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The hormonal herbicide, 2,4-dichlorophenoxyacetic acid, inhibits *Xenopus* oocyte maturation by targeting translational and post-translational mechanisms

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Abstract

The widely used hormonal herbicide, 2,4-dichlorophenoxyacetic acid, blocks meiotic maturation *in vitro* and is thus a potential environmental endocrine disruptor with early reproductive effects. To test whether maturation inhibition was dependent on protein kinase A, an endogenous maturation inhibitor, oocytes were microinjected with PKI, a specific PKA inhibitor, and exposed to 2,4-D. Oocytes failed to mature, suggesting that 2,4-D is not dependent on PKA activity and likely acts on a downstream target, such as Mos. *De novo* synthesis of Mos, which is triggered by mRNA poly(A) elongation, was examined. Oocytes were microinjected with radiolabelled *in vitro* transcripts of Mos RNA and exposed to progesterone and 2,4-D. RNA analysis showed progesterone-induced polyadenylation as expected but none with 2,4-D. 2,4-D-activated MAPK was determined to be cytoplasmic in localization studies but poorly induced Rsk2 phosphorylation and activation. In addition to inhibition of the G2/M transition, 2,4-D caused abrupt reduction of H1 kinase activity in MII phase oocytes. Attempts to rescue maturation in oocytes transiently exposed to 2,4-D failed, suggesting that 2,4-D induces irreversible dysfunction of the meiotic signaling mechanism.

Keywords: Oocyte maturation; Signal transduction; PKA; Mos; MAPK; Cytoplasmic polyadenylation; Endocrine disruptor; 2,4-D

1. Introduction

The formation of healthy eggs is crucial for successful fertilization, embryogenesis, and species viability in sexually reproductive organisms. Oogenesis is often a lengthy process that occurs over months and even years in which the growing oocyte is arrested at prophase I. Prolonged quiescence makes such cells highly vulnerable to exposure to factors that could perturb cellular, molecular and biochemical organization and thus compromise the subsequent process of meiotic maturation (egg formation) and reproductive health. Oocytes from the frog, *Xenopus laevis*, have historically served as an excellent *in vitro* model system for elucidating the mechanism of maturation, which is for the most part conserved among vertebrates [1,2]. Similarly, they are useful for examining the effects of exposure to exogenous factors such as suspected endocrine disruptors, chemicals that mimic, enhance or block hormone-driven events [3].

Meiotic maturation in *Xenopus* is induced by exposure to the steroid hormone, progesterone, which sets off a non-genomic signaling pathway characterized by partial meiosis whereby the full grown (stage VI) oocyte advances from prophase I to metaphase II, where it arrests. Under normal conditions the mature oocyte, which is easily identified by a white spot in the animal pole, indicative of nuclear envelope (germinal vesicle) breakdown (GVBD), is ovulated (the so-called egg) and completes meiosis in response to fertilization [4–6].

Progesterone-induced maturation is mediated through a membrane receptor that likely involves a G-coupled protein receptor [7–10] in addition to a traditional progesterone receptor [11,12] The oocyte's early response to progesterone is a transient drop in cAMP [13] that results in inactivation of the maturation inhibitor, cAMP-dependent protein kinase (PKA) [14,15]. This induces two parallel pathways that convert the pool of pre-maturation promoting factor (pre-MPF), a complex

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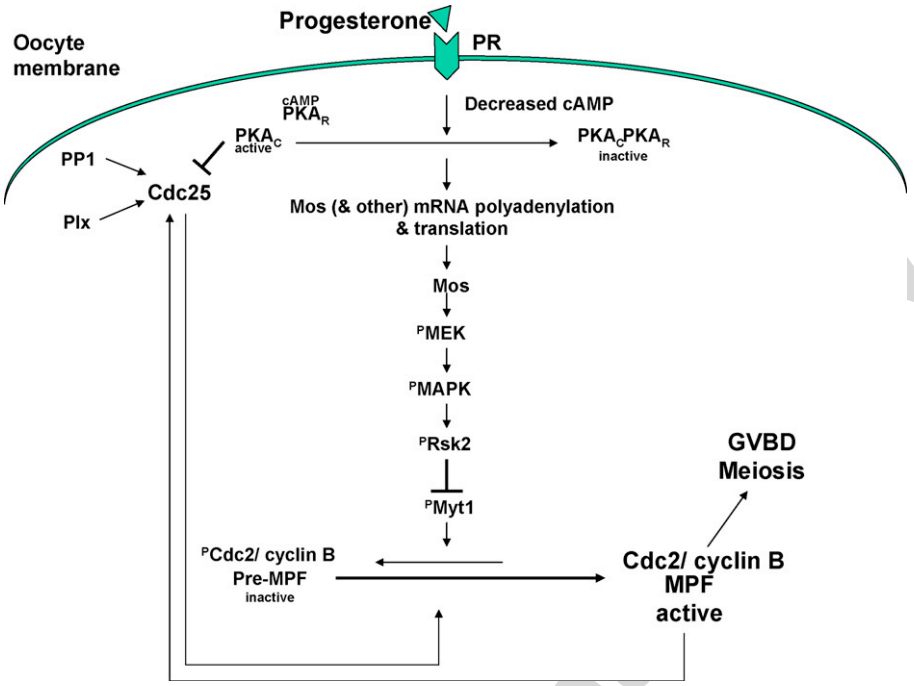

Fig. 1. Brief model of progesterone-stimulated meiotic maturation. Arrows denote activation steps while hammer heads denote inhibition. Predominant state of MPF is indicated by the relative size of the font and arrow. Progesterone receptor is abbreviated PR. PKA_C and PKA_R are catalytic and regulatory subunits, respectively. See the text for more details.

of the kinase, Cdc2, and cyclin B, to active MPF, which triggers chromosome condensation, spindle formation and GVBD (Fig. 1). One pathway is characterized by translational activation of certain maternal mRNAs, including Mos [16]. Mos, a ser/thr kinase, indirectly activates pre-MPF by blocking Myt1 kinase from adding inhibitory phosphates to Cdc2 at Tyr15. Mos works through the kinase signaling cascade, MEK/MAPK/Rsk2. In parallel, the phosphatase, Cdc25, is activated in part by the loss of PKA function, and removes the inhibitory phosphates from Cdc2. Both pathways are stimulated by MPF in positive feedback mechanisms [17].

Mos mRNA is translationally regulated by cytoplasmic polyadenylation [18]. During oogenesis, maternal Mos mRNA is transcribed and stored in a quiescent state with a short poly(A) tail. Progesterone induces poly(A) elongation and translation, which is regulated by the presence of two *cis* sequences in the 3′ untranslated region (UTR), the U-rich CPE (cytoplasmic polyadenylation element) and the nuclear hexanucleotide, AAUAAA. The CPE is bound by the CPE-binding protein (CPEB), whose regulation is responsible for polyadenylation and translation of CPE-containing mRNAs, including several cyclins [16].

2,4-Dichlorophenoxyacetic acid (2,4-D) is one of the most highly used herbicides globally [19,20]. A synthetic growth regulator, 2,4-D displays properties of a plant hormone within the auxin class and is routinely used in plant tissue culture to promote cell growth and division [21]. In high dosage, it is effective in deterring growth of broadleaf weeds and is applied to roadsides, fields, lawns, forests and aquatic environments [22]. Utilized since the 1940s [22], its long term and ubiquitous distribution suggest widespread exposure to non-target species over extended time, in particular amphibians, whose populations worldwide have contracted [23]. A variety of evidence suggests that such xenobiotics can have a negative effect on reproductive health, often acting as endocrine disruptors [24–28]. Previously, we hypothesized that 2,4-D may have endocrine disrupting properties on animal cells and reported that, indeed, exposed *Xenopus* oocytes fail to mature *in vitro* [29]. Our data showed that 2,4-D prevented a number of steps in the progesterone pathway including Mos expression, MPF activation, and GVBD. Interestingly, MAPK activation did occur through what appeared to be a Mos-independent pathway.

In this report, we have further characterized the molecular and biochemical responses to better elucidate the mechanism of maturation-block by 2,4-D. Because PKA is an endogenous inhibitor of maturation, we tested the hypothesis that it is required for 2,4-D's effects. Our data suggest that PKA is not involved and that perturbation of a downstream factor(s) is more likely. Since Mos kinase is required for meiosis and 2,4-D blocks its expression, we tested whether 2,4-D interfered with Mos mRNA polyadenylation and translation. Our results show that 2,4-D completely abrogated polyadenylation of Mos mRNA and likely promotes translationally quiescent CPEB-associated mRNA complexes. We then examined the localization and target of 2,4-D-induced MAPK, and show that MAPK remained cytoplasmic and only partially activated Rsk2. 2,4-D not only inhibits the G2/M transition but also dramatically reduced MPF activity in oocytes arrested in meiosis II. In addition, the failure of microinjected cyclin B or partially purified MPF to rescue transiently exposed oocytes suggests a breakdown in the Cdc2/cyclin B autoamplification mechanism.

2. Materials and methods

2.1. Oocyte isolation

Ovariectomies were performed on anaesthetized adult *Xenopus laevis* (Xenopus Express) through a small ventral incision. Oocytes were enzymatically defolliculated by gentle rocking at room temperature (~24 °C) for approximately 1 h in collagenase (2 mg/ml), dispase (1.2 mg/ml), 1× modified Barth's saline (MBS) (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.41 mM $CaCl_2{\cdot}H_2O$, 0.82 mM $MgSO_4{\cdot}7H_2O$, 2.4 mM $NaHCO_3$, 10 mM HEPES, pH 7.4). After rinsing extensively in 1× MBS, stage VI oocytes (the largest) were manually selected and allowed to equilibrate overnight in 1× MBS, pH 6.8 at 17 °C.

2.2. Oocyte incubation, microinjection and microdissection

Oocytes were incubated at room temperature in 1× MBS, pH 6.8 supplemented as indicated with 10 μM progesterone (dissolved in ethanol) (Sigma, St. Louis, MO); 10 mM 2,4-dichlorophenoxyacetic acid-sodium salt (dissolved in water) (Sigma) (as determined previously [29]); 50 μg/ml cycloheximide (dissolved in ethanol). Microinjections were performed with a Nanoject II Auto-Nanoliter Injector (Drummond Scientific Co., Broomall, PA) using borosilicate glass needles made with a P-30 vertical nichrome needle puller (Sutter Instrument Co., Novato, CA). Oocytes were injected with 4.6 nl of GST or GST-PKI (~3 ng/nl) or 46 nl of radiolabelled *in vitro* transcripts and then were incubated as described. Typically, oocytes were harvested at approximately 6–8 h from addition of treatment when at least 50% of the progesterone-treated oocytes displayed white spot formation (germinal vesicle breakdown) (GVBD). In some cases, oocytes were manually microdissected to verify the presence or absence of the GV (see below). In transient exposure studies, oocytes were subjected to a primary incubation in 1× MBS, progesterone or 2,4-D for 4 h, washed in 1× MBS, four times for 10 min and then incubated for another 8 h in the secondary treatment as indicated. Some oocytes were injected with 2.3 nl of a 10 μM solution of the fusion protein, maltose binding protein (MBP)-sea urchin cyclin B Δ90, a stable mutant of cyclin B, or 32.2 nl of a partially purified 20–40% ammonium sulfate precipitate of maturation promoting factor (MPF) (7.7 ng/nl) from unfertilized eggs (generous gifts from E. Shibuya). In controls, some oocytes were pre-incubated with and without cycloheximide followed by the indicated treatment for approximately 10 h at room temperature. In localization studies, after 8 h of the indicated treatment, oocytes were transferred to 0.1× MBS for 10 min and then manually microdissected with Dumont #5 fine forceps into GV and enucleated oocytes (cytoplasm) fractions. Typically, samples containing five oocytes each were frozen without buffer at −80 °C until further analysis. Each treatment included at least 20 oocytes and each experiment was repeated at least three times.

2.3. Preparation of glutathione-S-transferase (GST) and GST-PKI fusion protein

Bacterially expressed protein for oocyte microinjection was prepared from the plasmid, pGEX-KG-PKI (a generous gift from J. Ruderman; described in [30]). Bacterial strain BL-21 (a generous gift from J. Richter) was transformed with pGEX-KG-PKI using standard techniques [31]. A half liter of Terrific Broth (Genesee Scientific Corp., San Diego, CA) with 100 μg/ml ampicillin, sodium salt (Sigma) was inoculated with 5 ml of pGEX-KG-PKI overnight culture, incubated at 37 °C while shaking at 200 rpm for 2.5 h. Expression of GST-PKI was induced by addition of 0.1 mM IPTG (Amersham Biosciences, Piscataway, NJ) and a further 2 h incubation. Cells were pelleted and GST-PKI fusion protein isolated following the instructions in the GST purification kit (Amersham Biosciences). To prepare GST alone the pGEX-KG-PKI vector was digested with *Sma*I and *Sac*I (New England Biolabs, Beverly, MA) to remove the PKI insert. The *Sac*I overhang of the backbone was filled in with large Klenow fragment of DNA polymerase (New Biolabs) according to the manufacturer's instructions, ligated to the *Sma*I end of the backbone and transformed into XL1Blue (Stratagene, La Jolla, CA). The pGST construct was verified by a diagnostic double restriction digest with *Pst*I and *Bam*HI that showed the absence of the 225 base pair PKI *Sac*I/*Sma*I fragment relative to the original construct. After transforming it into BL-21 cells, GST expression was induced and the protein purified exactly as described above. The molecular weights of GST-PKI (~35 kDa) and GST (~27 kDa) were verified by SDS-polyacrylamide gel electrophoresis [31] and Western blot analysis with anti-GST antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Protein concentrations were determined by absorption at 280 nm ($1A_{280}$ = ~0.5 mg/ml). Purified GST (5 mg/ml) and GST-PKI (3 mg/ml) were stored in 10 μl aliquots at 4 and −20 °C with and without 5% glycerol. All samples were tested by oocyte microinjection. No differences in activity due to storage conditions were observed.

2.4. In vitro transcription reactions

The plasmid constructs, psmos and pscyclin B1 (described in [32]), were linearized with *Bam*HI and *Eco*RI, respectively (New England Biolabs). Radiolabelled transcripts were synthesized *in vitro* in a 20 μl reaction that contained 2 μg of linearized DNA, 0.5 mM $m^7G(5')ppp(5')G$ cap analog (Ambion, Austin, TX), 50 μCi α-UTP (800 Ci/mmol) (PerkinElmer, Shelton, CT), 500 μM ATP, 500 μM CTP, 50 μM GTP and 100 μM UTP (Ambion), 40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 2 mM spermidine, and 40 units SP6 RNA Polymerase-Plus, which contained SUPERaseIn™ (Ambion) for the psmos reaction or 10 units of T3 RNA Polymerase-Plus (Ambion) for the pscyclin B1 reaction. Samples were incubated at 37 °C for 1 h, extracted with phenol:chloroform (1:1), ethanol precipitated, resuspended in 10 μl deionized RNAse-free water and stored at −20 °C. Approximately 1/25 of the reaction was analyzed for RNA integrity by denaturing gel electrophoresis (4.5% acrylamide (29.2 acrylamide:0.2 bis-acrylamide)), 50% urea, 1× TBE (89 mM Tris–borate, 89 mM boric acid, 0.2 mM EDTA), pH 8 followed by autoradiography.

2.5. Extraction and analysis of RNA from oocytes

Oocytes microinjected with radiolabelled *in vitro* transcripts were homogenized by pipetting in PAS buffer (6% *p*-aminosalicylic acid-sodium salt (Sigma), 1% sodium dodecylsulfate, 0.1 M Tris, pH 7.6, 1 mM EDTA, pH 8) (100 μl/oocyte) (modified from [33]). Each sample contained five oocytes. Homogenates were pelleted at 14,000 rpm, 15 min at 4 °C. The supernatant was extracted twice with phenol:chloroform (1:1). RNA was ethanol precipitated, washed in 75% ethanol, dried and resuspended in 4 μl of 10× sequencing dyes (95% deionized formamide, 1 mM EDTA, pH 8, 0.1% bromophenol blue, 0.1% xylene cyanol). RNA samples were heat denatured at 90–100 °C for 10 min and fractionated by denaturing gel electrophoresis (4.5% acrylamide (29.2 acrylamide:0.2 bis-acrylamide), 50% urea, 1× TBE). Radiolabelled RNA was visualized by autoradiography.

2.6. Western blot analysis

Oocyte proteins were extracted, fractionated and electroblotted as previously described [29] with the exception of CPEB. In this case oocytes were homogenized in 5 μl/oocyte of 20 mM Tris pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 1 μM okadaic acid, 10 mM sodium β-glycerophosphate, 2 mM Na_3VO_4, 20 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml protease inhibitor cocktail (PIC) (Sigma Cat # 2714) (personal communication, S. Martinez). CPEB was resolved on 15% Anderson gels [34] and Rsk2 on 10% running gels at 100:1 (acrylamide:bis-acrylamide) instead of the typical 29:1 ratio. Primary antibodies were diluted as follows: 1/2000 of guinea pig anti-*Xenopus* CPEB IgG (a generous gift from L. Hake); 1/1000 of rabbit anti-human p44/42 MAP kinase (Thr202/Tyr204) IgG (Cell Signaling Technology, #9101); 1/1000 of mouse anti-human P-ERK2 (E-4) monoclonal IgG (Santa Cruz Biotechnology, sc-7383) (designated P-MAPK); 1/500 of rabbit anti-Mos^{Xe} (C237) IgG (Santa Cruz Biotechnology, sc-86) and of rabbit anti-human phospho-cdc2 (Tyr15) IgG (Cell Signaling Technology, #9111); 1/1000 of mouse monoclonal anti-human Rsk-2 (E-1) IgG (Santa Cruz Biotechnology, sc-9986); and 1/10 of mouse anti-goat nucleoplasmin monoclonal IgG (Developmental Studies Hybridoma Bank, Iowa City, IA). Secondary antibodies, either horseradish peroxidase-conjugated anti-rabbit IgG or goat anti-mouse IgG was diluted 1/2000, and goat anti-guinea pig IgG (Santa Cruz Biotechnology) was diluted 1/4000. Primary incubations were typically at 4 °C overnight and the secondary at room temperature for 1 h. Immunoreactive proteins were visualized by chemiluminescence using the ECL

reagents according to the manufacturer's instructions (Amersham Biosciences Corp.), and autoradiography. Five oocytes from each treatment were homogenized and typically 0.5–1 oocyte equivalent was analyzed. In localization studies, the oocyte equivalents were 0.6 for whole oocyte, 1.2 for cytoplasm, and 2.4 for GV.

2.7. Kinase reactions

Histone H1 kinase reactions were performed as described [29] except that homogenates were prepared in 8 μl instead of 20 μl/oocyte of H1 kinase buffer (80 mM sodium β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 0.5 mM Na_3VO_4, 10 μg/ml leupeptin and chymostatin). Rsk2 kinase assays were performed essentially according to [35]. Typically, five oocytes per sample were homogenized in up to 20 μl/oocyte of extraction buffer (20 mM Tris–HCl pH 7.5, 100 mM NaCl, 0.5% IGEPAL CA-630 (formerly NP-40) (Sigma), 10 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4, 1 μM okadaic acid, 10 μg/ml PIC). Homogenates were centrifuged at 14,000 rpm for 5 min at 4 °C, and the supernatants were pre-cleared by incubation with 20 μl resuspended protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) for 30 min at 4 °C, rocking. After removal of the beads by centrifugation at 1500 × *g* for 5 min at 4 °C, 2 μg of Rsk-2(E-1) mouse monoclonal antibody were added to the supernatant and incubated for 1 h at 4 °C. Twenty microliters of resuspended protein A/G beads were added and incubation continued for 4–24 h. The immunocomplex was pelleted and washed twice in extraction buffer and wash buffer (50 mM Tris–HCl pH 7.5, 750 mM NaCl), and once in kinase buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 5 mM EGTA, 1 mM β-mercaptoethanol). The kinase reaction was performed in 20 μl of supplemented kinase buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 5 mM EGTA, 1 mM β-mercaptoethanol, 10 mM $MgCl_2$, 100 mM ATP, 5 μCi γ-^{32}P-ATP (3000 Ci/mmol)) (PerkinElmer), 250 μM S6 kinase/Rsk2 substrate peptide 2 (Upstate Cell Signaling Solutions) for 10–60 min at 30 °C. After stopping the reaction on ice with 60 mM EDTA pH 8.0, 10 μl was spotted and dried onto P81 phosphocellulose squares (Upstate Cell Signaling Solutions) in duplicate, washed three times in 0.75% phosphoric acid and once with acetone for 5 min each, according to the manufacturer's instructions. Counts per minute were determined in a Beckman Coulter LS6500 scintillation counter. Relative incorporation was determined by normalizing the values to the progesterone internal control and is the mean of three independent experiments.

3. Results

3.1. 2,4-D does not require PKA activity to prevent maturation

In order to characterize the molecular mechanism by which 2,4-D blocks maturation, we asked whether 2,4-D required PKA activity, the endogenous maturation inhibitor. To examine this, we took advantage of the highly specific protein kinase inhibitor, PKI, to block the catalytic subunit (PKAc) and induce oocyte maturation. We predicted that if 2,4-D required PKA activity to inhibit maturation then oocytes microinjected with PKI would mature regardless of the presence of 2,4-D. However, if 2,4-D instead interfered with a factor (*s*) downstream of PKA in the maturation pathway, PKI would fail to induce maturation in 2,4-D's presence. Oocytes were microinjected with approximately 13 ng of GST-PKI fusion protein or GST alone and some were subsequently incubated with 2,4-D for various periods of time post-injection. Oocytes were harvested at time points up to 7 h post-injection and subjected to western blot analysis to detect two molecular indicators of maturation, Mos protein and the loss of inactive phospho-Cdc2 (P-Cdc2) due to dephosphorylation of Tyr 15. Active doubly phosphorylated MAPK (PP-MAPK) was also monitored by western analysis as an expected response to progesterone as well as 2,4-D [29]. Control oocytes showed that progesterone induced a typical maturation profile, the expression of Mos, the phosphorylation of MAPK and the dephosphorylation of Cdc2 (Fig. 2, compare lanes 1 and 2). In contrast, 2,4-D produced a unique profile not productive for maturation: no Mos expression, and relatively high levels of PP-MAPK and P-Cdc2 (Fig. 2, lane 3), which supports previous results [29].

To confirm that GST-PKI fusion protein does indeed induce maturation in the absence of progesterone, we performed a time course where oocytes were microinjected with GST-PKI, harvested at various time points and assayed for maturation markers. As expected, Mos was detected by hours 6 and 7. Concomitantly, PP-MAPK was detected while P-Cdc2 levels declined (Fig. 2, lanes 6–10). Oocytes injected with GST alone had no response thus resembling immature oocytes (Fig. 2, compare lanes 4 and 1). Furthermore, GST had no effect on oocyte responses to 2,4-D (Fig. 2, compare lanes 5 and 3). These results suggest that microinjected GST-PKI effectively blocks PKA activity, thereby stimulating maturation.

To test whether inhibition of PKA causes oocytes to be refractory to 2,4-D, oocytes were microinjected with GST-PKI, and exposed to 2,4-D at increasingly later time points for a total of 7 h post-injection. Our results show that oocytes exposed to 2,4-D within the first 4 h following microinjection were unable to undergo maturation as shown by the absence of Mos expression and the persistence of P-Cdc2. Oocytes were only refractory to 2,4-D at 6 h post-injection (Fig. 2, compare lanes 14 to 11–13).

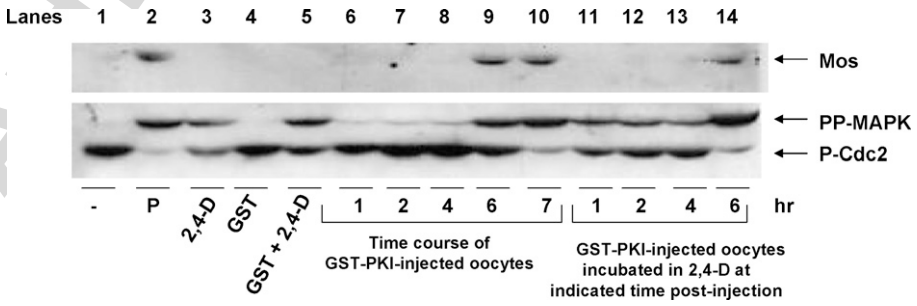

Fig. 2. PKI-injected oocytes fail to mature in the presence of 2,4-D. Oocytes were subjected to Western blot analysis for Mos, PP-MAPK, and P-Cdc2 (arrows on right) following incubation in 1× MBS (−) (lane 1); progesterone (P) (lane 2); 2,4-D (lane 3). Some oocytes were injected with GST and incubated in 1× MBS (lane 4) or 2,4-D (lane 5). Some oocytes were injected with GST-PKI, incubated in 1× MBS and harvested at the indicated times (h) (lanes 6–10). Other GST-PKI injected oocytes were exposed to 2,4-D at the time (h) indicated post-injection up to 7 h total (lanes 11–14). Unless otherwise stated, all other oocytes were harvested at 7 h.

Under these late conditions, Mos protein was expressed and P-Cdc2 levels were visibly reduced. These data suggest that 2,4-D does not require PKA or factors upstream of PKA to block maturation but targets a factor or factors(s) downstream of PKA that is required during at least the first 4–5 h of the signaling pathway (however, see below).

3.2. 2,4-D prevents the expression of Mos at the translational level

It is well established that both progesterone and PKI-induced maturation are dependent on translation [14,16,30]. Mos expression itself is regulated by both translational control and protein stabilization [36]. Preliminary experiments indicated that the level of Mos protein present in mature oocytes was unaffected by prolonged exposure to 2,4-D (data not shown). However, 2,4-D did block Mos expression during the first 4 h of progesterone exposure (data not shown). These data suggested that 2,4-D prevented *de novo* synthesis of Mos rather than promote protein degradation. Progesterone stimulates cytoplasmic polyadenylation and translation of Mos mRNA. To test whether 2,4-D inhibits this mechanism, oocytes were microinjected with radiolabelled *in vitro* transcripts of Mos 3′ untranslated region (UTR), which contains the polyadenylation signals, and exposed to progesterone and 2,4-D. Total RNA was subsequently isolated from oocytes, fractionated by denaturing gel electrophoresis and visualized by autoradiography. Poly(A) elongation is easily detected by the slower migration of longer polyadenylated radiolabelled transcripts, typically visible as an upward smear, relative to the uninjected and unadenylated transcript. Fig. 3a clearly shows that progesterone-induced polyadenylation of Mos RNA was completely eradicated by 2,4-D (compare lanes 3 and 5). This same effect was reproduced with microinjected cyclin B1 RNA (Fig. 3a), which is also under translational control via cytoplasmic polyadenylation during maturation [32], confirming previous northern blot analysis of endogenous cyclin B1 mRNA [29]. The lack of polyadenylation corresponds to the absence of Mos protein in oocytes as well as the presence of P-Cdc2, which is indicative of failures to translate Mos mRNA and mature (Fig. 3b). Interestingly, preliminary investigation of a key cytoplasmic polyadenylation factor, CPEB, which undergoes an upward size shift due to activating phosphorylations during maturation, did not occur in the presence of 2,4-D (Fig. 3b). These data strongly suggest that 2,4-D blocks expression of Mos and other proteins at the translational level by the inhibition of mRNA cytoplasmic polyadenylation machinery.

3.3. The effects of 2,4-D-induced MAPK activation

Although 2,4-D induces the double-phosphorylation of MAPK in a Mos-independent manner, neither activation of Cdc2 (e.g. Fig. 3b) nor GVBD (data not shown) occur [29]. We explored the possible effects of this from two different angles. Firstly, we asked whether 2,4-D-activated MAPK played a novel role in oocyte transcription, which might be reflected in translocation to the intact germinal vesicle [37]. To test this, 2,4-D-treated oocytes were microdissected, and MAPK local-

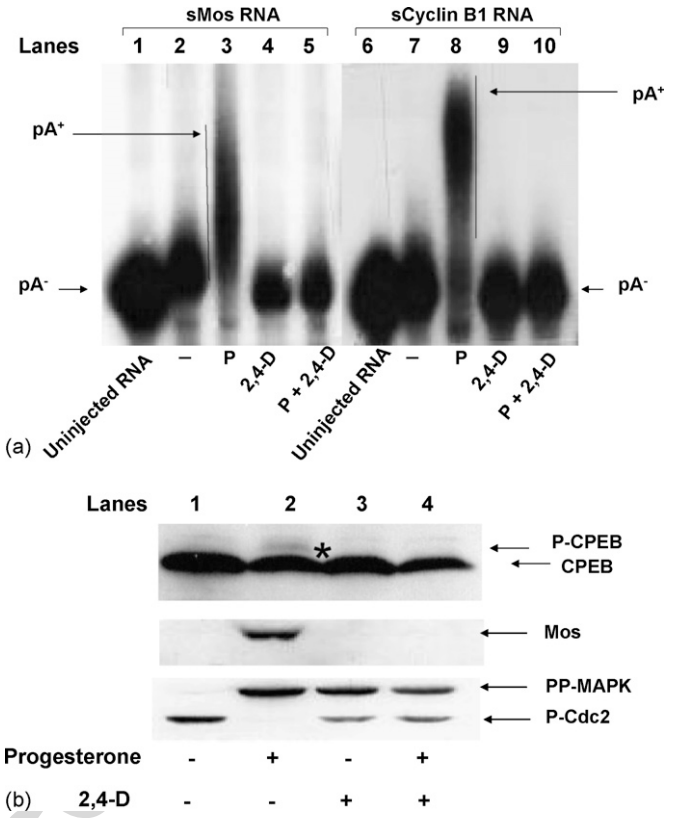

Fig. 3. Cytoplasmic polyadenylation of Mos and cyclin B RNAs inhibited by 2,4-D. (a) Oocytes were microinjected with radiolabelled *in vitro* synthesized sMos or scyclin B1 RNAs and incubated in 1× MBS (−) (lanes 2 and 7), progesterone (P) (lanes 3 and 8), 2,4-D (lanes 4 and 9), progesterone and 2,4-D (P + 2,4-D) (lanes 5 and 10). RNA was fractionated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. The specific RNAs are labeled at the top. Uninjected RNA (lanes 1 and 6) is indicated as unpolyadenylated (pA−) with an arrow. Polyadenylated RNA (pA+) is indicated with an arrow and vertical line. (b) Uninjected oocytes were analyzed by Western blot for CPEB, Mos, PP-MAPK and P-Cdc2 (right arrow). Oocyte treatments are indicated as 1× MBS (−), progesterone and 2,4-D (+). The asterisk denotes phosphorylated CPEB (P-CPEB).

ization was determined by western analysis of isolated germinal vesicles (GV) and cytoplasm (enucleated oocytes). Using nucleolin (data not shown) and nucleoplasmin as controls for nuclear proteins, we found that MAPK localized to the cytoplasm and little to none was detected in the nucleus in either immature or 2,4-D-treated oocytes (Fig. 4). Thus, 2,4-D-induced MAPK phosphorylation does not detectably shift the cytoplasmic pool to the nucleus, suggesting that a role in transcriptional regulation is unlikely.

Secondly, we tested the possibility that Rsk2, the immediate substrate of MAPK during maturation, may be less strongly activated in the presence of 2,4-D. Typically, progesterone induces hyperphosphorylation of Rsk2, which results in a relatively uniform species that migrates more slowly by SDS-PAGE as detected with an antibody specific for Rsk2 [38] (Fig. 5a, compare buffer and progesterone). In contrast, 2,4-D induced more heterogeneous lower molecular weight products (Fig. 5a) that were collectively diminished in intensity compared to the progesterone and buffer controls (Fig. 5b). Indeed, the kinase activ-

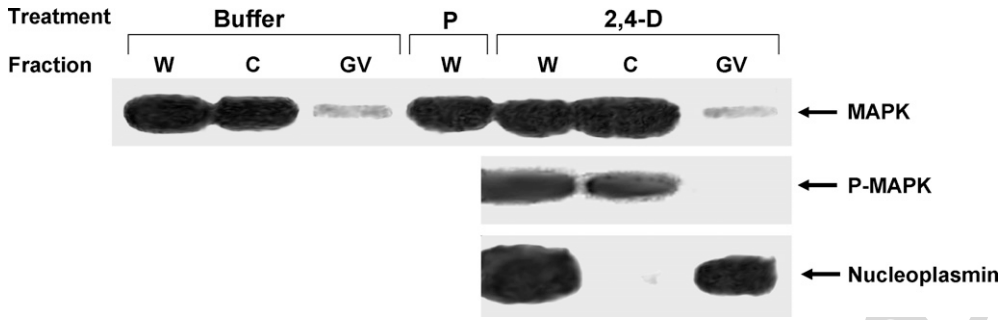

Fig. 4. Predominant cytoplasmic localization of MAPK is not perturbed by 2,4-D. Western blot analysis was performed on whole (W) oocytes, enucleated oocytes (cytoplasm) (C), and GVs. Oocyte treatments are indicated at the top: 1× MBS (buffer), progesterone (P), 2,4-D and immunodetected proteins on the right (arrows).

ity in Rsk2 immunoprecipitates from 2,4-D-treated oocytes was more than 50% reduced from progesterone controls (Fig. 5c). These data indicate that although 2,4-D induces some MAPK phosphorylation, it is not sufficient to cause robust activation of Rsk2.

3.4. 2,4-D reduces MPF activity in MII

Most of the inhibitory effects of 2,4-D we have examined thus far have focused on the transition from G2 to MI. Time course experiments (not shown) suggested that MII oocytes (∼4–6 h post-stimulus) were refractory to 2,4-D. Similar results were obtained when oocytes were induced to mature with PKI as shown by the persistence of maturation markers (Mos and dephosphorylated Cdc2) after the addition of 2,4-D at 6 h post-PKI (Fig. 2, lane 14). This suggested that 2,4-D had little effect on MII arrested oocytes. To examine this more closely, MPF activity was used as a more direct measure of maturation state. A comparative time course was performed with oocytes exposed to 2,4-D at G2/M ($t = 0$ h) or MII ($t \geq 5$ h post-progesterone). Oocytes were withdrawn hourly, homogenized and assayed for MPF activity using histone H1 as a substrate. As shown in Fig. 6a, the level of radiolabelled phosphate incorporated into histone H1 in progesterone-treated oocytes was well above background beginning at approximately 4 h and remained elevated over the ensuing 5 h (compare buffer and progesterone and 2,4-D panels) indicative of the transition from G2 into MI and MII. Because of the relatively long time points, these data did not capture the expected reduction of MPF activity as oocytes transit from MI to MII. As reported previously [29] and confirmed here, the addition of 2,4-D simultaneous to progesterone blocks histone H1 phosphorylation, in this case over a 9 h span. Importantly, MII phase oocytes with high MPF activity at 5 h, showed a significant sustained reduction in H1 kinase activity beginning at least 1 h after 2,4-D addition ($t = 5$ h). These data suggest that 2,4-D inhibits MPF activity at MII contrary to our previous preliminary conclusions based on western analysis alone (Fig. 2, lane 14).

In order to verify these seemingly conflicting results, oocytes in MI and MII were again treated with 2,4-D and examined for both H1 kinase activity as well as the presence of PP-MAPK and P-Cdc2. Fig. 6b shows that H1 kinase activity in progesterone-treated oocytes was detected above background beginning at 6 h and peaked at 8–9 h (lanes 4–12). MAPK phosphorylation and Cdc2 dephosphorylation were generally coincident with H1 kinase activity, with the detection of PP-MAPK slightly preceding P-Cdc2 dephosphorylation and H1 kinase activity (lane 8). As expected 2,4-D blocked H1 kinase activity when added with progesterone ($t = 0$ h) (Fig. 6b, lane 3), but did induce MAPK phosphorylation in the presence or absence of progesterone (lanes 2 and 3), while P-Cdc2 remained at levels similar to the G2/M control (Fig. 6b, lanes1–3). In contrast, MII oocytes exposed to 2,4-D at 7 h showed a dramatic reduction in H1 kinase activity, which confirms previous results (Fig. 6a) (data not shown). However, levels of phosphorylated MAPK remained high and no detectable rephosphorylation of tyr 15 on Cdc2 occurred. Other data (not shown) also indicate that Mos protein remained stable under these conditions. Taken together, these data suggest that in addition to blocking MPF activation at MI, 2,4-D significantly reduces MPF activity in MII through a mechanism that neither requires a decrease in the level of Mos and PP-MAPK nor Cdc2-tyr 15 rephosphorylation.

3.5. Irreversibility of maturation block

Finally, we asked whether maturation could be rescued. Oocytes were exposed transiently (4 h) to 2,4-D (primary treatment), and extensively washed. Following 8 h of secondary treatment, they were analyzed for Mos and PP-MAPK. Control oocytes that were incubated for 4 h in progesterone or 2,4-D showed characteristic protein profiles (Fig. 7a, lanes 1–3). Indeed, Mos and PP-MAPK persisted even after 8 h from the point when progesterone was removed (Fig. 7a, lane 6). In contrast, oocytes that were initially exposed to 2,4-D and washed, failed to express PP-MAPK at the end of the secondary buffer incubation (Fig. 7a, lanes 7 and 8), indicating that continuous exposure to 2,4-D is required for MAPK phosphorylation. Importantly, neither Mos nor PP-MAPK expression were induced upon secondary exposure to progesterone (Fig. 7a, lanes 9 and 10). This was not due to a general decline in responsiveness from prolonged incubation since Mos and PP-MAPK were strongly expressed in buffer-treated oocytes exposed to a secondary treatment of progesterone (Fig. 7a, lane 5). These results indicate that oocytes exposed transiently to 2,4-D become refractory to progesterone.

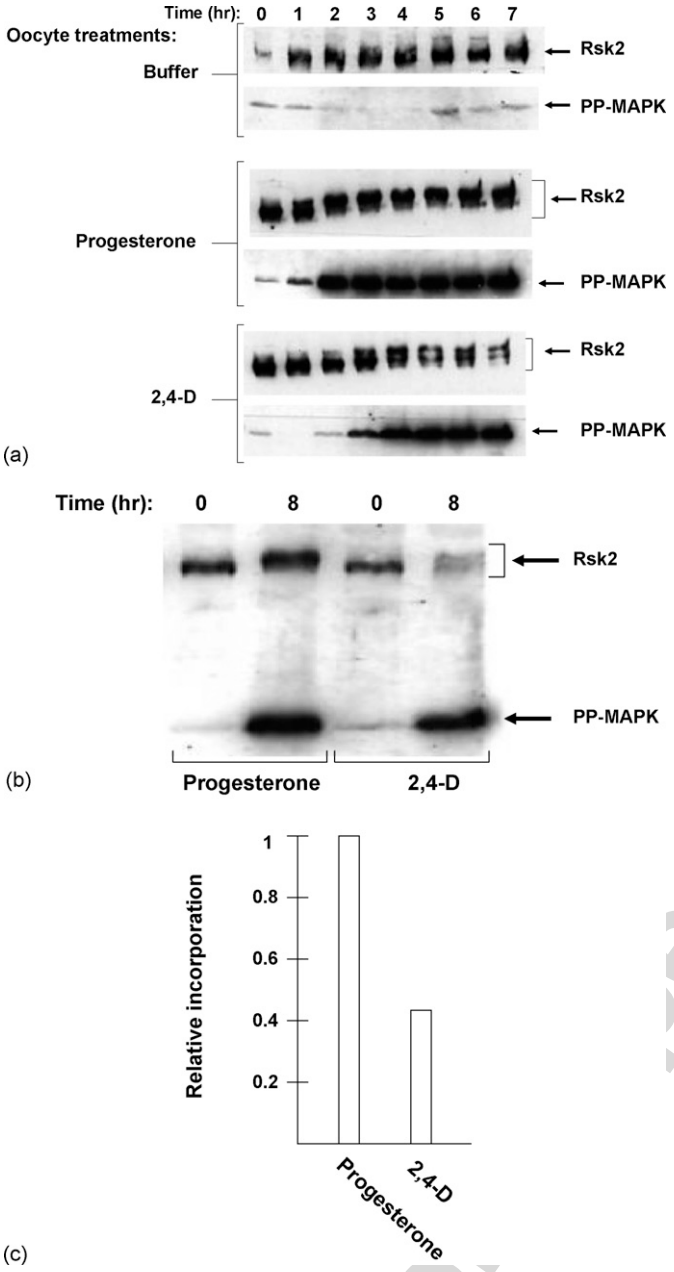

Fig. 5. Partial phosphorylation and activation of Rsk2 induced by 2,4-D. (a) Oocytes were exposed to 1× MBS (buffer), progesterone or 2,4-D (left) for the indicated times (top) and then analyzed for Rsk2 and PP-MAPK (right) by Western blot. Brackets on right highlight upward shift of Rsk2 due to phosphorylation. (b) Side-by-side comparison of Rsk2 and PP-MAPK kinase at 0 and 8 h of exposure to progesterone or 2,4-D. (c) Rsk2 kinase assays were performed using immunoprecipitated Rsk2 from progesterone and 2,4-D treated oocytes. Incorporation of radiolabelled phosphate into S6 peptide substrate was normalized to the progesterone control and represents an average of three independent experiments (±S.D.: 0.06).

To determine whether maturation could be rescued by a downstream effector, some oocytes were microinjected with a stable mutant of cyclin B (Δ90), which by itself induces maturation even in the absence of translation [39]. As expected, microinjected oocytes underwent GVBD (data not shown), and expressed Mos and PP-MAPK (Fig. 7b, lane 5). In contrast, oocytes pre-incubated in 2,4-D and washed were completely unresponsive to Δ90 (Fig. 7b, lanes 6 and 7). To further ensure that oocytes were responding appropriately to Δ90 even in the absence of translation, oocytes were pre-incubated with and without cycloheximide and assayed for the presence of PP-MAPK, P-Cdc2 and histone H1 kinase activity. In parallel, the same analysis was carried out in oocytes injected with a partially purified preparation of active MPF from unfertilized eggs. Indeed, GVBD (data not shown), and high levels of PP-MAPK and H1 activity were induced while Cdc2 was dephosphorylated (Fig. 7c, lanes 1–5), comparable to progesterone. As expected, oocytes pre-treated with cycloheximide did not undergo maturation in the presence of progesterone, indicated by the absence of GVBD (data not shown), as well as the lack of MAPK phosphorylation, P-Cdc2 dephosphorylation and H1 kinase activity (Fig 7c, lane 7). However, Cdc2 dephosphorylation was induced by Δ90 and MPF concomitant to stimulation of H1 kinase activity above background, albeit at lower levels than controls without cycloheximide (Fig. 7c, lanes 9 and 10). MAPK was not phosphorylated presumably due to the inhibition of Mos synthesis by cycloheximide (Fig. 7c, lanes 9 and 10). As reported previously [29], 2,4-D-induced MAPK phosphorylation was not blocked by cycloheximide, indicating it is a translation independent event. Taken together these controls show that two downstream maturation signaling factors, Δ90 and MPF, do indeed function as expected. However, consistent with previous results (Fig. 7b), and like progesterone they were unable to rescue maturation of oocytes transiently exposed to 2,4-D (Fig. 7c, lanes 12–14) as shown by the persistence of P-Cdc2 and the absence of H1 kinase activity relative to the positive control (Fig. 7c, lane 11). It is clear from these data that dysfunction persists in the maturation signaling pathway that includes a loss of MPF auto-amplification activity.

4. Discussion

Progesterone-induced meiotic maturation is controlled through complex signaling pathways that depend on translational and post-translational control mechanisms. These mechanisms are particularly crucial for the maturing oocyte and early embryo since transcriptional activity is silenced until the mid-blastula transition [40]. *In vitro* exposure of oocytes to the hormonal herbicide, 2,4-D, blocks maturation by interfering with both translational regulation and protein signaling. Firstly, our data suggest that 2,4-D does not require PKA activity but likely acts by interfering with downstream events in the progesterone pathway. This is based on experiments in which oocytes were microinjected with PKI to inactivate the catalytic subunit of PKA. This highly effective and selective competitive inhibitor functions in the subnanomolar range [41] without inhibiting other serine/threonine kinases [42]. We found that injecting oocytes with approximately 400 fmol of PKI induced at least 50% maturation in the absence of progesterone by approximately 6 h (data not shown). The maturation markers, Mos, active biphosphorylated MAPK and dephosphorylated Cdc2 were expressed, which corresponds well to other data [30,43]. These PKI-induced effects were all blocked by 2,4-D suggesting that PKA is not an essential mediator of 2,4-D function (Fig. 2).

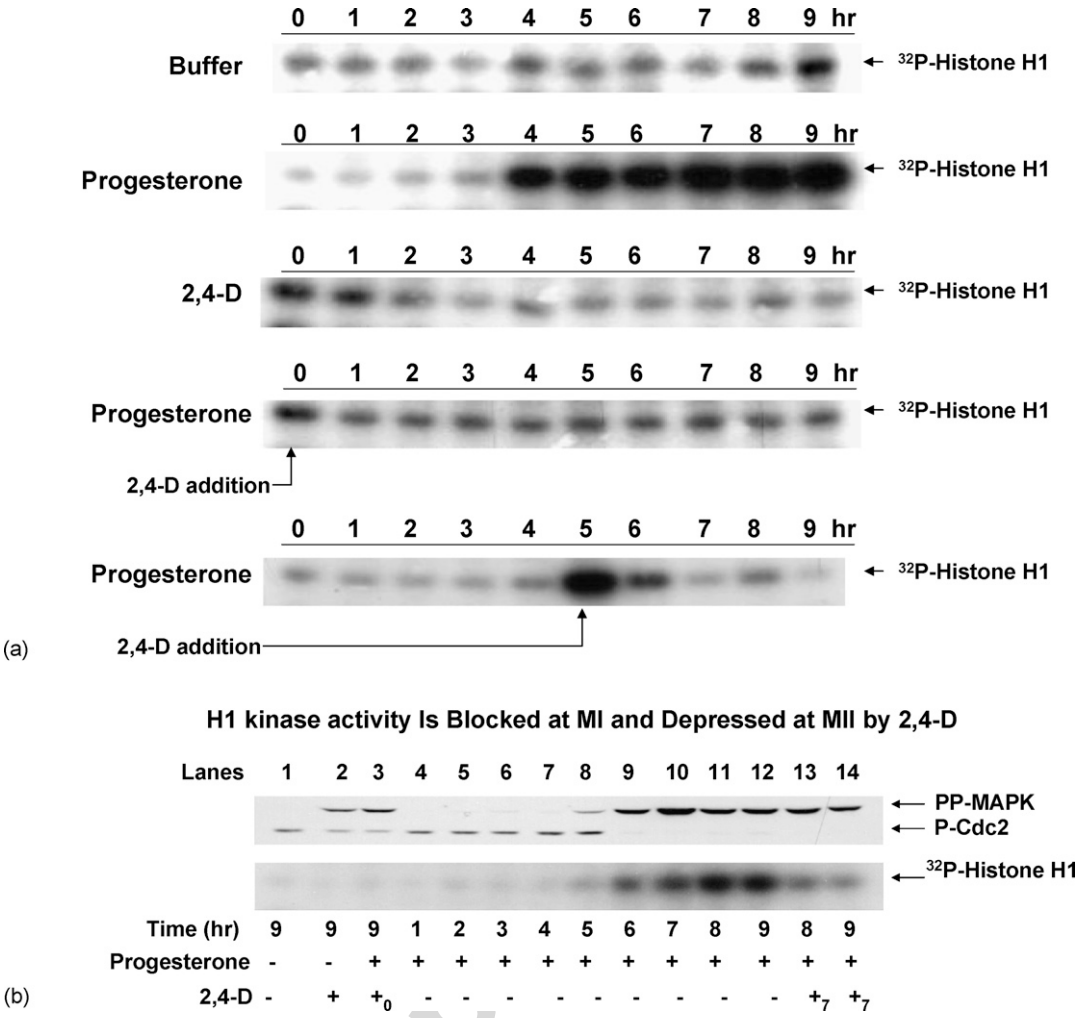

Fig. 6. Reduction of H1 kinase activity in MII oocytes exposed to 2,4-D. (a) Oocytes, all from a single female, were treated as indicated on the left and withdrawn hourly, as marked above each panel. Homogenates were prepared and assayed for H1 kinase activity. Proteins were fractionated by electrophoresis and visualized by autoradiography. Arrows indicate when 2,4-D was added to progesterone-treated samples: $t = 0$ h, concomitant to progesterone, and at $t = 5$ h, after oocytes had undergone GVBD (data not shown) and were likely in MII. (b) Oocytes from a different female were analyzed similarly as above except that extracts were further processed by western analysis as indicated on the right of the top panel. The duration of treatment (time) is indicated below each lane. The presence of progesterone and 2,4-D are indicated with (+). In lane 3, 2,4-D was added concomitant to progesterone ($+_0$) or in lanes 13 and 14 at $t = 7$ h ($+_7$).

We cannot rule out, however, the possibility that 2,4-D may weaken the binding between PKA and PKI, in which case sufficient PKAc may in fact be available to institute a maturation blockade.

Because both progesterone and PKI-induced maturation require protein synthesis [15,44], it is reasonable to suspect that 2,4-D may interfere with this mechanism. Mos is an excellent target since it is important for meiotic initiation and its synthesis is under translational control through progesterone-induced CPE-dependent polyadenylation. We have clearly shown that while progesterone induces poly(A) elongation of microinjected Mos 3′ UTR as well as cyclin B1 3′ UTR RNAs, which carry the requisite *cis*-acting polyadenylation sequences, polyadenylation is completely blocked by 2,4-D (Fig. 3a). Western blot analysis confirms that polyadenylation corresponds with synthesis of Mos protein while failure to polyadenylate negates accumulation. Though the specific mechanism of this block is unknown, a likely possibility is that 2,4-D inhibits the activation of CPEB, the primary regulator of polyadenylation. CPEB activity is controlled by differential phosphorylation during progesterone-induced maturation. When phosphorylation at Ser 174 by Eg2/Aurora is blocked, polyadenylation of Mos RNA is inhibited [45,46]. We speculate that 2,4-D may interfere with this process either by preventing Aurora activation or substrate recognition. Indeed, our preliminary data suggest that the diagnostic upward shift due to CPEB phosphorylation does not occur in oocytes exposed to 2,4-D (Fig. 3b). Another possibility is that 2,4-D may directly or indirectly perturb the more recently characterized guanine nucleotide exchange factor (XGef) that binds to CPEB in a Mos messenger ribonucleoprotein (mRNP) complex [47]. Antibodies against XGef block phosphorylation of CPEB [47], Mos mRNA polyadenylation, Mos protein expression and progesterone-induced maturation [48]. Since cyclin B RNA also fails to be polyadenylated as shown here and pre-

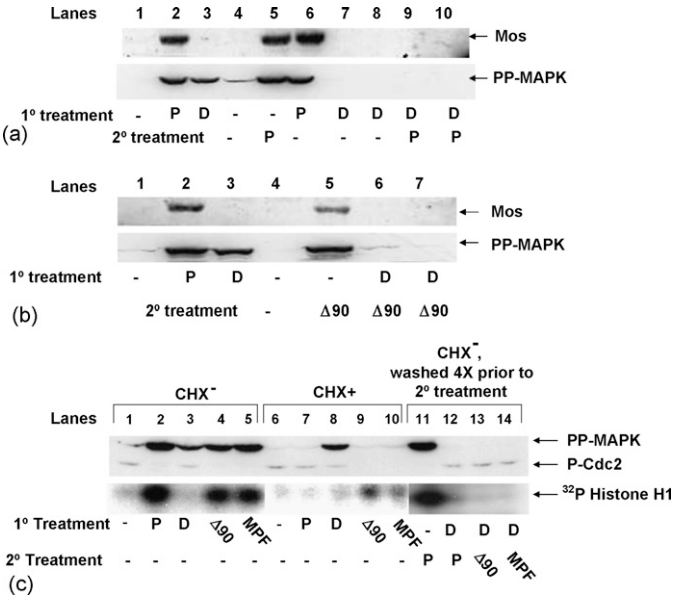

Fig. 7. Effects of transient exposure to 2,4-D on Mos and PP-MAPK expression. (a) Oocytes were incubated in 1× MBS (−), progesterone (P), or 2,4-D (D) for 4 h (1° treatment) and either immediately analyzed by Western blot (lanes 1–3) or washed and further incubated 8 h with a 2° treatment (lanes 5–10). (b) Same as above except that the microinjection of sea urchin cyclin B Δ90 (Δ90) comprised the 2° treatment. (c) Same as above with the addition of samples that were microinjected with MPF. Some oocytes were pre-treated for 1 h with cycloheximide (CHX^+). Other oocytes were incubated for 4 h in 1× MBS or 2,4-D and then washed 4× as indicated above lanes 11–14. All samples were analyzed by western blot analysis as labeled on the right of the top panel and assayed for H1 kinase activity (bottom panel).

viously [29], other RNAs under CPE-polyadenylation control, such as Ringo, a protein that activates Cdc2 and triggers GVBD [49], may also be silenced.

In the absence of Mos, one would predict that the MEK/MAPK/Rsk2/Myt1/MPF pathway would remain inactive (Fig. 7). This is confirmed in part by the persistence of inactive phospho-Tyr15-Cdc2 and the failure of oocytes to undergo GVBD (data not shown) in the presence of 2,4-D. However, a paradox exists in that MAPK is activated under these conditions (Fig. 3b, for example). Previous data showed that 2,4-D-induced MAPK required MEK but is independent of *de novo* protein synthesis [29], consistent with our data that support its uncoupling from Mos RNA polyadenylation and translation. In maturing oocytes, Mos-induced MAPK activates Rsk2, which then phosphorylates and blocks the Cdc2-inhibitory kinase, Myt1 (Fig. 1). Activated MAPK further stimulates Mos synthesis in a positive feedback loop to ensure a robust signal [51,52]. One model proposes that MAPK induces a series of direct and indirect phosphorylations on Rsk2, the latter involving Rsk2 autophosphorylation and phosphoinositide-dependent protein kinase (PDK1) [51]. These phosphorylations result in a noticeable upward shift in Rsk2 mobility as detected by Western blot analysis (Fig. 5). 2,4-D interferes with this process as shown by heterogeneous Rsk2 species that are somewhat lower in molecular weight and weaken over time (Fig. 5a). These observations indicate that MAPK does not target Rsk2 robustly and may even act on novel substrates under these conditions. Indeed, our results indicate that Rsk2 is activated to less than 50% of the progesterone controls (Fig. 5c). This may be insufficient to inactivate Myt1, and partially explain why the pool of phospho-inhibited Cdc2 is favored and maturation blocked by 2,4-D. To induce this weakened response what upstream MAPK activator might 2,4-D induce? Intriguingly, a response reminiscent of 2,4-D is reproduced in oocytes by the small GTPase, Ras, under specific conditions. Oocytes microinjected with constitutively active *Xenopus* H-RasV12 (Xe H-RasV12) undergo maturation. When translation is blocked, Xe H-RasV12 is unable to induce maturation but does stimulate phosphorylation of MAPK and Rsk2 at reduced levels relative to maturing oocytes [50]. Thus, Ras is a candidate upstream activator of MAPK and low level Rsk2 activation in translationally compromised 2,4-D-exposed oocytes (see model Fig. 8).

In somatic cells, MAPK can play a transcriptional role by phosphorylating transcription factors and co-regulators as well as by modifying chromatin [37]. We used nuclear localization as a preliminary test for a possible transcriptional function for 2,4-D-activated MAPK. Microdissection of oocytes into GVs and cytoplasm showed that MAPK, regardless of phosphorylation state, was predominantly cytoplasmic in both immature and 2,4-D-treated oocytes (Fig. 4). GVs from 2,4-D-treated oocytes tended to deform more readily relative to untreated oocytes and thus may leak, which could obscure low levels of nuclear import. In the absence of more direct and sensitive assays such as chromatin co-immunoprecipitation assays, these data show no dramatic relocalization of MAPK and thus no detectable nuclear function appears induced by 2,4-D.

To test therapeutic approaches to reversing maturation inhibition, several strategies were performed. Instead of chronic exposure, oocytes were incubated transiently (4 h) in 2,4-D and washed extensively. Further incubation in buffer alone caused the loss of phosphorylated MAPK suggesting that factors upstream of MAPK, possibly Ras (Fig. 8), must receive continual 2,4-D stimulation, suggestive of the absence of a positive feedback loop to sustain MAPK phosphorylation. Importantly, progesterone was unable to rescue Mos expression, MAPK activation, or GVBD in these oocytes indicating that one or more steps in the pathway are irreversibly disabled. That oocytes are refractory to progesterone could mean dysfunction at any step(s) of the signaling cascade starting with the receptor. In fact, 2,4-D can cause plasma membrane perturbations [53], which could lead to failure of productive hormone–receptor interactions. However, our data suggest that 2,4-D must interfere with other steps downstream of the receptor. Because 2,4-D blocks polyadenylation-induced translation, we tried an alternative rescue strategy that is not dependent on translation. Stable cyclin B induces maturation in the absence of protein synthesis through auto-amplification [39]. By binding monomeric Cdc2, active MPF is formed and through a positive feedback loop involving the phosphatase, Cdc25, inactive pre-MPF is converted into active MPF (Fig. 1). Although, cyclin B and crude MPF successfully induced maturation in untreated control oocytes, they were unable to rescue oocytes transiently treated with 2,4-D. This suggests that 2,4-D may exert an inhibitory effect on a translation-independent step such as Cdc25 activation. Although PKA has been shown to inhibit Cdc25 through phosphoryla-

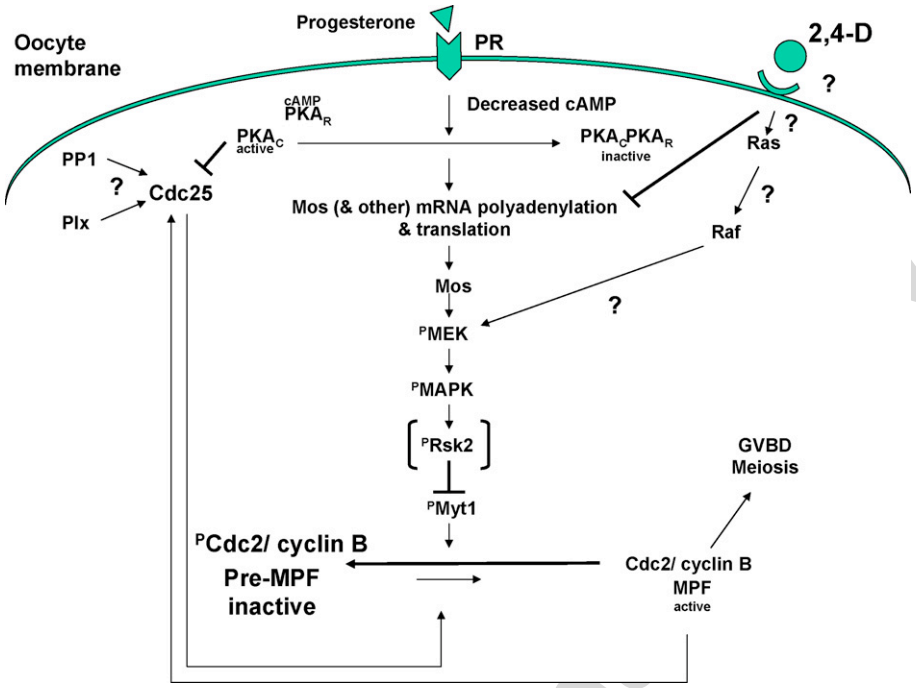

Fig. 8. Proposed model for maturation inhibition by 2, 4-D. As in Fig. 1, hammer heads indicate inhibition and arrow heads activation. The parentheses around Rsk2 indicate a decrease in phosphorylation and activation. Question marks label speculative interactions or pathways. Predominant state of MPF is indicated by the relative size of the font and arrow. Refer to Fig. 1 for other labels and the text for further explanation.

tion at S287, our data indicate that 2,4-D-induced effects do not require PKA. Instead, 2,4-D may act by blocking enzymes such as PP1 phosphatase [54] or Plx kinase [55], which play roles in activating Cdc25.

Finally, one particularly intriguing possibility to consider is the recent discovery of the 2,4-D/auxin receptor in plants, TRANSPORT INHIBITOR RESPONSE 1 (TIR1). This is the substrate-specific subunit of the ubiquitin ligase, Skp1/Cullin/F-box (SCF) that marks substrates for proteosomal degradation [56,57]. Does 2,4-D bind and activate a *Xenopus* oocyte counterpart, $SCF^{\beta TrCP}$, to induce APC/C (anaphase promoting complex/cyclosome) function, which is responsible for cyclin destruction and exit from meiosis [58,59]? This could explain why 2,4-D appears to cause dysfunction of the MPF positive feedback loop, which results in an inability to rescue transiently exposed oocytes (Fig. 7). Microinjection of small amounts of non-degradable cyclin or MPF are not likely to compensate for a declining cyclin store that is caused by degradation as well as a block on polyadenylation and translation of its mRNA. Indeed, such a model also could explain how 2,4-D abruptly suppresses MPF activity in MII phase oocytes without the inhibitory phosphorylation of Cdc2 at tyr 15 (Fig. 6b).

Because of the typically extended period of time between prophase I arrest and meiotic maturation, oocytes are particularly vulnerable to exposure to endocrine disruptors that interfere with hormone-regulated cell cycle events. These *in vitro* studies have elucidated new molecular and biochemical responses of oocytes to the maturation inhibitor, 2,4-D, and suggest avenues to further clarify the mechanisms that cause irreversible translational and post-translational dysfunction.

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