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INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR 1, A WIDESPREAD Ca^{2+}CHANNEL, IS A NOVEL SUBSTRATE OF POLO-LIKE KINASE 1 IN EGGS

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Abstract

To initiate embryo development, the sperm induces in the egg release of intracellular calcium ($[Ca^{2+}]_i$). During oocyte maturation, the inositol-1,4,5-trisphosphate receptor (IP_3R1), the channel implicated, undergoes modifications that enhance its function. We found that IP_3R1 becomes phosphorylated during maturation at an MPM-2 epitope and that this persists until the fertilization-associated $[Ca^{2+}]_i$ responses cease. We also reported that maturation without ERK activity diminishes IP_3R1 MPM-2 reactivity and $[Ca^{2+}]_i$ responses. Here, we show that IP_3R1 is a novel target for Polo-like kinase1 (Plk1), a conserved M-phase kinase, which phosphorylates it at an MPM-2 epitope. Plk1 and IP_3R1 interact in an M-phase preferential manner, and they exhibit close co-localization in the spindle/spindle poles area. This co-localization is reduced in the absence of ERK activity, as the ERK pathway regulates spindle organization and IP_3R1 cortical re-distribution. We propose that IP_3R1 phosphorylation by Plk1, and possibly by other M-phase kinases, underlies the delivery of spatially and temporally regulated $[Ca^{2+}]_i$ signals during meiosis/mitosis and cytokinesis.

Keywords

Calcium; mammalian eggs; ERK; inositol 1,4,5-trisphosphate receptor; phosphorylation; Polo

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INTRODUCTION

The activation of the egg is the first stage in the initiation of embryo development. It comprises a series of events that unfold soon after interaction of the gametes and that ends with the completion of meiosis and progression into the mitotic cell cycles (Ducibella et al., 2002; Schultz and Kopf, 1995). In all species studied to date, egg activation requires a fertilization-associated increase in the intracellular concentration of calcium ($[Ca^{2+}]_i$) (Stricker, 1999). In mammals, the fertilizing $[Ca^{2+}]_i$ signal consist of periodical rises, which are referred to as $[Ca^{2+}]_i$ oscillations (Miyazaki et al., 1993).

The type 1 inositol 1,4,5-trisphosphate receptor (IP_3R1) in mammals, or its homologue in other species, is responsible for the majority of Ca^{2+} release during fertilization (Miyazaki et al., 1993). IP_3R1, the most widely expressed isoform (Taylor et al., 1999), is distributed along the membrane of the endoplasmic reticulum (ER), the main Ca^{2+} reservoir in the cell (reviewed in Berridge, 2002). Production of inositol 1,4,5-trisphosphate (IP_3), the IP_3R1 ligand, is detected during fertilization (Stith et al., 1993), and entails hydrolysis of phosphatidylinostitol (4,5)-bisphosphate by the action of a phospholipase C (PLC; Rebecchi and Pentyala, 2000). During mammalian fertilization, a sperm-specific PLC isoform, PLCζ (Saunders et al., 2002), is reportedly responsible for IP_3 production.

During oocyte maturation, the capacity of IP_3R1s to mediate Ca^{2+} release and the oocytes' ability to mount $[Ca^{2+}]_i$ oscillations are enhanced (Terasaki et al., 2001; Jones et al., 1995a). For instance, prior to maturation, at the germinal vesicle (GV) stage, oocytes display low amplitude spontaneous $[Ca^{2+}]_i$ rises (Jones et al., 1995a), and show dampened responses to IP_3 (Fujiwara et al., 1993). Nonetheless, IP_3R1 sensitivity, which we define as the receptor's ability to conduct Ca^{2+} in response to IP_3, incrementally increases and becomes maximal by the time of fertilization (Fujiwara et al., 1993), which in mammals and in *Xenopus* eggs takes place at the metaphase of meiosis II (MII). Therefore, in eggs, maximal IP_3R1 sensitivity and maximal ability to mount $[Ca^{2+}]_i$ oscillations coexist. Fittingly, after fertilization and with progression into interphase both of these properties decline, suggesting an association between IP_3R1-mediated $[Ca^{2+}]_i$ oscillations and the M-phase stages of meiosis (Jellerette et al., 2004; Jones et al., 1995b).

Several subcellular events that develop simultaneously during maturation, such as an increased Ca^{2+} reservoir (Mehlmann and Kline, 1994; Tombes et al., 1992), a redistribution of the ER and IP_3R1 (reviewed in Stricker, 2006; Shiraishi et al., 1995; Kume et al., 1997), and an increase in IP_3R1 concentration and sensitivity (Fissore et al., 1999; Mehlmann et al., 1996) could explain the enhanced function of IP_3R1 in eggs. Furthermore, IP_3R1 becomes phosphorylated during maturation (Lee et al., 2006). Research shows that phosphorylation of IP_3R1 mostly enhances its Ca^{2+} release (reviewed in Patterson et al., 2004), and that it can be phosphorylated by numerous kinases, including protein kinase A and protein kinase C (Vermassen et al., 2004; DeSouza et al., 2002), protein kinase G (Koga et al., 1994), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Ferris et al., 1991), the tyrosine kinases Fyn (Jayaraman et al., 1996) and Lyn (Yokoyama et al., 2002), Rho kinase (Singleton and Bourguignon, 2002) and protein kinase B (Khan et al., 2006). The aforementioned kinases do not display association with the cell cycle and are therefore unlikely to enhance IP_3R1 function in MII eggs in a cell cycle-dependent manner. Recent studies on IP_3R1, however, have identified phosphorylation consensus sites for Cyclin Dependent Kinase 1 (Cdk1), also known as Maturation Promoting Factor (MPF), and for Extracellular Signal-Regulated Kinase (ERK), also known as Mitogen Activated Protein Kinase (MAPK), both of which are pivotal regulators of oocyte maturation (reviewed in Masui, 2001). *In vivo* and *in vitro* studies have shown that Cdk1 (Malathi et al., 2003) and ERK (Lee et al., 2006; Bai et al., 2006), phosphorylate several of these conserved

motifs, although their role in IP_3R1 phosphorylation during fertilization remains to be demonstrated.

In a previous study (Lee et al., 2006), we used the MPM-2 antibody, which specifically recognizes M-phase phosphoproteins with phosphorylated serine (Ser)/threonine (Thr) next to proline (Pro) (Westendorf et al., 1994; Davis et al., 1983), the basic phosphorylation motif of Cdk and ERK kinases, to examine the possible involvement of M-phase kinases on IP_3R1 phosphorylation in oocytes/eggs. We found that IP_3R1 first becomes MPM-2 phosphorylated at the time of meiosis resumption, the germinal vesicle breakdown stage (GVBD), and remains phosphorylated at the MII stage. Following fertilization, MPM-2 IP_3R1 phosphorylation decreases in a protracted manner, later than the decline in Cdk1 activity but in parallel with the decline in ERK activity. Consistent with this, inhibition of the ERK pathway during maturation reduced MPM-2 IP_3R1 phosphorylation in MII eggs as well as IP_3R1-mediated $[Ca^{2+}]_i$ oscillations (Lee et al., 2006). Although these results suggested a role for the ERK pathway in IP_3R1 MPM-2 phosphorylation in eggs, whether ERK is required for the initial phosphorylation and whether it directly phosphorylates the IP_3R1 MPM-2 epitope was not elucidated.

Another MPM-2-epitope generating kinase that is activated at the time of meiosis resumption (reviewed in Liu and Maller, 2005b) is Polo-like kinase-1 (Plk1) (do Carmo Avides et al., 2001; Kumagai and Dunphy, 1996). Polo function was first identified in *Drosophila melanogaster* larvae that showed cellular abnormalities in the organization of the spindle and spindle poles (Sunkel and Glover, 1988). Since then, research shows that Plk1 and its orthologs are serine/threonine kinases that play crucial roles in almost every phase of mitosis and cytokinesis (Lowery et al., 2007; reviewed in Barr et al., 2004). Of relevance to our studies is that Plk1 becomes activated at the onset of oocyte maturation, the GVBD stage, and remains active throughout maturation (Okano-Uchida et al., 2003; Pahlavan et al., 2000; Qian et al., 1998). Moreover, after fertilization, Plk1 becomes de-phosphorylated/inactivated well after extrusion of the second polar body (Pahlavan et al., 2000), which mirrors the profile of IP_3R1 MPM-2 reactivity during maturation and after fertilization (Lee et al., 2006). Collectively, these findings raise the prospect that Plk1 may be involved in MPM-2 IP_3R1 phosphorylation in eggs. In this study, therefore, our goal was to unravel those kinases commonly associated with oocyte maturation that are responsible for IP_3R1 MPM-2 phosphorylation, and elucidate the mechanism by which the ERK pathway regulates this phosphorylation. Our results show that IP_3R1 is a novel and hitherto unknown substrate of Plk1, and that the IP_3R1 MPM-2 epitope phosphorylation by Plk1 in MII eggs is regulated by the ERK pathway, which controls IP_3R1 redistribution and affects Plk1 spindle localization during oocyte maturation.

MATERIALS AND METHODS

Collection of oocytes/eggs and culture conditions

GV and MII eggs were collected from the ovaries and oviducts of 5 to 8 weeks-old CD-1 female mice, respectively, as previously described (Lee et al., 2006). GV oocytes were recovered in HEPES-buffered Tyrode-Lactate solution (TL-HEPES) supplemented with 5 % heat-treated fetal calf serum (FCS; Gibco, Grand Island, NY) and 100 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma, St. Louis, MO; all chemicals from Sigma unless otherwise specified). Oocytes were matured *in vitro* for 12–16 hr in a humidified atmosphere containing 6% CO_2 in Chatot, Ziomek, and Bavister (CZB) medium (Chatot et al., 1990) containing 3 mg/ml polyvinyl alcohol (PVA) at 36.5°C. MII eggs were recovered in TL-HEPES and, after removal of cumulus cells, eggs were transferred into 50 μl drops of KSOM (Potassium Simplex Optimized Medium; Specialty Media, Phillipsburg, NJ) and cultured as above. Animal handling and procedures were approved by the University's IACUC committee.

Microinjection procedures and preparation of Plk1 mRNA

Oocytes and eggs were microinjected as previously described (Lee et al., 2006). Reagents were diluted in injection buffer [100 mM KCl and 10 mM HEPES (pH= 7.0)], loaded into glass micropipettes by aspiration and delivered into the ooplasm by pneumatic pressure (PLI-100 picoinjector, Harvard Apparatus, Cambridge, MA); each egg received ~3–10 pl (1–3% of the total volume of the egg). Mouse Plk1 cDNA (Accession No. 18817) from mouse testis was amplified by PCR using previously reported primers (Clay et al., 1993) and cloned into the pCS^{2+} vector (Turner and Weintraub, 1994). A constitutively active (CA) form of the kinase was generated by substituting threonine (T) 210 by aspartate (D) (Jang et al., 2002) using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA); a construct encoding only the polo box domain of Plk1 was produced as described by the same authors and cloned into pCS^{2+}. The sequence encoding for Venus (a gift from Dr. Miyawaki, Riken, Japan to Dr. J. Ito), a yellow fluorescent protein variant (Nagai et al., 2002), was also subcloned into pCS^{2+}. cDNAs were *in vitro* transcribed using the mMESSAGE mMACHINE capping Kit (Ambion, Austin, TX) and generated mRNAs handled as previously reported (Lee et al., 2006).

Antibodies

Two polyclonal antibodies raised against the same peptide sequence on the C-terminal end of the molecule were used to detect IP_3R1. Rbt03 (Parys et al., 1995) was used for western blotting (WB) whereas CT1 (Wojcikiewicz et al., 1994), which was affinity purified, was used for immunolocalization studies. The MPM-2 monoclonal antibody (Upstate, Lake Placid, NY) was used to ascertain IP_3R1 phosphorylation as previously reported (Jellerette et al., 2004). Detection of total and phosphorylated forms of Plk1 was accomplished using a cocktail of mouse monoclonal antibodies against mPlk1 (Zymed, San Francisco, CA) and an anti-phospho (T210) Plk1 polyclonal antibody (Rockland Immunochemicals, Gilbertsville, PA), respectively.

Immunoblotting

40 to 50 mouse oocytes/eggs or cell lysates from 0.5 to 6.0 *Xenopus* eggs were mixed with 2xLaemmli sample buffer (2xSB) (Laemmli, 1970), boiled and loaded onto NuPAGE Novex 3–8% Tris-Acetate gels (Invitrogen, Carlsbad, CA), or onto 7.5% gels made with 120:1 acrylamide:bisacrylamide mixture (Hamanaka et al., 1995). After electrophoresis, proteins were transferred onto nitrocellulose membranes (Micron Separations, Westboro, MA) and successive probing of both MPM-2/IP_3R1 and phospho-Plk1/Plk1 was performed as described by our laboratory (Lee et al., 2006). Species-specific horseradish peroxidase-labeled secondary antibodies and chemiluminescence (NEN Life Science Products, Boston, MA) were used according to the manufacturer's instructions. Each nitrocellulose membrane was digitally captured and quantified using an imaging system (Kodak Imaging Station 440 CF, Rochester, NY); quantification was performed on the TIFF files before any rendering was carried out and was performed only between lanes from the same blot membrane. The relative intensities of MPM-2 and phospho-Plk1 immunoreactive bands and of phosphorylated substrate bands in kinase assays were plotted using Sigma Plot (Jandel Scientific Software, San Rafael, CA) using MII eggs as the normalizing value. Figures were prepared using ImageJ software (NIH; http://rsb.info.nih.gov/ij/) and Microsoft PowerPoint. To improve the order of presentation, some lane(s) within the same membrane were cut and pasted and two short parallel lines denote this action.

Immunofluorescence

Oocytes/eggs were prepared for immunofluorescence (IF) by removing the zona pellucida (acid Tyrode solution (pH= 2.7)) followed by washes in 0.1% BSA supplemented Dulbecco's PBS (DPBS-BSA). Cells were fixed in 3.7% paraformaldehyde + 0.02% Triton X-100,

permeabilized with 0.1% Triton X-100 DPBS-BSA, and blocked in 5% normal goat serum-DPBS. Primary antibodies, mPlk1 antibodies and the CT1 antibody were incubated overnight at 4°C. Secondary antibodies were Alexa fluor 488 goat anti-mouse IgG and Alexa fluor 555 goat anti-rabbit IgG (Molecular Probes, Eugene, OR), respectively, and were incubated for 1hr at room temperature (RT). Fixed specimens were mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). Slides were examined at RT with a confocal laser-scanning microscope (510 META, Carl Zeiss Microimaging, Inc., Germany) using an Axiovert 2 microscope outfitted with a 63x 1.4 NA oil immersion objective lens. Z-stack images were collected from cortical to equatorial planes every 2 to 5 μm and exported as TIFF files using Zeiss software, and TIFF files were processed using Photoshop (Adobe) and assembled in PowerPoint (Microsoft). Negative control eggs were incubated without the primary antibody but with both secondary antibodies, or with the primary antibody (CT1) pre-treated with the antigenic peptide (10 μg/ml).

Preparation of pharmacological inhibitors

Wortmannin (WTMN), a commonly used PI 3-kinase inhibitor was used to inhibit Plk1 (Liu et al., 2005; Liu et al., 2007). Wortmannin was dissolved in DMSO and further diluted in CZB-PVA. Wortmannin-supplemented medium was replaced every 4 hr (Kimura et al., 1994). U0126 (Calbiochem, San Diego, CA), a MEK-specific inhibitor, was prepared in DMSO and used at 25 μM; the inactive analog U0124 was used as a negative control. Doxorubicin (DXR) was diluted in H_2O and used at 1 μM in CZB-PVA (Jurisicova et al., 2006).

Histone 1 (H1) and myelin basic protein (MBP) kinase assays and kinase inhibitors

The activities of Cdk1 and ERK were assayed using lysates from 5 oocytes as described previously (Lee et al., 2006). Proteins were separated on 15% SDS-polyacrylamide gels, and H1 and MBP phosphorylations visualized by autoradiography. Autoradiographs were scanned and quantified as described for WB.

Preparation of *Xenopus* eggs/zygotes lysates and immunoprecipitation

Xenopus eggs were collected from mature females and after *in vitro* fertilization, as per standard protocols. For IP experiments, groups of 25 eggs were frozen on dry ice and solubilized with 500 μl cold embryo solubilization buffer containing 1.0% Triton X-100 (Cousin et al., 2000). After pelleting cellular debris, supernatants were incubated overnight at 4°C with non-immune serum, Rbt03 antibody, MPM-2 antibody or Plk1 antibody with head-over-head rotation. Incubation of protein A sepharose beads (Amersham) with immunocomplexes occurred for an additional 3 hr before several washes with PBS. Samples were denatured by addition of 2xSB and stored at −80°C until WB was performed.

Statistical analysis

Values from three or more experiments performed on different batches of oocytes, eggs or zygotes were used for evaluation of statistical significance. Statistical comparisons of band intensities after WB and kinase assays were performed using the Student's t-test or one-way ANOVA and, if differences were found between groups, they were resolved using the Tukey/Kramer test using the JMP-IN software (SAS Institute, Cary, NC). Differences were considered to be significant when $P<0.05$. Significance among groups/treatments is denoted in bar graphs by different superscripts (WB) or by the presence of one or two asterisks (kinase assays).

RESULTS

The ERK pathway is not required for the initial IP_3R1 MPM-2 epitope(s) phosphorylation during oocyte maturation

In a previous study we found that the IP_3R1 MPM-2 phosphorylation during oocyte maturation was greatly diminished when oocytes were matured to the MII stage in the presence of the MEK inhibitor U0126 (Lee et al., 2006); these results implicate the ERK pathway in IP_3R1 phosphorylation at the MPM-2 epitope(s). To determine whether the ERK pathway is required for IP_3R1 MPM-2 phosphorylation at earlier stages, we cultured oocytes in the presence or absence of U0126 and collected samples for WB 8 hr after the initiation of maturation, the time at which oocytes had reached the metaphase stage of meiosis I (MI). Oocytes at the GV stage were devoid of IP_3R1 MPM-2 reactivity and, as they matured, they became phosphorylated regardless of the presence of U0126 (Fig. 1A, upper panel), suggesting that the ERK pathway is not required for the initial MPM-2 IP_3R1 phosphorylation; probing for IP_3R1 after stripping of the membranes showed that receptor concentrations during this period remained stable (Fig. 1A., lower panel). Besides confirming that U0126 prevented ERK activity in these oocytes (data not shown), oocytes were also matured to the MII stage in the presence of U0126, replicating our previous experiments (Lee et al., 2006). Not unexpectedly, this procedure greatly decreased IP_3R1 MPM-2 reactivity in MII eggs (Fig. 1B, upper panel) without affecting IP_3R1 concentrations (Fig. 1B, lower panel); this last experiment was performed in the presence of colcemid to avoid the exit of metaphase stage that is known to occur in oocytes in which the ERK pathway is suppressed during maturation (Araki et al., 1996). Together, these results show that the ERK pathway is required to maintain IP_3R1 MPM-2 phosphorylation during the MI to MII transition, but it is not necessary earlier during maturation to establish IP_3R1 phosphorylation at the MPM-2 epitope(s).

To gain insight into the kinase responsible for IP_3R1 MPM-2 phosphorylation, we investigated when this phosphorylation first occurred during oocyte maturation. The first noticeable IP_3R1 MPM-2 immunoreactivity occurred 1 hr after initiation of maturation, continued to increase until the 2^{nd} hr, the time at which all oocytes had undergone GVBD, and reached maximal levels by 3 hr (Fig. 1C; upper panel), all this time ERK activity remains basal (Verlhac et al., 1994) and the levels of IP_3R1 remained unchanged (Fig. 1C; lower panel). The results therefore show that IP_3R1 MPM-2 phosphorylation is set early in oocyte maturation prior to ERK activation.

IP_3R1 MPM-2 phosphorylation during oocyte maturation coincides with activation of Plk1

The early appearance of IP_3R1 MPM-2 reactivity in oocytes leads to the expectation that the responsible kinase(s) must play a role early in oocyte maturation. Plk1 has been implicated in the G2-M transition in oocytes by virtue of phosphorylating and activating Cdc25C (Qian et al., 1998; Kumagai and Dunphy, 1996), the phosphatase responsible for Cdk1 activation, which in turn orchestrates the initiation of oocyte maturation (Nebreda and Ferby, 2000). Further, Plk1, or its *Xenopus* homologue Plx1, has been shown to generate MPM-2 reactive epitopes on target proteins (do Carmo Avides et al., 2001; Kumagai and Dunphy, 1996). To ascertain Plk1 activity in oocytes and zygotes, we evaluated two properties of Plk1 that correspond with its activation. First, because Plk1 activation coincides with its phosphorylation (Mundt et al., 1997), which in turn retards its migration during SDS-polyacrylamide gel electrophoresis (Hamanaka et al., 1995), we examined whether or not Plk1 undergoes mobility shifts during oocyte maturation and after egg activation. Second, specific phosphorylation on Thr210 of Plk1 in mammals, or the equivalent Thr201 residue of Plx1 in *Xenopus*, is indispensable for activation and i*n vitro* kinase activity of Plk1 (Jang et al., 2002; Qian et al., 1999); we therefore analyzed phosphorylation at this residue using an anti-phospho (Thr210) Plk1 antibody. By either method, Plk1 appeared dephosphorylated and therefore inactive at the GV stage, but

showed initial signs of phosphorylation by 1 hr of maturation and became progressively more phosphorylated for the next 3 hr of culture (Fig. 2A). Thereafter, Plk1 remained phosphorylated until the MII stage (Fig. 2B, left and center panels), although it underwent dephosphorylation after exposure to $SrCl_2$, which induced egg activation and PN formation (Fig. 2B, right panel). Our results therefore show that the profiles of Plk1 activity and IP_3R1 MPM-2 reactivity in oocytes and zygotes closely correspond.

To more precisely determine whether IP_3R1 MPM-2 epitope phosphorylation and Plk1 phosphorylation/activity temporally overlap, we simultaneously examined IP_3R1 MPM-2 reactivity and Thr210 Plk1 phosphorylation in the same groups of oocytes, eggs and zygotes. GV stage oocytes were devoid of both MPM-2 reactivity and phosphorylated Plk1 although, by the time of GVBD and through the MI and MII stages, high IP_3R1 MPM-2 reactivity was accompanied by increased levels of phospho-Plk1 reactivity (Fig. 2C); neither protein showed alteration in total concentrations (Fig. 2C). Following egg activation, both IP_3R1 MPM-2 and phospho-Plk1 reactivity decreased until the time of PN formation (Fig. 2D), although, while MPM-2 IP_3R1 reactivity remained low at mitosis I, phospho-Plk1 reactivity rebounded (Fig. 2D). Collectively, these results show that throughout meiosis and the early zygote stage, IP_3R1 MPM-2 reactivity corresponds with Plk1 phosphorylation/activity suggesting a role for Plk1 in IP_3R1 MPM-2 phosphorylation.

IP_3R1 MPM-2 phosphorylation occurs in oocytes matured in the absence of CDK1 activity

Cdk1/MPF activity also operates early during oocyte maturation (reviewed in Masui, 2001). Thus, to discern the possible impact of this kinase on IP_3R1 MPM-2 reactivity, we induced oocyte maturation while precluding Cdk1/MPF activity. Accordingly, oocytes were incubated with IBMX, a phosphodiesterase inhibitor that maintains the GV arrest, and treated for 1 hr with okadaic acid (OA), a phosphatase inhibitor; it is known that under these conditions oocytes undergo GVBD in the presence of negligible levels of Cdk1 activity (Phillips et al., 2002; de Vantery Arrighi et al., 2000). Our results indeed show that IBMX-imposed GV arrest can be relieved by OA treatment (Fig. 3A), and that resumption of meiosis is accomplished in the absence of Cdk1 activity as evidenced by the lack of H1 phosphorylation (Fig. 3B). Even under these conditions, IP_3R1s showed near normal levels of MPM-2 reactivity (Fig. 3C, left panel), suggesting that a kinase other than Cdk1 is responsible for this phosphorylation. While ERK undergoes robust and premature activation in IBMX-OA-treated oocytes (Fig. 3B), given our previous results, it is unlikely that it would be responsible for the IP_3R1 MPM-2 reactivity. Moreover, treatment of somatic cells with OA has been shown to lead to Plk1 activation in an ERK pathway-dependent manner (Liu et al., 2004a). Consistent with this observation, IBMX-OA-treated oocytes showed maximal Plk1 activation (Fig. 3C, right panel). Together, our results show that Cdk1 is not responsible for the IP_3R1 MPM-2 phosphorylation in oocytes and that, instead, Plk1 activity (as indicated by the p-Plk1 levels) seems more intimately associated with this phosphorylation.

Pharmacological inhibition of Plk1 activation precludes IP_3R1 MPM-2 phosphorylation

Two pharmacological inhibitors were used to ascertain whether IP_3R1 MPM-2 phosphorylation requires active Plk1. The first inhibitor tested was wortmannin, a well-known and broadly used phosphatidylinositol 3-kinase (PI3K) inhibitor (Cross et al., 1995). Research shows that at the concentrations routinely used to block PI3K in somatic cells, wortmannin also efficiently inhibits Plk1 activity (Liu et al., 2005). We therefore examined whether wortmannin was effective at blocking Plk1 activity in oocytes. First, a concentration-dependent study was performed to investigate the effects of wortmannin on meiotic progression. Wortmannin delayed initiation of GVBD (Fig. 4A) and progression to the MII stage in a concentration-dependent manner (Fig. 4B), although 0.1 and 1 μM wortmannin did not affect the total number of oocytes completing maturation (Fig. 4B); 10 μM wortmannin affected both

progression to GVBD and MII and, therefore, it was not used further (Figs. 4A, B). Consistent with the effect on meiotic progression, *in vitro* kinase assays revealed that 1 μM wortmannin delayed activation of Cdk1 and ERK, although both kinase activities fully recovered by the time oocytes had reached the MII stage (data not shown). Remarkably, under similar conditions, wortmannin prevented phosphorylation and activation of Plk1, and the acquisition of MPM-2 reactivity by IP_3R1 (Fig. 4C). As the PI3K/PKB pathway plays a role in meiotic progression (Hoshino et al., 2004) the latter effects of wortmannin could in theory be attributed to its inhibition of PI3K activity. Nevertheless, this is unlikely given that during oocyte maturation PKB activation is transient (Kalous et al., 2006), and that its phosphorylation consensus motif (Lawlor and Alessi, 2001) differs greatly from the MPM-2 epitope.

The second inhibitor used was the anti-tumor antibiotic doxorubicin, which induces DNA damage and triggers the DNA-damage checkpoint in somatic cells, reportedly inactivating and dephosphorylating Plk1 (Jang et al., 2007). We therefore examined the effect of 1μM doxorubicin on maturation-associated IP_3R1 MPM-2 phosphorylation. Although doxorubicin -treated oocytes resumed and progressed through meiosis, doxorubicin inhibited IP_3R1 MPM-2 phosphorylation as well as the occurrence of phospho-Plk1 at all of the time points examined (Fig. 4D). Together, the data provide evidence that active Plk1 is required for IP_3R1 MPM-2 phosphorylation during oocyte maturation.

Molecular manipulations of Plk1 influence IP_3R1 MPM-2 phosphorylation

Plk1, like other family members, consist of a canonical Ser/Thr kinase domain at the N terminus and two signature motifs, the polo boxes, at the C terminus, which form the polo box domain (PBD) (reviewed in Barr et al., 2004; Kumagai and Dunphy, 1996) (Fig. 5A). The PBD has been implicated in the regulation of the catalytic activity of the enzyme as well as in the targeting of Plks to specific cellular structures (Lee et al., 1998). Recent research showed that the PBD acts as a phosphopeptide-binding motif (Elia et al., 2003a) recognizing “primed phosphoepitopes” on Plk1 target proteins. It stands to reason that disruption of PBD function should interfere with the progression/completion of Plk1-controlled cellular events. Consistent with this notion, over-expression of a kinase dead Plx1 protein delayed activation of Cdc25C and initiation of GVBD in *Xenopus* oocytes (Liu et al., 2004b). Accordingly, we over-expressed in mouse oocytes a PBD-Plk1 mRNA to compete with endogenous Plk1 (PBD-Plk1; Fig. 5A). We found that while injection of PBD-Plk1 mRNA markedly delays GVBD, it does not affect the percent of oocytes that are able to complete this process (Fig. 5B). More specifically, we observed that while ~40% of buffer control injected oocytes or of the oocytes injected with mRNA encoding for the Venus fluorescent protein had undergone GVBD by 1.5 hr of maturation and nearly all oocytes had undergone GVBD by 2.5 hr, only 5% and 50%, respectively, of PBD-Plk1 mRNA injected oocytes had completed GVBD at those times of maturation (Fig. 5B). Injection of PBD-Plk1 mRNA also greatly prevents IP_3R1 MPM-2 phosphorylation by 2 hr of maturation (Fig. 5C, left panel), although it did not affect the levels of phospho-Plk1 (Fig. 5C, right panel) or the total amounts of IP_3R1 and Plk1 proteins (Fig. 5C, lower panels).

To further establish the role of Plk1 on IP_3R1 phosphorylation, we over-expressed the constitutively active (CA) form of Plk1, CA(T210D)-Plk1 (Fig. 5A) (Jang et al., 2002). Previously, injection of a similar construct into *Xenopus* oocytes induced premature activation of Cdc25C and GVBD (Qian et al., 1999). Similarly, injection of the CA(T210D)-Plk1 mRNA into mouse oocytes evoked premature GVBD (Fig. 6A). We next examined whether CA (T210D)-Plk1 mRNA injection induced IP_3R1 MPM-2 epitope phosphorylation. To circumvent the confounding effect of phosphorylation by endogenous kinases, all oocytes were kept in IBMX-supplemented media such that GVBD and activation of Cdk1 and ERK activities were precluded (de Vantery Arrighi et al., 2000). In the presence of IBMX, all groups remained

at the GV stage (Fig. 6B). Nonetheless, despite the arrest at the GV stage, CA(T210D)-Plk1-injected oocytes showed significant IP_3R1 MPM-2 reactivity, which was not the case for buffer injected oocytes (Fig. 6B, left panel). Injection of CA(T210D)-Plk1 mRNA did not alter the expression of IP_3R1 or phospho-Plk1, although it increased the total amount of Plk1, demonstrating that the CA(T210D)-Plk1 mRNA underwent *in vivo* translation in the oocyte (Fig. 6B, right panel). Collectively, these data suggest that manipulation of Plk1 activity influences IP_3R1 MPM-2 phosphorylation, directly implicating this kinase in IP_3R1 phosphorylation.

IP_3R1 and Plx1 interact in a cell cycle-stage preferential manner in Xenopus eggs/zygotes

We next performed immunoprecipitation (IP) studies to investigate whether Plk1 and IP_3R1 interact. For these studies we chose *Xenopus* egg extracts because of the large availability of material in this species, and because in *Xenopus* eggs Plx1 also becomes dephosphorylated/inactivated after fertilization and transition into interphase (Descombes and Nigg, 1998) (Fig. 7A). IP of *Xenopus* MII egg extracts using an anti-IP_3R1 antibody brought down Plx1 (Fig. 7B, left part), demonstrating direct association of these two molecules; IP of the same extracts using non-immune serum (far left lane) or with beads alone (far right lane) failed to do so. We next examined whether this interaction occurred in a cell cycle-preferential manner. We found that association of IP_3R1 and Plx1 was higher at the MII stage than at the interphase stage (Fig. 7B, bar graphs), which is similar to the preferential association between Plk1 and other substrates during M-phase stages of the cell cycle (Elia et al., 2003b). In addition, we found that the Plk1 antibody precipitated equal amounts of Plx1 during the cell cycle (Fig. 7B), suggesting that Plx1 concentrations remain stable in zygotes and therefore, per se, cannot account for the cell cycle-specific IP_3R1 MPM-2 phosphorylation. Together, these results suggest that Plk1 binds and phosphorylates IP_3R1 in vertebrate oocytes/eggs in a cell cycle preferential manner.

Inactivation of the ERK pathway prevents IP_3R1 cortical cluster formation and MPM-2 IP_3R1 phosphorylation at the MII stage

While our data collectively show that Plk1 is the IP_3R1 MPM-2 generating kinase, the finding that abrogation of the ERK pathway inhibits IP_3R1 MPM-2 phosphorylation after the MI stage suggests that the ERK pathway has another, novel regulatory role on IP_3R1 phosphorylation. For this purpose, we examined whether its inhibition prevented phosphorylation/activation of Plk1. Contrary to previous observations in starfish oocytes (Okano-Uchida et al., 2003), inactivation of the ERK pathway using U0126 did not affect Plk1 phosphorylation (data not shown). We therefore examined whether inactivation of the ERK pathway might affect IP_3R1 and Plk1 localization, and by doing so might compromise receptor phosphorylation. We considered this possibility because research shows that the ERK pathway is required for the egg's cortical reorganization (Deng et al., 2005), for the migration of the meiotic spindle to the oocyte cortex (Verlhac et al., 2000), and because several ERK substrates contribute to maintain the configuration of the MII spindle (Terret et al., 2003; Lefebvre et al., 2002). Given that IP_3R1 attains prominent cortical cluster organization in MII eggs (Melhmann et al., 1996; Shiraishi et al., 1995) and that Plk1 accumulates to the spindle poles in these cells (Wianny et al., 1998), it is possible that abrogation of the ERK pathway during maturation might interfere with the normal distribution of these molecules and therefore decrease their functional association. Accordingly, oocyte maturation in the presence of U0124 did not alter IP_3R1 distribution, cortical cluster formation (Fig. 8Aa; arrows; C; circles), or accumulation of Plk1 to the spindle poles (Fig. 8Ab; arrowheads). Nevertheless, oocyte maturation in the presence of U0126 diminished the numbers of IP_3R1 clusters in the ooplasm and prevented IP_3R1 cortical cluster formation (Fig. 8Ba; D; arrowheads); U0126 treatment also altered Plk1 localization, as several additional Plk1 accumulations were seen around the spindle, leading to a generally decreased IP_3R1 and Plk1 overlap (Fig. 8Bb, c; and panels displaying signal

quantification (Ad and Bd). IP_3R1 and Plk1 signals were absent in negative control samples, which were treated without primary antibodies but with both secondary antibodies (E), or with the CT1 antibody pretreated with the appropriate antigenic peptide (F). These results reveal that during maturation the ERK pathway plays a novel regulatory role in the localization of IP_3R1 and Plk1, which is essential for IP_3R1 MPM-2 phosphorylation in MII eggs.

DISCUSSION

A consequence of the extensive biochemical and morphological modifications that oocytes of vertebrate species undergo during maturation is an enhancement of the egg's $[Ca^{2+}]_i$ releasing mechanisms required for fertilization. Here we show that one of these modifications involves active MPM-2 epitope(s) phosphorylation of IP_3R1, and that Plk1 is the kinase responsible for this phosphorylation. We found that IP_3R1 and Plk1 associate in a cell cycle-preferential manner, that they display diffuse distribution throughout the ooplasm, and that they co-localize more prominently on areas around the spindle/spindle poles. We demonstrate that while the ERK pathway is dispensable for IP_3R1 MPM-2 phosphorylation during the early stages of maturation, it is required for this phosphorylation at the MII stage. We also determined that the ERK pathway regulates IP_3R1 cellular distribution during the late stages of meiosis. Altogether, our results in oocytes show that Plk1 and possibly other M-phase kinases involved in the reinitiation of meiosis are also involved in its completion via regulation of the Ca^{2+} release channels necessary for fertilization.

Plk1 is the MPM-2 epitope IP_3R1 kinase

The precise nature of the molecular mechanisms underlying the increased sensitivity of IP_3R1s during oocyte maturation remains to be elucidated. Nonetheless, IP_3R1 phosphorylation is likely to play a pivotal role in this process, as it has been associated with increased Ca^{2+} release in numerous other cell systems (reviewed in Patterson et al., 2004; Krizanova and Ondrias, 2003). In this study we extend our previous findings (Lee et al., 2006) by showing that the temporal development of IP_3R1 MPM-2 phosphorylation coincides with the progressive increase in IP_3R1 sensitivity that occurs during oocyte maturation (Mehlmann and Kline, 1994; Fujiwara et al., 1993). Because a number of M-phase kinases are activated in close succession during oocyte maturation, we used a combination of pharmacological and molecular reagents to ascertain the kinase responsible for IP_3R1 MPM-2 phosphorylation. Here we show that IP_3R1 is a novel target for Plk1, that it phosphorylates IP_3R1 at an MPM-2 epitope, and that both proteins interact in a cell-cycle preferential manner. The association of Plk1 and IP_3R1 is likely to be mediated by the PBD of Plk1, which binds phosphorylated epitopes and reportedly targets Plk1 to its substrates (Elia et al., 2003b). The preferred binding epitope of Plk1 is Ser-pSer/pThr-Pro/X (Elia et al., 2003a), and a Ser-Ser-Pro motif is present in IP_3R1 (amino acids 1491–1493). This Ser-Ser-Pro site lies within a larger consensus sequence remarkably reminiscent of the epitope recognized by the MPM-2 antibody (Yaffe et al., 1997), raising the prospect that Plk1 might phosphorylate this site. If this were so, it would imply that Plk1 acts as its own priming kinase, which has been shown to be the case with other Plk1 substrates (Neef et al., 2003). Our finding that injection of CA (T210D)-Plk1 mRNA induces IP_3R1 MPM-2 reactivity in the absence of other M-phase kinases supports this possibility. Nonetheless, in the absence of Cdk1 activity, such as it occurs in OA-IBMX induced maturation, IP_3R1 MPM-2 phosphorylation is noticeably lower than in control oocytes, suggesting that other kinases assist Plk1-dependent phosphorylation of IP_3R1.

Proteins displaying a PBD binding motif are likely to host Plk1 phosphorylation sites (Lowery et al., 2007). The consensus sequence for Plk1 phosphorylation was first described by Nakajima et al. (2003) and consists of the following residues E/D-X-Ser/Thr-ψ-X-D/E, where D/E stand for either of the acidic amino acids and ψ stands for a hydrophobic amino acid; an expanded

motif has recently been described by Johnson et al. (2007). Examination of the IP_3R1 sequence reveals that it contains three highly conserved Plk1 phosphorylation sites centered on residues Thr1048, Ser1890, and Thr2656. Moreover, several minimal consensus sites for Plk1 (Lowery et al., 2007) are scattered throughout the receptor sequence. It is therefore likely that besides the MPM-2 epitope(s), Plk1 phosphorylates one or several of these sites in oocytes and eggs. Nonetheless, further research will be needed to ascertain how many sites are phosphorylated *in vivo* by Plk1, and how this affects IP_3R1 function. It is worth noting that while Cdk1 or ERK do not phosphorylate the IP_3R1 MPM-2 epitope in eggs, they could still act on IP_3R1 by phosphorylating their own target sites (Lee et al., 2006; Bai et al., 2006; Malathi et al., 2003). If this were the case, it would underscore the importance of IP_3R1 phosphorylation to promote maximal Ca^{2+} release at fertilization to ensure completion and exit of the meiotic program.

IP_3R1 MPM-2 phosphorylation, Plk1 and IP_3R1 cellular distribution and the role of ERK

A second mechanism that might underlie the increased IP_3R1 sensitivity in oocytes after maturation is the differential redistribution of the receptor that culminates with the formation of conspicuous cortical clusters (Stricker et al., 2006; reviewed in Kline, 2000). Here, we show that the ERK pathway is required for IP_3R1 cortical redistribution, as eggs matured in the presence of the MEK inhibitor U0126 were devoid of IP_3R1 cortical clusters, although the receptor dispersed from its spindle localization at MI. Inhibition of the ERK pathway also reduced the overlap of Plk1 and IP_3R1 signals on the spindle poles, and this might explain, at least in part, the decreased IP_3R1 MPM-2 phosphorylation found in these eggs (present data and Lee et al., 2006). Whether the reduced IP_3R1 MPM-2 phosphorylation is due to ERK's ability to impose priming phosphorylation(s) on IP_3R1, or is merely due to this pathway's regulatory role on cytoskeletal/cortical re-organization (Deng et al., 2005; Verlhac et al., 1994) remains to be elucidated. Likewise, whether the reported inability of eggs maturated in the presence of U0126 to mount normal $[Ca^{2+}]_i$ oscillations (Matson and Ducibella, 2007; Lee et al., 2006) is due to reduced IP_3R1 phosphorylation, or to the absence of cortical clusters or to both remains to be determined. It is worth noting that these effects of U0126 likely reflect specific suppression of the ERK pathway, as during mitosis I, which is naturally devoid of ERK activity, IP_3R1s show minor MPM-2 reactivity (this study and Lee et al., 2006), the ER does not organize in cortical clusters (FitzHarris et al., 2003), and zygotes display limited $[Ca^{2+}]_i$ oscillatory ability (Jellerette et al., 2004). Therefore, our results provide the framework for future studies to discern the contribution of cortical clusters to IP_3R1 sensitivity and $[Ca^{2+}]_i$ oscillatory capacity.

Plk1, Ca^{2+} release, cell cycle progression and cytokinesis

Plk1 plays essential roles in almost every phase of the cell cycle, from promoting G2/M transition in oocytes, to making possible the completion of meiosis after fertilization (Liu and Maller, 2005b; Rauh et al., 2005), to being a critical regulator of mitosis and cytokinesis (Liu and Maller, 2005a; reviewed in Barr et al., 2004). Remarkably, the unfolding of several of these Plk1-regulated events is accompanied by Ca^{2+} release, and requires activation of Ca^{2+}-dependent kinases. Nevertheless, whether Plk1 function and Ca^{2+} release are interdependent as well as the molecular underpinning of this association remain unexplored. Here, we offer the first demonstration that Plk1 associates with and phosphorylates IP_3R1 thereby providing a direct molecular link between the cell cycle machinery and the Ca^{2+} release system. For instance, it is well known that the function of Plx1, a Plk ortholog, is required for MII exit in *Xenopus* eggs (Descombes and Nigg, 1998), although earlier studies had shown that this event also requires Ca^{2+} release and activation of CaMKII (Lorca et al., 1993). The association between these kinases, however, remained unclear until the recent demonstration that CaMKII introduces a priming phosphorylation for Plx1 on Emi2, the Anaphase Promoting Complex/Cyclosome (APC/C) egg inhibitor (Madgwick et al., 2006; Tung et al., 2005). Following Plx1 phosphorylation, Emi2 is degraded, which is necessary for eggs to escape the MII arrest (Liu

and Maller, 2005b; Rauh et al., 2005). It is therefore logical to envision a regulatory role for Plx1 on the Ca^{2+} channel responsible for the priming phosphorylation of a pivotal substrate. Similarly, the distinct nuclear-associated IP_3-mediated [Ca^{2+}] rises observed in syncytial *Drosophila* embryos (Parry et al., 2005) might be under the regulation of Polo, given that in these embryos as mitosis approaches both the ER (Parry et al., 2005) and Polo (Moutinho-Santos et al., 1998) gather on the spindle poles, which we show are sites of IP_3R1 and Plk1 co-localization. Lastly, the demand for Ca^{2+} release during cytokinesis in *Drosophila* spermatoocytes (Wong et al., 2005) and in *Xenopus* embryos (Muto and Mikoshiba, 1998) might also be under a similar regulation, as both the presence and function of Plk1/Polo and IP_3R1 are required for cleavage furrow formation and stability (Muto and Mikoshiba, 1998; Mitsuyama and Sawai, 2001; Wong et al., 2005; Santamaria, et al., 2007) and, by this late stage of mitosis, most other M-phase kinases have been inactivated (Neef et al., 2003).

In summary, our results reveal that IP_3R1, a widespread Ca^{2+} channel responsible for the majority of $[Ca^{2+}]_i$ release in non-muscle cells is a novel substrate for Plk1, a M-phase kinase with a broad range of functions during meiosis/mitosis and cytokinesis. We propose that IP_3R1 phosphorylation by Plk1, and possibly by other M-phase kinases, is responsible for the delivery of spatially and temporally regulated $[Ca^{2+}]_i$ signals that ensure faithful progression and completion of meiosis/mitosis and cytokinesis.

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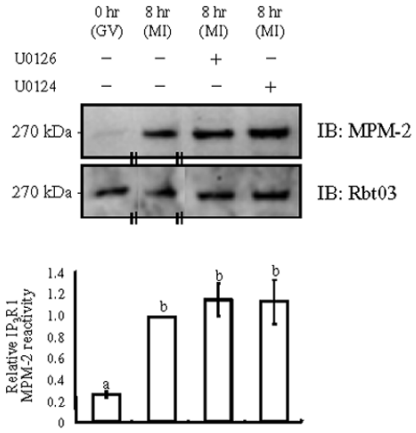
Fig. 1A



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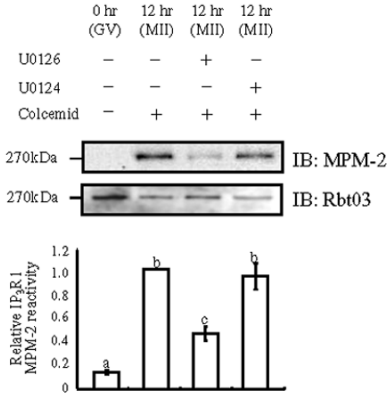


Fig. 1C

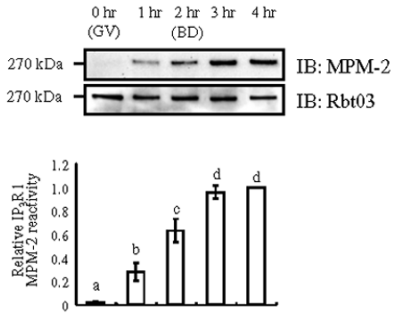
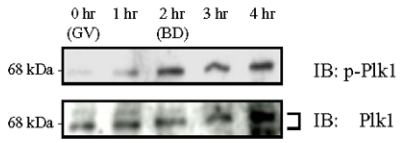


Fig. 1. MPM-2 phosphorylation of IP_3R1 is regulated independently of the ERK pathway during the early stages of maturation

(A) Immunoblotting of oocyte lysates collected at GV (0hr), MI (8hr) and MI (8hr) after treatment with U0126 or U0124 for the indicated times and probed with the MPM-2 antibody (upper panel) and, after stripping of the blot, with the IP_3R1 antibody Rbt03 (lower panel). Quantification of IP_3R1 MPM-2 reactivity is shown in the graph below the immunoblotting panels. Data are presented as means ± s.e.m., and bars with different superscripts are significantly different ($P<0.05$) both here and elsewhere. The pair of short parallel lines indicates a lane that was moved from within the same blot. **(B)** Immunoblotting of lysates of GV, MII (12hr) eggs in the presence of colcemid and treated with U0126 or U0124 and **(C)** of

oocytes cultured for 0 (GV), 1, 2 (BD), 3 and 4hr were probed as described above. For **B**, similar results were observed without colcemid.

Fig. 2A



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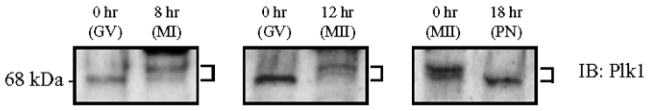

Fig. 2C

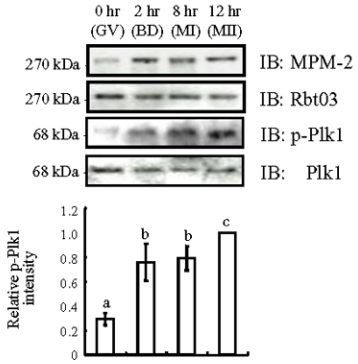

Fig. 2D

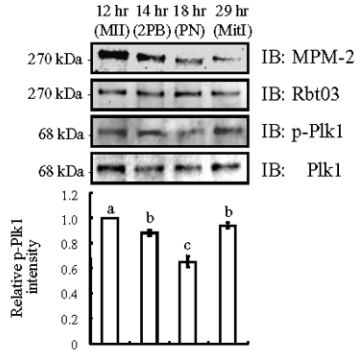

Fig. 2. IP_3R1 MPM-2 reactivity and Plk1 phosphorylation correspond during oocyte maturation and the early zygotic stage

(A) Immunoblotting of oocyte lysates collected at 0 (GV), 1, 2 (BD), 3 and 4 hr probed with a phospho-Plk1 (p-Plk1) antibody (upper panel) and, after stripping, re-probed with a Plk1 antibody (lower panel). **(B)** Immunoblotting of lysates of GV (0hr), MI (8hr), MII (12hr) oocytes and PN (18hr) zygotes were probed with a Plk1 antibody. In A and B, proteins were separated on 7.5% gels made with 120:1 acrylamide:bisacrylamide mixture, and upper arm of the bracket at the right side of the blot points to the phosphorylated Plk1 variant. **(C)** Immunoblotting of lysates of GV, BD, MI and MII oocytes and **(D)** of MII (12hr), 2PB (14hr), PN (18hr) and MitI (29hr) eggs/zygotes show MPM-2 reactivity (upper panel) and p-Plk1

reactivity (3th panel). After stripping, IP_3R1 reactivity (Rbt03; 2nd panel) and Plk1 reactivity (lower panel) was demonstrated. p-Plk1 intensity is shown in the graph below the immunoblotting panels.

Fig. 3A

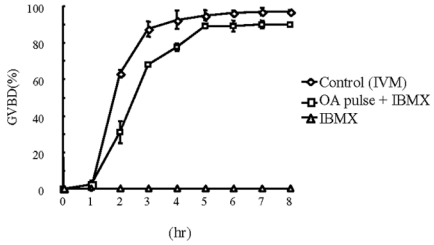

Fig. 3B

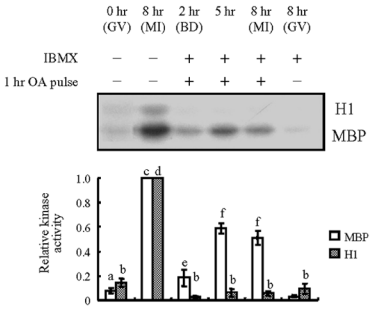

Fig. 3C

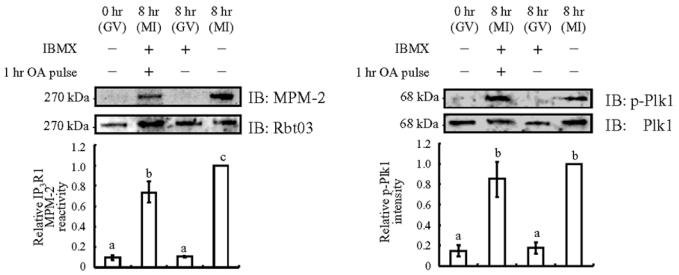

Fig. 3. Completion of GVBD and IP_3R1 MPM-2 reactivity occur in the absence of Cdk1 activity. OA activates ERK and Plk1 in the presence of IBMX

(A) Percentage (%) of oocytes (control, 1hr OA pulse + IBMX, and IBMX) that underwent GVBD at different times of *in vitro* maturation; at least 30 oocytes examined per treatment and time point. **(B)** Kinase assays. H1 and MBP phosphorylation indicate Cdk1 and ERK activities, respectively. Oocytes were cultured for 0, 2, 5, 8hr ± IBMX and ± 1hr OA pulse. Quantification of kinase activities is shown below the autoradiograph. BD under the 2 hr time point stands for GVBD. **(C)** Immunoblotting of oocyte lysates collected at 0hr (GV), 8hr (MI) treated with IBMX + 1hr OA pulse, 8hr (GV) with IBMX and 8hr control (MI) and probed with MPM-2 antibody (left panel) and p-Plk1 antibody (right panel) and after stripping re-probed with IP_3R1 (Rbt03) and Plk1 antibodies, respectively. Quantification of IP_3R1 MPM-2 reactivity and p-Plk1 intensity is shown in the graph below the immunoblotting panels.

Fig. 4A

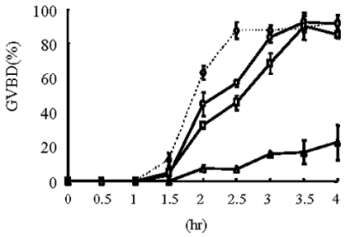

Fig. 4B

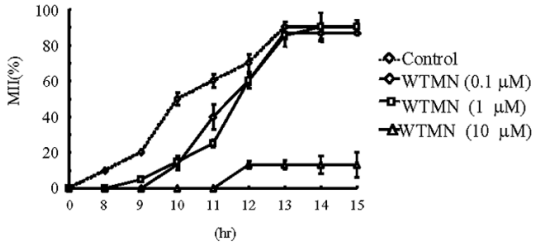

Fig. 4C

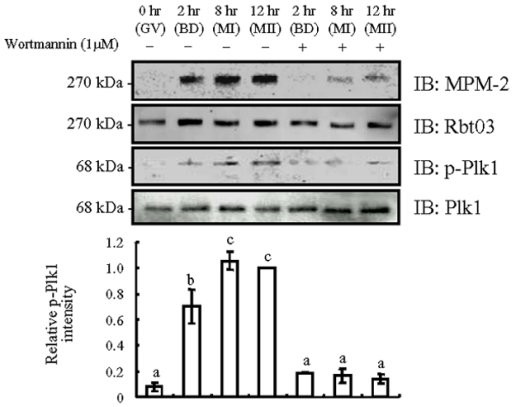

Fig. 4D

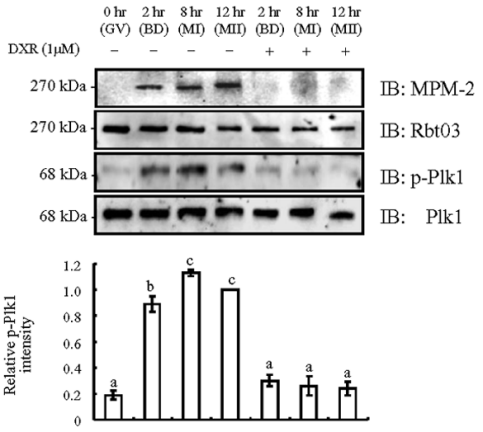

Fig. 4. Inhibition of Plk1 activity by Wortmannin (WTMN), or Doxorubicin (DXR) abolishes IP_3R1 MPM-2 reactivity and delays progression to GVBD and MII

Percentage of oocytes (control, 0.1, 1 or 10μM WTMN) that underwent progression to GVBD **(A)** and MII **(B)** during maturation; at least 30 oocytes were examined per concentration and time point. Immunoblotting of oocyte lysates collected at 0, 2, 8 and 12h ± 1μM WTMN **(C)** or ± 1μM DXR **(D)** were probed with MPM-2 (upper panel) antibody and p-Plk1 antibody (3^{th} panel) and, after stripping, re-probed with IP_3R1 antibody (Rbt03; 2^{nd} panel) and Plk1 (lower panel), respectively. Relative p-Plk1 intensity is shown for both (4C and 4D) in the graphs below the immunoblotting panels.

Fig. 5A

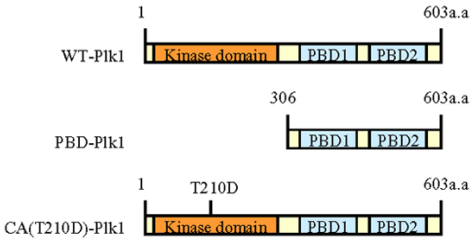

Fig. 5B

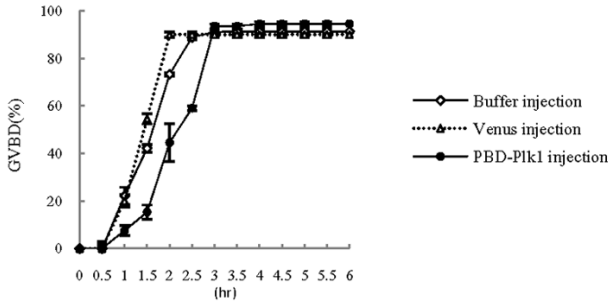

Fig. 5C

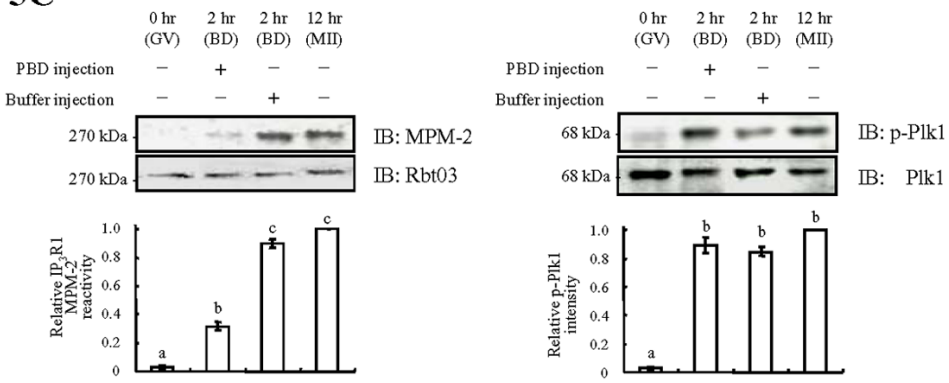

Fig. 5. Overexpression of PBD-Plk1 delays GVBD and inhibits IP_3R1 MPM-2 reactivity

(A) Schematic overview of the different Plk1 constructs. Wild type Plk1 (WT-Plk1) consists of a kinase domain and two Polo Boxes. PBD-Plk1 encodes only for the 2 Polo Boxes, and the CA Plk1 has residue Thr 210 substituted by Asp (CA(T210D)-Plk1). **(B)** Percentage of oocytes injected with buffer, venus mRNA (control) or with PBD-Plk1 mRNA that underwent GVBD after initiation of maturation; at least 20 oocytes were examined per group and time point. **(C)** Immunoblotting of oocyte lysates obtained after injection with PBD-Plk1 mRNA or buffer and collected at GVBD (2hr) or from buffer-injected GV and MII oocytes. Immunoblottings were probed with MPM-2 (left panel) or p-Plk1 antibody (right panel) and after stripping with

IP_3R1 (Rbt03) or Plk1 antibody, respectively. Quantification of MPM-2 or p-Plk1 intensity is shown in graph below the immunoblotting.

Fig. 6A

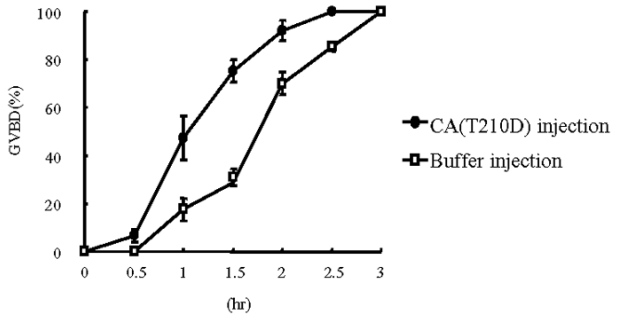

Fig. 6B

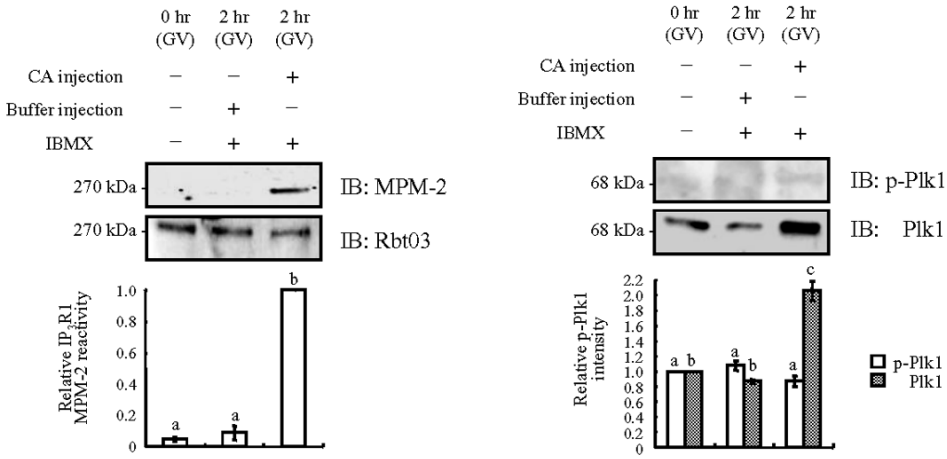

Fig. 6. Overexpression of CA(T210D)-Plk1 accelerates GVBD and induces IP_3R1 MPM-2 reactivity

(A) Percentage of oocytes (buffer-injected and injected with CA(T210D)-Plk1 mRNA) that underwent GVBD after initiation of maturation; at least 20 oocytes were examined per group and time point. **(B)** Immunoblotting of lysates from uninjected GV oocytes, or from GV oocytes injected with CA(T210D)-Plk1 mRNA or buffer (all in IBMX) and collected at 2hr. Immunoblottings were probed with MPM-2 (left panel) or p-Plk1 antibody (right panel) and after stripping with IP_3R1 (Rbt03) or Plk1 antibody, respectively. Quantification of MPM-2 or p-Plk1/Plk1 intensity is shown in graph below the immunoblotting.

Fig. 7A

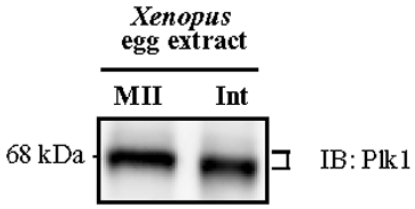

Fig. 7B

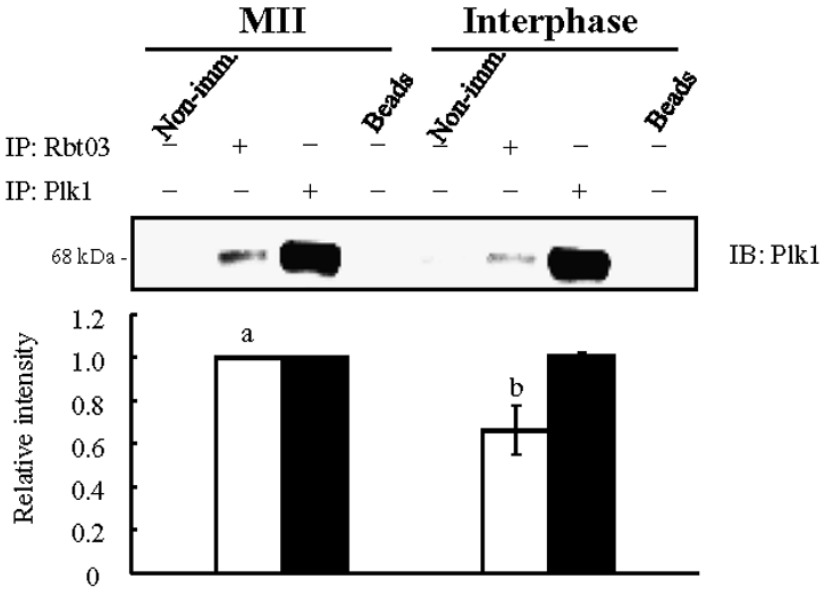

Fig. 7. Plx1 is active and interacts maximally with IP_3R1 in MII *Xenopus* egg extracts

(A) Immunoblotting of MII and Interphase (Int) extracts of *Xenopus* eggs probed with Plk1 antibody. Arms of the bracket at the right of immunoblotting denote upper and lower location of Plx1 bands **(B)** IP of Plx1 (~68kDa) with IP_3R1 (Rbt03; lanes 2 and 6) or Plk1 antibody (lanes 3 and 7) in both MII (left) and Int (right) extracts; IP with non-immune (Non-imm) serum (lanes 1 and 5) or beads alone (lanes 4 and 8) were used as controls. Quantification of Plx1 after IP is shown in graph below the immunoblotting.

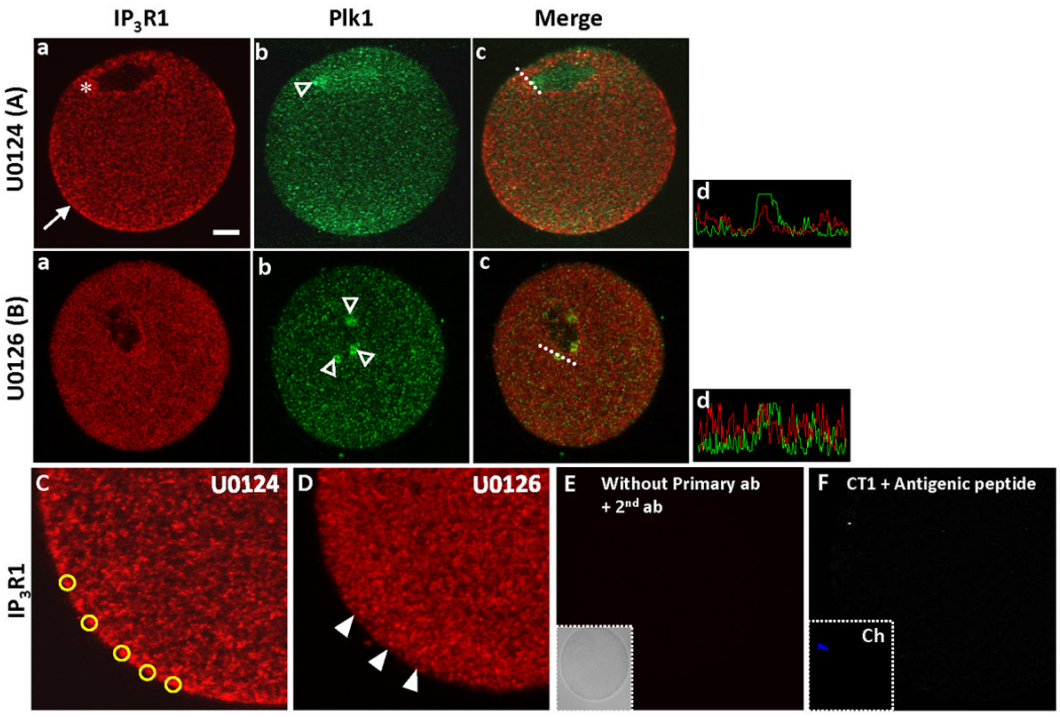

Fig. 8. The ERK pathway regulates IP_3R1 redistribution and formation of IP_3R1 cortical clusters during oocyte maturation

IF confocal images of IP_3R1 (**a**; red; CT1 antibody), Plk1 (**b;** green), and merged images (**c**) in MII oocytes treated with U0124 **(A, C)** or U0126 **(B, D)**. Asterisks and open arrowheads denote accumulations of IP_3R1 and Plk1, respectively. White arrows denote IP_3R1 cortical clusters (**Aa**), and closed arrowheads denote their absence in U0126 matured eggs (**D**). Broken white lines in merged images denote the traces used for intensity quantification of IP_3R1 and Plk1 signals (intensity panels on the right side of the figure; **Ad**, U0124, and **Bd**, U0126). Negative controls were stained without primary antibody but in the presence of both secondary antibodies **(E;** inset shows bright field image**),** or with the CT1 antibody pre-incubated with antigenic peptide **(F)**. Yellow circles encircle representative IP_3R1 clusters, which we defined as having an area of $\geq 0.7\mu m^2$ or a diameter of $\geq 1\mu m$. Ch in (**F**) denotes chromosomes (DNA staining with TO-PRO-3 (blue)). Scale bar (**Aa**) indicates 10μm.