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Report

The PP2A Inhibitor I2PP2A Is Essential for Sister Chromatid Segregation in Oocyte Meiosis II

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Summary

Haploid gametes are generated through two consecutive meiotic divisions, with the segregation of chromosome pairs in meiosis I and sister chromatids in meiosis II. Separase-mediated stepwise removal of cohesion, first from chromosome arms and later from the centromere region, is a prerequisite for maintaining sister chromatids together until their separation in meiosis II [1]. In all model organisms, centromeric cohesin is protected from separase-dependent removal in meiosis I through the activity of PP2A-B56 phosphatase, which is recruited to centromeres by shugoshin/MEI-S332 (Sgo) [2–5]. How this protection of centromeric cohesin is removed in meiosis II is not entirely clear; we find that all the PP2A subunits remain colocalized with the cohesin subunit Rec8 at the centromere of metaphase II chromosomes. Here, we show that sister chromatid separation in oocytes depends on a PP2A inhibitor, namely I2PP2A. I2PP2A colocalizes with the PP2A enzyme at centromeres at metaphase II, independently of bipolar attachment. When I2PP2A is depleted, sister chromatids fail to segregate during meiosis II. Our findings demonstrate that in oocytes I2PP2A is essential for faithful sister chromatid segregation by mediating deprotection of centromeric cohesin in meiosis II.

Results and Discussion

PP2A is a heterotrimer, consisting of a scaffold subunit (A), a regulatory subunit (B), and a catalytic subunit (C). In mammals there are two catalytic subunits, two scaffold subunits, and several isoforms of regulatory subunits (at least 13 regulatory subunits exist in humans), giving a large array of possible heterotrimers [6]. Phosphatase activity can be detected already upon assembly of only the A and C subunits, and this so-named Core-PP2A enzyme was shown to exist in vivo [7]. The PP2A-B56 heterotrimer is thought to confer protection of centromeric cohesin in mitosis by preventing cohesin removal by the prophase pathway before metaphase-to-anaphase transition [3]. In mammalian meiosis I, PP2A-C is present in a Sgo2-dependent manner at centromeres [5], but it is still unknown whether all three subunits of the PP2A-B56 holoenzyme colocalize at the metaphase I centromere. According to the current model, Sgo2 is removed from its localization at centromeres due to the bipolar tension applied on sister kinetochores, in late metaphase II. It was proposed that, as a consequence of Sgo2 delocalization, PP2A is moved sufficiently far away from Rec8 to allow Rec8's phosphorylation at the metaphase II-to-anaphase II transition and subsequent cleavage by separase [5, 8, 9]. Thus, the differences in attachment and tension applied on metaphase I or II kinetochores generated by microtubule-pulling forces have been proposed as the mechanism leading to loss of centromeric cohesin protection. However, in *S. cerevisiae* monopolin mutants and in *S. pombe* mutants undergoing achiasmatic meiosis I, sister chromatids biorient and come under tension during meiosis I, yet centromeric cohesion is maintained until meiosis II [10–13]. Furthermore, artificially targeting Sgo1 and PP2A to meiosis II centromeres in fission yeast does not hinder sister chromatid segregation [14]. These results and localization of the PP2A-C subunit to the centromere also in metaphase II oocytes [5] prompted us to further examine the localization of all three PP2A subunits and, therefore, potentially functional PP2A in oocytes in order to get insights into the mechanisms underlying centromeric cohesin deprotection.

To determine whether and when all three PP2A subunits colocalize with the meiotic cohesin subunit Rec8, we performed mouse oocyte metaphase I (germinal vesicle breakdown [GVBD] + 6–7 hr) and metaphase II (GVBD + 14–16 hr) chromosome spreads. Metaphase II spreads were performed at a time when oocytes have extruded the first polar body (PB) and are competent to undergo metaphase II-to-anaphase II transition upon activation ("late metaphase II"). Immunostaining of endogenous proteins shows that PP2A-A, PP2A-B56, and PP2A-C colocalize with CREST in both meiosis I and II, and all three subunits are also detected between the two centromere dots in meiosis II (Figures 1A and 1B). The expected PP2A-A and PP2A-C colococalization in metaphase I and II is shown in Figures 1C and 1D. Next, we analyzed localization of PP2A relative to Rec8 in meiosis I and II. Colocalization of PP2A-C with Rec8 in meiosis I was confirmed (Figure 1E). Crucially, in meiosis II, PP2A-C was found at the centromere exactly where centromeric Rec8 is localized (Figure 1F). Sgo2 localization in meiosis I and II was identical to PP2A localization relative to CREST staining (see Figures S1A and S1B available online). It is important to note that our spreading technique allows us to maintain the tension applied on sister kinetochores in meiosis II, because sister kinetochore dots are still clearly separated in a microtubule-dependent manner (Figure S1C). Therefore, our localization data are not in accordance with a model where PP2A is removed from Rec8 in metaphase II due to the bipolar tension applied on sister kinetochores.

Presence of the PP2A holoenzyme between sister chromatid arms in meiosis II prompted us to ask whether some unknown mechanism prevents dephosphorylation of Rec8 to allow sister chromatid segregation in meiosis II. To identify candidates required for PP2A inhibition in meiosis II, we

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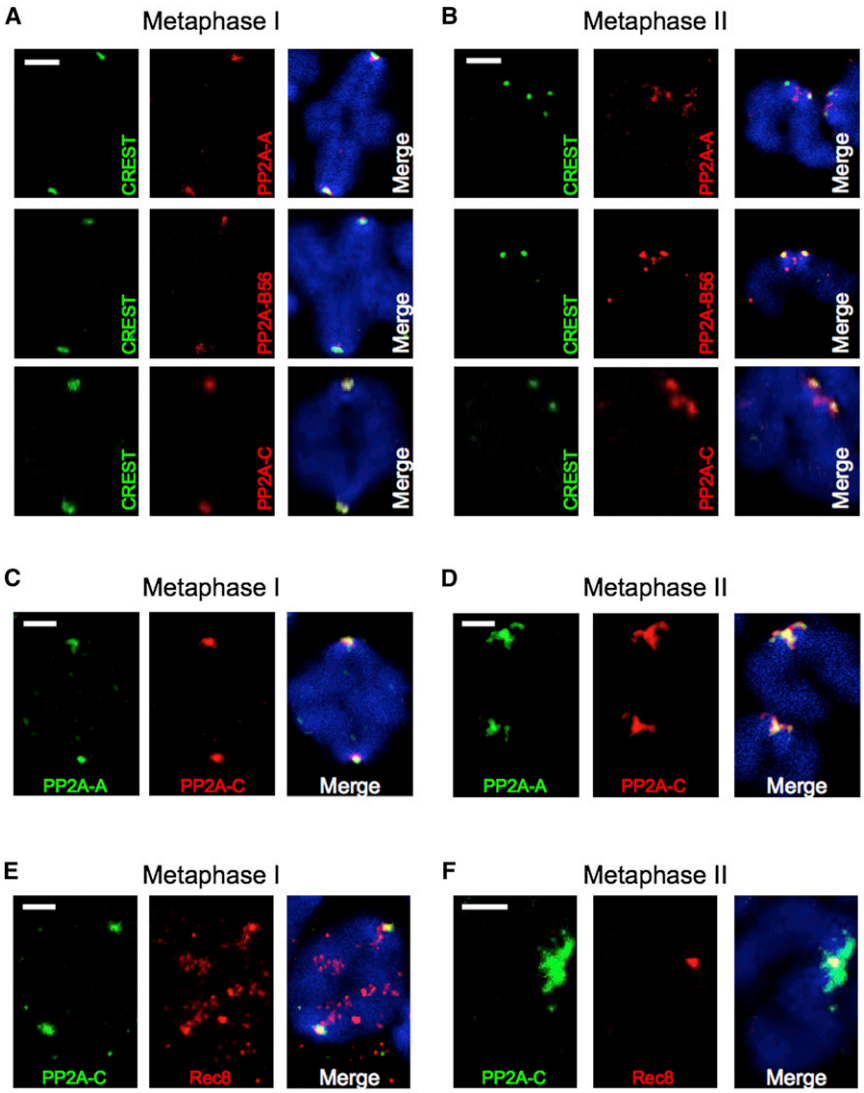

Figure 1. All Three PP2A Subunits Localize to the Centromere in Metaphase I and II Where Centromeric Rec8 Is Found

(A and B) Approximately 30 metaphase plates were analyzed for each staining. Acquisitions were obtained by confocal microscopy. The pseudocolors attributed to each staining are indicated. Chromosomes were stained with Hoechst (blue). Scale bars represent 2 μm. Metaphase I (A) and metaphase II (B) spreads were stained with antibodies against the different PP2A subunits (red), CREST (green), and Hoechst (DNA, blue).

(C and D) Metaphase I (C) and metaphase II (D) spreads were stained with PP2A-A (green) and PP2A-C (red) antibodies.

(E and F) Metaphase I (E) and metaphase (F) II spreads were stained with PP2A-C (green) and Rec8 (red) antibodies.

(See Figure S1.)

switched to ascidian oocytes. In the ascidian species used in this study, *Ciona intestinalis* (suborder of phlebobranchs), only one Sgo ortholog is present: Sgo1 [15]. The huge number of oocytes that can be obtained per animal allowed the construction of a *C. intestinalis* oocyte library (Proquest, Invitrogen) to perform a yeast two-hybrid screen, using Ci-Sgo1 as bait. We identified Ci-I2PP2A, a homolog to a previously identified mammalian PP2A inhibitor [16], which is also a subunit of the INHAT complex (inhibitor of acetyltransferases [17], also termed SET, PHAP-II, or TAF-1β; see [18–20] and references therein), as an interaction partner of Ci-Sgo1 (Table S1). Ci-I2PP2A is expressed in oocytes during meiotic maturation (Figure S1D) and has the capacity to inhibit PP2A in vitro (Figure S1E), as has been shown for its mammalian homolog, which interacts with and inhibits the catalytic subunit of PP2A [16]. This led us to hypothesize that I2PP2A inhibits PP2A to allow sister chromatid separation in oocyte meiosis II. Interestingly, I2PP2A was detected in hSgo1 immunoprecipitations [3] and was very recently shown to be part of a PP2A-B56 network, together with hSgo1 in human mitotic cells [21], suggesting that I2PP2A may play a role as a PP2A inhibitor in mitosis to protect centromeric cohesin from removal by the prophase pathway.

I2PP2A is found in diverse multicellular eukaryotes (Figure S1F), and its amino acid sequence is well conserved between ascidians and mouse (78% homology and 55% identity, data not shown). We performed western blots from mouse oocyte extracts and found that I2PP2A is expressed in meiosis I and II in mouse oocytes (Figure S1G). To get insight into I2PP2A's potential role in mammalian female meiosis, we analyzed I2PP2A's localization in mouse oocytes relative to the localization of the centromere marker CREST and the different PP2A subunits. I2PP2A was localized in the centromere region in meiosis I and II, but it was only in meiosis II that the I2PP2A signal overlapped with the entire CREST signal and was also found between the two centromeres (Figures S2A and S2B). I2PP2A staining is shifted away from the different PP2A subunits in meiosis I, but it colocalizes with the three PP2A subunits in meiosis II (Figures 2A–2D; Figures S2C and S2D). The different localization patterns of PP2A and I2PP2A in metaphase I were confirmed by super-resolution structured illumination microscopy (SIM) (Figure S2E). Triple labeling of I2PP2A with PP2A-A and PP2A-C subunits further shows that the core PP2A enzyme does not overlap with I2PP2A in meiosis I but does overlap in meiosis II (Figures S2F–S2H). I2PP2A colocalization relative to the PP2A subunits, as well as PP2A-A and PP2A-C colocalization (not shown), was quantified and shows that a substantial fraction of PP2A is not colocalizing with I2PP2A in meiosis I and is therefore free to dephosphorylate Rec8 (Figure S2I).

Now the key question was to determine where I2PP2A is localized relative to Rec8. I2PP2A does not colocalize with Rec8 in meiosis I, but it does so in meiosis II (Figures 2E and 2F; quantitation in Figure S2I). Our localization data are therefore in agreement with a role for I2PP2A in mediating deprotection in meiosis II.

Does the difference in I2PP2A staining between meiosis I and II depend on bipolar or monopolar configuration of kinetochores and, in particular, the differences in tension exerted

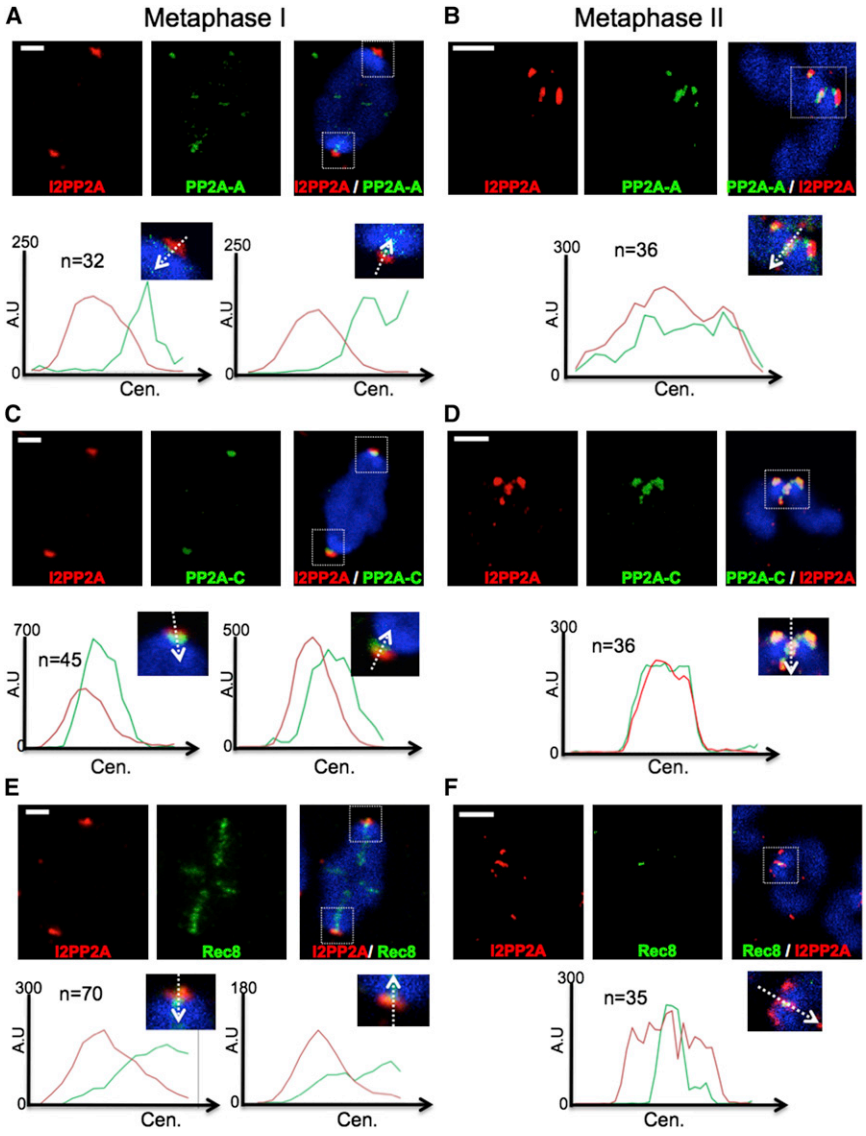

Figure 2. I2PP2A Colocalizes with PP2A-A, PP2A-C, and Centromeric Rec8 in Metaphase II

Approximately 30 metaphase plates were analyzed for each staining. Acquisitions were obtained by confocal microscopy. The pseudo-colors attributed to each staining are indicated. Chromosomes were stained with Hoechst (blue). Scale bars represent 2 μm. Representative line scans for the stainings of the corresponding images are shown. n indicates the number of centromere stainings analyzed by line scans from multiple metaphase plates.

(A and B) Metaphase I (A) and metaphase II (B) spreads were stained with antibodies against I2PP2A (red) and PP2A-A (green).

(C and D) Metaphase I (C) and metaphase (D) II spreads were stained with antibodies against I2PP2A (red) and PP2A-C (green).

(E and F) Metaphase I (E) and metaphase II (F) spreads were stained with antibodies against I2PP2A (red) and Rec8 (green).

(See Figure S2.)

on sister kinetochores in meiosis I and II? To answer this question, we examined I2PP2A localization on bivalent chromosomes that are attached in a monopolar fashion in meiosis II. For this, mouse oocytes were injected with mRNAs coding for nondegradable securin (Δ91-securin), which inhibits separase [22] but still allows oocytes to progress into meiosis II without chromosome segregation, as shown by securin-YFP degradation and reaccumulation (Figure 3A), similarly to oocytes devoid of separase [23]. Monopolar attachment of bivalent chromosomes was shown by CREST staining on chromosome spreads (Figure 3B, CREST signals of sister kinetochores appear as one dot) and spindle staining of cold-stable microtubules in whole oocytes (Figure 3C). Even though bivalents are attached in a monopolar fashion, I2PP2A relocalized as in meiosis II, and it now colocalized with PP2A-C (which did not change its localization) (Figure 3D). This relocalization at the centromere was also observed when CREST and I2PP2A double labeling was performed (Figure 3E). Our result shows that the relocalization of I2PP2A at the centromere in metaphase II occurs independently of the tension applied through bipolar attachment in meiosis II and is regulated through meiotic cell-cycle progression.

If I2PP2A were required for sister separation in meiosis II, loss of endogenous I2PP2A was expected to abrogate sister chromatid separation. We performed morpholino-mediated knockdown experiments to address whether sister chromatids are separated without I2PP2A. Germinal vesicle (GV) stage mouse oocytes were injected with morpholino or control oligos and incubated for up to 24 hr. Oocytes were induced to undergo meiotic maturation until metaphase II. Knockdown efficiencies were determined by whole oocyte immunofluorescence (Figure 4A). Chromosome spreads showed that only dyads were present in metaphase II (Figure 4B). Metaphase II oocytes were artificially activated to induce metaphase-to-anaphase transition of meiosis II. Control oocytes harbored only single CREST dots on separated sister chromatids in the oocyte. Importantly, 100% of spreads that were negative for I2PP2A staining (31 out of 88) showed the presence of paired CREST dots (Figure 4C) and, therefore, paired sister chromatids. The percentage of sister chromatids that remained paired varied between individual knockdown oocytes. Spreads that remained positive for I2PP2A staining did not show paired CREST dots. The presence of separated sisters only in oocytes that were activated indicates that metaphase-to-anaphase transition of meiosis II took place. Furthermore, the formation of pronuclei and decondensation of chromatin indicative of meiosis II exit was observed in control and knockdown oocytes (Figure 4D). Importantly, I2PP2A knockdown to analyze meiosis I, with morpholino oligo (MO) incubation times comparable to the ones used to analyze meiosis II, did not affect meiotic maturation (data not shown) and had no effect on chromosome segregation in meiosis I or on spindle formation (Figures S3A and S3B).

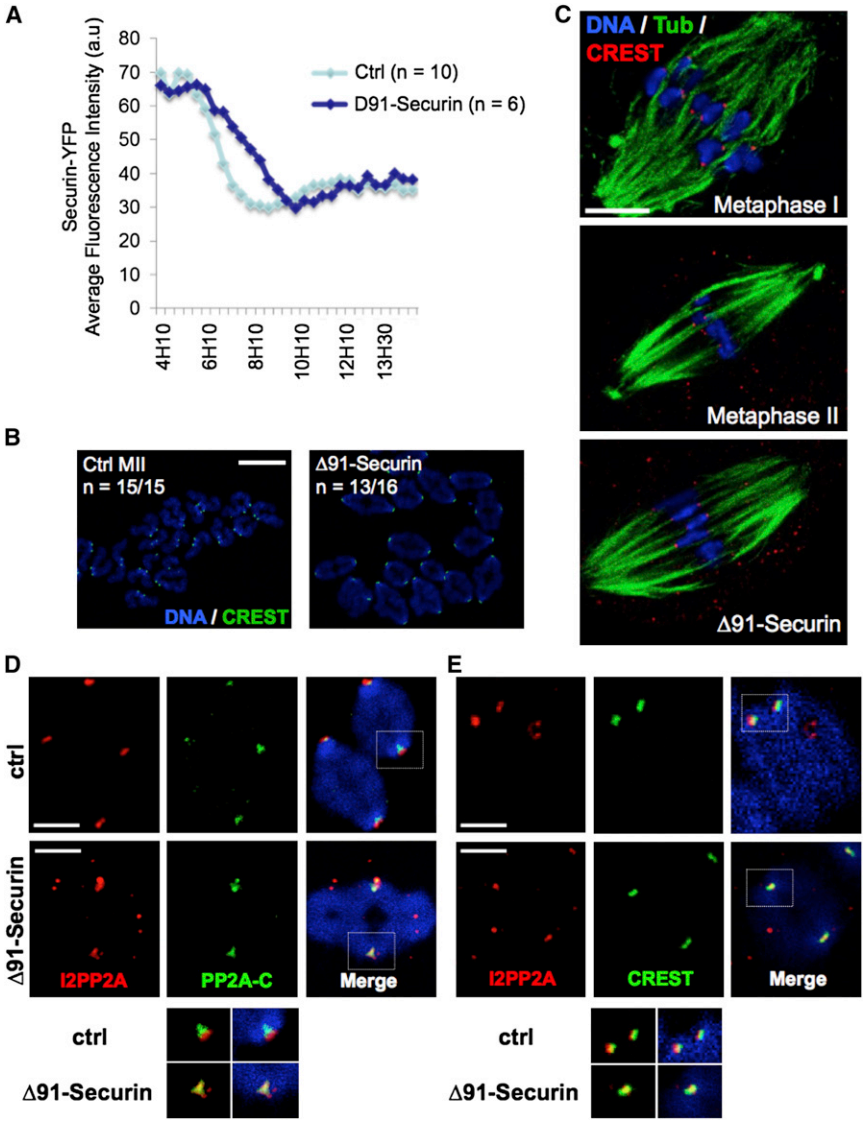

Figure 3. I2PP2A Changes Its Localization from Metaphase I to Metaphase II at the Centromere Independently of Chromosome Structure and Tension Exerted on Sister Kinetochores

(A) A representative graph of YFP-securin fluorescence in a Δ91-securin-expressing oocyte, showing that Δ91-securin does not block cell-cycle progression.

(B) Chromosome spread of a metaphase II (control) and a metaphase II oocyte expressing Δ91-securin, stained with CREST (green) and showing chromosomes stained with Hoechst (blue). Scale bar represents 10 μm.

(C) Examples of whole-mount immunofluorescence images of a metaphase I, a metaphase II, and a metaphase II oocyte expressing Δ91-securin. Cold-stable microtubules were stained with anti-β-tubulin antibody (green), kinetochores with CREST (red), and chromosomes with Hoechst (blue). One z section of 75 sections of 0.13 μm (confocal microscope) is shown. Scale bar represents 35 μm.

(D and E) Metaphase I spreads (control) and metaphase II spreads of oocytes expressing Δ91-securin where indicated, stained with I2PP2A antibody (red) and counterstained (in green) with either PP2A-C antibody or CREST antiserum. I2PP2A is localized exactly where centromeric PP2A-C (D) and CREST (E) is found on bivalent chromosomes in MII after Δ91-securin expression. Controls: metaphase I bivalents. Insets show the shift observed on bivalent chromosomes in metaphase I control oocytes, with equivalent bivalents at metaphase II. Scale bar represents 4 μm.

Our data strongly suggest that I2PP2A locally inhibits PP2A and, therefore, allows Rec8 phosphorylation. We hypothesize that, in mouse oocytes, changes in I2PP2A's localization relative to Rec8 and PP2A and posttranslational modifications, such as phosphorylation of I2PP2A or PP2A itself, ensure the inhibitor's activity toward PP2A only in meiosis II. Indeed, human I2PP2A has to be phosphorylated on two serine residues in βAR signaling to bind and inhibit PP2A [24]. Future work will aim at identifying the signaling pathways regulating I2PP2A's localization and activity toward PP2A in a meiosis II-specific manner. It remains to be determined whether the recently identified role of cyclin A2 for sister chromatid segregation requires I2PP2A [25].

The relocalization of I2PP2A is regulated by cell-cycle progression, independently of chromosome structure (bivalent or dyad) or attachment to microtubules. In contrast, it has been shown that Sgo2's localization depends on bipolar versus monopolar attachment [5, 9]. It has been assumed that PP2A would therefore be displaced from Rec8 in meiosis II as well. Intriguingly, in a recent study, Sgo1/2-independent localization of PP2A-B56 to the mitotic centromere has been described, indicating that Sgo1/2 localization does not necessarily reflect PP2A localization under certain conditions, at least in mitosis [26]. We show here that PP2A colocalizes with Rec8 in meiosis II. We propose that I2PP2A locally inhibits PP2A exactly where centromeric cohesin is found in metaphase II, though we do not exclude an additional mechanism of physical removal of PP2A at anaphase II onset [5, 9], which is additionally required for complete PP2A inhibition toward Rec8. The localization of I2PP2A itself is regulated in a cell-cycle-dependent manner, independent of mono- or bipolar attachment. Our findings that I2PP2A is essential for sister chromatid segregation in meiosis II adds a new layer of complexity to the regulation of PP2A. Our results allow us to propose a novel regulatory mechanism of centromeric PP2A inhibition by I2PP2A that is controlled in space and time in oocyte meiosis.

Female meiosis is error-prone in humans—it is estimated that 20% of oocytes are aneuploid, and errors in meiotic chromosome segregation account for one-third of all miscarriages [27]. Tight regulation of cohesin removal is a prerequisite for correct chromosome segregation in oocytes, and weakening of cohesion with age has been associated with meiotic missegregations in mice [28–30]. Precocious loss of centromeric cohesin protection will also cause the generation of aneuploid oocytes; therefore, the mechanisms underlying cohesin protection and deprotection have to be elucidated to understand why female meiosis goes awry so frequently.

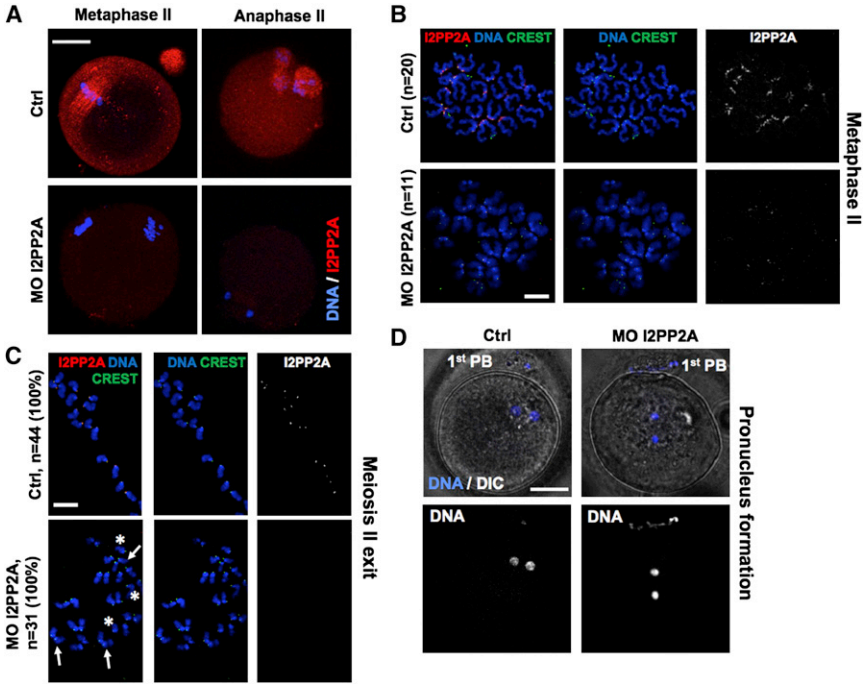

Figure 4. Endogenous I2PP2A Is Required for Faithful Chromosome Segregation in Mouse Oocytes

(A) Morpholino-mediated knockdown of I2PP2A in mouse oocytes was confirmed by immunofluorescence (I2PP2A in red, chromosomes in blue) in MII and AII oocytes. Scale bar represents 25 μm.

(B) Chromosome spreads of control or MO-injected oocytes that have extruded the first PB, before activation at GVBD + 16 hr. Chromosomes were stained with CREST antibody (green), Hoechst (blue), and I2PP2A antibody (red) to control for knockdown efficiencies in individual oocytes. No single sisters can be detected in control or MO-injected oocytes. Scale bar represents 10 μm.

(C) Chromosome spreads of activated control or MO-injected mouse oocytes that underwent anaphase II as judged by the presence of single sister chromatids. Spreads were stained as in (B). Scale bar represents 8 μm. Arrows indicate examples of sisters that have not been separated; asterisks indicate examples of single sister chromatids.

(D) Pronucleus formation after activation of control or MO-injected oocytes. Extrusion of the second PB was prevented through cytochalasin B treatment for better visualization of the two decondensed chromosome masses in the oocyte. Live images of oocytes incubated with Hoechst (blue) are shown. Scale bar represents 25 μm.

(See Figure S3.)

Experimental Procedures

Mouse Oocyte Collection and Microinjection

GV oocytes were obtained as described [31] from adult female mice. MI (meiosis I) oocytes were obtained around 6–7 hr, and MII (meiosis II) oocytes 14–16 hr, after entry into meiosis. mRNAs for injections were transcribed using the mMessage mMachine T3 Kit (Ambion), according to the manufacturer's protocol, and injected into GV oocytes as described previously for exogenous protein expression [31]. For Δ91-securin expression, GV stage oocytes were injected with Δ91-securin mRNA [25] and released to enter meiosis I after 2–3 hr incubation. Oocytes were used for metaphase II spreads or fixation 14–16 hr after GVBD. Movies to quantify securin-YFP were done as described in [31]. MetaMorph software was used to quantify fluorescence signals in individual oocytes, which were subtracted from background fluorescence. Parthenogenetic activations and anaphase II spreads were done as described in [25, 32]. Extrusion of a second PB was observed after 45 min, at which time oocytes were spread. For pronucleus formation, oocytes were activated in the presence of 5 mg/ml cytochalasin B (which prevents extrusion of the second PB) and 2 ng/ml bisbenzimide H33342 (Hoechst 33342) and were imaged after 3 hr incubation. Morpholino oligos (5′ ACAGAGCCCAGAGCCCTGATGTTCA 3′ against I2PP2A, Gene Tools) and control morpholino oligos (unrelated sequence, Gene Tools) were injected at a concentration of 300 nM into GV oocytes, which were incubated for up to 24 hr before being induced to enter meiosis, unless otherwise indicated. To control for knockdown efficiencies, spreads were stained with anti-I2PP2A antibody (see Supplemental Experimental Procedures), and acquisitions by spinning-disk confocal microscopy were performed with the same settings for control and knockdown oocytes. Images to assess knockdown efficiencies were not adjusted for brightness or contrast.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.02.004.

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