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CORRECTION

Correction: CIP2A acts as a scaffold for CEP192-mediated microtubule organizing center assembly by recruiting Plk1 and aurora A during meiotic maturation (doi:10.1242/dev.158584)

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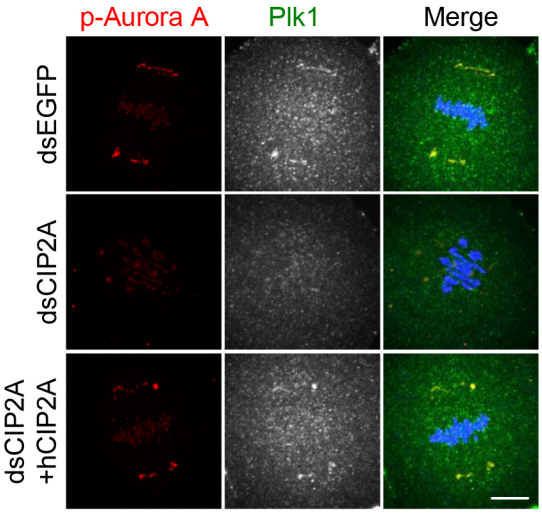
There was an error published in *Development* (2017) **144**, 3829-3839 (doi:10.1242/dev.158584).

In Fig. 4E, the incorrect Plk1 image was displayed for dsCIP2A+hCIP2A (it was a duplicate of the dsEGFP image). The corrected Fig. 4E appears below.

This error does not affect the conclusions of the paper. The authors apologise to readers for this mistake.



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RESEARCH ARTICLE

CIP2A acts as a scaffold for CEP192-mediated microtubule organizing center assembly by recruiting Plk1 and aurora A during meiotic maturation

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ABSTRACT

In somatic cells spindle microtubules are nucleated from centrosomes that act as major microtubule organizing centers (MTOCs), whereas oocytes form meiotic spindles by assembling multiple acentriolar MTOCs without canonical centrosomes. Aurora A and Plk1 are required for these events, but the underlying mechanisms remain largely unknown. Here we show that CIP2A regulates MTOC organization by recruiting aurora A and Plk1 at spindle poles during meiotic maturation. CIP2A colocalized with pericentrin at spindle poles with a few distinct cytoplasmic foci. Although CIP2A has been identified as an endogenous inhibitor of protein phosphatase 2A (PP2A), overexpression of CIP2A had no effect on meiotic maturation. Depletion of CIP2A perturbed normal spindle organization and chromosome alignment by impairing MTOC organization. Importantly, CIP2A was reciprocally associated with CEP192, promoting recruitment of aurora A and Plk1 at MTOCs. CIP2A was phosphorylated by Plk1 at S904, which targets CIP2A to MTOCs and facilitates MTOC organization with CEP192. Our results suggest that CIP2A acts as a scaffold for CEP192-mediated MTOC assembly by recruiting Plk1 and aurora A during meiotic maturation in mouse oocytes.

KEY WORDS: Oocyte meiosis, CIP2A, CEP192, Plk1, Aurora kinase A, Microtubule organizing center

INTRODUCTION

Assembly of a bipolar spindle is essential to ensure accurate chromosome segregation and to prevent aneuploidy. Centrosomes, by organizing microtubules at the spindle poles in animal somatic cells, are crucial for this process. Aurora A and Plk1 are key kinases involved in the centrosome maturation and separation required for spindle assembly (Berdnik and Knoblich, 2002; Hannak et al., 2001; Lane and Nigg, 1996). However, these kinases are not directly targeted to the centrosomes. Instead, they form a complex with the centrosomal protein CEP192, which is then targeted to centrosomes. The accumulation of CEP192 complexes at centrosomes triggers the activation of aurora A, which in turn phosphorylates and activates Plk1. The active aurora A and/or Plk1 in CEP192 complexes can render γ-tubulin capable of microtubule nucleation, driving the formation of microtubule organizing centers (MTOCs) and subsequent spindle assembly (Joukov et al., 2010, 2014). Therefore, by scaffolding aurora A and Plk1 at the centrosome, CEP192 is likely to be a key protein for MTOC formation and spindle assembly.

In contrast to somatic cells, oocytes lack canonical centrosomes (Manandhar et al., 2005). Instead, spindle microtubules are assembled by multiple, discrete MTOCs around chromosomes (Schuh and Ellenberg, 2007). These MTOCs contain many of the pericentriolar material components, including γ-tubulin and pericentrin (PCNT), but lack centrioles (Carabatsos et al., 2000; Gueth-Hallonet et al., 1993; Palacios et al., 1993; Szollosi et al., 1972). During meiotic maturation, multiple acentriolar MTOCs are fragmented into a large number of small MTOCs, redistributed toward the spindle poles, and merged into two equal spindle poles (Clift and Schuh, 2015). Although aurora A and Plk1 have been suggested to be involved in these processes (Clift and Schuh, 2015; Ding et al., 2011; Solc et al., 2012, 2015), the molecular mechanisms underlying MTOC assembly at spindle poles during meiotic maturation are not well understood.

Cancerous inhibitor of PP2A (or cell proliferation regulating inhibitor of PP2A) (CIP2A) has been identified as an oncogene that inhibits endogenous protein phosphatase 2A (PP2A) activity and thereby activates multiple oncogenes, such as *c-Myc* and *Akt*, in several types of cancer cells (Come et al., 2009; Junttila et al., 2007; Khanna et al., 2009; Lucas et al., 2011; Ma et al., 2011; Teng et al., 2012). Inhibition of CIP2A reduces cell proliferation and tumor growth by enhancing the catalytic activity of PP2A in various types of cancer cells (Chen et al., 2011; Junttila et al., 2007; Khanna et al., 2013a; Lucas et al., 2011; Yu et al., 2014). However, accumulating evidence suggests that CIP2A has non-canonical functions that are unrelated to PP2A activity (Jeong et al., 2014; Kim et al., 2013; Niemelä et al., 2012; Peng et al., 2015). Indeed, recent studies show that depletion of CIP2A impairs mitotic progression, independent of PP2A activity (Jeong et al., 2014; Kim et al., 2013). However, the functions of CIP2A during cell cycle progression are not fully understood.

In this study we show that CIP2A is a novel MTOC component and is required for spindle assembly during meiotic maturation, independent of PP2A activity. We demonstrate that CIP2A is reciprocally associated with CEP192, promoting the recruitment of aurora A and Plk1 at MTOCs. Moreover, Plk1-mediated phosphorylation of CIP2A at the S904 residue targets CIP2A into MTOCs, facilitating MTOC organization with CEP192. Therefore, our results uncover a novel function of CIP2A in the regulation of MTOC and spindle assembly during oocyte meiotic maturation.

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RESULTS

CIP2A is a novel component of MTOCs

To investigate the function of CIP2A during meiotic maturation, we evaluated the expression and subcellular localization of CIP2A in mouse oocytes. Oocytes were fixed after 0, 4, 8 and 13 h of culture, corresponding to the germinal vesicle (GV), GV breakdown (GVBD), metaphase I (MI) and metaphase II (MII) stages, respectively, and stained with CIP2A antibody. CIP2A was mainly distributed in the cytoplasm, with several distinct foci around the nucleus during the GV stage. After GVBD, CIP2A was localized at multiple discrete foci around the condensing chromosomes. During MI and MII, CIP2A specifically localized at the spindle poles (Fig. 1A). Weak signals were also detected on the condensed chromosomes during the MI and MII stages. Moreover, CIP2A colocalized with PCNT and γ-tubulin, suggesting that CIP2A is localized to MTOCs during oocyte meiosis (Fig. 1B,C).

To clarify the correlation between CIP2A and MTOCs, oocytes were treated with taxol, a microtubule-stabilizing drug. After taxol treatment, the microtubules were excessively polymerized, and the spindles were enlarged with numerous asters in the cytoplasm. In this context, CIP2A specifically colocalized with PCNT at the spindle poles and cytoplasmic asters (Fig. 1D). Ectopic overexpression of human CIP2A-GFP also revealed that CIP2A colocalized with PCNT, corroborating that CIP2A is localized to MTOCs during oocyte meiosis (Fig. 1E). A similar colocalization of CIP2A with PCNT or PCM1 was also observed in somatic cells (Fig. S1). Therefore, these results not only indicate that CIP2A is a novel component of MTOCs, but also suggest that the functions of CIP2A are conserved in mitosis.

Overexpression of CIP2A does not override meiotic arrest

It has long been known that the phosphatase inhibitor okadaic acid (OA) induces meiotic resumption by inhibiting PP1 and/or PP2A activity (Alexandre et al., 1991; Maton et al., 2005; Picard et al., 1989). Considering that OA has a 100-fold greater potency toward PP2A compared with PP1, it is likely that PP2A is the main phosphatase responsible for regulating meiotic resumption in oocytes (Shi, 2009). Because CIP2A has been identified as an endogenous inhibitor of PP2A in multiple cancers (Junttila et al., 2007; Khanna et al., 2013b), we assumed that CIP2A would override meiotic arrest by inhibiting PP2A activity. However, overexpression of CIP2A did not induce meiotic resumption, whereas OA induced a significantly higher incidence of meiotic resumption (Fig. 2A,B). Once released from meiotic arrest, the oocytes overexpressing CIP2A typically underwent polar body extrusion (PBE) without any discernible defects in chromosomes or spindles (Fig. 2C,D). By contrast, oocytes treated with OA after GVBD failed to undergo PBE and showed impaired chromosome alignment and spindle organization (Fig. 2C,D). These results suggest that CIP2A is not associated with PP2A activity during meiotic maturation in mouse oocytes.

Depletion of CIP2A impairs chromosome alignment and spindle organization

To explore the role of CIP2A during meiotic maturation, CIP2A expression was downregulated using double-stranded RNA against *Cip2a* (dsCIP2A). EGFP double-stranded RNA (dsEGFP) was used as a control. The expression of CIP2A was significantly reduced by dsCIP2A injection (Fig. 3A,B). CIP2A knockdown oocytes underwent meiotic resumption with kinetics comparable to those

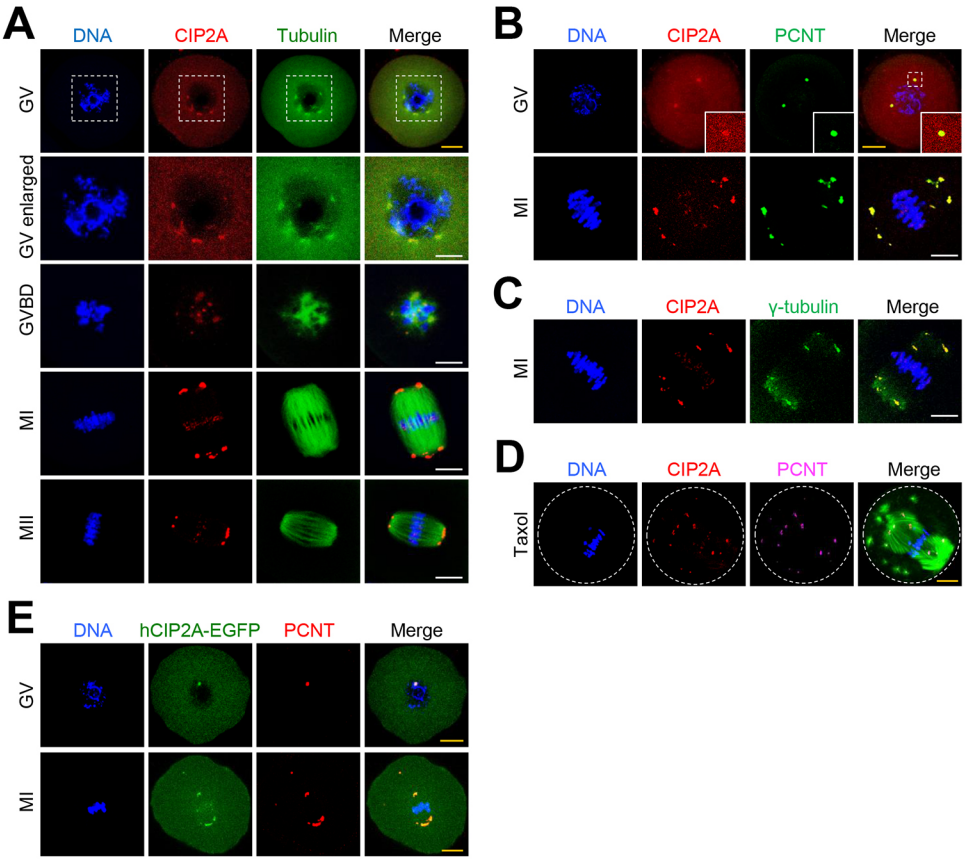

Fig. 1. CIP2A is a novel MTOC component in mouse oocytes. (A) Oocytes at the GV (0 h), GVBD (4 h), MI (8 h), and MII (13 h) stages were immunolabeled with anti-CIP2A antibody and counterstained with anti-α-tubulin-FITC and Hoechst to visualize spindles and DNA, respectively. (B,C) GV or MI oocytes were co-stained with anti-CIP2A and either anti-PCNT or anti-γ-tubulin antibodies. (D) Oocytes at the MI stage were incubated in M16 medium containing 10 µM taxol for 45 min and then stained with anti-CIP2A, anti-PCNT, anti-α-tubulin-FITC and Hoechst. (E) Oocytes injected with a low concentration of cRNA encoding human CIP2A-EGFP were fixed and double stained with anti-PCNT antibody and Hoechst. Scale bars: 20 µm (yellow); 10 µm (white).

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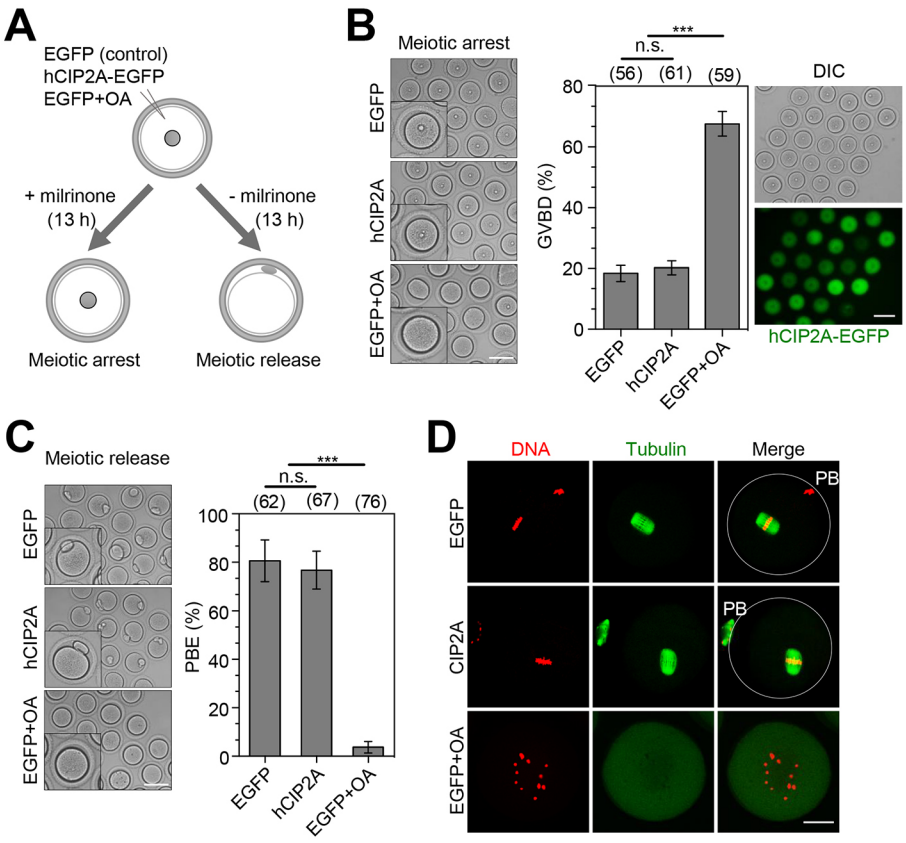

Fig. 2. Overexpression of CIP2A does not override meiotic arrest or polar body extrusion. (A) Experimental scheme for CIP2A overexpression. GV oocytes were injected with human (h) CIP2A-EGFP or EGFP (control) cRNA or were treated with okadaic acid (OA; 400 nM) after EGFP cRNA injection. Following microinjection, oocytes were cultured in M16 medium with (+) or without (–) 2.5 µM milrinone for 13 h for meiotic arrest or meiotic release, respectively. (B) The incidence of GVBD was scored after 13 h in the presence of milrinone. Representative DIC images and expression of hCIP2A-EGFP are shown. (C) The incidence of PBE was scored after 13 h in the absence of milrinone and is shown in representative images. (B,C) The number of oocytes analyzed is specified in parentheses. ***P<0.001; n.s., not significant. Error bars indicate s.e.m. (D) Oocytes were fixed and double stained with anti-α-tubulin-FITC and Hoechst. PB, polar body. Scale bars: 20 µm.

of control oocytes, supporting the idea that CIP2A does not act as a PP2A inhibitor during meiotic resumption in mouse oocytes (Fig. 3C). However, the PBE rate decreased significantly after CIP2A knockdown (Fig. 3D), suggesting that CIP2A does not have any essential role during meiotic resumption, but is required for meiotic maturation to the MII stage.

To further analyze the function of CIP2A during meiotic maturation, we examined chromosome and spindle morphology by confocal microscopy. Whereas a barrel-shaped bipolar spindle was normally formed in control oocytes, spindle assembly was impaired in CIP2A knockdown oocytes, displaying diverse abnormalities including small, nonpolar or multipolar spindles (Fig. 3E-H, Fig. S2). In addition, chromosomes were misaligned and scattered on the microtubules after CIP2A knockdown (Fig. 3E, H, Fig. S2). Moreover, kinetochore-microtubule attachments were partially impaired after CIP2A knockdown, thereby increasing the incidence of aneuploidy (Fig. S3). Notably, these spindle and chromosome defects were repaired and, consequently, the PBE rate was restored by overexpressing human CIP2A, excluding the possibility of off-target effects of CIP2A knockdown (Fig. 3E-I). These results suggest that CIP2A localized at MTOCs regulates chromosome alignment and spindle organization during meiotic maturation.

CIP2A is essential for MTOC organization

Given the evidence that CIP2A is localized at spindle poles during mitosis in somatic cells (Jeong et al., 2014; Kim et al., 2013), we hypothesized that depletion of CIP2A would perturb MTOC organization during meiotic maturation, thereby disturbing spindle organization and chromosome alignment. Indeed, the level of PCNT at the MTOCs was markedly reduced following CIP2A knockdown (Fig. 4A,B). Also, the number of MTOCs decreased dramatically in CIP2A knockdown oocytes (Fig. 4A,C). This phenotype was specific to CIP2A depletion, as the level of PCNT and MTOC number were restored when human CIP2A was overexpressed (Fig. 4A-C).

Because aurora A and Plk1 localized at the spindle poles play key roles in centrosome maturation and spindle assembly (Berdnik and Knoblich, 2002; Hannak et al., 2001; Lane and Nigg, 1996), we examined their levels in CIP2A knockdown oocytes. Similar to PCNT, the levels of aurora A, phospho-aurora A and Plk1 at MTOC were significantly reduced in CIP2A-depleted oocytes, and these levels were rescued by overexpressing human CIP2A (Fig. 4D-H). Consistent with our results, CIP2A has been shown to regulate Plk1 localization at spindle poles during mitosis (Kim et al., 2013). Therefore, these findings suggest that CIP2A is a key element that recruits MTOC components, including PCNT, aurora A and Plk1, during meiotic maturation.

CIP2A is associated with centrosomal protein CEP192

We next investigated how CIP2A regulates the recruitment of aurora A and Plk1 at MTOCs during meiotic maturation. Since CEP192 is known to be an essential centrosomal protein that is positioned at the top of the hierarchy of aurora A and Plk1 recruitment during centrosome maturation (Joukov et al., 2014), we investigated whether CIP2A regulates CEP192 during meiotic maturation. CIP2A and CEP192 colocalized at the spindle poles (Fig. 5A). Moreover, their localization at spindle poles was abolished and the number of MTOCs decreased when either CIP2A or CEP192 was depleted (Fig. 5A,B). The levels of CEP192 decreased following CIP2A knockdown, but were rescued by overexpression of human CIP2A (Fig. 5C,D). Notably, the CIP2A level decreased in CEP192

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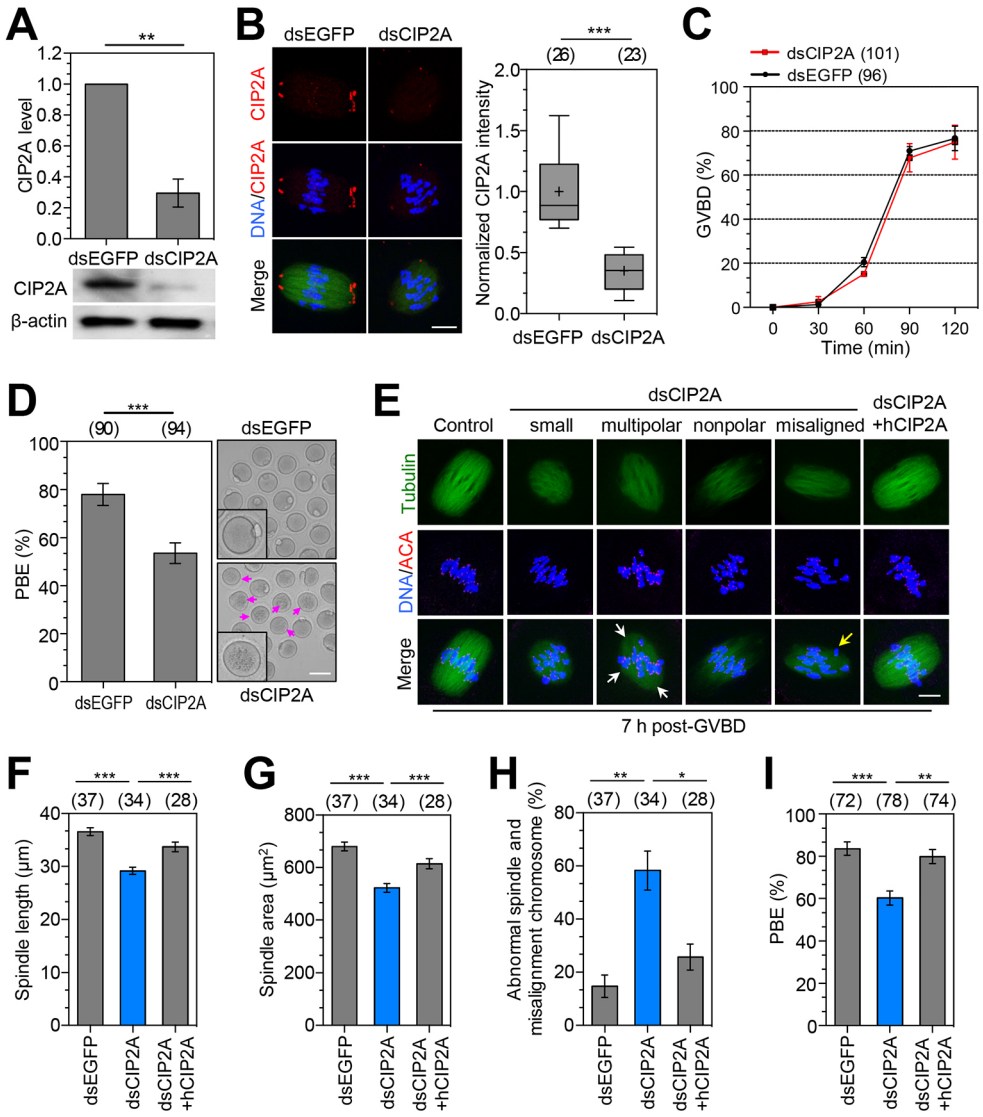

Fig. 3. CIP2A depletion causes spindle and chromosome abnormalities. GV oocytes injected with *Cip2a* (dsCIP2A) or *EGFP* (dsEGFP) dsRNA were cultured for 24 h in the presence of 2.5 µM milrinone. (A) Knockdown of CIP2A was confirmed by immunoblot analyses. β-actin was used as a loading control. Each lane contains the equivalent of 250 oocytes. Normalized expression of CIP2A was quantified from two independent experiments. (B) Oocytes microinjected with dsCIP2A or dsEGFP were incubated in M16 medium containing 2.5 µM milrinone for 24 h and then transferred to milrinone-free M16 for 8 h. Knockdown of CIP2A was confirmed by immunostaining with anti-CIP2A antibody, α-tubulin-FITC and Hoechst. Quantification of CIP2A intensity at MTOCs is shown together with representative images. The number of oocytes analyzed is specified in parentheses. (C,D) Timing of GVBD and percentage of first PBE were determined after CIP2A knockdown. Data are the mean±s.e.m. of at least three independent experiments. The number of oocytes analyzed is specified in parentheses. Representative images of PBE are shown. Arrows indicate oocytes arrested at MI stage. (E-I) Oocytes injected with dsCIP2A or dsEGFP were cultured in M16 medium containing 2.5 µM milrinone for 24 h and then transferred to milrinone-free M16 for 8 h. For rescue experiments, hCIP2A cRNA was co-injected with dsCIP2A. Kinetochores, spindle, and DNA were stained with anti-centromere antibody (ACA), anti-α-tubulin-FITC, and Hoechst, respectively. Representative images of spindle morphologies and chromosome alignment are shown. White and yellow arrows indicate spindle poles and lagging chromosome, respectively. Spindle length (F), spindle area (G), abnormal spindle and chromosome misalignment (H), and PBE rate (I) were quantified. Data are mean±s.e.m. of at least three independent experiments with the indicated number of oocytes. **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bars: 10 µm in B,E; 100 µm in D.

knockdown oocytes, but was rescued by overexpressing human CEP192 (Fig. 5E,F). This implies that CIP2A and CEP192 are mutually required for their localization and stabilization during meiotic maturation.

To further clarify the relationship between CIP2A and CEP192, we performed an *in situ* proximity ligation assay (PLA), which can visualize the *in vivo* interactions between two proteins (Söderberg et al., 2006). Whereas no signal was detected in the negative control, strong positive signals were observed throughout the cytoplasm, indicating a direct interaction between CIP2A and CEP192 in mouse oocytes (Fig. 5G). Furthermore, we confirmed the interaction between CIP2A and CEP192 by *in vitro* pull-down assay and *in situ* PLA analysis in somatic cells (Fig. 5H, Fig. S4). Our results suggest that reciprocal interactions between CIP2A and CEP192 are essential for MTOC assembly during meiotic maturation.

Plk1-mediated phosphorylation of CIP2A is required for MTOC localization

Since MTOC assembly was impaired after aurora A inhibition and the CEP192-organized aurora A-Plk1 cascade is crucial for

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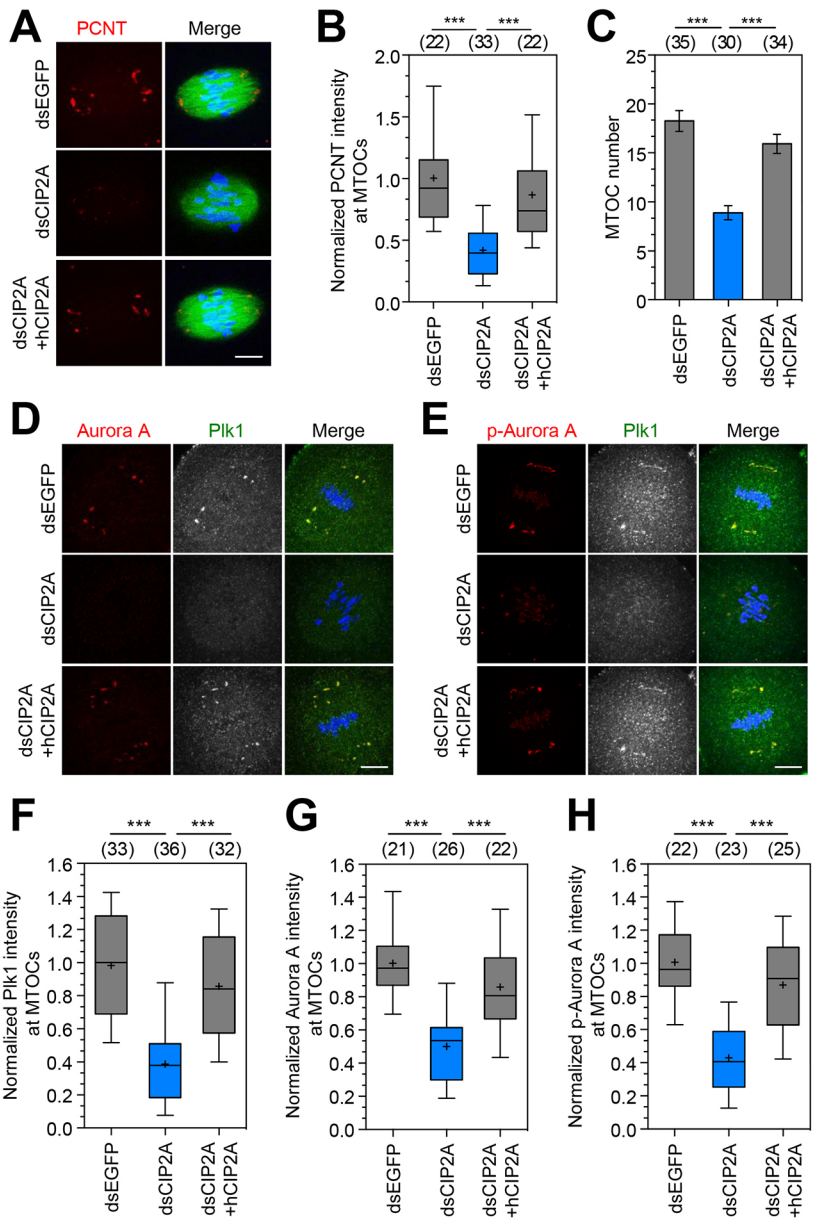

Fig. 4. CIP2A is essential for MTOC organization during meiotic maturation. (A) Oocytes injected with dsEGFP, dsCIP2A, or dsCIP2A+hCIP2A were cultured in M16 medium containing 2.5 µM milrinone for 24 h and then transferred to milrinone-free M16 for 8 h. Representative images of oocytes labeled with anti-α-tubulin, anti-PCNT and Hoechst are shown. (B,C) PCNT intensity and MTOC number were quantified. Error bars indicate s.e.m. (D,E) Oocytes injected with dsEGFP, dsCIP2A, or dsCIP2A+hCIP2A were labeled with anti-aurora A, anti-Plk1 or anti-phospho-aurora A antibodies. (F-H) The levels of Plk1 (F), aurora A (G) and phospho-aurora A (H) at MTOCs were quantified. The number of oocytes analyzed is specified in parentheses. ***P<0.001. Scale bars: 10 µm.

centrosome maturation and spindle assembly (Ding et al., 2011; Joukov et al., 2014; Saskova et al., 2008; Solc et al., 2012), we further examined CIP2A expression and localization after either aurora A or Plk1 inhibition with the kinase-specific inhibitor MLN8237 or BI2536, respectively. In this context, the CIP2A level at spindle poles was decreased by aurora A or Plk1 inhibition (Fig. 6A,B). Interestingly, however, CIP2A relocated to chromosomes when Plk1 was inhibited (Fig. 6A,B), implying that CIP2A recruitment to MOTCs is dependent on Plk1 activity. Because Plk1 is a well-known mitotic kinase that phosphorylates numerous substrates during mitosis, we assumed that CIP2A could be phosphorylated by Plk1, which might be important for the recruitment of CIP2A to MTOCs. Indeed, the results of *in vitro* kinase assays demonstrated that CIP2A is directly phosphorylated by recombinant active Plk1 (Fig. 6C), indicating that CIP2A is a substrate of Plk1. It is also noteworthy that Plk1 activity was increased proportionally by increasing the amount of CIP2A, suggesting that the regulation between CIP2A and Plk1 occurs in a cooperative manner.

The pericentriolar material (PCM) scaffold is known to form via the assembly of coiled-coil proteins such as PCNT and CEP192 (Woodruff et al., 2015). The coiled-coil domain of centrosomal proteins is crucial for mediating the homo-oligomerization and self-assembly that recruit downstream PCM proteins (Woodruff et al., 2015). Because CIP2A has a coiled-coil domain in the C-terminal region (Wang et al., 2017), we asked whether this domain is required for CIP2A centrosomal localization. A deletion construct of CIP2A lacking the coiled-coil domain showed nuclear localization both in somatic cells and mouse oocytes, suggesting that the localization of CIP2A is mediated by the coiled-coil domain (Fig. S5).

Sequence analysis of CIP2A identified two putative phosphorylation sites for Plk1 (T761 and S904) on the coiled-coil domain of CIP2A. To ascertain the role of Plk1-mediated phosphorylation of CIP2A in MTOC assembly, we overexpressed phospho-resistant mutants of CIP2A and observed their localization and MTOC organization. Whereas no discernible defect was observed in oocytes expressing the CIP2A-T761A mutant, MTOC assembly and subsequent spindle assembly and

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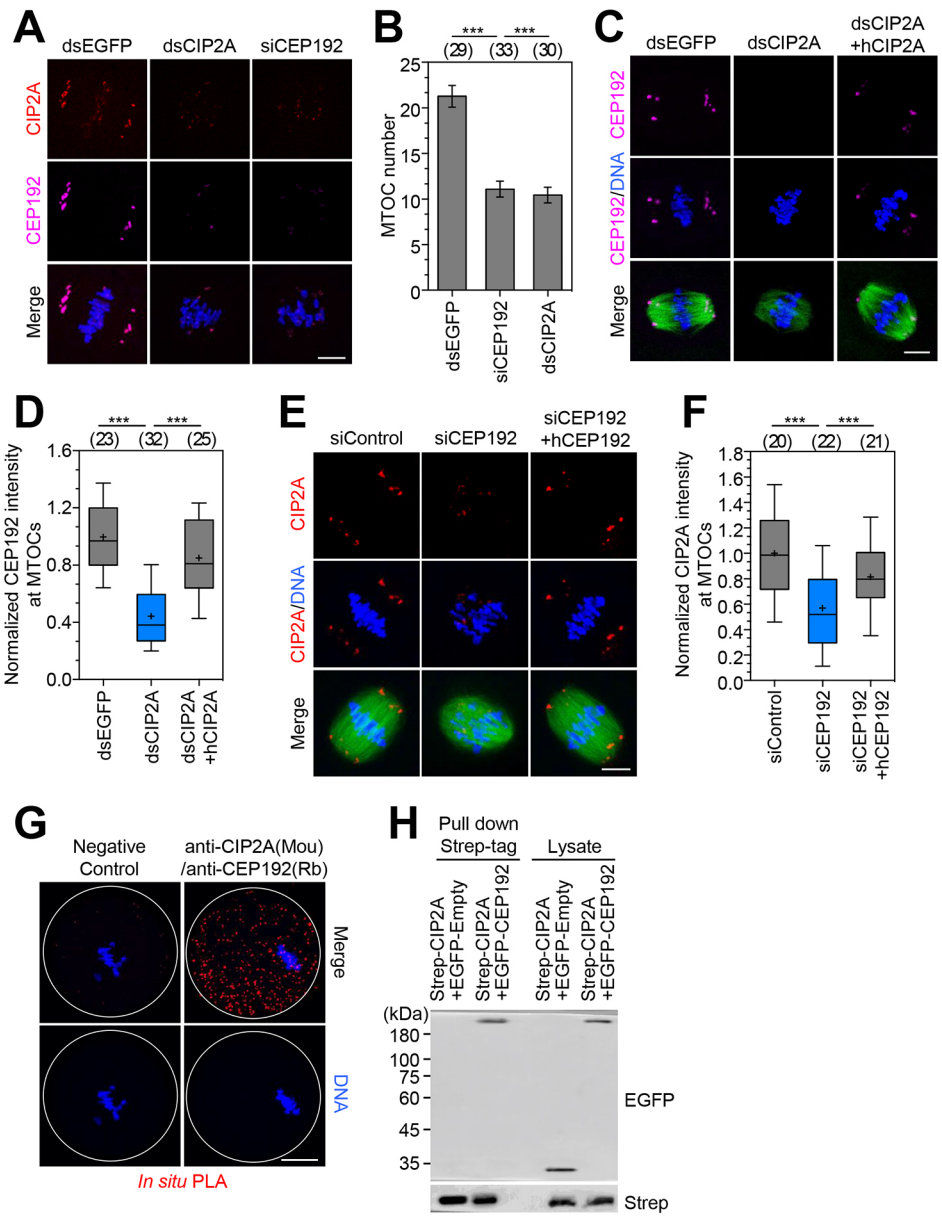

Fig. 5. CIP2A regulates CEP192-mediated MTOC organization. (A) Oocytes injected with dsEGFP, dsCIP2A or siCEP192 were cultured in M16 medium containing 2.5 µM milrinone for 24 h and then transferred to milrinone-free M16 for 8 h. Oocytes were then immunostained with anti-CIP2A antibody, anti-CEP192 antibody and Hoechst. (B) MTOC number was quantified. Error bars indicate s.e.m. (C) Oocytes injected with dsEGFP, dsCIP2A, or dsCIP2A+hCIP2A were cultured in M16 medium containing 2.5 µM milrinone for 24 h and then transferred to milrinone-free M16 for 8 h. Oocytes were then immunostained with anti-CEP192 antibody, anti-α-tubulin and Hoechst. (D) The level of CEP192 at MTOCs was quantified. (E) Oocytes injected with control siRNA (siControl), siCEP192, or siCEP192+hCEP192 were cultured in M16 medium containing 2.5 µM milrinone for 24 h and then transferred to milrinone-free M16 for 8 h. Oocytes were immunostained with anti-CIP2A antibody, anti-α-tubulin antibody and Hoechst. (F) The level of CIP2A at MTOCs was quantified. (G) Oocytes at the MI stage were fixed and incubated with mouse anti-CIP2A antibody together with rabbit anti-CEP192 antibody, followed by *in situ* PLA analysis. Oocytes injected with dsCIP2A were used as a negative control. (H) HEK293 cells were transfected with Strep-CIP2A, EGFP-Empty, or EGFP-CEP192 for 24 h. Lysates of HEK293 cells were pulled down with Strep-Tactin beads. The EGFP-CEP192 protein associated with Strep-CIP2A was detected by immunoblotting with EGFP antibody. The number of oocytes analyzed is specified in parentheses. ***P<0.001. Scale bars: 10 µm in A,C,E; 20 µm in G.

chromosome alignment were impaired in oocytes expressing a CIP2A-S904A mutant (Fig. 6D). Importantly, the CIP2A-S904A mutant was mainly detectable at the chromosomes (Fig. 6D), as in the case of Plk1 inhibition. Moreover, overexpression of the CIP2A-S904A mutant did not rescue the decreased level of PCNT and CEP192 in CIP2A knockdown oocytes, whereas PCNT and CEP192 levels were restored by overexpressing the CIP2A-T761A mutant (Fig. 6E,F). Similarly, the abnormalities in spindle organization and chromosome alignment observed in CIP2A knockdown oocytes were not rescued by overexpressing the CIP2A-S904A mutant, but were rescued by CIP2A-T761A mutant overexpression (Fig. 6E,F). These results suggest that Plk1-mediated phosphorylation of CIP2A at S904 is important for CIP2A targeting to MTOCs and subsequent MTOC organization and spindle assembly during meiotic maturation.

Collectively, it is likely that Plk1-mediated phosphorylation promotes targeting of CIP2A, possibly with CEP192, into MTOCs, which in turn leads to recruitment of aurora A and Plk1 at spindle poles (Fig. 7).

DISCUSSION

In this study, we revealed that CIP2A is a novel MTOC component regulating MTOC organization and spindle assembly during meiotic maturation. It is known that aurora A and Plk1 are essential kinases that regulate MTOC assembly. We found that CIP2A regulates the recruitment of these kinases by associating with CEP192. Moreover, we found that CIP2A is phosphorylated by Plk1, which targets CIP2A to MTOCs. Our results provide the first evidence that CIP2A is a novel MTOC component and is required for MTOC formation and spindle assembly during oocyte meiotic maturation.

Our data showed that CIP2A colocalizes with MTOCs during meiotic maturation in mouse oocytes. Consistent with this, a recent report suggested CIP2A as a candidate novel centrosome and centriolar satellite protein through a proximity-dependent biotinylation technique (Gupta et al., 2015). Therefore, it is likely that CIP2A is a novel MTOC component during meiotic maturation in mouse oocytes and might correspond to a pericentrosomal protein in somatic cells.

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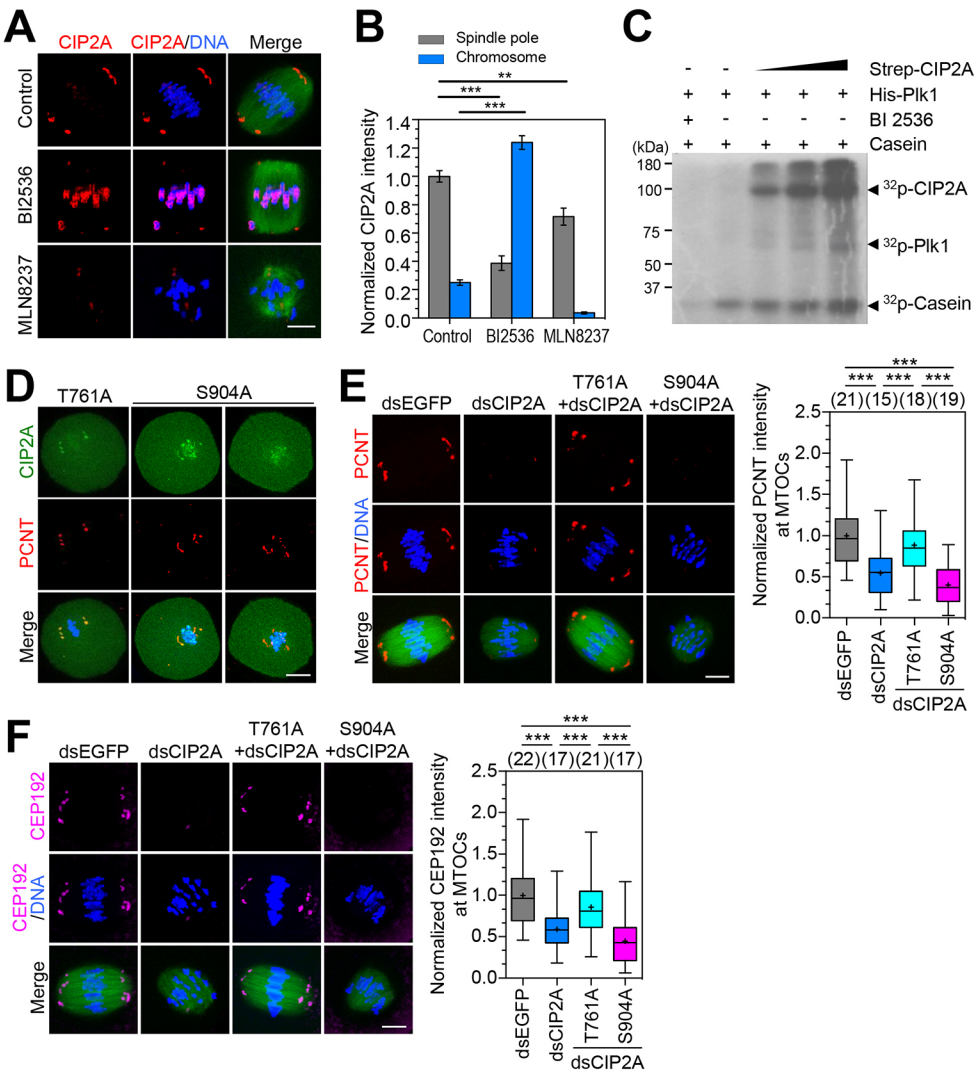

Fig. 6. Phosphorylation of CIP2A by Plk1 is required for MTOC organization. (A) GV oocytes cultured in fresh M16 medium for 4 h (GVBD) were further cultured with either BI2536 or MLN8237 for 4 h (MI stage). Oocytes were immunostained with anti-CIP2A antibody, anti-α-tubulin antibody and Hoechst. Representative images from at least three experiments are shown. (B) The intensity of CIP2A at spindle poles or chromosomes was quantified. Error bars indicate s.e.m. (C) Recombinant His-Plk1 was incubated with increasing amounts of purified Strep-CIP2A protein (0.25, 0.5 or 1 µg) for 30 min. Casein was used as a Plk1 substrate to determine Plk1 activity. (D) Oocytes injected with cRNAs encoding the CIP2A-T761A or CIP2A-S904A mutants were cultured in M16 medium containing 2.5 µM milrinone for 3 h and then transferred to milrinone-free M16 for 8 h. Oocytes were stained with anti-GFP antibody, anti-PCNT antibody and Hoechst. (E,F) Oocytes injected with dsEGFP, dsCIP2A, dsCIP2A+hCIP2A-T761A, or dsCIP2A+hCIP2A-S904A were cultured in M16 medium containing 2.5 µM milrinone for 3 h and then transferred to milrinone-free M16 for 8 h. (E) Oocytes were immunostained with anti-PCNT antibody, anti-α-tubulin antibody and Hoechst. The PCNT level at MTOCs was quantified and is shown in representative images. (F) Oocytes were stained with anti-CEP192 antibody, anti-α-tubulin antibody and Hoechst. Representative images are shown from two independent experiments. The CEP192 level at MTOCs was quantified. The number of oocytes analyzed is specified in parentheses. $^{**}P<0.001$, $^{***}P<0.001$. Scale bars: 10 µm in A,E,F; 20 µm in D.

Our data suggest that CIP2A is not associated with PP2A activity during meiotic maturation. It is well known that inhibition of PP2A activity stimulates meiotic resumption but causes aberrant spindle organization (Alexandre et al., 1991). However, and in contrast to the effect of OA treatment, we did not observe any discernible change in meiotic resumption or spindle organization in oocytes overexpressing human CIP2A, suggesting that CIP2A is not involved in the regulation of PP2A activity during meiotic maturation in mouse oocytes. Interestingly, similar to our observation, previous studies have suggested that CIP2A regulates mitotic progression and centrosome separation independently of PP2A activity in human cancer cells (Jeong et al., 2014; Kim et al., 2013). However, many studies have shown that inhibition of CIP2A increases the catalytic activity of PP2A and hence inhibits cell proliferation and tumor growth in many types of cancer cells (Chen et al., 2011; Junttila et al., 2007; Khanna et al., 2013a; Lucas et al., 2011; Yu et al., 2014). Thus, it is possible that the non-canonical function of CIP2A, which is not involved in PP2A activity, might be associated with specific cell cycle stages such as meiosis and mitosis or MTOC localization. Because PP2A is a heterotrimer consisting of two catalytic subunits, two scaffold subunits, and several isoforms of regulatory subunits, it is also possible that CIP2A might regulate a different isoform of PP2A that is not required for oocyte meiosis or that is involved in distinct localization during meiotic maturation (Barr et al., 2011). Therefore, it is of interest to determine the specific PP2A isoform regulated by CIP2A that is responsible for meiotic maturation.

In mouse oocytes, a bipolar spindle is assembled by fragmenting the acentriolar MTOCs into a large number of small MTOCs, which then merge into two equal spindle poles (Clift and Schuh, 2015). During this meiotic maturation, Plk1 is known to play an essential role not only in MTOC fragmentation, but also in MTOC assembly (Clift and Schuh, 2015; Solc et al., 2015). In addition to Plk1, aurora A is known to regulate MTOC number and spindle bipolarization (Ding et al., 2011; Solc et al., 2012). However, the mechanism by which oocytes assemble a bipolar spindle from multiple MTOCs without a canonical centrosome remains largely unknown. Our data revealed that depletion of CIP2A significantly decreased MTOC number, with reduced levels of both Plk1 and aurora A, which were rescued by overexpressing human CIP2A. This indicates that CIP2A is crucial for the recruitment of Plk1 and aurora A at the MTOC, thereby regulating MTOC organization and spindle bipolarization during meiotic maturation. Indeed, the spindle defects and chromosome misalignment observed following inhibition of Plk1 or aurora A were the major phenotypes of CIP2A depletion reported in mouse oocytes (Ding et al., 2011; Solc et al., 2012, 2015).

Our data showed that CIP2A reciprocally regulates CEP192 localization and function via direct interaction during meiotic

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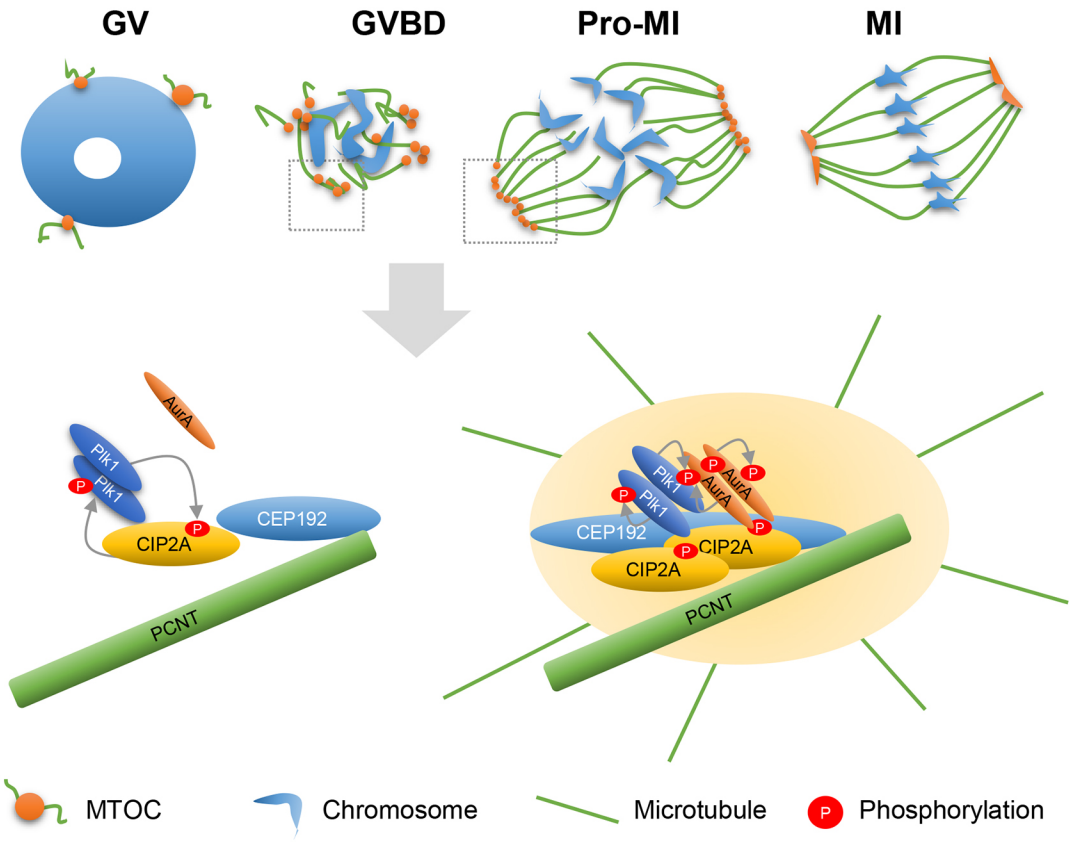

Fig. 7. Model of CIP2A-mediated acentriolar MTOC assembly during meiotic maturation. Upon meiotic resumption, spindle microtubules are assembled by multiple, discrete MTOCs around chromosomes. During these processes, Plk1 phosphorylation promotes CIP2A targeting to the MTOCs, which in turn facilitates the interaction with CEP192. The CIP2A-CEP192 complex in turn leads to recruitment of other MTOC components including aurora A and Plk1 at spindle poles, promoting subsequent spindle assembly.

maturation. Depletion of CIP2A or CEP192 reduced the CEP192 or CIP2A level, and these phenotypes were rescued by overexpressing human CIP2A or CEP192, respectively. It is known that CEP192 binds to and activates aurora A, which in turn activates Plk1 to promote centrosome maturation and bipolar spindle assembly in egg extracts and in mammalian cells (Joukov et al., 2014; Nikonova et al., 2013). In addition, this CEP192-organized aurora A-Plk1 complex was proposed to be attained in a cooperative manner (Meng et al., 2015). Our previous report suggested that CIP2A binds to and activates Plk1 during mitosis as a mitotic scaffold protein, and CIP2A depletion caused Plk1 mislocalization during mitosis (Kim et al., 2013). Thus, it is possible that CIP2A binds to and activates Plk1, which reciprocally promotes the association between CEP192 and CIP2A in a cooperative manner during the onset of meiotic maturation and mitosis. Regarding the CIP2A-CEP192 complex, we also showed that these two proteins can bind each other in mouse oocytes and somatic cells. Consistent with our data, CIP2A has recently been reported to be a binding partner of CEP192 and other centrosomal proteins, including CEP152, CEP128, CEP104, CNTROB and OFD1 (Gupta et al., 2015; Pallai et al., 2015). Collectively, these observations suggest that the CIP2A-CEP192 complex acts as a scaffold recruiting Plk1 and aurora A to promote centrosome maturation and bipolar spindle assembly.

Our data revealed that Plk1 phosphorylates S904 of CIP2A, and this is responsible for CIP2A targeting to MTOCs. Consistent with our results, S904 of CIP2A has been identified as a mitotic phosphorylation site in Hela S3 cells (Daub et al., 2008; Dulla et al., 2010). It is well established that Plk1-mediated phosphorylation of centrosomal scaffold proteins such as CEP192 and PCNT is essential for their localization and function during centrosome maturation in somatic cells and during meiotic maturation in oocytes (Clift and Schuh, 2015; Conduit et al., 2015; Joukov et al., 2014). Similar to CEP192 regulation by Plk1, our data indicated that Plk1 inhibitor treatment, but not aurora A inhibitor treatment, results in relocalization of CIP2A from MTOCs to chromosomes. Given that the phospho-inactive mutant (S904A) of CIP2A delocalized in oocytes, it is likely that Plk1-mediated phosphorylation of the coiled-coil domain of CIP2A is essential for CIP2A localization and function during meiotic maturation. Furthermore, using an *in vitro* kinase assay, we observed that purified CIP2A protein also enhances recombinant Plk1 activity. This indicates that CIP2A-Plk1 regulation is cooperative, similar to the CEP192-organized aurora A-Plk1 cascade (Joukov et al., 2014; Meng et al., 2015). Importantly, we also found that a CIP2A-S904A mutant caused multiple defects in MTOC organization, indicating that CIP2A is a functional substrate of Plk1 in regulating MTOC structure in oocytes. Since MTOCs in mouse oocytes lack centrioles, the centrosomal linker protein C-Nap1 (Cep250) cannot act as a functional centriolar linker (Clift and Schuh, 2015). Interestingly, another group has reported that CIP2A regulates centrosome separation in somatic cells (Jeong et al., 2014). Therefore, it is possible that CIP2A regulates not only MTOC assembly, but also MTOC fragmentation during meiotic maturation.

In conclusion, our data collectively indicate that CIP2A acts as a scaffold for CEP192-mediated MTOC assembly by recruiting Plk1 and aurora A in mouse oocytes. Considering not only that acentriolar MTOC assembly in oocytes is similar to mitotic centrosome

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assembly in somatic cells (Conduit et al., 2015), but also that CIP2A is localized at the centrosome in mitotic cells (Jeong et al., 2014), this newly discovered function of CIP2A seems to be evolutionarily conserved. It is also noteworthy that multiple centrosomes are frequently observed in cancer cells (Leber et al., 2010). Given that CIP2A is known to be upregulated in many cancer cells, we are tempted to speculate that the oncogenic properties of CIP2A are associated with centrosome amplification and chromosomal instability. Taken together, our results provide new insights into the mechanism and regulation of MTOCs and spindle assembly.

MATERIALS AND METHODS

Reagents and antibodies

All reagents and media were purchased from Sigma-Aldrich unless stated otherwise. Antibodies used in this study were anti-CIP2A (Santa Cruz, sc-80662; 1:500), anti-γ-tubulin (Abcam, ab11317; Sigma-Aldrich, T3320; 1:500), anti-PCNT (Abcam, ab4448; BD Transduction, 611814; 1:500), anti-PCM1 (Cell Signaling Technology, 5259; 1:500), anti-Strep tag (Qiagen, 34850; 1:1000), anti-α-tubulin-FITC (Sigma-Aldrich, F2168; 1:1000), anti-α-tubulin (Sigma-Aldrich, T9026; 1:1000), anti-Plk1 (Santa Cruz, sc-17783; 1:100), anti-aurora A (Cell Signaling Technology, 12100; 1:100), anti-phospho-aurora-A/B/C pT288/232/198 (Cell Signaling Technology, 2914; 1:200), anti-CEP192 (AbFrontier, AR07-PA0001; 1:200), anti-β-actin (Cell Signaling Technology, 3700; 1:500) and anti-centromere (Antibodies Inc, 15-234-0001; 1:100).

Oocyte collection and culture

Animal care and handling were conducted according to policies issued by the ethics committee of the Department of Animal Science, Chungbuk National University. GV oocytes were collected from ovaries of 5-week-old CD-1 mice and were placed in M2 medium (Sigma) supplemented with 2.5 µM milrinone to prevent GVBD. The oocytes were placed in M16 medium under liquid paraffin oil at 37°C in an atmosphere containing 5% CO_2 for specific periods of additional culture.

RNA isolation and RT-PCR

Total RNA was extracted from oocytes using the Dynabead mRNA DIRECT Kit (Ambion). First-strand cDNA was generated using a cDNA synthesis kit (LeGene). KAPA SYBR FAST qPCR Kits (Kapa Biosystems) were used in combination with a CFX Connect Real-Time PCR Detection System (Bio-Rad). PCR was performed using the following primers (5′-3′, forward and reverse): *Cip2a*, AGGCACTTGGAGGTAGTTTCT and TGAAGCACTTATGTTTGGGTCTT; *Cep192*, TCCCTTGCCTTTGAAA AATG and AGGCTTTGGAATCCTTTGGT; *Gapdh*, ACCACAGTCCA TGCCATCAC and TCCACCACCCTGTTGCTGTA.

Plasmids, siRNA, and preparation of mRNAs and dsRNAs

The full-length cDNA sequence encoding human CIP2A (purchased from Origene) was subcloned into pcDNA3.1-EGFP (Addgene). pEGFP-CEP192 was kindly provided by Dr Kyung S. Lee (NCI/CCR, National Institutes of Health, USA). Non-phosphorylatable mutants of CIP2A (T761A and S904A) were generated by PCR. Amplified PCR products were subcloned into the pRN3-GFP vector (Addgene). *In vitro* transcription and poly(A) tailing were performed using the T3/T7 mMessage mMachine Kit (Life Technologies) and the Poly(A) Tailing Kit (Life Technologies).

For dsCIP2A generation, PCR products were used as template for *in vitro* transcription using the MEGAscript Kit (Ambion). Primers were (5′-3′, forward and reverse): *Cip2a*, ATTAATACGACTCACTATAGGGA GAATACTGCTTGAAGCCTCTC and ATTAATACGACTCACTATAG GGAGACAAGTTCCATGTAGGACTT; *EGFP*, ATTAATACGACTAA CTATAGGGAGAATGGTGAGCAAGGGCGAG and ATTAATACGAC TCACTATAGGGAGAGCTCGTCCATGCCGAGAG.

Cep192-targeted small interfering RNAs (siCEP192) were purchased from Thermo Fisher Scientific. siRNA sequences were (5′-3′): sense, CCAGAGAGUUUGUUUCUUAtt; antisense, UAAGAAACAAACUC UCUGGgt.

Microinjection and treatment

Approximately 5-10 pl dsRNA/siRNA/cRNA was microinjected into the cytoplasm of oocytes using an Eppendorf microinjector, and the procedure was completed within 1 h. After injection, the oocytes were cultured for 24 h in M16 medium containing milrinone. They were then transferred to fresh medium and cultured under mineral oil at 37°C in an atmosphere of 5% CO_2.

For treatment, a solution of MLN8237 (aurora A inhibitor) or BI2536 (Plk1 inhibitor) in dimethyl sulfoxide (DMSO) was diluted in M16 medium to 20 µM or 100 nM, respectively. GVBD stage oocytes were then cultured in M16 medium containing MLN8237 or BI2536 for 4 h. Controls were cultured in M16 medium containing an equivalent volume of DMSO. Okadaic acid dissolved in DMSO was diluted in M16 medium to 400 nM and added to the culture medium.

Immunofluorescence analysis

Oocytes were fixed in 4% paraformaldehyde (PFA) in PBS for 30 min and then transferred to a membrane permeabilization solution (0.5% Triton X-100) for 20 min incubation. After 1 h incubation in blocking buffer (PBS containing 1% BSA), the oocytes were incubated overnight at 4°C with primary antibody, followed by Alexa Fluor 488/594/647-conjugated secondary antibodies (Sigma; 1:5000) for 1 h at room temperature. Hoechst 33342 (10 mg/ml in PBS) was used for DNA counterstaining. Oocytes were mounted on glass slides and examined under a laser-scanning confocal microscope (Zeiss LSM 710 META) with a 40× or 63× oil-immersion objective lens. Images were captured with the same laser power to measure fluorescence intensity. For quantification of fluorescence intensity, the mean intensities within the MTOC regions were measured, unless otherwise specified. Data were analyzed using ImageJ software (National Institutes of Health, USA) under the same processing parameters.

Chromosome spreads

For chromosome spreads, MII oocytes were exposed to acidic Tyrode's solution (Sigma) for 1 min to remove the zona pellucida. After brief recovery in the M2 medium, the oocytes were fixed in 1% PFA in distilled H_2O (pH 9.2) containing 0.15% Triton X-100 and 3 mM dithiothreitol. The slides were dried slowly in a humid chamber for several hours, and then blocked with 1% BSA in PBS for 1 h at room temperature. The oocytes were then incubated with anti-centromere antibody (1:50) overnight at 4°C and then with secondary antibody at room temperature. DNA on the slides was stained with Hoechst 33342, and the slides were mounted for observation by immunofluorescence microscopy.

Immunoblotting

In total, 250 mouse oocytes per sample were mixed with 2× SDS sample buffer and boiled for 5 min at 100°C for SDS-PAGE. Western blotting was performed as described previously (Wang et al., 2016), using the following antibody dilutions: anti-CIP2A, 1:500; anti-β-actin, 1:2000.

Strep pull-down

Strep-tagged CIP2A proteins were pulled down using Strep-Tactin beads (IBA TAGnologies), washed five times, and eluted with desthiobiotin according to the manufacturer's protocol. The eluted samples were analyzed by electrophoresis and detected using immunoblotting.

Plk1 kinase assay

The Plk1 kinase assay was conducted as previously described (Kim et al., 2013). Strep-tagged CIP2A from HEK293 cells expressing Strep-CIP2A was purified using the Strep tag purification method. Various concentrations of purified Strep-CIP2A protein (0.25, 0.5 or 1 µg) were subjected to the *in vitro* kinase assay with active His-Plk1 (0.1 µg; R&D Systems). *In vitro* kinase assays were conducted in kinase buffer (20 mM HEPES pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol, 5 mM NaF, 0.2 mM Na_3VO_4) supplemented with 50 mM ATP and 2.5 µCi [γ-^{32}P] ATP at 30°C for 30 min in the presence of 2 µg dephosphorylated α-casein (Sigma). The reaction mixture was size fractionated by electrophoresis and detected by autoradiography. BI2536 was used as a positive control to inhibit Plk1 activity.

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Proximity ligation assay (PLA)

To detect protein-protein interactions between CEP192 and CIP2A in mouse oocytes or somatic cells, a proximity ligation assay (PLA) was performed using the *in situ* Red Starter Kit Mouse/Rabbit (DUO92101-1KT, Sigma). Mouse anti-CIP2A and rabbit anti-CEP192 antibodies were conjugated with PLA PLUS and PLA MINUS probes, respectively. The oocytes injected with dsCIP2A or cells transfected with CIP2A siRNA were used as negative controls. The PLA signals were visualized using a confocal laser-scanning microscope (Zeiss LSM 710 META).

Statistical analysis

Statistical analysis was performed using GraphPad Prism. Data are the average of at least three independent experiments unless otherwise specified. Values were analyzed by one-way ANOVA or Student's *t*-test. The data are expressed as mean±s.e.m. $P<0.05$ was considered statistically significant. Box plots show the median (line), mean (+), and 25th and 75th percentiles (boxes), and the whiskers show the minimum and maximum.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.N., J.-S.K., J.S.O.; Methodology: H.W., M.H.C., I.-W.L.; Validation: H.W., M.H.C.; Formal analysis: H.W., I.-W.L.; Investigation: H.W., M.H.C., I.-W.L.; Data curation: S.N.; Writing - original draft: J.-S.K., J.S.O.; Writing - review & editing: J.-S.K., J.S.O.; Visualization: H.W.; Supervision: J.-S.K., N.-H.K., J.S.O.; Project administration: N.-H.K., J.S.O.; Funding acquisition: J.-S.K., N.-H.K., J.S.O.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.158584.supplemental

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