

Dual-mode regulation of the APC/C by CDK1 and MAPK controls meiosis I progression and fidelity

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Female meiosis is driven by the activities of two major kinases, cyclin-dependent kinase 1 (Cdk1) and mitogen-activated protein kinase (MAPK). To date, the role of MAPK in control of meiosis is thought to be restricted to maintaining metaphase II arrest through stabilizing Cdk1 activity. In this paper, we find that MAPK and Cdk1 play compensatory roles to suppress the anaphase-promoting complex/cyclosome (APC/C) activity early in prometaphase, thereby allowing accumulation of APC/C substrates essential for meiosis I. Furthermore,

inhibition of MAPK around the onset of APC/C activity at the transition from meiosis I to meiosis II led to accelerated completion of meiosis I and an increase in aneuploidy at metaphase II. These effects appear to be mediated via a Cdk1/MAPK-dependent stabilization of the spindle assembly checkpoint, which when inhibited leads to increased APC/C activity. These findings demonstrate new roles for MAPK in the regulation of meiosis in mammalian oocytes.

Introduction

Meiosis in mammalian oocytes is controlled by changes in the activity of Cdk1–cyclin B and MAPK. Oocytes remain arrested in a gap phase 2 (G2)/prophase-like state for much of their existence and are only stimulated to progress into metaphase of meiosis I (metaphase I) by an increase in Cdk1–cyclin B activity (Hashimoto and Kishimoto, 1988; Conti et al., 2012). Continued progression through meiosis I is driven by a slow rise in Cdk1–cyclin B activity that results in a protracted prometaphase I: 6–7 h compared with tens of minutes in somatic cells (Polanski et al., 1998; Ledan et al., 2001; Jones, 2008; Davydenko et al., 2013). On reaching metaphase I, a transient decrease in Cdk1–cyclin B activity leads to exit from metaphase I and extrusion of the first polar body (PB1). Oocytes then proceed directly to metaphase of the second meiotic division (metaphase II) at which MAPK activity and Emi2 (early mitotic inhibitor 2) cooperate to inhibit the anaphase-promoting complex/cyclosome (APC/C), thereby stabilizing Cdk1 activity (Tung et al., 2005; Shoji et al., 2006; Inoue et al., 2007; Nishiyama et al., 2007; Wu et al., 2007).

The APC/C is an E3 ubiquitin ligase that targets key regulatory proteins, such as securin and cyclin B, for degradation by the 26S proteasome via polyubiquitinating them (Peters, 2006; Pines, 2011; Homer, 2013). The APC/C-mediated destruction of securin and cyclin B activates separase and inhibits Cdk1–cyclin B, respectively, thereby providing a mechanism of coordinating chromosome segregation and cell cycle exit (Clute and Pines, 1999; Zur and Brandeis, 2001; Peters, 2002; Herbert et al., 2003; Terret et al., 2003; Madgwick et al., 2004). The timely activation of the APC/C is controlled via direct binding of two coactivators, Cdc20 (cell division cycle 20) and Cdh1 (Cdc20 homologue 1; Visintin et al., 1997). The APC/C is also subject to other regulatory mechanisms, such as binding to the components of spindle assembly checkpoint (SAC), which inhibits the APC/C until the establishment of stable bipolar microtubule attachments (Homer et al., 2005a; Musacchio and Salmon, 2007; McGuinness et al., 2009; Hoffmann et al., 2011).

In mitosis, phosphorylation of key elements, such as the APC/C subunits, its coactivators, and the SAC proteins, plays key roles in regulation of the APC/C (Hershko et al., 1994; Kotani

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Abbreviations used in this paper: APC/C, anaphase-promoting complex/cyclosome; CREST, calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia; GV, germinal vesicle; GVBD, GV breakdown; IBMX, 3-isobutyl-1-methylxanthine; SAC, spindle assembly checkpoint.

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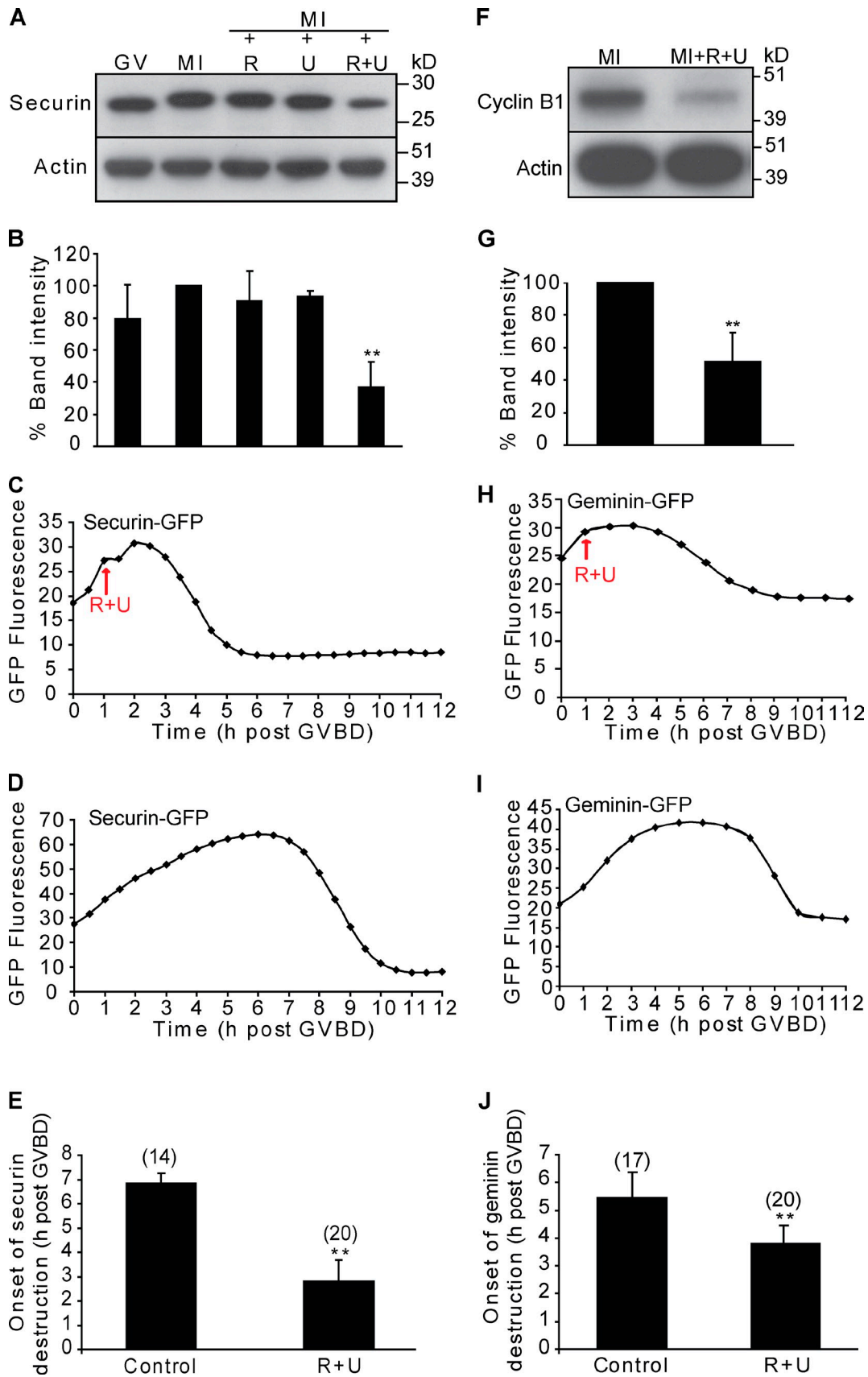


Figure 1. **Inhibition of CDK1 and MAPK in prometaphase I induces premature destruction of APC/C substrates.** (A and B) Western blot (20 oocytes/lane; A) and analysis of securin (B) in oocytes during GV arrest, meiosis I (MI), and meiosis I after treatment with roscovitine (R), UO126 (U), or both roscovitine and UO126 (R + U). (C and D) Representative fluorescence traces of oocytes injected with securin-GFP in the presence (C) or absence (D) of roscovitine

et al., 1998; Zachariae et al., 1998; Jaspersen et al., 1999; Chung and Chen, 2003; D'Angiolella et al., 2003; Kraft et al., 2003; Wassmann et al., 2003a; Zhao and Chen, 2006; Kim et al., 2010). In mammalian meiosis, however, this remains largely unexplored. Here, we investigate the role of Cdk1 and MAPK on APC/C activity and uncover new roles for Cdk1/MAPK in regulation of meiotic progression.

Results and discussion

The roles of Cdk1 and MAPK in regulation of APC/C activity in prometaphase I were investigated by applying specific inhibitors during a 2-h window, 3–5 h after release from prophase I arrest. Inhibition of the kinases was performed using small molecule inhibitors, roscovitine and UO126, which have been used widely in many cell types, including oocytes (Meijer et al., 1997; Favata et al., 1998; Phillips et al., 2002; Gorr et al., 2006; Yu et al., 2007; Nabti et al., 2008). Western blots performed on these oocytes demonstrate that inhibition of Cdk1 or MAPK during this window did not impact on securin levels, suggesting that APC/C activity was not affected. In contrast, inhibition of Cdk1 and MAPK caused an ~60% decrease in securin level (Fig. 1, A and B). To verify this role of Cdk1 and MAPK, we used alternative approaches to inhibit the kinases (Fig. S1). Cdk1 activity was inhibited with flavopiridol (Potapova et al., 2006; Holt et al., 2012) and MAPK by depletion of Mos, an oocyte-specific MAPK kinase, through injection of a Mos morpholino (Coonrod et al., 2001). We confirmed in positive control experiments that flavopiridol inhibits germinal vesicle (GV) breakdown (GVBD), and Mos morpholino-treated oocytes progress through metaphase II (Fig. S1, A and B). Western analysis of securin levels in flavopiridol and Mos morpholino-treated oocytes revealed a similar decrease in protein levels as seen with roscovitine and UO126 (Fig. S1, C and D). Together, these findings provide strong evidence that Cdk1 or MAPK activity is essential for securin accumulation during prometaphase I.

Next, we used time-lapse microscopy of oocytes injected with securin-GFP cRNA to examine whether the loss of securin was caused by premature destruction. In oocytes treated with roscovitine and UO126, securin-GFP destruction is initiated ~3 h after GVBD compared with 7 h in untreated control oocytes (Fig. 1, C–E). We then examined whether two other APC/C substrates, cyclin B1 and geminin (McGarry and Kirschner, 1998; Herbert et al., 2003), also undergo premature destruction on Cdk1 and MAPK inhibition. Western analysis of cyclin B1 in roscovitine- and UO126-treated oocytes showed a similar premature decrease in protein levels as seen with securin (Fig. 1, F and G). Similarly, time-lapse imaging of inhibitor-treated oocytes expressing geminin-GFP showed destruction starting ~3 h after GVBD

compared with 8 h in control oocytes, a pattern very similar to that seen with securin (Fig. 1, H–J). These data demonstrate that Cdk1/MAPK activity is essential for suppression of APC/C activity early in prometaphase I, which is essential to allow the accumulation of cyclin B1 to levels that are sufficient to achieve a metaphase state (Polanski et al., 1998; Gavet and Pines, 2010).

To prove that the APC/C is the site of Cdk1/MAPK action, we set out to test whether phosphorylation was the mode of regulation and whether the 26S proteasome was necessary for increased destruction. Western analysis of roscovitine- and UO126-treated oocytes that were co-incubated with the phosphatase inhibitor okadaic acid or the proteasome inhibitor MG132 shows a complete reversal of the premature securin destruction (Fig. 2, A and B). To provide a genetic evidence that securin destruction was mediated by the APC/C, we examined securin levels in roscovitine- and UO126-treated oocytes lacking APC/C activity as a result of conditional deletion of the *Apc2* gene (McGuinness et al., 2009). The experiments revealed that inhibition of MAPK and Cdk1 in *Apc2*-deleted (*Apc2*^{-/-}) oocytes was without effect on securin stability during prometaphase I (Fig. 2, C and D). This confirms that the APC/C is responsible for the observed decrease in securin when M-phase kinases are inhibited.

Next, we set out to determine how phosphorylation by Cdk1/MAPK suppresses APC/C activity during prometaphase I. Given the major role of the SAC in suppressing APC/C activity, we focused on a role for phosphorylation in suppressing APC/C via spindle checkpoint regulation (Schwab et al., 2001; Wassmann et al., 2003a; Zhao and Chen, 2006; Kim et al., 2010; Morin et al., 2012). As a readout of the SAC status, we examined the localization of the checkpoint protein Mad2, which binds to unattached kinetochores and is released upon stable microtubule–kinetochore attachments (Waters et al., 1998; Wassmann et al., 2003b; Homer et al., 2009). The effect of inhibition of Cdk1 and MAPK on Mad2 localization was examined using the same treatment regimen that destabilizes APC/C substrates. At 5 h after release, bipolar spindles are forming, and as previously reported (Homer et al., 2009), strong Mad2 staining is seen at virtually every kinetochore (99%; Fig. 3, A–C). After inhibition of Cdk1 or MAPK, Mad2 was present on 70% of kinetochores (Fig. 3, A and B), whereas inhibition of both Cdk1 and MAPK resulted in Mad2 localization on only 5% of kinetochores, including those that lacked microtubule attachments (Fig. 3, A–C). This strongly links Cdk1/MAPK to SAC function in this prometaphase period.

A strong candidate of the SAC that may be a substrate of Cdk1/MAPK activity is Mps1 (monopolar spindle 1). Recent work in *Xenopus laevis* egg extracts has shown phosphorylation by Cdk1 or MAPK is important for kinetochore localization of the SAC proteins, including Mad2 (Zhao and Chen, 2006; Morin et al., 2012). Also, mouse oocytes depleted of Mps1 show premature

and UO126. In C, the inhibitors were added at 1 h after GVBD (red arrow). (E) Time of onset of securin-GFP destruction in the treated (roscovitine + UO126; *n* = 20) and nontreated oocytes (control; *n* = 14). The peak of the trace was taken as a marker for the start of destruction. (F and G) Western blot (30 oocytes/lane; F) and analysis of cyclin B (G) in oocytes at meiosis I in the presence or absence of roscovitine and UO126. (H and I) Representative fluorescence traces of oocytes injected with geminin-GFP in the presence (H) or absence (I) of roscovitine and UO126, as in C and D. (J) Time of onset of geminin-GFP destruction in the inhibitor-treated (roscovitine + UO126; *n* = 20) and nontreated oocytes (control; *n* = 17 from two experiments); data were analyzed as in E. In B and G, results are compared with the control meiosis I lane, which is set at 100%. Error bars show SDs. **, *P* < 0.01.

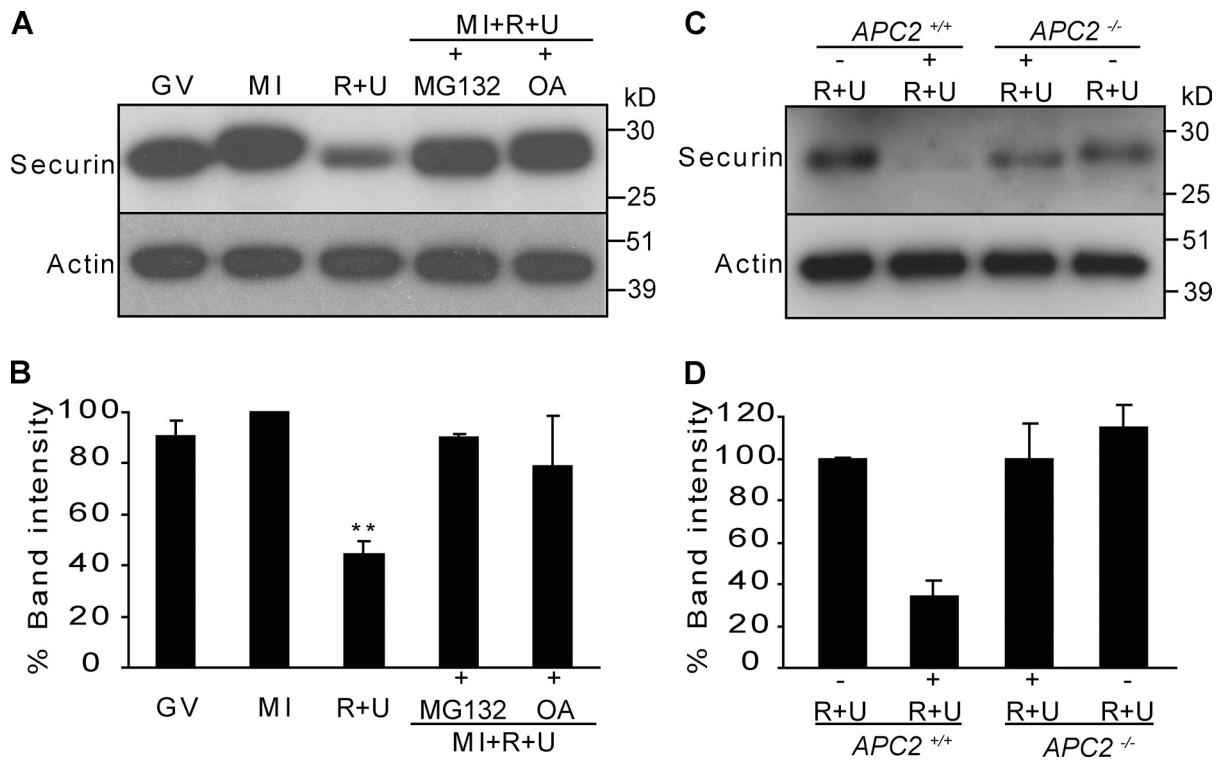


Figure 2. **Proteasome, phosphatase inhibitor, and APC/C deletions restore securin levels.** (A and B) Western blot (20 oocytes/lane; A) and analysis of securin (B) in oocytes during GV arrest, meiosis I (MI), and meiosis I after incubation with roscovitine (R), UO126 (U), MG132, or okadaic acid (OA). Results are compared with the meiosis I lane, which is set at 100%. (C and D) Western blot (20 oocytes/lane, $n = 2$; C) and analysis of securin (D) in *Apc2*-deleted (*Apc2*^{-/-}) and control (*Apc2*^{+/+}) oocytes at meiosis I in the presence or absence of roscovitine and UO126. Error bars show SDs. **, $P < 0.01$.

activation of APC/C (Hached et al., 2011). Here, we have asked whether inhibition of Mps1 using the well-characterized inhibitor AZ3146 (Hewitt et al., 2010), during the same prometaphase window, phenocopies the effects of inhibition of Cdk1 and MAPK. The data clearly show that inhibiting Mps1 causes loss of kinetochore-localized Mad2 (Fig. 3 D) and also phenocopies the effect of inhibiting Cdk1 and MAPK in causing a similar premature destruction of securin (Fig. 3, E and F). This study strongly implicates Mps1 as the downstream target for Cdk1/MAPK-mediated suppression of the APC/C.

The redundancy of Cdk1 and MAPK in regulating APC/C activity early in prometaphase I raises the question as to whether MAPK plays a role in restraining APC/C activity during the meiosis I to meiosis II (MI–MII) transition when Cdk1 activity is declining. We therefore designed experiments to target this window of APC/C activity during meiosis I. To this end, oocytes were treated with UO126 at 7 h after release, close to the time APC/C activity increases, and the MI–MII transition is initiated in the majority of oocytes (Fig. 1 D). Securin levels were analyzed over the next 3 h to examine whether MAPK inhibition modulates APC/C activity. Western blots show that in control oocytes only 10% of endogenous securin is degraded at 8 h compared with nearly 50% in UO126-treated oocytes (Fig. 4, A and B). This difference is maintained at 9 h when ~20% and just over 80% of securin is degraded in control and UO126-treated oocytes, respectively. By 10 h, securin destruction has reached a maximum and is similar in both groups (Fig. 4, A and B). Time-lapse imaging of securin-GFP in oocytes treated at 7 h with UO126

show that the faster destruction observed in Western blots appears to be caused by an earlier onset of securin destruction rather than an increased rate of destruction (Fig. 4, C and D). This early onset of APC/C activity is consistent with inhibition of MAPK causing early satisfaction of the SAC. Subsequent to our findings in early prometaphase, we have inhibited Mps1 over the 7–10-h time window of the MI–MII transition and found that it causes premature securin destruction, similar to the effects of inhibiting MAPK (Fig. 4 E).

To further implicate MAPK-dependent regulation of the SAC in the timing of meiosis, we tested whether inhibition of MAPK from 7 h leads to premature loss of Mad2 from kinetochores and acceleration of polar body extrusion. In control oocytes, Mad2 is lost from kinetochores over the 7–9-h treatment window, whereas in UO126-treated oocytes, Mad2 loss from kinetochores is significantly accelerated by 8 h (Fig. 4 F). Consistent with this premature satisfaction of the SAC, analysis of the timing of PB1 extrusion reveals that PB1 extrusion was consistently faster in UO126-treated oocytes (Fig. 4 G). Thus, inhibition of MAPK from 7 h after release causes premature securin destruction, accelerated loss of kinetochore-associated Mad2, and faster progression through meiosis I. These results suggest that MAPK, acting via the SAC, restrains APC/C activity and therefore refines the timing of meiosis I.

Accelerated progression through meiosis I in mouse oocytes has been associated with chromosome segregation errors during anaphase I, leading to aneuploid metaphase II–arrested eggs (Homer et al., 2005b; Reis et al., 2007). Therefore, we next

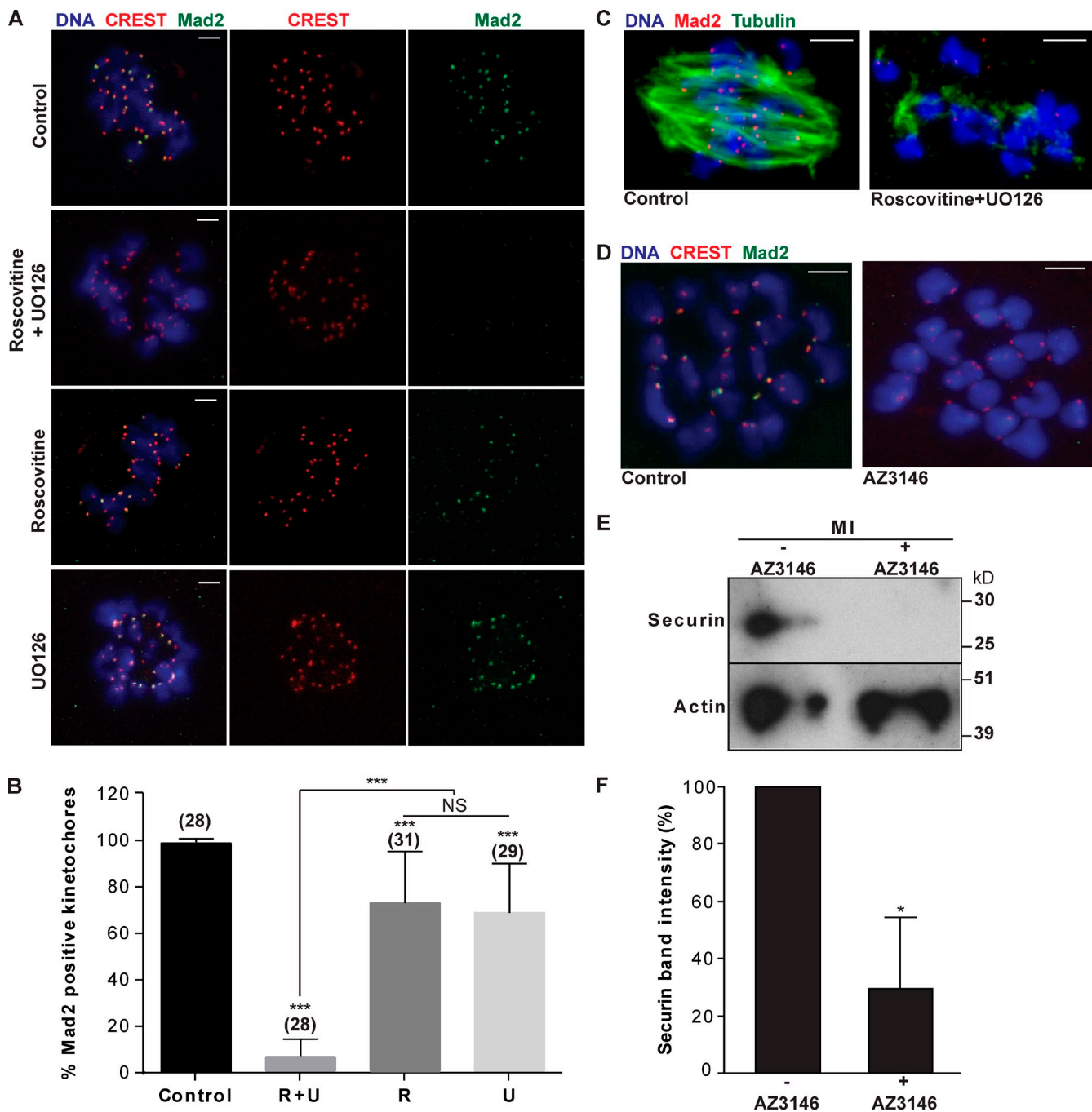


Figure 3. Inhibition of CDK1 and MAPK induces Mad2 dissociation from kinetochores. (A) Representative images of immunostaining for DNA, CREST, and Mad2 in nontreated control oocytes and oocytes treated with roscovitine, UO126, or both roscovitine and UO126. (B) Proportion of Mad2-positive kinetochores in control ($n = 28$), both roscovitine- and UO126 (R + U; $n = 28$), roscovitine (R; $n = 31$), and UO126 (U; $n = 29$)-treated oocytes. (C) Representative images of meiotic spindles from control nontreated meiosis I oocytes ($n = 10$) and oocytes treated with roscovitine and UO126 ($n = 20$). (D) Representative images of control nontreated meiosis I oocytes ($n = 10$) and oocytes treated with the Mps1 inhibitor AZ3146 ($n = 10$) immunostained for DNA, CREST, and Mad2. (E and F) Western blot (20 oocytes/lane; E) and analysis of securin (F) in oocytes at meiosis I in the presence or absence of AZ3146. In B and F, results were compared with the nontreated control or to each other where indicated and expressed as SEMs. NS, $P > 0.05$; *, $P < 0.05$; ***, $P < 0.001$. Bars, 5 μ m.

examined whether inhibition of MAPK at 7 h leads to an increase in aneuploidy in oocytes after completion of meiotic maturation. The majority of controls had a normal chromosome count of 20 monovalents, with only 7% being hyperploid. In contrast, in the UO126-treated oocytes, hyperploidy was observed in 16% of the oocytes, whereas 7% were hypoploid, leading to a total of 23% being aneuploid. Thus, chromosome missegregation had

occurred in nearly a quarter of the UO126-treated oocytes, a three- to fourfold increase over controls (Fig. 5, A–D). Furthermore, we observed that in 69% of the UO126-treated aneuploidy oocytes, there were single chromatids, a sign of premature sister separation. This was also observed in 9.4% of the UO126-treated euploid oocytes (Fig. 5 E), suggesting that inhibition of MAPK resulted in a reduced cohesion level. Subsequent measurement of

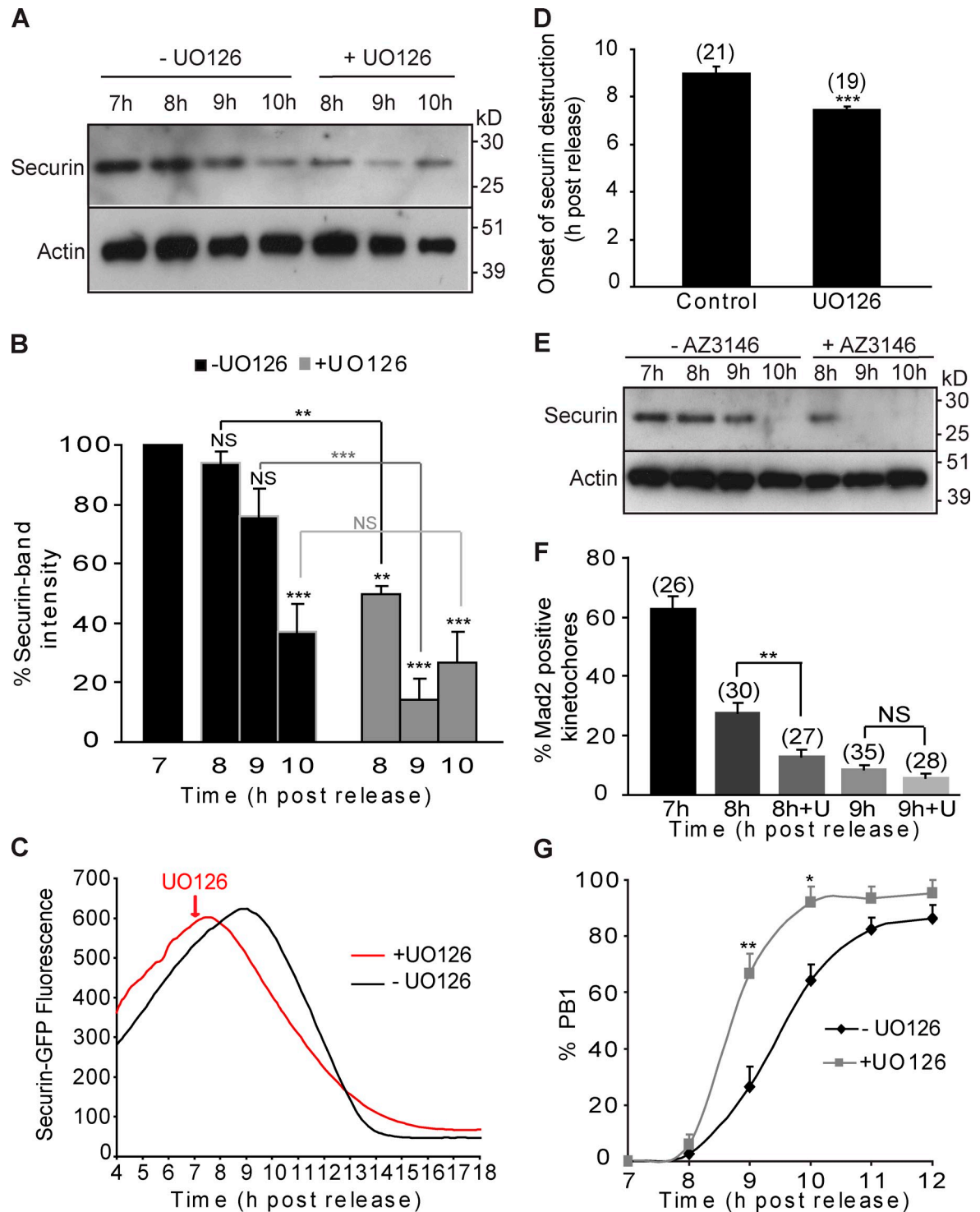


Figure 4. **Inhibition of MAPK in late prometaphase I induces early destruction of securin, faster Mad2 dissociation, and accelerated PB1 extrusion.** (A and B) Western blot (20 oocytes/lane; A) and analysis of securin (B) in oocytes at 7, 8, 9 and 10 h after release from IBMX, in the presence or absence of UO126. (C) Representative fluorescence traces of oocytes injected with securin-GFP in the presence or absence of UO126. (D) Time of onset of securin-GFP destruction in the UO126-treated oocytes ($n = 19$ from two experiments) and nontreated/control oocytes ($n = 21$ from two experiments). The peak of the graph was taken as a marker for the start of destruction. (E) Western blot (20 oocytes/lane, $n = 2$) of securin in oocytes at 7, 8, 9, and 10 h after release from IBMX, in the presence or absence of AZ3146. (F) The proportion of Mad2-positive kinetochores at 7, 8, and 9 h after release from IBMX, in the presence or absence of UO126 (U). (G) Timing of PB1 extrusion in control ($n = 116$) and UO126-treated oocytes ($n = 112$). In B, D, F, and G, results were compared with the nontreated controls or to each other where indicated and expressed as SEMs. UO126 and AZ3146 were added to the media at 7 h after release from IBMX. NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

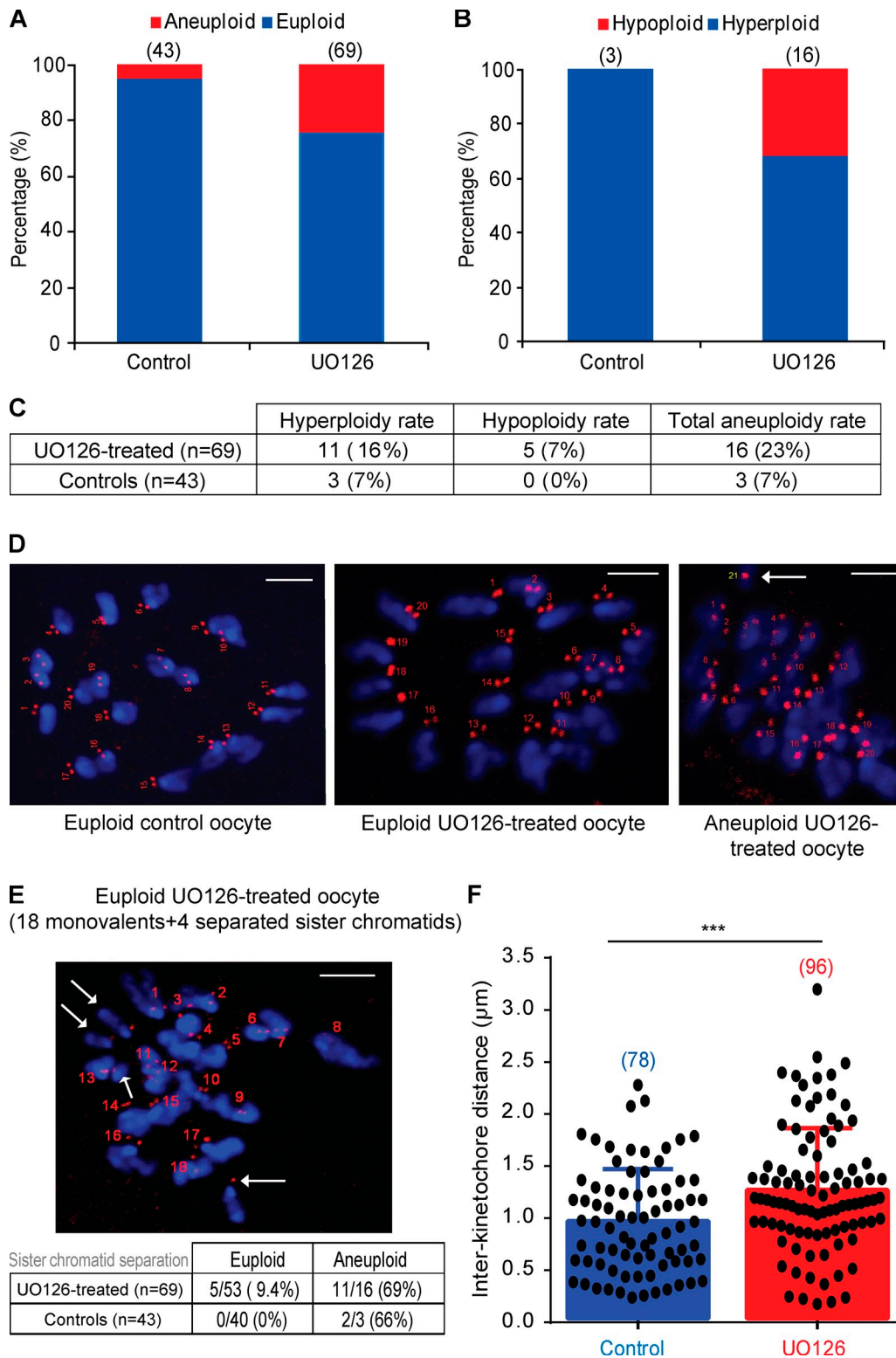


Figure 5. Inhibition of MAPK in late prometaphase I causes higher rates of aneuploidy and premature sister chromatid separation in metaphase II-arrested eggs. (A) Chromosome count in metaphase II-arrested eggs, at 14 h after release from IBMX, in the presence (UO126, $n = 69$) or absence (control, $n = 43$) of UO126. UO126 was added to the media at 7 h after release. (B) Rate of hypoploidy and hyperploidy within the aneuploid UO126-treated eggs ($n = 16$) and the aneuploid nontreated controls ($n = 3$). (C) Table summarizing the rate of hyperploidy, hypoploidy, and total aneuploidy in UO126-treated ($n = 69$) and control ($n = 43$) eggs. (D) Representative examples of the chromosome counting assay demonstrating a euploid control egg (20 monovalents, numbered in red), a euploid UO126-treated egg (20 monovalents), and an aneuploid UO126-treated egg (20 monovalents + one single chromatid pointed out with an arrow). DNA is shown in blue, and CREST-labeled kinetochores are shown in red. (E) Representative example of a euploid UO126-treated egg featuring premature sister chromatid separation (18 monovalents + four single chromatids pointed out with arrows) and a table summarizing the rates of premature sister separation within UO126-treated ($n = 69$) and control eggs ($n = 43$). (F) The mean distance between sister kinetochores in UO126-treated eggs ($n = 96$ pairs of kinetochores from 17 eggs) and controls ($n = 78$ pairs of kinetochores from 15 eggs). The results are mean + SEMs. ***, $P < 0.001$. Bars, 5 μm .

the interkinetochore distance revealed a significantly increased separation of kinetochores in UO126-treated oocytes (mean distance of 1.23 μm) compared with nontreated controls (mean distance of 0.93 μm ; Fig. 5 F). On the basis of these findings, we propose that MAPK activity during the MI–MII transition is required for normal segregation and can regulate chromosome cohesion. The increased rate of aneuploidy is probably caused by overriding the SAC, leading to a more rapid activation of the APC/C as well as a loss in SAC-mediated control over the fidelity of the first meiotic division. The increased occurrence of sister separation and increased interkinetochore distance may also be a result of premature degradation of securin, leading to aberrant separase activity (Yamamoto et al., 1996; Ciosk et al., 1998; Nabti et al., 2008). Alternatively, an effect of MAPK on the localization of I2PP2A, an endogenous inhibitor of PP2A (protein phosphatase 2A), cannot be discounted. I2PP2A was shown to colocalize with PP2A at metaphase II, during which it counteracts PP2A-mediated protection of centromeric cohesion (Chambon et al., 2013). Analysis of the mouse I2PP2A sequence has identified a consensus motif (serine 30) for MAPK phosphorylation, thereby providing a possible mechanism linking MAPK to cohesion.

Conclusions

Cdk1 activity drives oocytes through meiosis I, whereas MAPK is best known for promoting arrest at metaphase II. Here, we show that Cdk1 and MAPK act in a complementary manner during prometaphase I to restrain APC/C activity and thereby drive the accumulation of APC/C substrates, including cyclin B1, which is required for completion of meiosis I (Polanski et al., 1998; Ledan et al., 2001; Davydenko et al., 2013). The mechanism, as revealed by a loss of kinetochore-associated Mad2 and a phenocopy of the effects by inhibition of Mps1, appears to be mediated via an effect on the ability to establish or stabilize the SAC. Therefore, our data provide a new feedback loop whereby Cdk1/MAPK activity promotes stabilization of cyclin B1, which is then available to further drive an increase in Cdk1 activity.

A second role uncovered for MAPK is during the MI–MII transition when persistent MAPK activity maintains the SAC as shown by its ability to modulate the timing of securin destruction, the localization of Mad2 at kinetochores, and the timing of progression through the MI–MII transition. Modulation of the timing of meiosis I by this MAPK-mediated maintenance of the SAC ensures a faithful segregation of chromosomes at meiosis I. These observations, together with the finding that Mps1 inhibition phenocopies premature APC/C activation, are consistent with a model in which MAPK-dependent phosphorylation of Mps1 maintains the SAC and controls the timing and fidelity of meiosis I. Finally, our data suggest an additional role for MAPK in the protection of centromeric cohesion, thereby preventing premature sister separation. This study may lead to new insights into the mechanisms of aneuploidy in human oocytes in which an increase in premature sister separation is a common feature (Angell, 1991, 1997; Nagaoka et al., 2012).

Materials and methods

Oocytes collection and culture

GV oocytes were collected from 21–24-d-old MF1 mice (Harlan). The females were superovulated by intraperitoneal injection of 7.5 IU pregnant mare's serum gonadotropin (Intervet). Mice were killed by cervical dislocation at 46–48 h after pregnant mare's serum gonadotropin injection. The ovaries were removed and immediately transferred to dissection medium, consisting of M2 medium supplemented with 200 μM 3-isobutyl-1-methylxanthine (IBMX), to keep the oocytes arrested at the GV stage. The cumulus-enclosed oocytes were isolated by mechanical perforation of the ovaries with a 27-gauge needle. The cumulus cells were removed by repeated mouth pipetting using narrow-bore glass Pasteur pipettes. For longer term incubation, the oocytes were cultured in M16 in a 5% CO_2 humidified incubator at 37°C.

Treatments

For treatments, oocytes were incubated in culture media (M2 or M16) containing the following drugs: 50 μM roscovitine (EMD Millipore), 50 μM UO126 (Promega), 50 μM MG132 (EMD Millipore), 1 μM okadaic acid (EMD Millipore), or 2 μM AZ3146 (Santa Cruz Biotechnology, Inc.). Because roscovitine is oil soluble, no mineral oil was added to the media, and the culture dish was covered with a lid to prevent evaporation. All drug treatments were performed between 3 and 5 h after release from IBMX, unless otherwise stated.

Microinjection and imaging

All microinjections of GV stage oocytes were performed in M2 medium on the stage of an inverted microscope (DM IRB; Leica). In brief, fabricated micropipettes were inserted into cells using the negative capacitance overcompensation facility on an electrophysiological amplifier (World Precision Instruments), whereas cells were immobilized with a holding pipette (Hunter Scientific). A precise injection volume (2–5% of the total egg volume) was achieved using a pressure regulator (Pneumatic PicoPump; World Precision Instruments). Epifluorescence images of oocytes incubated in M2 medium at 37°C were recorded using an objective (20 \times , 0.75 NA) on an inverted microscope (Axiovert 100; Carl Zeiss) equipped with an interline cooled charge-coupled device camera (Princeton Instruments MicroMAX; Roper Scientific). GFP-tagged securin and geminin were imaged using an FITC filter set at band pass 450–490 nm for excitation, dichroic mirror 510 nm, and band pass 520 nm for emission. Oocytes were imaged every 10 min to minimize photobleaching and photodamage. MetaMorph and MetaFluor software 6.1 (Molecular Devices) were used for image capture and data analysis.

cRNA

The cRNA for GFP-tagged securin and GFP-tagged geminin were prepared from the T3 promoter of a pRN3-GFP vector, using a transcription kit (T3 mMACHINE; Ambion). The cRNA was then polyadenylated and purified in nuclease-free water to a concentration of $\sim 1 \mu\text{g}/\mu\text{l}$ before microinjection.

Western blotting

Meiosis I oocytes, at 5 h after release from IBMX, unless otherwise stated, were washed in PBS with 1% polyvinylpyrrolidone solution and then heated at 95°C for 5 min with 5 \times sample buffer. Proteins were fractionated at 200 mV for 50 min on an X Cell II Blot Module (Invitrogen) using a 4–12% NuPAGE Bis-Tris precast gel (Invitrogen) and MOPS running buffer. Proteins were blotted onto polyvinylidene fluoride membranes for 1.5 h at 100 mV. Mouse anti-securin (ab3305; 1:1,000; Abcam), mouse anti-cyclin B1 (ab72; 1:400; Abcam), and mouse anti- β -actin (ab3280; 1:400; Abcam) were used for Western blotting. For primary antibody detection, we used a goat HRP-conjugated anti-mouse secondary antibody (Sigma-Aldrich). Standard ECL techniques (GE Healthcare) were used for secondary antibody detection according to the manufacturer's instructions. The densitometric analysis of the blots involved the measurement of the intensity of each band using Photoshop CS2 (Adobe), which was then normalized against the relevant actin loading control.

Immunofluorescence

Meiosis I oocytes, at 5 h after release from IBMX, unless otherwise stated, were fixed and permeabilized in PHEM buffer (60 mM Pipes, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl_2) containing 4% paraformaldehyde and 0.5% Triton X-100 and then labeled with calceinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia (CREST) serum, a human centromere antiserum, (1:300; a gift from G. Fitzharris,

University College London, London, England, UK), rabbit anti-Mad2 (PRB-452C; 1:300; Covance), mouse anti- β -tubulin (T4026; 1:1,000; Sigma-Aldrich), and 10 μ g/ml Hoechst 33342 (Sigma-Aldrich). Serial z sections of fixed oocytes in PBS were acquired at room temperature using a Plan ApoChromat 63 \times , 1.4 NA oil differential interference contrast objective and a laser-scanning confocal microscope imaging system (LSM 510 META; Carl Zeiss) with the following band pass emission filters in nm 385–470 (Hoechst 33342), 505–530 (Alexa Fluor 488), and 585–615 (Alexa Fluor 546). Z sections were analyzed and projected into one picture using the LSM image browser (Carl Zeiss). The images were then assembled by Illustrator CS2 (Adobe).

Chromosome counts

Chromosome counts were performed as previously described (Duncan et al., 2009; Illingworth et al., 2010). In brief, spindles were collapsed using a 90-min pulse of 200 μ M monastrol (EMD Millipore). Oocytes were fixed and permeabilized in PHEM buffer, labeled with CREST serum and Hoechst, and mounted on slides in PBS. Serial z sections were acquired at 0.5- μ m intervals at room temperature using a Plan ApoChromat 63 \times , 1.4 NA oil differential interference contrast objective and a laser-scanning confocal microscope imaging system (LSM 510 META) with the following band pass emission filters (nm): 385–470 (Hoechst 33342) and 585–615 (Alexa Fluor 546). Z sections were analyzed and projected into one picture using the LSM image browser. The images were then assembled by Illustrator CS2. All chromosome counts were scored by two independent people. To measure the interkinetochore distance, we used ImageJ software (National Institutes of Health). In brief, a linescan was drawn across each pair of CREST-labeled sister kinetochores. The interkinetochore distance was then measured as the distance between the two peaks of fluorescence, which represent the center of each kinetochore. However, when the sister kinetochores were not in the same plane of focus, we used the Pythagorean theorem to calculate the actual interkinetochore distance.

Statistics and data analysis

Data analysis was performed using appropriate statistical tests in Prism software (GraphPad Software). Analysis was performed for at least three replicate experiments and represented as means and SDs, unless otherwise stated. The level of significance is indicated in the figures and figure legends by NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Online supplemental material

Fig. S1 shows that flavopiridol and Mos morpholino cause a similar decrease in securin levels as seen with roscovitine and UO126 in early prometaphase I. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201305049/DC1>.

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