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Temporal and SUMO-specific SUMOylation contribute to the dynamics of Polo-like kinase 1 (PLK1) and spindle integrity during mouse oocyte meiosis

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Abstract

During mammalian meiosis, Polo-like kinase 1 (PLK1) is essential during cell cycle progression. In oocyte maturation, PLK1 expression is well characterized but timing of posttranslational modifications regulating its activity and subcellular localization are less clear. Small ubiquitin-related modifier (SUMO) posttranslational modifier proteins have been detected in mammalian gametes but their precise function during gametogenesis is largely unknown. In the present paper we report for mouse oocytes that both PLK1 and phosphorylated PLK1 undergo SUMOylation in meiosis II (MII) oocytes using immunocytochemistry, immunoprecipitation and *in vitro* SUMOylation assays. At MII, PLK1 is phosphorylated at threonine-210 and serine-137. MII oocyte PLK1 and phosphorylated PLK1 undergo SUMOylation by SUMO-1, -2 and -3 as shown by individual *in vitro* assays. Using these assays, forms of phosphorylated PLK1 normalized to PLK1 increased significantly and correlated with SUMOylated PLK1 levels. During meiotic progression and maturation, SUMO-1-SUMOylation of PLK1 is involved in spindle formation whereas SUMO-2/3-SUMOylation may regulate PLK1 activity at kinetochore-spindle attachment sites. Microtubule integrity is required for PLK1 localization with SUMO-1 but not with SUMO-2/3. Inhibition of SUMOylation disrupts proper meiotic bipolar spindle organization and spindle-kinetochore attachment. The data show that both temporal and SUMO-specific-SUMOylation play important roles in orchestrating functional dynamics of PLK1 during mouse oocyte meiosis, including subcellular compartmentalization.

Keywords

Cell cycle; SUMOylation; Meiosis; Kinetochore; Centrosome; Spindle; MTOC

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INTRODUCTION

Mammalian oocytes reach prophase of the first meiosis around the time of birth, and remain arrested at this stage until puberty, when meiosis resumes in response to the Luteinizing Hormone (LH) surge shortly before ovulation. During meiotic resumption, the oocyte undergoes nuclear and cytoplasmic changes, completing the first meiotic division in a process called “oocyte maturation”. Oocyte maturation consists of three stages; 1) resumption of meiotic cell cycle including germinal vesicle breakdown (GVBD), chromosome condensation, and spindle formation, 2) the transition between meiosis I and meiosis II without S-phase, and 3) further arrest in meiotic metaphase II. Following the arrest at the metaphase II, oocytes remain at this stage until fertilization triggers resumption and completion of meiosis II. These maturation processes are precisely controlled by various protein kinases and the posttranslational regulation of essential proteins are critical to control multiple processes during oocyte maturation.

Among the multiple kinases involved in cell cycle regulation during oocyte maturation, the Polo-like kinase 1 (PLK1) protein plays a key role (Pahlavan et al., 2000; Tong et al., 2002; Wianny et al., 1998). PLK1 is a member of the polo-like kinases, a conserved family of serine– threonine kinases (Lowery et al., 2005). Like other PLKs, PLK1 is characterized by a C-terminal non-catalytic region containing the Polo-box domain (PBD), which has been implicated in interactions with phosphoserine/phosphothreonine-containing motifs, coordinating protein– protein interactions and the subcellular localization of proteins (Lee et al., 1998; Qian et al., 1998; van de Weerdt et al., 2008). PLK1 activity controls a number of processes throughout the cell cycle such as centrosome maturation (Lane and Nigg, 1996; Lee and Rhee, 2011; Ruan et al., 2012), mitotic entry (Abrieu et al., 1998; Arnaud et al., 1998; Sumara et al., 2004), chromosome segregation (Godinho and Tavares, 2008; Kang et al., 2006), kinetochore function (Liu et al., 2012; Suijkerbuijk et al., 2012), spindle formation (Eot-Houllier et al., 2010; Zhu et al., 2013), and cytokinesis (Burkard et al., 2007; Petronczki et al., 2007).

Although PLK1 function is relatively well characterized in oocyte maturation, the mechanisms that control its activity and/or localization is yet not well understood. It is known that PLKs undergo Post-Translational Modifications (PTM) such as phosphorylation and ubiquitination-mediated degradation. In specific cell types, critical temporal and cellular compartment-coordinated activities can involve protein modifications during the process of SUMOylation. SUMOylation and de-SUMOylation modifications are orchestrated by members of the small ubiquitin-related modifier (SUMO) protein family. Differential SUMOylation regulates several diverse protein-driven cellular processes such as cell cycle regulation, gene transcription, differentiation, cellular localization and DNA repair.

Vertebrates express four SUMO isoforms (SUMO-1 through -4), each with the potential to act as unique modifiers by interacting with distinct downstream target proteins (Kerscher, 2007). SUMO-2 and SUMO-3 herein are indicated as SUMO-2/3 based on their 97% protein sequence identity and immunoreagent specificity. The SUMOs are conjugated to distinct target substrates *in vivo* and differ in their ability to form SUMO chains following the initial SUMO tagging of a target. SUMO-1, -2 and -3 expression patterns have been

characterized in human and rodent gametes but, to date, little is known about their precise function during gametogenesis (Brown et al., 2008; La Salle et al., 2008; Vigodner et al., 2006; Vigodner and Morris, 2005). In the human testis, we demonstrated that SUMOylation of the synaptonemal complex and SUMO involvement occurs during meiotic progression in spermatocytes (Brown et al., 2008).

To understand the intrinsic mechanisms that control PLK1 activity during oocyte maturation, we designed experiments to evaluate the potential role of PLK1 SUMOylation during this developmental process. Here, we show for the first time that PLK1 is dynamically and temporally modified by SUMO proteins. The data presented here demonstrate distinct functional roles for PLK1 modification by SUMO-1 and SUMO-2/3. Importantly, our present findings indicate that while PTM by SUMO-1 is related to PLK1 function in microtubule and spindle pole organization, the localization and function of PLK1 at the kinetochore is regulated by modification by SUMO-2/3.

MATERIALS AND METHODS

Animal use and study regulatory compliance

All animals were supplied by the Jackson Laboratory (Bar Harbor, Maine). The mice used in the present study were housed and maintained in The Rockefeller University's Comparative Biosciences facility that is approved by the American Association for the Accreditation of Laboratory Animal Care. In compliance with all US Federal and New York State regulatory requirements, The Rockefeller University (RU) has appointed an Institutional Animal Care and Use Committee (IACUC), which oversees all components of The Rockefeller University's program of animal care, including the review and approval of all proposed animal work. For the studies described herein, all experiment protocols were in accordance with the Guidelines for Care and Use of Laboratory Animals set forth by NIH, and protocols received approval by The Rockefeller University's Institutional Animal Care and Use Committee (RU IACUC protocol 13659).

Mouse oocyte collection and culture

Fully grown GV-stage oocytes were collected from the ovaries of 6- to 10-week-old C57BL/6J female mice (Jackson Laboratories). Females were injected with 5I Units pregnant mare serum gonadotropin (PMSG; EMD Biosciences/Calbiochem). Cumulus-intact GVs were recovered 42-46h post PMSG into HEPES–buffered Tyrode's lactate solution (TL-HEPES) containing 1 mg/ml bovine serum albumin (BSA; Sigma) supplemented with 100μM 3-isobutyl-1-methylxanthine (IBMX; EMD Biosciences/ Calbiochem, 410957) to block spontaneous progression of meiosis. Cumulus cells were removed by repeated pipetting using a fine tip capillary glass. Denuded oocytes were rinsed to remove IBMX and matured in vitro for 12h in Chatot, Ziomek, and Bavister medium (CZB) (Chatot et al., 1989) supplemented with 3 mg/ml (BSA) at 36.5°C under a humidified atmosphere of 5% CO_2. In vivo matured oocytes were obtained from 6- to 10 week-old C57BL/6J female mice induced to ovulate by an injection of 5I Units human chorionic gonadotropin (hCG; Sigma, CG5) 44-48h after PMSG stimulation. At 12–14h post-hCG, MII oocytes were collected from the oviducts into TL-HEPES supplemented with 1mg/ml

BSA; cumulus cells were removed by a brief incubation with 0.1% bovine testis hyaluronidase in TL-HEPES.

Pharmacological inhibitors

All drugs were prepared as stock solutions by dissolving in dimethyl sulfoxide (DMSO; Fisher Scientific, D128-500) to the following stock solutions: Nocodazole (Sigma, M1404), 20mM; Taxol (Sigma, T7402), 5mM; IBMX, 50mM (Calbiochem, 410957); Ginkgolic acid (GA; EMD-Milipore, 345887), 25mM. All stock solutions were stored at −20°C until further dilution in maturation medium (CZB-BSA) to their final working concentrations.

Immunofluorescence-based bioimaging and confocal microscopy

Zona pellucidae (ZP) were removed from oocytes by a brief treatment with 0.5% Pronase (Sigma, P8811). ZP-free oocytes were washed three times in 0.1% BSA in Dulbecco's phosphate-buffered saline (PBS–BSA) and then fixed in 3.7% paraformaldehyde (Electron Microscopy Sciences, 15710) in PBS-BSA (30min) and permeabilized with 0.1% Triton X-100 in PBS–BSA (5min). After rinsing three times in 0.1% PBS–BSA, oocytes were then blocked in 1% BSA in PBS for at least 2h at 4°C followed by incubation with primary antibody overnight (4°C). Oocytes were then washed with PBS–BSA, and incubated with secondary antibody for 1h at room temperature (RT). Next, oocyte DNA was counterstained with 4′, 6-diamidino-2-phenylindole (DAPI; 10μg/ml, 10min; Sigma, D9542), and the samples mounted with DABCO (1,4-Diazabicyclo[2.2.2]octane; Sigma, D2522) on glass slides (Thermo Scientific, 2960-001). Oocytes were examined using an inverted LSM 510 laser scanning confocal microscope (Carl Zeiss Microimaging Inc., Oberkochen, Germany) using a 40x/1.20 N.A., C-Apochromat water immersion objective lens.

In the experiment where the localization of phosphorylated (*p*) PLK1 (Ser137) with SUMO-1 or SUMO-2/3 was evaluated, as all secondary antibodies had a rabbit IgG isotype, immunofluorescence protocols were carried out in two-steps. Oocytes were prepared and incubated with either the primary antibody anti-SUMO-1 or SUMO-2/3 followed by incubation with the secondary antibody. Next, oocytes were washed and blocked in 1% PBS-BSA for at least 2h at 4°C followed by incubation with primary anti-phosphoPLK1 (Ser137) antibody then secondary antibody. The negative immunofluorescence control were carried out by omitting the primary antibody.

Immunoreagents

For immunofluorescence analyses, the rabbit polyclonal anti-SUMO-1 (Cell Signaling, 4930) antibody and rabbit monoclonal anti-SUMO-2/3 antibody (Cell Signaling, 4971) were used at a dilution of 1:50 each. A mouse monoclonal antibody against PLK1 (Invitrogen/ Life Technologies, 37-7000) was used at dilution of 1:100. To confirm our SUMO findings, additional anti-SUMO-1 (PW9460) and SUMO-2/3 (PW9465) antibodies for immunoblotting were obtained from ENZO Life Sciences (Farmingdale, NY). The rabbit polyclonal antibody raised against phospho-PLK1 (Ser137; Upstate/EMD-Millipore, 07-1348) was diluted 1:100. The same dilution was used for the mouse monoclonal antibody anti-phosphorylated PLK1 (pT210) conjugated with Alexa Fluor® 488, (BD Bioscience, 558446). For tubulin detection, a rabbit monoclonal α-tubulin antibody was used at dilution

1:200 (Cell Signaling, 2125) for double-immunofluorescence experiments. For triple-immunofluorescence, rat monoclonal anti-α-tubulin antibody (Abcam, 6161) was used at 1:200. Secondary goat antibodies (Alexa Fluor®, Life Technologies) were as follows: 488, anti-mouse (A-11017; 1:200); 633, anti-rabbit (A-21072; 1:100-400); 555 anti-rabbit (A-21429; 1:100-200); 555 anti-rat (A-21434;1:400).

Immunoprecipitation and Western Analysis of SUMOylated PLK1

Mouse MII oocytes (total: 852) were lysed using extraction buffer (10mM Tris-HCl (pH7.8), 150mM NaCl, 1mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1mM DTT, 1mM Na3VO4) with a protease inhibitor cocktail (Sigma, P8340). The lysates were subjected to centrifugation (15,000×g, 15min, and 4°C). Immunoprecipitation was performed using 10 μg anti-PLK1 antibody (Life Technologies, clone 35-206) and Dynabeads® Protein G Immunoprecipitation Kit following the manufacturer's protocol (Life Technologies, 10007D). Immuno-selected proteins were then eluted. In brief, the beads were suspended in 20 μl Elution Buffer and incubated under denaturing conditions (10min, 70°C) and the resulting supernatant saved.

Proteins in the supernatant was then subjected to separation by polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE® 10% Bis-Tris gel (Life Technologies, NP0315BOX). The electrophoresed proteins were next transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, ProtranBA85 10402680). Membrane-bound proteins were sequentially hybridized with specific primary antibodies in 5% non-fat dry milk (BioRad, 170-6404XTU) in 1X TBS/Tween-20, 2h at RT or overnight, 4°C. Western blots were developed using horseradish peroxidase-conjugated secondary antibody and ECL2 Western reagents for detection of specific protein signals (Thermo Scientific, 32132).

In Vitro SUMOylation Assays

Two sets of whole cell protein lysates were prepared from 423 and 408 MII oocytes (MII OC) respectively and the extracts then pooled. Each set was lysed using 150 μl extraction buffer (10mM Tris-HCl (pH7.8), 150mM NaCl, 1mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1mM DTT, 1mM Na3VO4) and a protease inhibitor cocktail (Sigma, P8340). The lysates were subjected to centrifugation (15,000xg for 15 min at 4°C).

In vitro SUMOylation assays were performed according to manufacturer's instructions using an assay kit (Enzo Biosciences, BML-UW8955-0001). Briefly, 20 μl of the MII OC protein extraction was incubated in SUMOylation buffer with a reaction mixture containing recombinant E1 enzyme, UBC9 enzyme, and the specific recombinant SUMO protein (SUMO 1, 2, or 3) in the presence or absence of ATP for 2h at 37°C. The resulting proteins from each condition were subjected to separation by polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE 4-12% Bis-Tris gels (Life Technologies, NP0335BOX).

The electrophoresed proteins were next transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, 10402680). The membrane-bound proteins were sequentially hybridized with specific primary antibodies overnight at 4°C in 5% non-fat dry milk (BioRad, 170-6404XTU).The Western blots were developed using horseradish peroxidase-conjugated secondary antibody and ECL2 Western blotting reagents for detection of specific

protein signals (Thermo Scientific, 32132). Densitometry was performed after scanning multiple exposures within the linear range of the films using the personal computer version of National Institutes of Health Image software (Scion Image). Replicates representing two or three membranes for each assay were used for densitometry. For Western analysis, particular signal intensities of phosphorylation for the specific proteins in each lane were then normalized with those for the "total specific protein" on the same membrane. Data was expressed as arbitrary units relative to control, set as a value of 1.

RESULTS

Dynamics of PLK1 subcellular localization during mouse oocyte maturation

PLK1 and tubulin were visualized by indirect immunofluorescence-based detection using mouse oocytes temporally matured *in vitro* to obtain oocytes at germinal vesicle (GV), germinal vesicle break-down (GVBD), metaphase-I (MI), telophase and metaphase-II (MII) stages.

In the GV oocyte, the surrounded nucleolus (SN) displays condensed chromatin around the nucleolus and a punctate pattern for PLK1 (Fig. 1; a–d) that is mainly associated with heterochromatic regions (Fig. 1a, arrow and arrow head, respectively). No PLK1 localization with alpha (α)-tubulin was observed; α-tubulin was detected as dots in the nucleus. After GVBD, PLK1 is detected as foci on condensed chromosomes (Fig. 1; e–h) and coincident with α-tubulin at the organizing spindle poles (Fig. 1; arrows, e–g). The data suggests the involvement of PLK1 in the organization of the cytoplasmic microtubule-organizing center (MTOCs) that are characteristic for rodent MII oocytes and will form the two poles. Consistent with this evidence, at the MI stage PLK1 is coincident at the spindle poles with α-tubulin (Fig. 1, arrows i–k). At MI (Fig. 1; i–l), PLK1 is concentrated at the centromeric region, findings that suggest that the kinase may participate in establishing proper attachment of kinetochores to the microtubules. At telophase (Fig. 1; m–p), although centromeric PLK1 remains in close proximity with the chromatids (Fig. 1m, large box), the location of PLK1 has changed from the spindle poles to the spindle mid-zone, data which is consistent with PLK1 activity during cytokinesis. In MII, PLK1 is observed once again at the spindle poles (Fig. 1; q–t), findings reflective of a role for this kinase in the establishment of a bipolar spindle. In MII oocytes, PLK1 is also detected in the centromeric region, data suggestive of activity involving the proper attachment of chromosomes to the mitotic spindle microtubules. Note that PLK1 is coincident with α-tubulin staining at the centromeric region (Fig. 1q, box).

SUMO-1 and PLK1 are coincident during oocyte meiosis

To delineate the spatiotemporal dynamics of SUMO-1 and its connection with PLK1 during oocyte maturation, *in vitro* matured oocytes at GV, GVBD, MI, telophase and MII stages were subjected to immunodetection and fluorescence analyses.

In GV oocytes, SUMO-1 signals were detected predominantly in the nuclear membrane and clearly associated with heterochromatic regions (Fig. 2; a–d), and SUMO-1 is coincident with PLK1 (*see* Fig. 2; insets, a–d). In contrast, SUMO-1 was not coincident with PLK1 in

the heterochromatin rim. There is little-to-none detectable transcriptional activity in the GV oocyte and SUMOylation based PTMs of transcription factors and cofactors often lead to transcription repression. Taken together, the present findings suggest the involvement of SUMO-1-mediated SUMOylation and PLK1 in the transcriptional repression of gene expression at this stage of maturation.

Following GVBD, SUMO-1 is coincident with PLK1 at the organizing spindle poles (Fig. 2; e–h, arrows in e–g). Strikingly, SUMO-1 is not coincident with PLK1 on the condensed chromosomes. As the oocyte progresses into the cell cycle to the MI stage, SUMO-1 is localized to the spindle, and mainly concentrated at the spindles poles where SUMO-1 is coincident with PLK1 (Fig. 2; i–l; arrows, i–k, arrows). Interestingly, in the centromeric region SUMO-1 was not detected. At telophase, SUMO-1 is coincident with PLK1 in the spindle mid-zone (Fig. 2; m–p, arrow, m–o), findings suggesting the involvement of SUMO-1 in the PLK1 function in the contractile ring during cytokinesis. At the MII stage, SUMO-1 and PLK1 are detected together at the spindle poles (Fig. 2; q–t).

SUMO-2/3 and PLK1 dynamics during oocyte maturation

To understand better how PLK1 localization and/or activity are regulated, we next focused on the spatiotemporal dynamics of SUMO-2/3 and PLK1 during oocyte maturation. Oocytes were matured *in vitro* and evaluated at the GV, GVBD, MI, telophase and MII stages using immunofluorescence.

At the GV stage (Fig. 3A; a–d), SUMO-2/3 is detected in heterochromatic regions coincident with PLK1 (Fig. 3A; a, box), findings similar to those observed for SUMO-1. SUMO-2/3 was also detected with the chromatin occupying the entire volume of the GV. However, SUMO-2/3 was not detected on the nuclear membrane in contrast to that observed for SUMO-1.

Interestingly, after GVBD the dynamic patterns for SUMO-2/3 are strikingly different from SUMO-1. Following GVBD, SUMO-2/3 begins to localize in punctate dots on the chromatin coincident with PLK1 (Fig. 3A; e–h, box). Interestingly, SUMO-2/3 was not detected at the organizing spindle poles. By MI, PLK1 localizes at the spindle poles and is coincident with SUMO-2/3 in the centromeric regions (Fig. 3A; box, i–l). In telophase stage (Fig. 3A; m–p), SUMO-2/3 is coincident with PLK1 in the spindle mid-zone and with the chromatids (Fig. 3A; box, m, o). During progression to the MII stage, PLK1 is observed at the spindle poles and to the centromeric region where it is coincident with SUMO-2/3 (Fig. 3A; box, q–t). The finding that SUMO-2/3 is coincident with PLK1 in the centromeric region during oocyte maturation is consistent with the involvement of SUMO-2/3 in the regulation of its activity and/or the localization of PLK1 in the process of attachment of chromosomes to the mitotic spindle (see Fig.3B, MII oocytes, in detail).

Posttranslational modifications of PLK1 by phosphorylation and SUMOylation during oocyte meiosis

PLK1 is a serine/threonine kinase and its phosphorylation at serine-137 and threonine-210 is known to have distinct effects on spindle checkpoint activity (Jang et al., 2002). Therefore, we next sought to evaluate the effect(s) of threonine and serine phosphorylation on the

subcellular localization of PLK1 as well as determining whether PLK1 forms are SUMOylated by SUMO-1 and/or SUMO-2/3. First, this study determined that PLK1 is phosphorylated at both serine-137 and threonine-210 in mouse oocytes (Fig. 4A). Serine-phosphorylated PLK1 was detected along the entire spindle. At telophase, it is found in the contractile ring at telophase and in the spindle pole at MII (Fig. 4A; b and f, respectively). A distinctive threonine-210 phosphorylation pattern was noted (Fig. 4A; c and g). In contrast to $^{Ser\text{-}137}$PLK1, threonine phosphorylation was only weakly observed at the spindle but is markedly associates with chromatin arms. Interestingly, $^{Thr\text{-}210}$PLK1 extends along chromatin arms, suggesting its involvement with proteins associated with the kinetochore.

Next, potential localization patterns for $^{Ser\text{-}137}$PLK1 with SUMO-1 and SUMO-2/3 were evaluated (Fig. 4B). We determined that $^{Ser\text{-}137}$PLK1 is coincident with SUMO-1 over the spindle, and markedly concentrates together at the spindle pole (Fig. 4B; a–c, arrows; e). SUMO-2/3 is visualized as punctata with the chromosomes aligned at the midplate (Fig. 4B, e). PLK1 and SUMO-2/3 are coincident at some of the kinetochores (inset, 4B, white box in e).

We also examined the SUMO proteins patterns together with $^{Thr\text{-}210}$PLK1 (Fig. 4C). The $^{Thr\text{-}210}$PLK1 is coincident with SUMO-1 at the spindle poles (Fig. 4C; a–c, arrows and white box in a) but in contrast to SUMO-1, it is at the centromeric region that $^{Thr\text{-}210}$PLK1 is coincident with SUMO-2/3 (Fig. 4C; e–g, double arrows and box in e). These results, when taken together, suggest the involvement of SUMO-1 and SUMO-2/3 and both forms of phosphorylated-PLK1 in the attachment and movements of chromosomes on the meiotic spindle.

PLK1 kinase is posttranslationally modified by SUMOylation

Total proteins were extracted from MII mouse oocytes (MII OC) and pooled as the starting material for analyses. Aliquots of these MII OC proteins were either directly evaluated (non-IP) or following immunoprecipitation (IP) with anti-PLK1. SDS-PAGE electrophoresis was used to separate and transfer proteins to the immunoblotting membrane, which was then subjected to sequential Western blotting as indicated (Figure 5A; WB lane). In addition, matched aliquots were subjected to immunoprecipitation (IP) with anti-PLK1 antisera before SDS/PAGE and immunoblotting (Figure 5A; IP lanes). Using pan-PLK1 and phosphorylation-specific PLK1 immunoreagents and conditions for immunodetection, PLK1 in MII oocytes is clearly present in non-IP samples, including its Thr-210-phosphorylated and Ser-137-phosphorylated forms (Figure 5a, WB Lane). Using denaturing conditions, the IP data show that each form of PLK1 is associated with the SUMOs (Figure 5a, IP lane 2 compared to IP-control, lane 1). For SUMO-1, a light band and one more prominent band detected by anti-SUMO-1 following PLK1 enrichment by immunoprecipitation were observed. These SUMO-1 results were similar using two additional SUMO-1 immunoreagents (data not shown). Similarly, two bands were observed for SUMO-2/3 in the IP-PLK1 (IP lane 2 compared to IP-control, lane 1). Immunodetection using three different ubiquitin-specific antibodies revealed no ubiquitinated PLK1 in the PLK1 complexes at this stage (data not shown).

Individual *in vitro* SUMOylation assays specific for SUMO-1, SUMO-2 and SUMO-3 were performed using whole MII OC protein extracts and components of the SUMOylation cascade as indicated in Methods. In Figure 5b, Western analyses are shown for PLK1 forms in the matched starting material (Lane 1, substrate, $time_0$), PLK1 forms following a 2h incubation assay with substrate and indicated reaction conditions (Lanes 2, 3) in comparison to the reaction materials without MII OC (Lane 4, non-substrate control). Lane 1 illustrates the total PLK1 and both threonine and serine-phosphorylated (*p*PLK1) detected in the starting substrate material ($time_0$). Representative results are shown for the individual SUMO-1, SUMO-2, or SUMO-3 specific *in vitro* assays with MII OC protein extracts as substrates (Lanes 2, 3). The two-hour assay did not significantly decrease the overall immunodetected PLK1 observed at the end of the incubation but, as expected, did markedly diminish the detected amount of the phosphorylated PLK1 forms (Lane 2 compared with 1). In the presence of substrate, reaction mix and ATP (2h), a single newly generated SUMOylated band was demonstrated for PLK1 and for threonine-phosphorylated PLK1 (Lane 3 compared with Lane 2). Serine-phosphorylated PLK1 showed a single upper SUMOylated form in the presence of substrate and reaction mix in the absence of ATP (Lane 2), with multiple SUMOylated species detected when the incubation included ATP. Immunodetection of the same membranes using the anti-tubulin antisera was negative for *in vitro* SUMOylation (data not shown). It was noted that levels of site-specific PLK1 phosphorylation normalized to pan-PLK1 significantly increased in the presence of sumoylation and ATP (Fig. 5c). Taken together, these biochemical findings demonstrate that MII OC PLK1 can be SUMOylated by each of the three SUMO proteins.

SUMO-1-PLK1 localization is dependent on spindle integrity and is involved in MTOC organization

To further investigate the relationship of SUMO-1 with PLK1 at the spindle poles, we next investigated whether an intact spindle was necessary for this event. MII *in vivo* matured oocytes were treated with Nocodazole, an anti-mitotic agent with high affinity for tubulin that reversibly disrupts microtubules and inhibits new spindle assembly. Oocytes treated with either 0.1 or 20-μM Nocodazole for 10min were subjected to immunofluorescence analyses immediately following treatment or a 1h rescue period in the absence of drug.

Treatment with the lower dose of Nocodazole (0.1μM) partially disrupts the meiotic spindle (Fig. 6A; e–h) compared to that of control vehicle-matched oocytes (Fig. 6A; a–d). However, incomplete spindle disruption does not affect the coincident pattern of PLK1 with SUMO-1 at the remaining spindle poles (Fig. 6A, arrows, e–g). In comparison, when oocytes are treated with the higher dose of Nocodazole (20μM), the microtubules are completely disassembled without any intact spindles observed (Fig. 6A, i–l). Interesting, in the absence of an organized spindle, PLK1 remains at the kinetochores but no longer coincident with SUMO-1 at the poles.

To confirm whether proper spindle organization is required for SUMO-1 localization with PLK1, Nocodazole-treated oocytes (0.1 and 20-μM) were rinsed and then maintained in culture (1h) to allow microtubule re-assembly. Such rescues allow spindle reorganization

following either Nocodazole concentration and, in addition, the restoration of the SUMO-1 together with PLK1 at the poles (Fig. 6A, m–p: 0.1μM; q–t, 20μM).

At the spindle poles, SUMO-1 is coincident with PLK1 in a spindle integrity-dependent way. Based on these observations, our data suggest that SUMO-1 localization with PLK1 may be involved in spindle assembly, importantly with such a role at the poles or MTOCs. To further delineate the correlation between SUMO-1 and PLK1 with microtubule dynamics, oocytes at MII stage were treated with Taxol, a microtubule-stabilizing drug (10μM, 30min).

Taxol induced a distinctive increase in SUMO-1 localization over the spindle (Fig. 6B; a, c). Taxol treatment also resulted in the formation of numerous asters in the cytoplasm (Fig. 6B; see asters, asterisks). Interestingly, at the center of these microtubule cytoplasmic asters, SUMO-1 specifically is coincident with PLK1 (Fig. 6B; a–b). These data routinely showed a ring-shaped pattern of tubulin, a pattern strikingly similar to that for SUMO-1 and PLK1 (*see* enlargement, Fig. 6C). This coincident pattern of SUMO-1 with PLK1 focally at the center of the Taxol-induced microtubule cytoplasmic asters is consistent with involvement of SUMOylation-mediated modification of the PLK1 kinase and tubulin in the organization of MTOCs (Fig. 6C; a–d).

Independent of spindle attachment or tension, centromeric SUMO-2/3 and PLK1 remain coincident

PLK1 is coincident with SUMO-1 at the spindle and spindle pole but it is at the centromeric region that SUMO-2/3 is coincident with PLK1. Therefore, we next determined whether SUMO-2/3-PLK1 localization at the kinetochore is dependent on proper spindle assembly. More specifically, disruption of the spindle assembly was employed to determine whether this coincident pattern depends on an intact attachment between microtubule and kinetochore. Spindle assembly in MII *in vivo* matured oocytes was disrupted using Nocodazole (0.1 or 20-μM; 10min) and oocytes evaluated using immunodetection analyses immediately after treatment or after a 1h rescue period without drug.

Both Nocodazole doses affected spindle structure compared to control vehicle-matched oocytes (Fig. 7A; a–d). Whereas the lower dose of Nocodazole (0.1μM) partially disrupts the meiotic spindle (Fig. 7A; e–h), the higher dose of Nocodazole (20μM) completely disrupted the spindle (Fig. 7A; i–l). These Nocodazole-induced effects were reversed after rescue, which allowed microtubule re-assembly (Fig. 7A; m–p, 0.1μM; q–t, 20μM treatment). Interestingly, neither partial nor total spindle disassembly affects SUMO-2/3 localization with PLK1 at the kinetochores (Fig. 7A, e, i). Nocodazole disorganizes the attachment between microtubules and kinetochores but does not affect SUMO-2/3 localization with PLK1.

Therefore, it was necessary to further characterize the association between tubulin, PLK1 and SUMO-2/3, by comparing MII oocytes treated with either Nocodazole to disrupt the spindle or Taxol (10μM; 30min.) to release spindle tension on kinetochores.

In the MII oocyte, chromosomes are aligned with SUMO-2/3 co-distributed with PLK1 at kinetochores (Fig. 7B; a–d). However, spindle disruption with Nocodazole results in the disorganization of centromeric region and chromosomal misalignment but does not affect SUMO-2/3 localization with PLK1 (Fig. 7B; e–h). In comparison to Nocodazole, Taxol had a stronger negative effect on chromosomes alignment, resulting in chromosomes segregated into the cytoplasm (Fig. 7B; i–l). However, strikingly even after chromosomes segregation induced by Taxol, SUMO-2/3 is still coincident with PLK1 at the chromatin. Thus, SUMO-2/3 localization with PLK1 apparently functions in spindle attachment at the chromosome (Fig. 7C; a, c) and the release of spindle tension does not affect the coincident pattern of SUMO-2/3 with PLK1 (Fig. 7C; b, d and b', d').

Taken together, these results indicate that although the spindle assembly is needed for correct chromosome alignment, it is not required for the maintenance of the SUMO-2/3 coincident with PLK1. Moreover, their coincident localization pattern at the chromosomes is independent of both proper spindle attachment and tension.

SUMOylation is required for proper spindle organization

Our data show that SUMO-1 and SUMO-2/3 are involved with PLK1 localization during spindle organization and attachment to kinetochores. In addition to our findings with PLK1, our data are consistent with an important role for SUMOylation in chromosome segregation and spindle and kinetochore assembly. To define the role of SUMO in these events, we next treated MII oocytes with Ginkgolic acid (GA, 100μM), which at this dose inhibits protein posttranslational modification by SUMO proteins as previously reported (Fukuda et al., 2009; Wu et al., 2012; Yao et al., 2011).

MII oocytes were cultured with 100μM GA for 1h. Following treatment, the oocytes were incubated with Nocodazole 20μM (10min) to disrupt the spindle. Following removal of Nocodazole, treated oocytes were exposed to GA (100μM, 2h) and spindle reorganization then compared with that of matched Nocodazole, but non-GA-treated, oocytes. Most of the further SUMOylation-inhibited oocytes were unable to recover from the Nocodazole-induced spindle disruption. Blocking SUMO-modifications of target proteins significantly affected spindle re-organization. GA-treated oocytes exhibited various abnormalities in the meiotic spindle and an increase in spindle defects (75%) compared to control oocytes (matched DMSO-Nocodazole but not GA-treated vehicle; 25%).

Very few oocytes properly reorganize the spindle after GA, and most fail to exhibit SUMO-1 at the spindle poles (Fig. 8A; a, b), showing instead aberrant, and perhaps residual, localization with tubulin (Fig. 8A; c, d). In addition, SUMOylation inhibition resulted in the misplacement of SUMO-1-PLK1 (Fig. 8A, e–g).

Similar results are observed with SUMO-2/3. Most oocytes did not reorganize their spindle following GA treatment and did not exhibit SUMO-2/3 at the kinetochore (Fig. 8B; a–d). Interestingly, even with the chromosomes spread in the cytoplasm, SUMO-2/3 localization with PLK1 was only partially affected by GA treatment (Fig. 8B; e–h).

DISCUSSION

Regulation of PLK functions involves temporal and spatial control by transcription, phosphorylation and ubiquitination, as well as, protein-protein and protein-chromatin interactions (Ferris et al., 1998; Lee et al., 1998; Macůrek et al., 2008; Uchiumi et al., 1997). PLK1 dynamic in the present work was similar to those observed by previous works in mouse oocyte (Pahlavan et al., 2000; Tong et al., 2002; Wianny et al., 1998), but to our knowledge, our findings indicate for the first time that SUMOylation may play a role in regulating PLK1 activity in the mouse oocyte. Our findings show that SUMO-1 as well as SUMO-2/3 is coincident with PLK1 during oocyte maturation, suggesting that PLK1 SUMOylation by these SUMO proteins may be involved in PLK1 subcellular compartmentalization and/or function during maturation. The data indicate that SUMO-1 as well as −2/3 are coincident with serine-137 and threonine-210 phosphorylated PLK1. Moreover, immunoprecipitation and SUMOylation biochemical assays using freshly-extracted MII oocyte proteins and sequential Western analyses confirmed that PLK1 can be SUMOylated by SUMO-1, -2 and -3.

SUMO-1 and PLK1

In the GV oocyte, SUMO-1 is nuclear, and its signal observed mainly at the nuclear envelope, a highly regulated and organized double membrane with functions as diverse as nuclear assembly, integrity, replication and transcription. Studies in somatic cells have implicated SUMO as an important regulator of nuclear envelope protein localization for components such as lamin and nuclear pore proteins (Chow et al., 2012; Goeres et al., 2011; Zhang and Sarge, 2008). Our data suggests that in GV oocytes SUMOylation may be involved in nuclear envelope composition and its membrane gating function. SUMO-1 also localizes at the perinucleolar heterochromatin rim and is coincident with PLK1 at nuclear bodies. In somatic cells, SUMOylation is critical for assembly of PML nuclear bodies and protein recruitment (Bernardi and Pandolfi, 2007; Nacerddine et al., 2005; Shen et al., 2006). In immortalized HeLa and 293 cells, SUMOylation correlates with both chromatin regulation and transcriptional repression. In oocyte growth, the transition from Non-Surrounded Nucleolus (NSN) to Surrounded Nucleolus (SN) is temporally coordinated with global repression of transcription (Bouniol-Baly et al., 1999). Our present findings suggest that SUMO-1-mediated SUMOylation is not involved in transcriptional repression within the nucleolus of the GV oocyte.

From GVBD, SUMO-1-PLK1 correlation is consistent with its functional activity relating to spindle assembly and cytokinesis. During spindle formation, centrosomes are the central sites of microtubule polymerization and consequently spindle assembly. For centrosomes formation, the centrioles assemble and organize a matrix of pericentriolar material (PCM) around themselves (Conduit et al., 2015). Mouse oocytes, however, have acentriolar centrosomes and spindle assembles from multiple MTOCs that reorganize progressively into a bipolar spindle during oocyte maturation (Schuh and Ellenberg, 2007; Severson et al., 2016). Although mouse oocytes do not have centrioles, MTOCs do contain several PCM associated proteins, such as γ-tubulin, pericentrin, Nuclear Apparatus (NuMA) and astrin (Ou et al., 2010; Severson et al., 2016; Yuan et al., 2009). As a centrosomal kinase, PLK1 is

involved in centrosome maturation and spindle assembly in both mitosis and meiosis (Haren et al., 2009). It plays a similar function in oocyte MTOCs (Ou et al., 2010). Reduced PLK1 activity is known to adversely affect protein recruitment to spindle poles resulting in abnormal spindles (Donaldson et al., 2001; Gonzalez et al., 1998). Similar to PLK1, SUMO is implicated in mitotic spindle dynamics, and in HeLa cells inhibition of SUMOylation results in defective, multi-polar spindles (Pérez de Castro et al., 2011). In these cells SUMO-1 overexpression causes centrosomal proteins hNinein, pericentrin and γ-tubulin to switch their localization (Cheng et al., 2006). Interestingly, γ-tubulin recruitment to the poles is also regulated by protein kinases such as PLK1(Haren et al., 2009).

PLK1 is involved in cytokinesis in both mitotic and meiotic cells (Hu et al., 2012; Lindon and Pines, 2004; Petronczki et al., 2008; Wianny et al., 1998). Our data suggests that SUMOylation participates in PLK1-mediated cytokinesis, findings consistent with transfection studies in immortalized cell lines (Di Bacco et al., 2006). Together the findings indicate that SUMO-1 involvement in cytokinesis could be mediated, at least in part, by PLK1-SUMOylation.

PLK1 localization at the poles is dependent on spindle integrity. We treated oocytes with spindle-disrupting drugs to evaluate any inter-dependence of proper spindle assembly and SUMO-1-PLK1-SUMOylation. SUMO-1 and PLK1 are no longer detected on the spindle and poles following complete spindle disruption. In contrast, when the spindle is partially disrupted, SUMO-1 persists with PLK1 at the remaining poles. This result suggests that even a small portion of organized spindle microtubule correlates with SUMO-1 coincident with PLK1, findings consistent with a dependence on an intact spindle microtubular network and pole. Dependence of spindle integrity on SUMO-1-PLK1 localization provides further support for the hypothesis that PLK1 modification by SUMO-1 participates in spindle organization and MTOC formation. MTOC contains a γ-tubulin ring configuration within a large multi-protein complex that plays an important role in microtubule nucleation (Johmura et al., 2011; Wiese and Zheng, 2006). Moreover, at MTOC PLK1 is involved in γ-tubulin recruitment to the ring complexes that serves as a template for microtubular nucleation (Casenghi et al., 2003). In Taxol-induced cytoplasmic MTOC, we observed a PLK1 ring shape similar to those previously described for γ-tubulin and PLK1 (Haren et al., 2006; Wiese and Zheng, 2006). Interestingly, the SUMO-1 pattern is observed in a similar ring shape structure at MTOC and is coincident with PLK1. Furthermore, microtubules as detected by α-tubulin radiate from the ring structure formed by SUMO-1-PLK1, findings suggestive that SUMO-1-SUMOylation may be involved in regulating PLK1 functions in MTOC and microtubular organization at the spindle.

The centrosomes organize at the spindle poles to nucleate microtubules and establish the meiotic spindle. However, it uses different mechanisms in rodent oocyte compared to oocytes from non-rodent species (Schatten and Sun, 2015, 2011). The acentriolar centrosomes containing ɣ-tubulin (MTOC) nucleate and organize the cytoplasmic asters in mouse oocytes (Gueth-Hallonet et al., 1993; Schuh and Ellenberg, 2007), in which several preexisting smaller cytoplasmic asters assemble to form the spindle poles (Henson et al., 2008). In contrast, non-rodent oocyte do not contain cytoplasmic asters (Kim et al., 1996; Long et al., 1993), in which have been suggested that the centrosome organization at the

spindle poles is mediated by NuMA that bundles microtubules and by ɣ-tubulin (Lee et al., 2000; Liu et al., 2006; Sedo et al., 2011). In this way, more studies are needed to determine the PLK1-SUMO-1 involvement in MTOC formation and spindle organization in oocyte from non-rodent species.

SUMO-2/3 and PLK1

At GV, except for the notable absence of SUMO-2/3 at the nuclear envelope, its distribution pattern was similar to that observed for SUMO-1, suggesting that it may also be involved in SN organization. From GVBD and despite PLK1 association at cytokinesis, SUMO-2/3 has a distinctive focal pattern markedly different from SUMO-1. SUMO-2/3 and PLK1 are coincident in punctate dots and distinct foci on chromosomes condensed after GVBD; in MI and MII oocytes they temporally are coincident at the centromeric region. These findings suggest that PLK1 modification by SUMO-2/3 regulates its localization and thereby function during the process of chromosome attachment at the mouse meiotic spindle. Indeed, previous studies showed that SUMO-2/3 localizes at centromeres and kinetochore during mitosis (Azuma et al., 2005; Wang et al., 2010; Zhang et al., 2008). The centromere/kinetochore is defined by four structurally-distinct regions. In somatic cells, different SUMO-2/3-SUMOylated proteins are associated with these regions. SUMO-2/3 posttranslationally modifies proteins such as inner centromere Aurora B, Borealin and Topoisomerase Iiα (Ban et al., 2011; Klein et al., 2009; Ryu et al., 2010), inner kinetochore CENP-H and CENP-I (Mukhopadhyay et al., 2010), outer kinetochore Nuf2 (Zhang et al., 2008), and outermost kinetochore domain BubR1 and CENP-E (Yang et al., 2012; Zhang et al., 2008).

SUMOylation is known to promote protein-protein interactions on chromatin. In this study, the demonstrated localization at the centromere/kinetochore of SUMO-2/3 with PLK1 during maturation further suggests that PLK1-SUMOylation may likewise promote PLK1 interactions with oocyte centromere/kinetochore proteins. Such SUMOylation-promoted proteins interactions can be simple heterodimeric associations, or they can also potentially enhance assembly of large multi-protein complexes. Notably, the over one hundred different proteins identified with the centrosome/kinetochore are known to be arranged in multiple complexes (Santaguida and Musacchio, 2009).

At metaphase, kinetochores mediates the chromosome attachment to microtubules from the bipolar spindle. It places kinetochores under tension generated by the spindle microtubules that pull the chromatids in direction to the opposite poles against the multi-subunit protein complex that holds the sister chromatids together (Pinsky and Biggins, 2005). The spindle assembly checkpoint (SAC) prevents anaphase until all chromosomes/kinetochores are properly aligned and attached to the spindle, which is regulated by both chromosomes attachment to kinetochore microtubules and tension exerted on kinetochores (Zhou et al., 2002). Since PLK1 is directly involved in maintaining efficient Kinetochore and SAC signaling (Lera et al., 2016; O'Connor et al., 2016), we treated the oocytes with Nocodazole that disrupt the spindle and Taxol that reduces the tension on kinetochores.

In the present study, SUMO-2/3 modification of PLK1 is consistent with potential regulatory involvement in its function(s) at kinetochores during microtubule attachment. In our study

with Nocodazole-treated oocytes, kinetochores were not attached to microtubules and chromosomes misaligned due to lack of spindle attachment. In these oocytes, PLK1 still coincident with SUMO-2/3 at kinetochores, demonstrating that kinetochore-microtubule attachment is important for chromosome alignment, but not for maintenance of SUMO-2/3 coincident with PLK1. Taxol permits microtubule attachment without tension exerted on kinetochores, which leads to chromosome misalignment but PLK1-SUMO-2/3 is still observed at the kinetochore. Together these data show that neither attachment nor tension is required for the coincident pattern of PLK1 with SUMO-2/3.

SUMOylation and oocyte spindle organization

As SUMO is involved in proper spindle organization in somatic cells, we also evaluated the effect(s) of inhibition of the SUMOylation process on meiotic spindle organization. We treated oocytes with GA, which globally inhibits new protein modification by SUMO in an ATP-dependent manner by selectively targeting E1 SUMO-activating enzyme. Up to 6h, there was no effect of GA on spindle organization or SUMO localization with PLK1 (data not shown). Since treatment does not affect previously SUMO-modified proteins, one possible explanation for this finding may be residual half-life of prior SUMOylated proteins. To address this possibility, we treated oocytes with acute GA to block the conjugation pathway, and then with acute Nocodozole to completely disrupt the spindle. Oocytes were rescued, and then treated for a second time with or without GA. Notably, inhibition of new SUMOylation significantly decreased the oocyte's ability to reorganize the spindle properly after rescue. Almost 75% of the oocytes presented with spindle defects compared to matched-vehicle control oocytes (25%). The most common spindle defects observed after inhibition of SUMOylation were failure to organize a bipolar spindle and chromosomal misalignment/scatter throughout the cytoplasm, findings consistent with an important role for SUMOylation of essential MTOC organizing proteins in the assembly of a bipolar spindle and proper kinetochore attachment to microtubules. However, since centrosome and microtubule dynamics and organization as well as spindle formation during meiosis in mouse oocyte use different mechanisms compared to non-rodent oocytes (Schatten and Sun, 2011), further studies are needed to clarify if SUMOylation is involved in these process in other species. These results are consistent with earlier studies which show that SUMO protease SENP2 overexpression results in defective spindle formation in mature oocytes and proper kinetochore attachment to microtubules (Wang et al., 2010).

In somatic cells, a lack of spindle attachment or tension increases PLK1 activity at kinetochores, a response to facilitate their attachment to microtubules. In the mouse oocyte, our data suggest that inhibition of SUMOylation inhibits new SUMO-modification of PLK1 and potentially other regulators, abrogating a response mechanism to promote microtubule attachment to kinetochores following loss of spindle attachment. It is important to consider that any subcellular alterations in PLK1 localization and functionality after globally inhibiting the SUMOylation pathway could be a direct result of changes in SUMOylation or phosphorylation of PLK1 or, an indirect effect mediated by interfering with the PTMs of PLK1 activity regulators such as Aurora A kinase and its activator Borealin (Macůrek et al., 2008; Pérez de Castro et al., 2011; Seki et al., 2008).

In summary, the data presented demonstrate that posttranslational modifications by SUMO are involved in temporal and spatial PLK1 functions during mouse oocyte meiosis, including modification of essential MTOC organizing proteins. The findings further suggest that SUMOylation may affect the levels of phosphorylated forms of PLK1 in the MII oocyte. Therefore, SUMO proteins potentially play multiple critical roles in mouse oocyte meiosis including those of cell cycle progression, spindle assembly, kinetochore function and cytokinesis.

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Highlights

- Plk1 is posttranslationally modified by SUMO
- SUMO-1 SUMOylates Plk1 at spindle and spindle poles
- SUMO-2/3 SUMOylates Plk1 at kinetochore
- Plk1 SUMOylation by SUMO-1 is spindle-dependent whereas SUMOylation by SUMO-2/3 is spindle independent
- SUMOylation is important for proper oocyte spindle organization

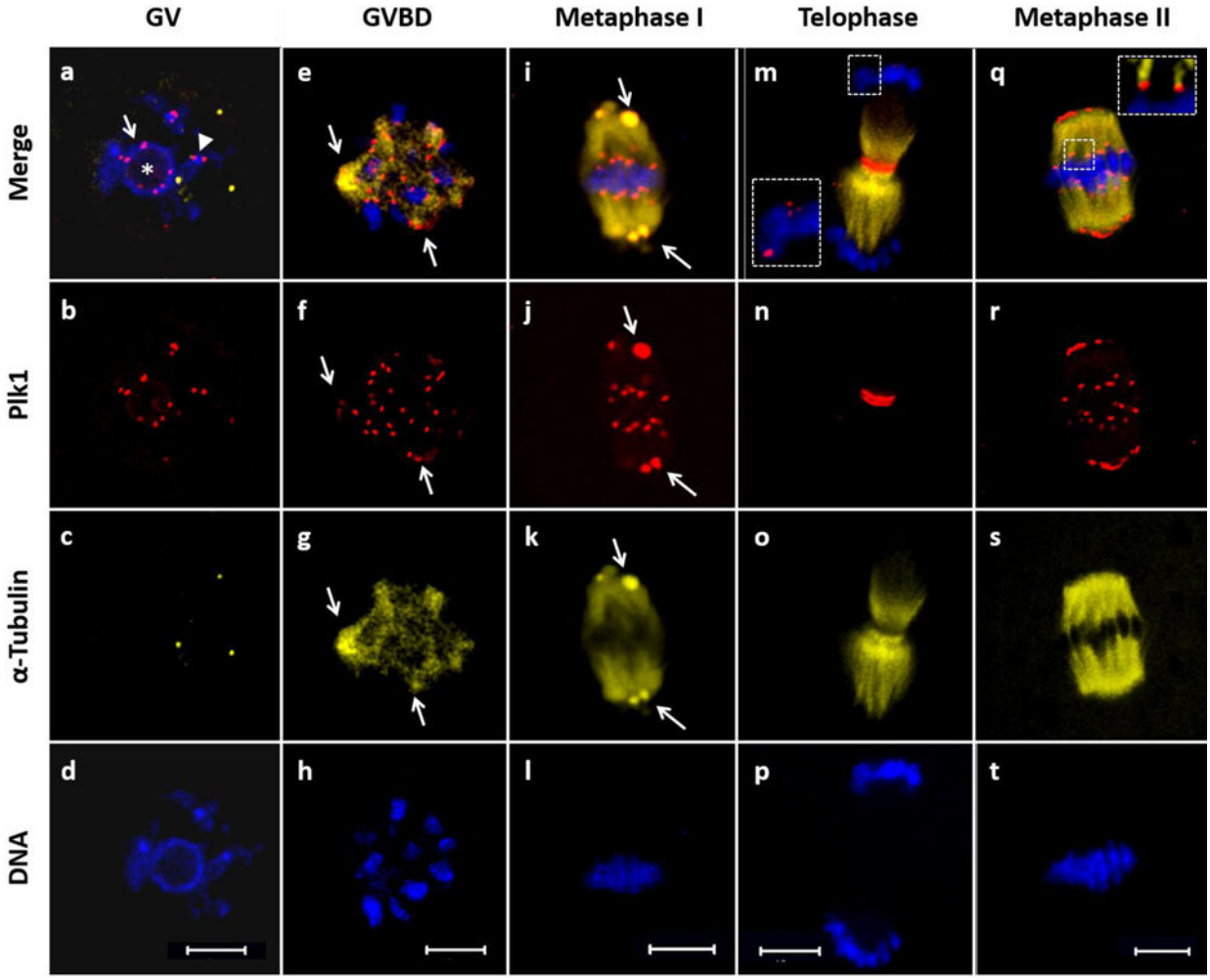

Figure 1. The pattern of subcellular PLK1 is dynamic during oocyte maturation

Mouse oocytes were matured *in vitro* and the following stages harvested: 0h (germinal vesicle, GV), 4h (GV breakdown, GVBD), 8h (metaphase-I) and 12h (telophase and metaphase-II). PLK1 (b, f, j, n, r) and alpha-tubulin (c, g, k, o, s) were localized in mouse oocytes using confocal microscopy following immune-fluorescence labeling with mouse anti-PLK1 and rabbit anti-alpha-tubulin antibodies. DNA was counterstained with DAPI (d, h, l, p, t); merged images shown (a, e, i, m, q). Large white squares shown in “m” and “q” are enlargements of each corresponding small box. At GV (a–d), PLK1 is mainly observed in nuclei with a punctate staining pattern. After GVBD, PLK1 is seen as foci on condensed chromosomes (e–h) and is coincident with alpha-tubulin at organizing spindle poles (see arrows, e–g). At metaphase I, PLK1 concentrates at centromeres and is coincident with alpha-tubulin in the spindle poles (i–l; arrows, i–k). At telophase, PLK1 is observed at the spindle mid-zone, a change from its localization at the poles (m–p); however, PLK1 still localizes with chromatids (large box, m). After telophase, PLK1 localizes to the centromeric region and spindle poles in metaphase II (q–t). Note, PLK1 is coincident with the microtubules at the centromeric region (box, q). Bar, 10μM.

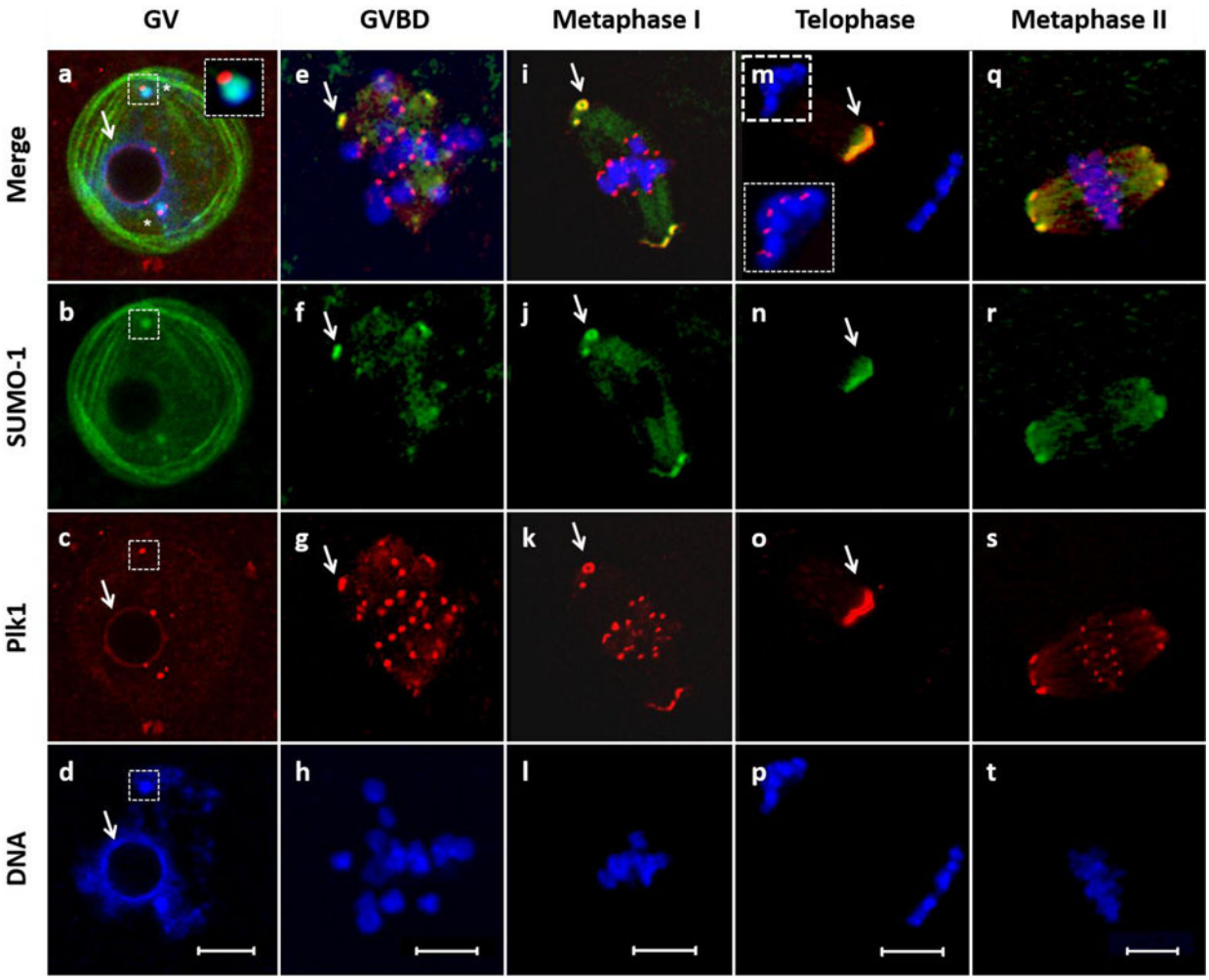

Figure 2. PLK1 and SUMO-1 are coincident during meiosis

Oocytes were staged and harvested as in Figure 1. Subcellular localization for SUMO-1 (b, f, j, n, r) and PLK1 (c, g, k, o, s) were visualized by bioimaging using confocal microscopy, following immunolabeling with mouse anti-PLK1 and rabbit anti-SUMO-1 antibodies. DNA was counterstained with DAPI (d, h, l, p, t); merged images are shown (a, e, i, m, q). White large square boxes shown in “a” and “m” are enlargements of each corresponding smaller box. At GV, SUMO-1 is in the nucleus, predominantly localized at the nuclear membrane and non-nucleolar heterochromatin (a–d). Non-nucleolar SUMOylated heterochromatin closely associate with PLK1 (boxes, a–d). After GVBD (e–h), SUMO-1 is detected in the organizing spindle poles and is coincident with PLK1 (see arrows in e–g). At MI, SUMO-1 localizes over the spindle with a marked intensity at the spindle poles (i–l; arrows, i–k), where SUMO-1 is coincident with PLK1. As oocyte progress to telophase (m–p), SUMO-1, similar to that observation with PLK1, now locates from the spindle poles to the mid-zone where it overlaps with PLK1 (arrows, m–o); however, PLK1 still localizes with chromatids (large box, m). At MII, SUMO-1 localizes on the spindle coincident with PLK1, most prominently at the poles (q–t). Bar, 10μM.

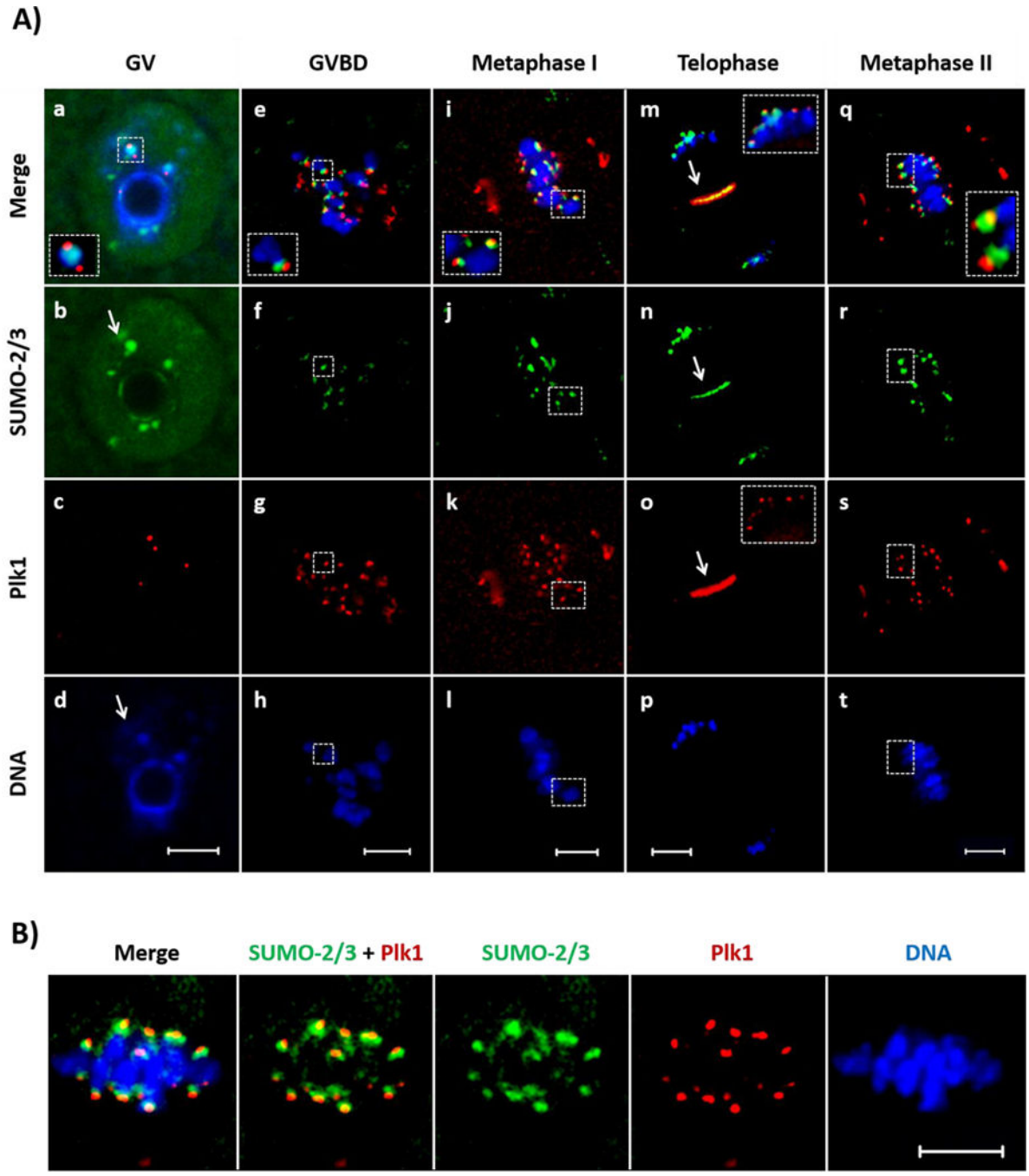

Figure 3. SUMO-2/3 and PLK1 dynamics during oocyte maturation

(A) GV, GVBD, MI, telophase and MII stages of matured oocytes were collected as above. SUMO-2/3 (b, f, j, n, r) and PLK1 (c, g, k, o, s) were immuno-fluorescently labeled with mouse anti-PLK1 and rabbit anti-SUMO-2/3 antibodies and visualized by confocal microscopy. DNA was counterstained with DAPI (d, h, l, p, t), and merged images are shown (a, e, i, m, q). The white larger square boxes show enlargements of each corresponding small dotted box. At GV (a–d), SUMO-2/3 is diffusely distributed throughout the nuclei. Prominent perinucleolar chromatin rim and non-nucleolar heterochromatin

staining is observed (arrows, b, d) where PLK1 overlaps with SUMO (white box, a). After GVBD (e–h), SUMO-2/3 is coincident with PLK1 in foci on condensed chromosomes (box, e–h). At MI, SUMO-2/3 is coincident with PLK1 adjacent to centromeres (i–l; see box, i–l). At telophase (m–p), SUMO-2/3 is coincident with PLK1 at chromatids (box, m, o) and spindle mid-zone (arrows, m–o). In MII (q–t), SUMO-2/3 is again detected with PLK1 at the centromeres (boxes, q–t). Bar, 10μM. (B) SUMO-2/3 is observed between chromatin and PLK1. Chromatin enlargement of mouse oocyte in metaphase I showing in detail SUMO-2/3 coincident with PLK1 at kinetochore.

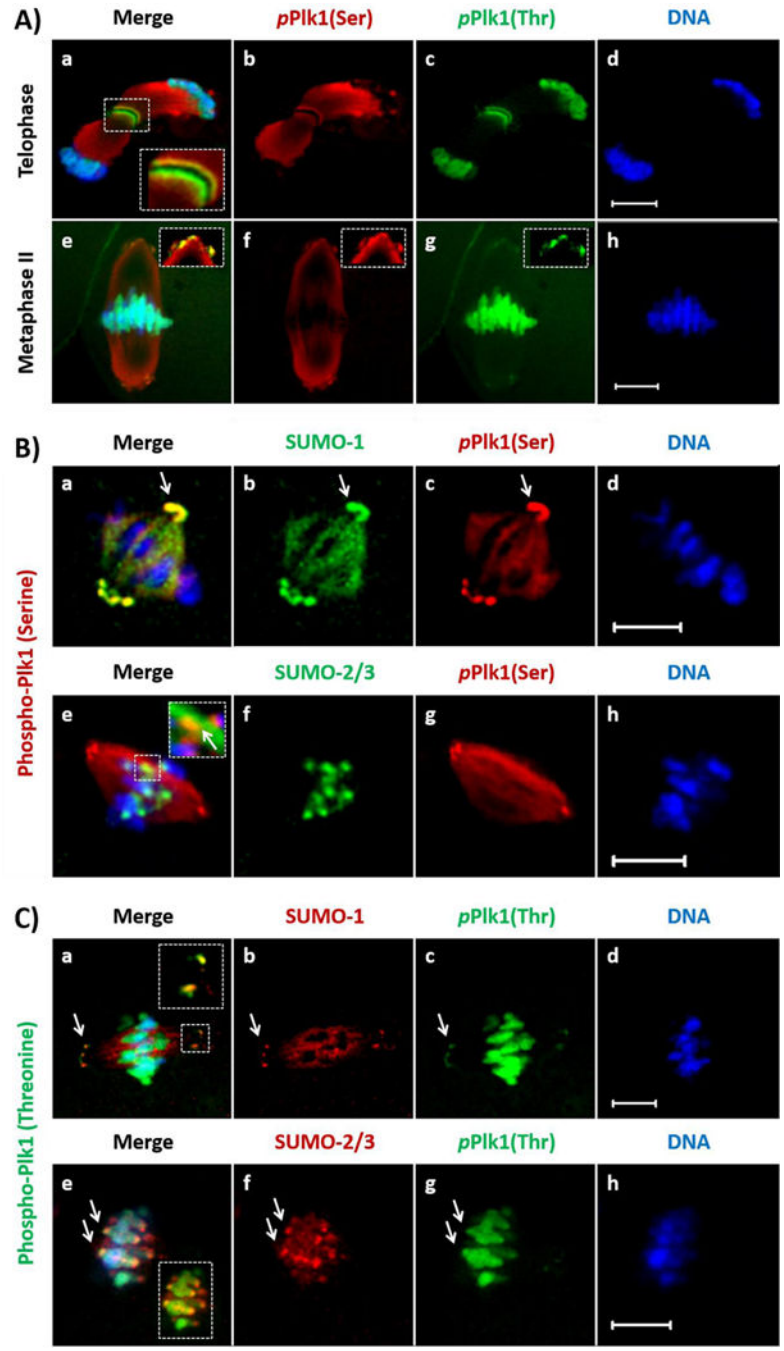

Figure 4. Shared meiotic patterns of phosphorylated-PLK1 kinases and SUMO proteins

Oocytes matured *in vivo* were employed to delineate temporal cellular localizations for phosphorylated (*p*) PLK1 proteins *p*-threonine-PLK1(T210) and *p*-serine-PLK1(S137), SUMO-1 and SUMO-2/3. Subcellular localization was determined using high-resolution bioimaging following immunodetection using phosphorylation-specific antibodies. DAPI was used to counterstain DNA. (A) Oocytes in telophase (a- d) and metaphase II (e- h) were labeled with anti-*p-serine*-PLK1 (b, f), and anti-*p-threonine*-PLK1 (c, g); DNA (d, h). Merged images are shown (a, e). (B) MII mouse oocytes were labeled with anti-SUMO-1

(b), anti-SUMO-2/3 (f) and anti-*p*-serine-PLK1 (c, g) antibodies; DNA (d, h). Merged images are shown (a, e). *p*-serine-PLK1 markedly is coincident with SUMO-1 at the spindle poles (arrows, a–c) and appears coincident over the spindle apparatus. At the centromere, some overlap with SUMO-2/3 is observed (e; inset, arrow). (C) Patterns for SUMO-1 (b), SUMO-2/3 (f) and *p*-threonine-PLK1 (c, g) were evaluated in MII oocytes. DNA (d, h) and the merged images are shown (a, e). Only a modest *p*-threonine-PLK1 signal is detected with SUMO-1 at the spindle pole (arrows, a–c). In contrast, a pronounced signal with SUMO-2/3 is observed at centromeres (arrows, e–g and box, e). Bar, 10μM.

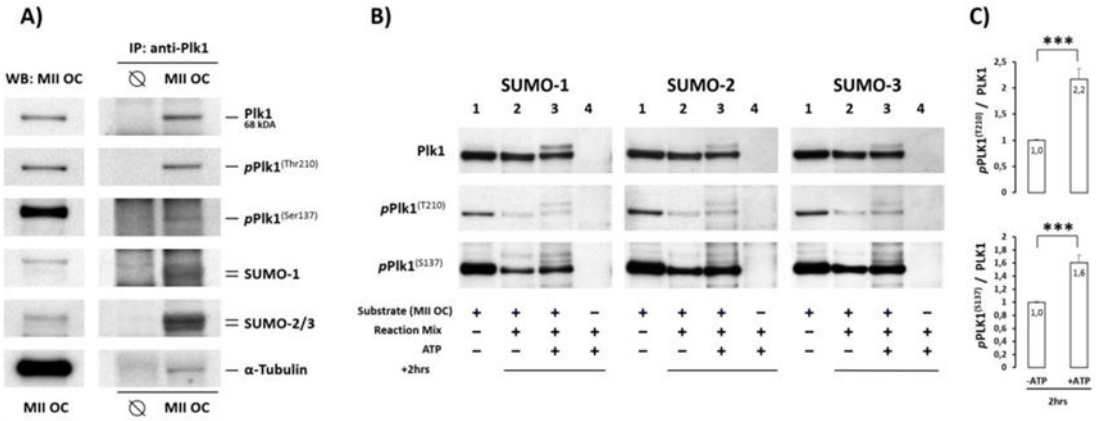

Figure 5. PLK1 and phosphorylated PLK1 kinases are posttranslationally modified by SUMOylation

(A) Proteins extracted from freshly harvested MII oocytes (MII OC) and those subsequently subjected to immunoprecipitation (IP) with anti-PLK1 antibody were simultaneously separated using gel electrophoresis under denaturing conditions and transferred to the same membrane as described. As a control, IP was conducted with the antibody but without MII proteins added (Ø). Sequential immunodetection analyses were performed to determine specific PLK1 posttranslational modifications and associations (see left-sided labels). An illustrative single membrane is shown with sequential hybridizations with particular antibodies. Although a linear range of film exposures were made for each protein, representative signal intensities determined the exposure used for illustration purposes. (B) *In vitro* sumoylation assays specific for SUMO-1, SUMO-2 and SUMO-3 were each performed using the protein extract equivalent from 55-freshly harvested MII oocytes incubated with components of the sumoylation cascade for 2h incubation at 37°C as indicated in Methods. For each SUMO-specific assay, Western analyses are shown for PLK1 forms for the matched "starting material" (Lane 1, substrate, time$_0$), PLK1 forms following a 2h assay with substrate and indicated reaction conditions (Lanes 2, 3) in comparison to the reaction materials without MII OC (Lane 4, non-substrate control). Immunodetection of proteins on the same membranes using sequential hybridizations with the specific anti-PLK1 and phosphorylated (*p*) PLK1 proteins *p*-threonine-PLK1(T210) and *p*-serine-PLK1(S137) antisera for Western analyses is shown on a representative membrane for each. The order of hybridizations for each duplicate experiment was randomized. Immunoblotting with the anti-α-tubulin antisera was negative for modification in the assay (data not shown). (C) Densitometry analyses of serine- or threonine-phosphorylated PLK1 levels following normalization with those of pan-PLK1. Results in the presence or absence of ATP for 2h are illustrated, representing densitometric measurements from either triplicate or duplicate membranes.

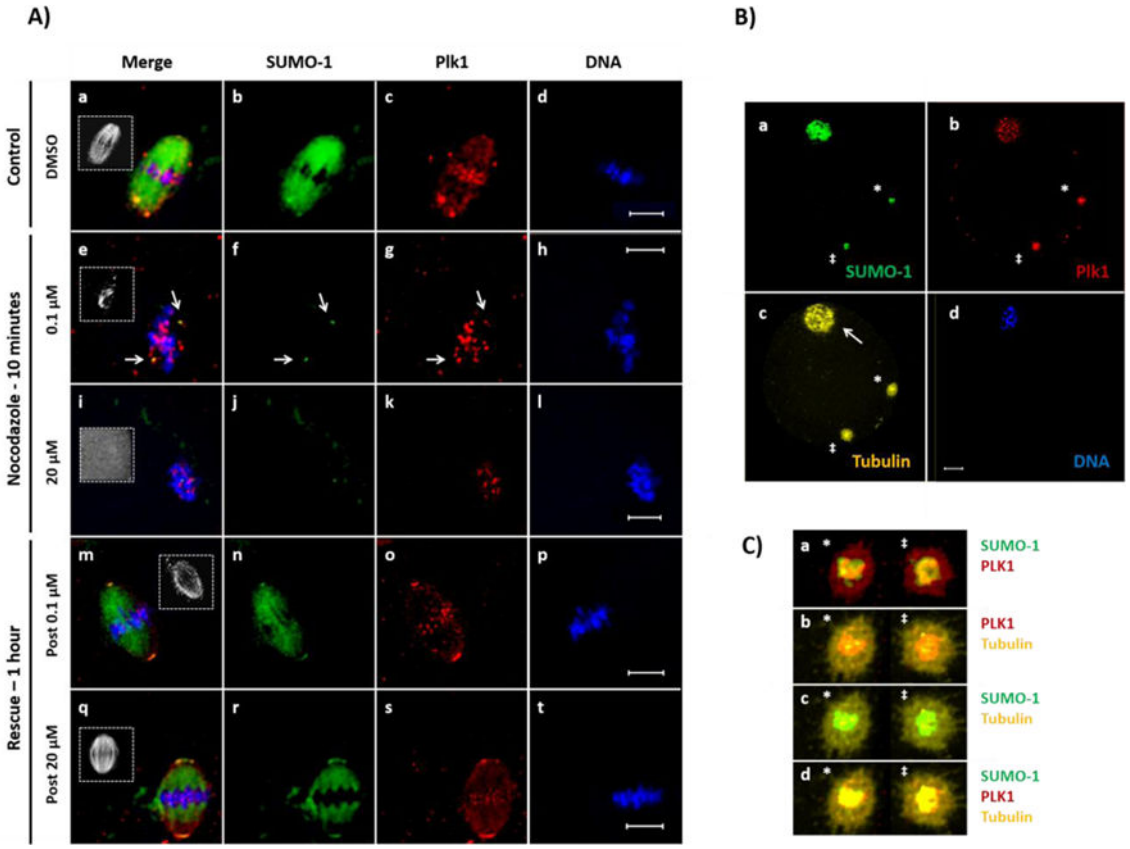

Figure 6. SUMO-1-PLK1 localization depends on spindle integrity and is involved in MTOC organization

MII oocytes matured *in vivo* were treated with microtubule-disturbing drugs followed by immunofluorescence bioimaging using confocal microscopy. DNA was detected using DAPI. (A) Mouse oocytes were cultured with Nocodazole (0.1 or 20μM; 10min), or without (DMSO-matched vehicle controls). Nocodazole-treated or vehicle-matched control MII oocytes were either processed immediately for immuno-bioimaging or rinsed and cultured for an additional 1h to allow microtubule re-assembly (rescue). Oocytes were immunostained using rabbit anti-SUMO-1 (b, f, j, n, r), mouse anti-PLK1 (c, g, k, o, s) and rat anti-α-tubulin (white boxes, a, e, i, m, q) antibodies; DNA (d, h, l, p, t). The merged image is shown (a, e, i, m, q); tubulin signals are not shown to better visualize SUMO-1 localization with PLK1. In vehicle-matched control oocytes, intact spindles are observed (box, a). PLK1 localizes at centromeres and is coincident with SUMO-1 at the spindle poles (a–d). Treatment with 0.1μM Nocodazole partially disassembled microtubules (box, e) and PLK1 localizes both at centromeres and coincident with SUMO-1 at the remaining spindle poles (arrows, e–g). Treatment with 20μM Nocodazole completely disorganized oocyte microtubules and no intact spindles are observed (see absence of any spindle structure, box, i). PLK1 remains at the centromeres but no longer coincident with SUMO-1 (i–l). A 1h rescue after Nocodazole treatment with 0.1μM (m–p) or 20μM (q–t) Nocodazole allowed spindle reorganization and SUMO-1-PLK1 localization at the poles. Bar, 10μM. (B) MII oocytes were treated with Taxol (10μM, 30min) to initiate microtubule organizing centers (MTOC) formation. Oocytes were labeled with specific antibodies for SUMO-1 (a), PLK1

(b), and α-tubulin (c); DNA (d). Treatment induced spindle relaxation (arrow, c) and formation of cytoplasmic microtubule asters (ǂ, asterisk; a, b, c). (C) Representative cytoplasmic aster (merged images, d) shows PLK1 localized with SUMO-1 (a) and each with tubulin (b, c).

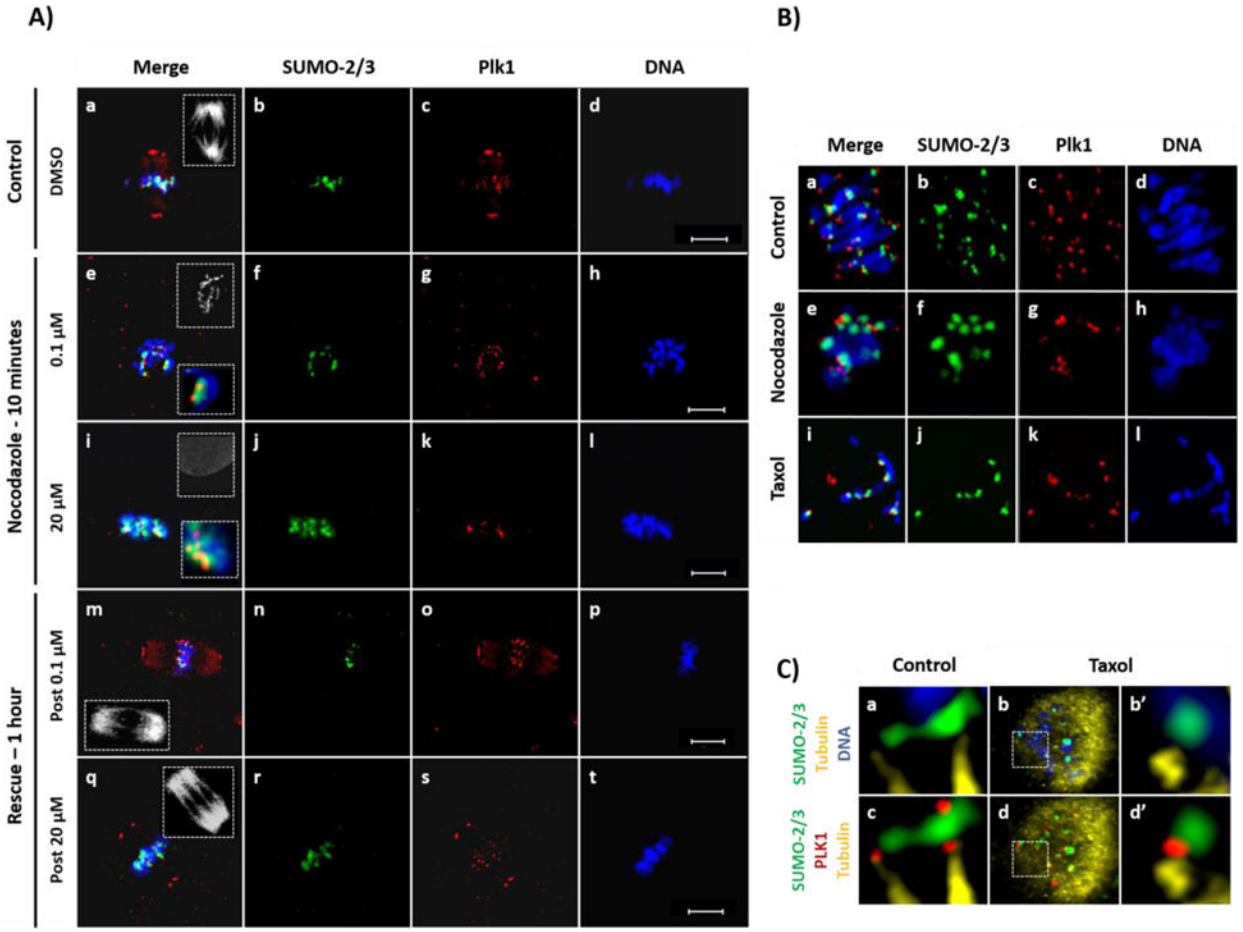

Figure 7. Independent of spindle attachment or tension, centromeric SUMO-2/3 and PLK1 remain coincident

Spindle dependency was evaluated using pharmacological disruptors and immuno-bioimaging. (A) MII mouse oocytes were treated (10min) with Nocodazole (0.1 or 20μM) or without (DMSO-matched vehicle controls) and either processed immediately for immuno-bioimaging or rinsed and cultured for an additional 1h to allow microtubule re-assembly (rescue). Oocytes were analyzed by confocal microscopy after immunolabeling using rabbit anti-SUMO-2/3 (b, f, j, n, r), mouse anti-PLK1 (c, g, k, o, s) and rat anti-α-tubulin (white squares, a, e, i, m, q) antibodies; DNA counterstained with DAPI shown (d, h, l, p, t). The merge for DNA, SUMO-2/3 and PLK1 is shown without tubulin for better visualization of SUMO with PLK1 (a, e, i, m, q). In MII vehicle-matched control oocytes, intact spindles are observed (box, a); PLK1 is coincident with SUMO-2/3 at the centromeres (a–d). Treatment with 0.1μM Nocodazole partially disrupts microtubules (box, e) but at the centromeres PLK1is still coincident with SUMO-2/3 (e–h). 20μM Nocodazole completely disorganizes oocyte microtubules and no intact spindles are observed (box, i). PLK1 remains centromeric with SUMO-1 (i–l). Spindle reorganization was observed following 1h rescue after either 0.1μM (m–p) or 20μM (q–t) Nocodazole. Bar, 10μM. (B) MII oocytes matured *in vivo* were treated with Nocodazole to disrupt the spindle or Taxol to reduce spindle tension. In matched-vehicle treated control oocytes, SUMO-2/3 (b) and PLK1 (c) are observed with overlapping signals at centromeres (a). Treatment with 20μM Nocodazole (10min) does not

alter SUMO-2/3 (f) and PLK1 (g) coincident pattern (e). Similarly, Taxol-treatment of oocytes (10μM, 30min) does not affect SUMO-2/3 (j), PLK1 (k) or their coincident pattern (i) at the centromeric region. (C) Illustration represents a control (a, c) or Taxol-treated (b, d) oocyte and the localization of tubulin with DNA and SUMO-2/3 (a, b, b') and SUMO-2/3 with PLK1 (c, d, d'). White boxes (b, d) enlarged respectively (b' and d'). SUMO-2/3 localization with PLK1 is unaffected by Taxol-induced spindle relaxation.

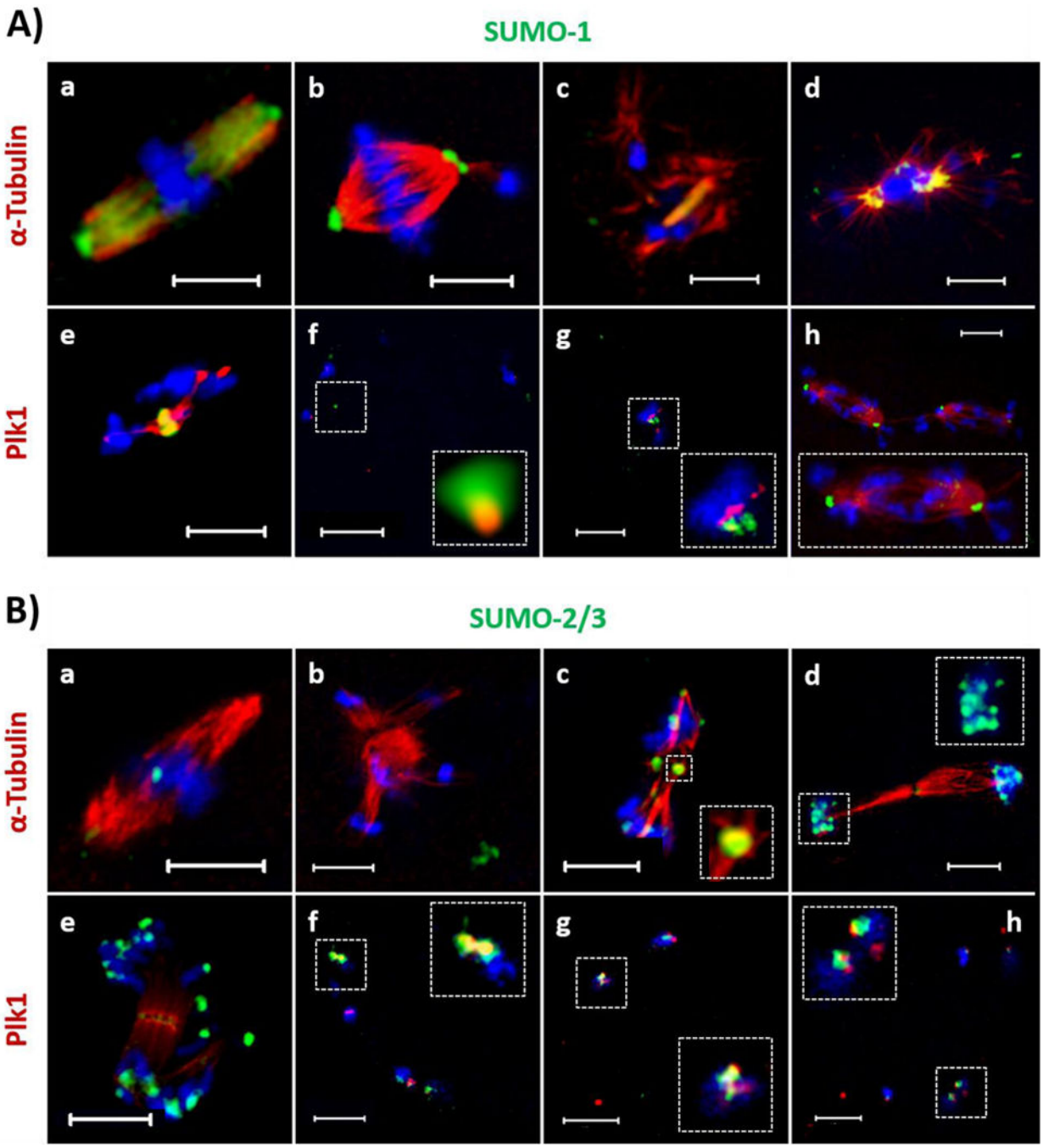

Figure 8. SUMOylation is required for proper spindle organization

MII in vivo-matured oocytes were incubated with 100μM Ginkgolic Acid (GA; 1h), then treated with 20μM Nocodazole (10min). Nocodazole was removed and oocytes rinsed and incubated again with 100μM GA (2h). After treatment with GA or matched-vehicle control, oocytes were analyzed by bioimaging analysis as described previously. Rabbit anti-SUMO-1 (A, a–h) SUMO-2/3 (B, a–h), rat anti-α-tubulin (A, B; a–d) and mouse anti-PLK1 (A, B; e–h) antibodies were used for immunodetection; DNA counterstained by DAPI. GA inhibition of SUMOylation prevents proper spindle organization after Nocodazole (A, B). In

(A), very few oocytes show typical SUMO-1 at spindle poles (a, b), with most demonstrating no localization or scattered signals on the disorganized spindle (c, d). Following GA treatment, aberrant SUMO-1 localization with PLK1 is observed (e–h). In (B), most GA-treated oocytes are not able to reorganize their spindle properly and abnormal patterns for SUMO-2/3 are observed at the chromatin (a–d). In contrast, even with chromosomes spread in the cytoplasm after GA, SUMO-2/3 is coincident with PLK1 and still associated with DNA.