to support downstream developmental processes such as meiosis, fertilization, and embryonic development. Because AURKB and AURKC are similar in sequence and function, it is plausible that RNA regulates AURKC localization and activity in mammalian oocytes, as it does AURKB in mitosis. To investigate the potential of RNA in regulating AURKC-CPC function, we compared the meiotic phenotypes (meiotic progression, alignment of chromosomes, and spindle formation) of oocytes depleted of maternal RNA with those where AURKC in inhibited [7, 10, 12]. To deplete maternal RNA, we selected RNase A, whose efficiency to degrade RNA in mouse oocytes was previously reported [23]. First, we microinjected three different concentrations of RNase A into meiotically competent oocytes. After in vitro maturation to Met II, we evaluated completion of meiotic progression by examining polar body extrusion (PBE). Similar to the phenotype when AURKC is perturbed [7], depletion of maternal RNA impaired meiotic progression in a dose-dependent manner. The percentage of PBE was significantly decreased when 50 μg/ml (16.86 ± 9.45) or 100 μg/ml (7.94 ± 2.06) of RNase A was microinjected into oocytes than in PBS-injected controls (82.86 ± 2.85, Figure 1A). Using qRT-PCR, we evaluated the efficiency of the RNase A treatment using the lowest effective dose (50 μg/ml) that perturbed meiotic progression. We found efficient depletion (~80%) of the housekeeping gene, *GAPDH* within 4 h after microinjection, confirming the efficiency of RNase A to deplete mRNA in mouse oocytes (Supplemental Figure S1A).

Because RNase A injection significantly impaired PBE, we therefore examined these oocytes live to visualize this defect in real time. Time-lapse imaging showed an increased incidence of cytokinesis defects (PB retraction), in which RNA-depleted oocytes, but not PBS-injected controls, began to extrude a PB but subsequently retracted it (Supplemental Movie S1and S2; Figure 1B and C). When we examined the resulting chromosomes and spindle configurations, we observed a collapsed-like chromosome phenotype in RNA-depleted oocytes, presumably due to the failure in cytokinesis and inability to reform a spindle (Supplemental Movie S2; Figure 1D and E). Chromosomes that resembled a ball were considered collapsed. When we re-evaluated the behavior of the chromosomes in the live images, we found that those oocytes with cytokinesis failure had visible aggregates of chromatin, suggesting that chromosomes of RNA-depleted oocytes collapsed after the attempt at cytokinesis (Figure 1C, lower panel).

We next investigated other perturbations that depletion of RNA had on MI. Because RNA regulates microtubule spindle assembly in *Xenopus* and in human cells [3, 22], we examined spindle morphology at Met I in more detail. Oocytes with barrel-shaped spindles containing clearly defined poles were considered normal. Depletion of RNA significantly increased the percentage of oocytes that formed abnormal spindles at Met I when compared to controls (Figure 2A and B). In general, spindle abnormalities in RNA-depleted oocytes ranged from mild aberrant spindle formation where there was a decreased spindle length-to-width (L/W) ratio (Figure 2A and C) to more severe phenotypes such as monopolar spindles (Figure 2A and D) or a failure to assemble a spindle, characterized by the absence of spindle microtubules in the vicinity of chromosomes (Figure 2A and E) consistent with previous data showing that maternal RNA being

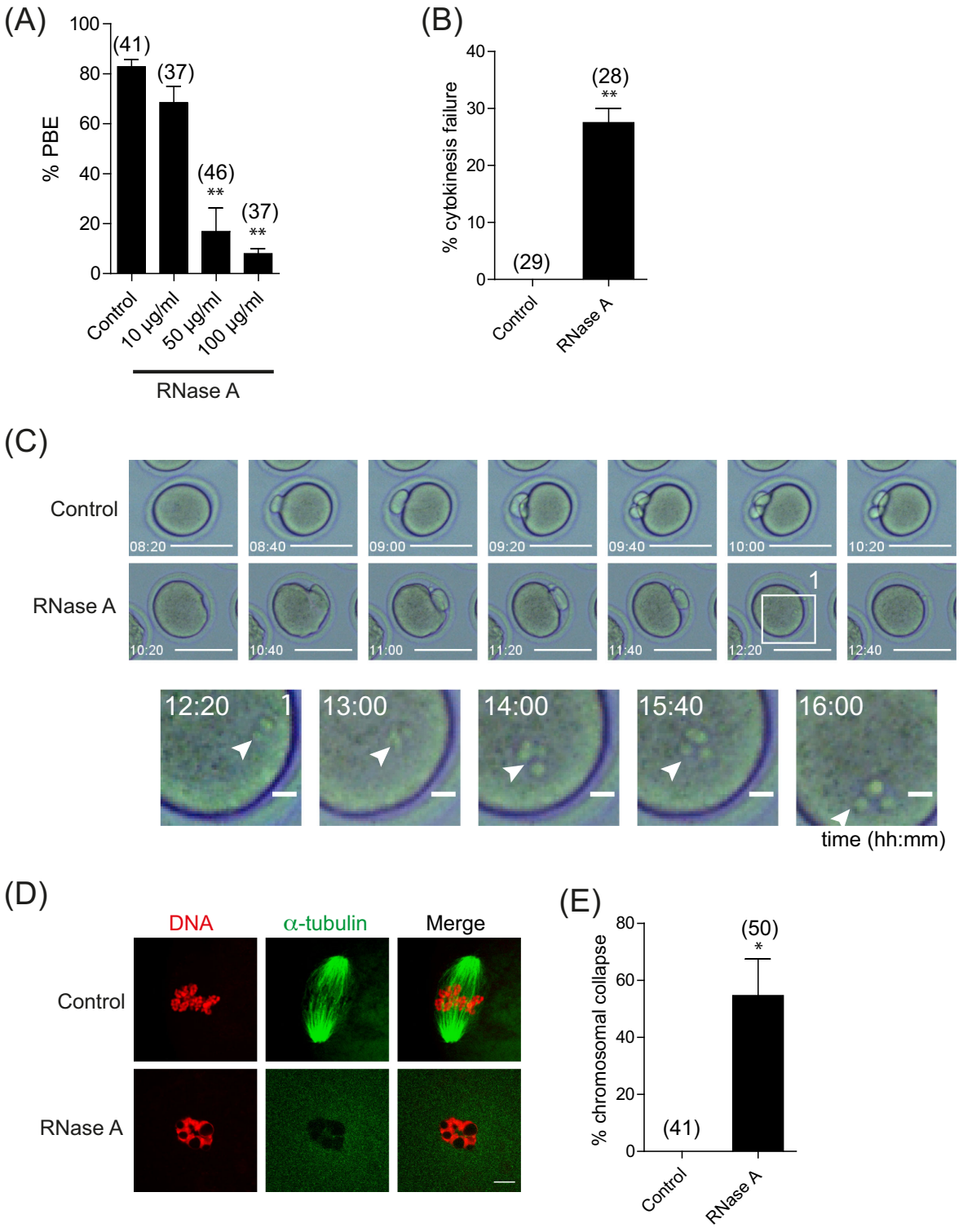

Figure 1. Depletion of maternal RNA impairs oocyte maturation. Full-grown prophase I-arrested oocytes were injected with PBS or RNase A followed by maturation in vitro for 16 h. (A) First polar body extrusion (PBE) was scored to assess meiotic progression. (B) Quantification of cytokinesis defects based on live cell imaging (n = 21, 26). (C) Representative live cell images showing retraction of the extruded polar body in RNA-depleted oocytes; scale bar represents 100 μm. White arrowheads in the lower panel indicates chromatin aggregation; scale bar, 10 μm. (D) Confocal microscopy images of Met II eggs stained with an anti-α-tubulin antibody to label spindle (green) and DAPI to label DNA (red). Representative images are shown. (E) Quantification of chromosomal collapse phenotype. The experiments were carried out at least three times and the total numbers of oocytes examined are indicated above the graph bars. The data are expressed as mean ± SEM, and Student *t*-test was used to analyze the data except (A) where one-way ANOVA was used to analyze the data. Values with asterisks vary significantly, $^{*}P < 0.05$, $^{**}P < 0.01$.

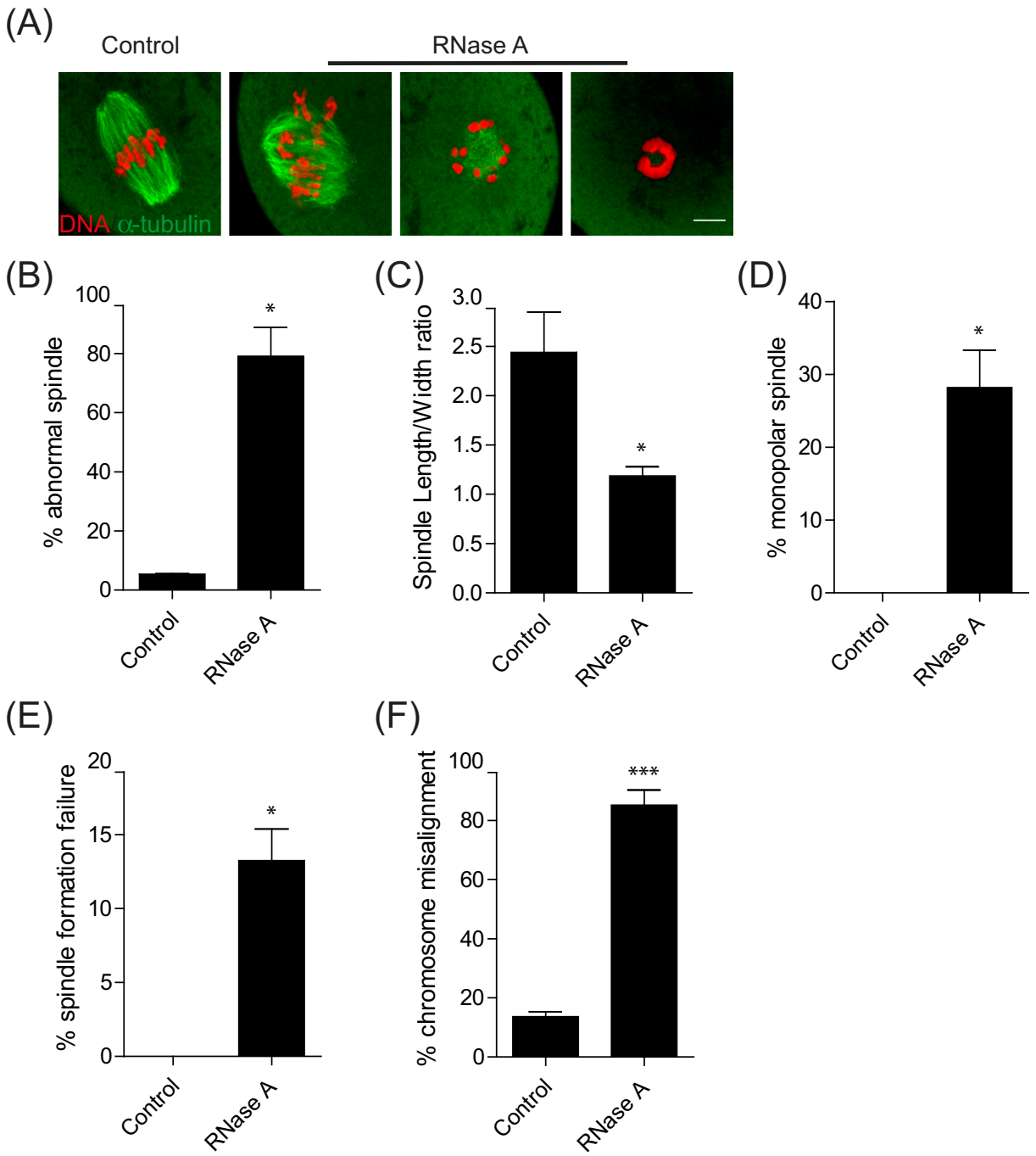

Figure 2. Maternal RNA perturbs bipolar spindle assembly. Full-grown, prophase I-arrested oocytes were injected with PBS or RNase A followed by in vitro maturation to Met I. (A) Met I oocytes were fixed and stained with an anti-α-tubulin antibody to label spindle microtubules (green) and DAPI to label DNA (red) followed by confocal imaging. Representative images are shown. Scale bar represents 10 μm. (B) Quantification of abnormal spindle morphology (C) Quantification of spindle length/width ratio. (D) Quantification of the number of oocytes with a monopolar spindle. (E) Quantifications of oocytes that failed to form a spindle. (F) Quantification of the number of oocytes with misaligned chromosomes. The experiment was carried out three times, and the total number of oocytes examined were 30 and 34 oocytes in the control and RNase A groups, respectively. The data are expressed as mean ± SEM and Student *t*-test was used to analyze the data. Values with asterisks vary significantly, $^{*}P < 0.05$, $^{***}P < 0.001$.

required for normal meiotic spindle assembly in mouse oocytes [19, 31, 32]. Finally, we examined alignment of chromosomes at the Met I plate according to the parameters described previously [33]. We found that in contrast to controls, where 13.65 ± 1.66% of oocytes had misaligned chromosomes, RNA-depleted oocytes had a significant increase in misalignment (85.19 ± 5.08%, Figure 2F). Taken together, these results indicate that maternal RNA, similar to AURKC, has an important role to regulate meiotic progression, bipolar spindle

assembly, chromosome alignment, and cytokinesis during oocyte meiosis.

Maternal RNAs are required for Aurora kinase C-chromosomal passenger complex localization and activity during meiosis I

To determine if RNA regulates AURKC localization in mouse oocytes, we performed immunocytochemistry in RNA-depleted oocytes. In control-injected oocytes, AURKC localized to kinetochores and along the interchromatid axes of Met I bivalents as previously reported [5–7]. Importantly, depletion of maternal RNA perturbed chromosomal AURKC localization (Figure 3A and B). Although some AURKC puncta could be visualized, we did not observe any "cross"-like structures that we routinely see localizing along arms of Met I bivalents. The absence of such cross-like pattern in all bivalents was considered mislocalization. Translocation of the CPC from Met I chromosomes to the spindle midzone and midbody is critical for completion of cytokinesis [2, 7, 8, 34]. Consistent with loss of AURKC on chromosomes, and, in contrast to controls, depletion of maternal RNA resulted in loss of AURKC localization to the midbody at Telo I (Figure 3C). Collectively, these results suggest that maternal RNA is required for AURKC localization throughout MI.

In mitotic and meiotic cells, the CPC consists of INCENP, borealin, survivin, and an Aurora kinase catalytic subunit [8, 35, 36]. We previously demonstrated that AURKC is required to retain CPC localization during MI in mouse oocytes [7]. To investigate whether depleting maternal RNA disrupts CPC localization, we assessed the localization of survivin in RNA-depleted oocytes. Consistent with loss of AURKC localization, kinetochore and interchromatid axis localization of survivin was perturbed in RNase A-injected oocytes when compared to injected controls (Figure 4A and B). These data suggest that maternal RNA is also required for CPC localization. AURKC binds the "IN box" domain of INCENP [37]. This binding is prerequisite for phosphorylation of INCENP (pINCENP) at serines 893 and 894 by AURKC and subsequent activity of the CPC [7, 38]. To investigate whether maternal RNA also regulates CPC activity, we evaluated both pINCENP and phosphorylated histone H3 at serine 10 (H3pS10), another AURK substrate, in RNA-depleted oocytes. As expected, phosphorylation of INCENP was significantly decreased in RNA-depleted oocytes when compared to controls (Figure 4C and D). Consistent with loss of pINCENP, oocytes lacking RNA showed significantly reduced levels of H3pS10 (Figure 4E and F).

This reduction in CPC activity upon RNA depletion could be an artifact of a failure of the antibody to access the epitope because of the collapsed chromosome configuration (Figure 1D). To exclude this possibility, we examined the timing of loss of CPC activity (H3pS10) to the timing of the change in DNA architecture during meiotic maturation (6, 8, and 12 h time points). After 6 h of maturation, while the chromosome configuration remained normal, AURKC-CPC activity was beginning to decrease (Supplemental Figure S2A). Importantly, complete CPC perturbation was observed by 8 h (Supplemental Figure S2B), which preceded the time that it took for the chromosomes to collapse (~12 h) in RNA-depleted oocytes (Supplemental Figure S2C). Therefore, these data indicate that AURKC-CPC is lost from the chromosomes prior to their collapse and support the model that maternal RNAs regulate localized AURKC-CPC activity. Furthermore, these data suggest that loss of AURKC-CPC is causative of the chromosome collapse phenotype.

Aurora kinase C regulation by maternal RNA is independent of translation during meiosis I

In fully grown oocytes, mRNA constitutes about one-fifth of the volume of total RNA [15]. Moreover, it is well established that maternal mRNA is recruited for translation during oocyte maturation, and that this recruitment is essential for many cellular processes during MI [19]. To eliminate the possibility of a translation-dependent effect, we examined the effect of inhibiting translation by cycloheximide (CHX) treatment, a protein biosynthesis inhibitor, on AURKC-CPC localization and activity. We first assessed the efficiency of CHX as an inhibitor of translation in this system. Full-grown oocytes were microinjected with *Gfp* cRNA and subsequently matured in vitro either in the presence or absence of CHX. Incubation of the microinjected oocytes with 10 μg/ml CHX significantly decreased GFP protein levels at Met I (Figure 5A and B). Importantly, similar to control oocytes incubated in CHX-free medium, AURKC localized to kinetochores and interchromatid axes, and had normal levels of H3pS10 at Met I in CHX-treated oocytes (Figure 5C–F). These data suggest that maternal RNA regulates AURKC function independent of a role in translation.

To further eliminate the possibility of a translation-dependent effect, we microinjected RNase A into oocytes at Met I (6 h) followed by incubation in in vitro maturation medium for an additional 2 h. This short incubation was enough to induce RNA degradation as evidenced by the significant decrease of *GAPDH* mRNA level (Supplemental Figure S1B). After 6 h of in vitro maturation, AURKC is localized to chromosomes, a bipolar spindle is established, and messages have been recruited for translation [6, 8, 39]. Strikingly, acute depletion of RNA (~2 h) in Met I oocytes perturbed AURKC localization at chromosomes when compared to injected controls (Figure 6A and B). Moreover, depletion of RNA resulted in a significant increase of abnormal spindles with wider spindle poles (51.3 ± 5.8%) when compared to controls (5.0 ± 5.0%; Figure 6A and C). To further confirm the results, we conducted the same experiment using Met II-arrested eggs. Similar to Met I oocytes, acute depletion (~2 h) of RNA in Met II oocytes (~14 h of in vitro maturation) perturbed AURKC localization at chromosomes and perturbed spindles (Figure 6D–F). Nearly 15% of RNase A-injected oocytes showed complete depolymerization of the Met II spindle (Figure 6D, lower panel). Toxic insult results in cytotoxicity-mediated cell death [40, 41]. Cell death has two major pathways either apoptosis or necrosis. Interestingly, the death and fragmentation of ovulated oocytes was proven to be an unequivocal example of apoptosis but not necrosis [42, 43]. To eliminate the possibility that AURKC perturbation in RNase A-injected oocytes, at least under acute depletion condition, is a consequence of a nonspecific toxic effect, we conducted a TUNEL assay. Similar to control oocytes, RNase A-injected Met II eggs did not show any evidence of apoptotic cell death (Supplemental Figure S3A), suggesting that AURKC perturbation and spindle depolymerization is independent of cytotoxicity and DNA fragmentation. Consistent with not causing apoptosis, when we assessed oocyte viability in injected oocytes matured for a longer time period (16–18 h) via propidium iodide uptake, no RNase A-injected oocytes (0/34) stained positive for the intercalating agent (Supplemental Figure S3B). This result is in contrast to 100% of positive controls (40/40) that were matured in the presence of hydrogen peroxide to generate reactive oxygen species that stained positive. Taken together, these results indicate that maternal RNA is essential not only for AURKC localization and bipolar spindle assembly but also for maintaining their stability, and provide further evidence that these functions are regulated independently of translation during MI.

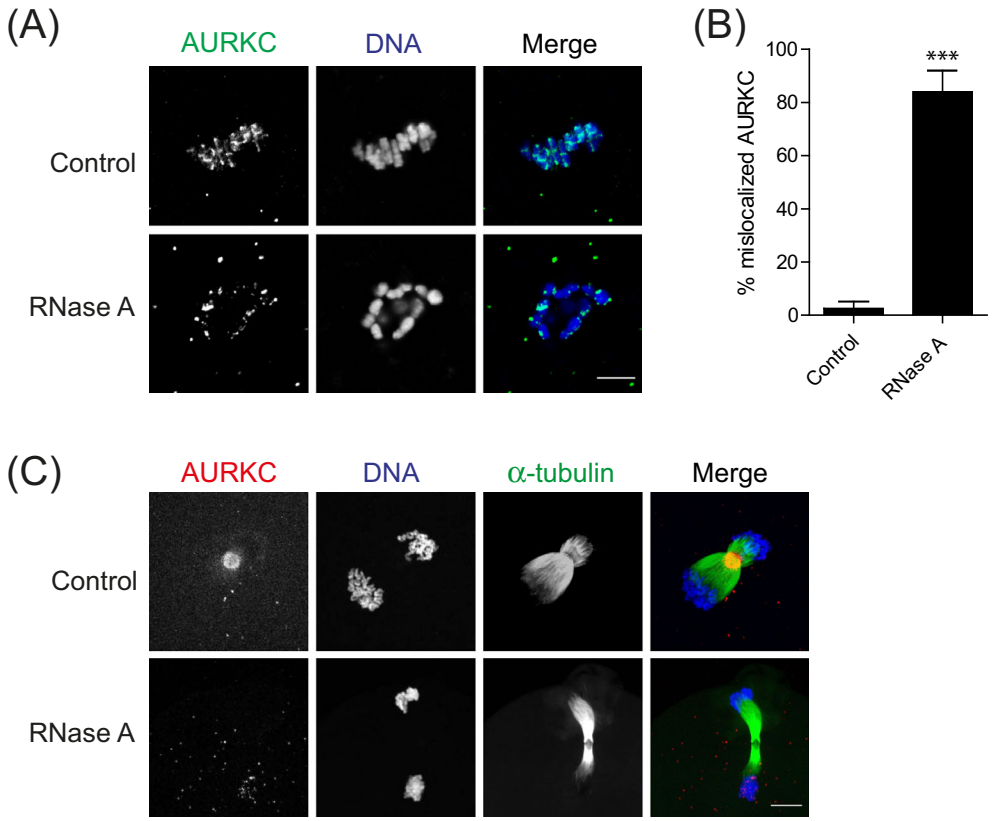

Figure 3. Maternal RNA perturbs AURKC localization during oocyte maturation. Full-grown prophase I-arrested oocytes were injected with PBS or RNase A followed by in vitro maturation. (A) Met I oocytes (6 h) were fixed and immunostained with an anti-AURKC antibody (green in merge). DNA was detected with DAPI (blue in merge). (B) Corresponding quantification of oocytes with localized AURKC in (A). (C) Telophase I oocytes were fixed and immunostained with an anti-AURKC antibody (red in merge) and an anti-α-tubulin antibody (green in merge). DNA was labeled with DAPI (blue in merge). The scale bar represents 10 μm, and representative images are shown. The experiments were carried out two times and the total number of oocytes examined were 20 and 24 oocytes in the control and RNase A groups, respectively. The data are expressed as mean $\pm$ SEM, and Student *t*-test was used to analyze the data. Values with asterisks vary significantly, $^{***}P < 0.001$.

Discussion

In mammalian oocytes, AURKC carries out many cellular functions that are critical for faithful chromosome segregation [6–10, 12]. However, less is understood about how AURKC is regulated in mammalian oocytes to carry out these functions. In this study, we uncover a translation-independent roles of maternal RNA to regulate AURKC localization and activity, and bipolar spindle assembly in mouse oocytes.

During oocyte maturation, selected pools of mRNAs including *Orc6l, Sin3a, Dazl,* and *Aurkc* are recruited for translation [6, 31, 44, 45]. We eliminated the possibility that the phenotypes observed via RNA depletion were due to a lack of translating key regulators by two ways. First, acute RNA depletion after cells had reached metaphase still perturbed AURKC-CPC localization. Second, the AURKC-CPC pertubations were not observed when translation was inhibited by CHX treatment. Although we did not investigate the molecular mechanism of this regulation, two models could be considered: (1) either maternal RNA regulates AURKC indirectly through promoting histone phosphorylation (possibly at H3 T3) and histone deacetylation that are prerequisite for AURKC localization during MI or (2) maternal RNA has a direct affinity to bind and regulate AURKC localization. This direct model is supported by the finding that RNA, in mitotic cells, binds directly to and activates AURKB to form RNP complexes [3, 27]. This interaction is likely through a sequence-specific RNA-binding domain. This domain is not in the N-terminus of the kinase because only full-length AURKB had the affinity to bind RNA in vitro [3].

We previously demonstrated that AURKC has overlapping functions with AURKB to regulate the SAC response and cytokinesis during MI [7]. The high incidence of cytokinesis defects in RNA-depleted oocytes suggests that RNA regulates both AURKs. This hypothesis is supported by the facts that both proteins belong to a conserved serine-theronine kinase family, have a high amino acid sequence identity, and share interacting proteins [46, 47]. Furthermore, in mitotic cells, Jambhekar *et al.* demonstrated that RNA binds the CPC through at least two RNA-binding domains: one in AURKB and another in the regulatory subunits (Borealin or Survivin) [3]. Given that both AURKs acquire their activities through binding the CPC [8, 38, 47], it is highly possible that the CPC instability induced by RNA depletion perturbed both AURKs functions. However, further research is required to uncover whether AURKs are the primary targets within the CPC for maternal RNA in mammalian oocytes.

Spindle MTs play indispensible roles to capture chromosomes thereby regulating chromosome alignment and segregation during the cell cycle. To accomplish these tasks in female meiosis, MTs require functional MTOCs and MT-associated proteins. Accordingly,

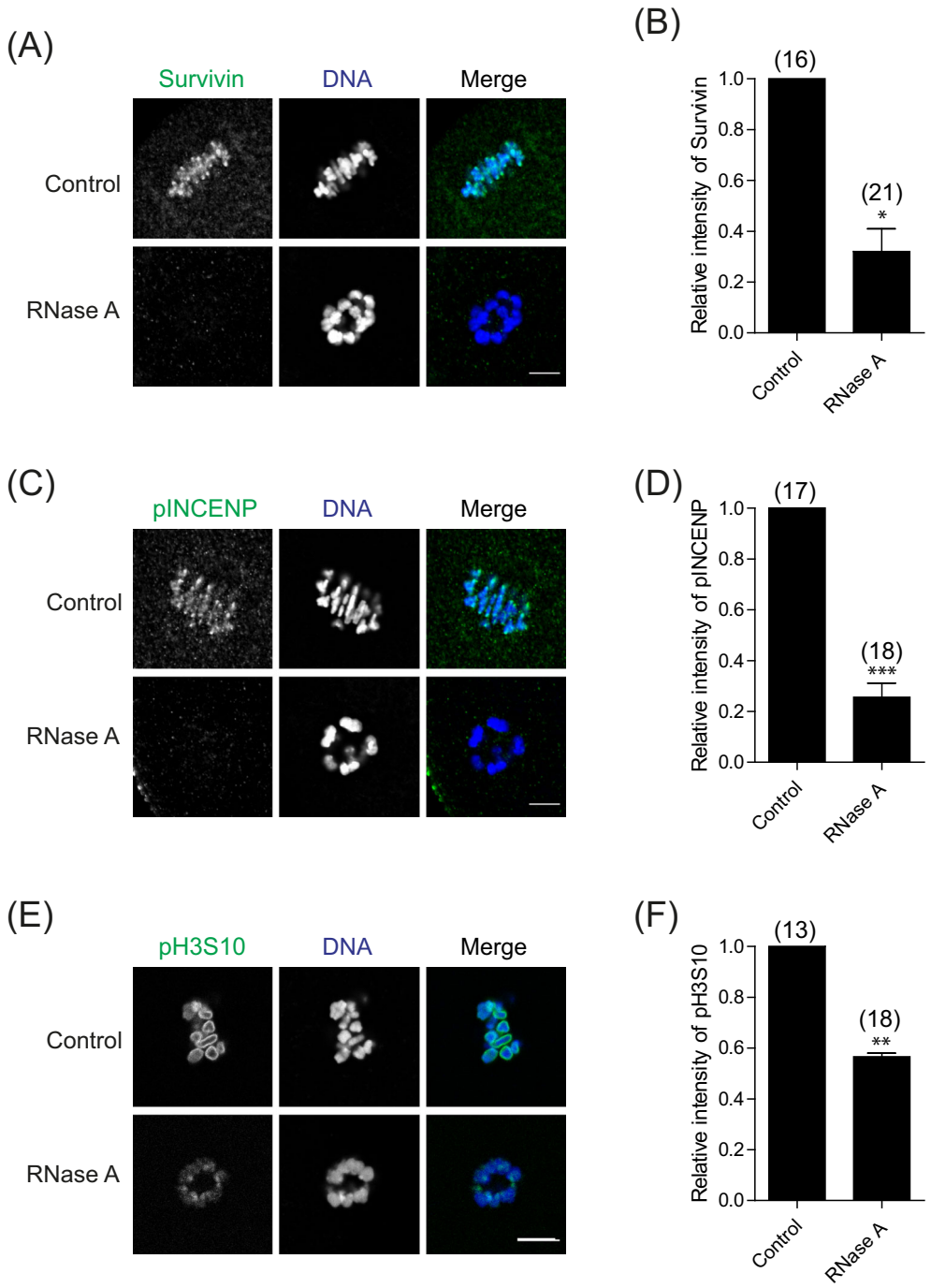

Figure 4. Maternal RNA perturbs CPC localization and activity during oocyte maturation. Full-grown prophase I-arrested oocytes were injected with PBS or RNase A followed by in vitro maturation to Met I. Met I oocytes (6 h) were fixed and immunostained (green in merge) with an anti-survivin antibody (A), anti-pINCENP antibody (C), and anti-H3pS10 antibody (E). DNA was detected with DAPI (blue in merge). Representative images are shown, scale bar represents 10 μm. (B), (D) and (F) Corresponding quantifications of fluorescence intensities of (A), (C) and (E), respectively. The experiments were carried out two times and the total numbers of examined oocytes are indicated above the graph bars. The data are expressed as mean ± SEM, and Student *t*-test was used to analyze the data. Values with asterisks vary significantly, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

knockdown of these proteins using siRNA-mediated mRNA degradation resulted in disorganized spindles (reviewed in [48]). Interestingly, cDNA microarray analysis revealed that maternal mRNAs encoding spindle proteins are preferably enriched along MTs in Met II eggs. This translation of localized mRNA is critical to support spindle assembly by providing a high concentration of the required proteins at the site of action [19]. Consistent with the role of mRNA translation in spindle assembly, inhibition of protein synthesis using CHX treatment induced abnormal spindle formation in Met I oocytes (unpublished observations). In this study, we demonstrated a translation-independent role of maternal RNA to regulate bipolar spindle assembly. This finding is consistent with the results in

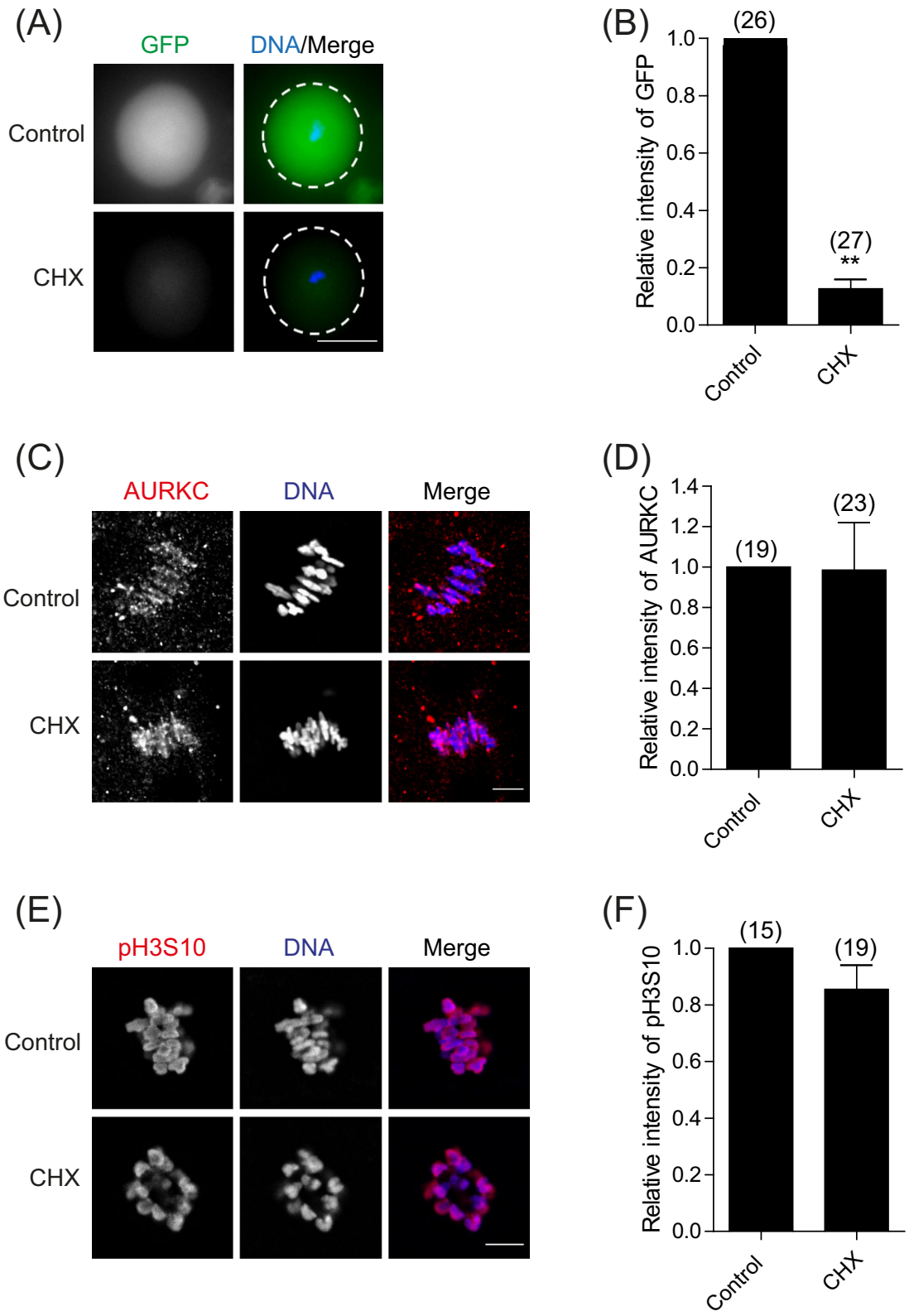

Figure 5. Translation of maternal RNA is not required for AURKC localization and activity. Full-grown prophase I-arrested oocytes either noninjected or injected with *Gfp* cRNA were in vitro matured in the presence of PBS or 10 μg/ml cycloheximide for 6 h. Met I oocytes were fixed and examined for GFP expression (A; green in merge) or immunostained (red in merge) with an anti-AURKC antibody (C), or anti-H3pS10 antibody (E). DNA was detected with DAPI (blue in merge). The scale bar represents 50 μm for (A) and 10 μm for (C and E). Representative images are shown. The experiments were carried out two times and the total numbers of examined oocytes are indicated above the graph bars. (B), (D), and (F) Corresponding fluorescence intensity quantifications for (A), (C), and (E), respectively. The data are expressed as mean ± SEM, and Student *t*-test was used to analyze the data.

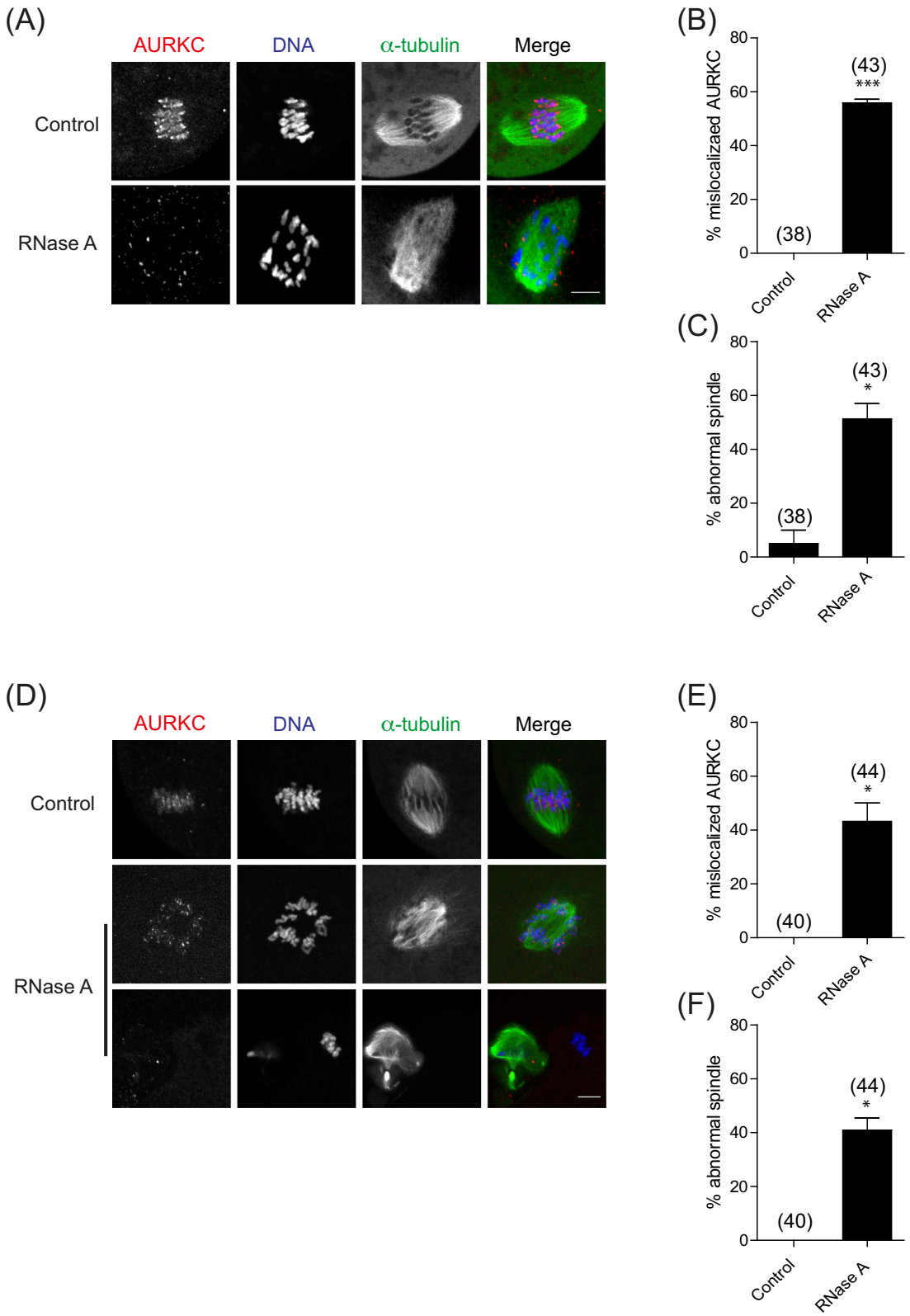

Figure 6. Regulation of AURKC and meiotic spindle by maternal RNA during oocyte maturation is independent of translation. Full-grown prophase I-arrested oocytes were matured in vitro for 6 h (Met I) or 14 h (Met II). Met I and Met II oocytes were injected with PBS or RNase A followed by incubation in the same maturation condition for an additional 2 h. Met I (A-C) and Met II (D-F) injected oocytes were fixed and immunostained with an anti-AURKC antibody (red in merge) and an anti-α-tubulin antibody (green in merge). DNA was labeled with DAPI (blue in merge). The scale bar represents 10 μm, and representative images are shown. (B, C) Corresponding quantifications of oocytes in A. (E, F) Corresponding quantifications of eggs from D. The experiments were carried out two times and the total numbers of examined oocytes are indicated above the graph bars. The data are expressed as mean ± SEM, and Student *t*-test was used to analyze the data. Values with asterisks vary significantly, $^{*}P < 0.05$, $^{***}P < 0.001$.

Xenopus extracts where RNA depletion resulted in aberrant MT organization [22]. These extracts do not require transcription or translation to assemble the spindle, supporting the model that maternal RNA has a translation-independent role to regulate spindle assembly. The translation-independent role could have two possible explanations. First, RNAs associate tightly and directly to the cytoskeleton to hold the filaments together with subsequent promoting its structural integrity and maintaining its stability [25, 26]. Second, RNA may regulate MT spindle assembly through being a component in RNP complexes to activate certain spindle regulators such as Rae1 and AURKB [3, 22]. Because both AURKs have a high-sequence similarity, and because AURKC perturbation in mouse oocytes resulted in abnormal spindle formation [12], it is possible that RNA regulates bipolar spindle assembly in mouse oocytes, at least partially, through regulating AURKC function. However, the abnormal spindle phenotype that we found here in RNA-depleted oocytes is much more severe than that in AURKC-perturbed oocytes [12], suggesting that RNA has different pathways to regulate meiotic spindle in mouse oocytes.

It is now well established that advanced maternal age is accompanied by alteration in global gene and protein expressions [49, 50] and is positively correlated with aneuploidy during MI, with subsequent reduced fertility in females [11, 51]. Interestingly, aging of mouse oocytes leads to reduction in YBX2, an essential regulator of maternal mRNA stability in the oocyte [52]. Moreover, postovulatory aging of in vivo and in vitro Met II eggs leads to deadenylation and shortening of polyadenylation tails of many transcripts, resulting in their precocious degradation [53, 54]. Because we identified maternal RNA as an important regulator of AURKC function, and because AURKC is an essential regulator for faithful chromosome segregation during MI [7, 10], it is tempting to ask whether the age-associated aneuploidy of mammalian oocytes could be, at least in part, due to RNA degradation-mediated AURKC perturbation. Therefore, investigating whether AURKC has a direct role in age-related aneuploidy will be a step to better understand the phenomenon of maternal age effect on female reproduction.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Information contains Supplemental Experimental Procedures, three figures, two movies and one table are available online.

Supplemental Figure S1. RNase A efficiently depletes maternal RNA in mouse oocytes. (A) Relative abundance of *GAPDH* transcripts in RNase A-injected prophase I-arrested oocytes. Full-grown prophase I-arrested oocytes were injected with PBS or RNase A followed by incubation in CZB medium containing milrinone for 4 h. (B) Relative abundance of *GAPDH* transcripts in RNase A-injected Met I oocytes. Met I oocytes (6 h) were injected with PBS or RNase A followed by incubation in the same maturation condition for an additional 2 h. Levels of mRNA were measured by quantitative RT-PCR. Data were normalized against exogenously added Gfp message and expressed relative to that in control oocytes. The experiments were carried out two times and 40 oocytes were sampled in each group. The data are expressed as mean ± SEM, and Student *t*-test was used to analyze the data. Values with asterisks vary significantly, $^{**}P < 0.01$, $^{***}P < 0.001$.

Supplemental Figure S2. CPC perturbation precedes the chromosomal collapse phenotype in RNA-depleted oocytes. Full-grown, prophase I-arrested oocytes were injected with PBS or RNase A followed by in vitro maturation. Injected oocytes at different time points (6, 8, and 12 h) of meiotic maturation were fixed and immunostained with an anti-H3pS10 antibody (green in merge). DNA was detected with DAPI (blue in merge). The scale bar represents 10 μm, and representative images are shown. The experiments were carried out two times, and the total number of oocytes examined were 59 and 64 oocytes in the control and RNase A groups, respectively.

Supplemental Figure S3. RNase A treatment does not cause cytotoxicity. (A) Full-grown prophase I-arrested oocytes were matured in vitro for 14 h (Met II). Met II oocytes were injected with PBS or RNase A followed by incubation in the same maturation condition for an additional 2 h. Met II oocytes were fixed and assessed for early stages of apoptosis using TUNEL assay (green in merge). DNA was detected with DAPI (blue in merge). The experiments were carried out two times and the total number of oocytes examined were 19 and 14 oocytes in the control and RNase A groups, respectively. (B) Full-grown prophase I-arrested oocytes were microinjected with PBS or RNase A followed by incubation in the same maturation condition for an additional hour. After the incubation, oocytes were matured for 16–18 h, treated with propidium iodide (PI) and Hoescht for 30 min, and imaged to detect the uptake of the nuclear stains. The experiments were carried out two times, and the total number of oocytes examined were 48 and 34 oocytes in the control and RNase A groups, respectively. Trans, transmitted light. The scale bars represent 50 μm. Representative images from each experiment are shown.

Supplemental Movie S1. Time-lapse microscopic analysis of a living oocyte injected with PBS. Imaging of meiotic maturation began at prometaphase I. Time in hours and minutes (h: min) is shown. Acquisitions were taken every 20 min. Scale bar represents 100 μm.

Supplemental Movie S2. Time-lapse microscopic analysis of a living oocyte injected with 50 μg/μl RNase A. Imaging of meiotic maturation began at prometaphase I. Time in hours and minutes (h: min) is shown. Acquisitions were taken every 20 min. Scale bar represents 100 μm.

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Competing interests

The authors declare no competing interests.

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