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A critical balance between Cyclin B synthesis and Myt1 activity controls meiosis entry in *Xenopus* oocytes

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SUMMARY

In fully grown oocytes, meiosis is arrested at first prophase until species-specific initiation signals trigger maturation. Meiotic resumption universally involves early activation of M phase-promoting factor (Cdc2 kinase-Cyclin B complex, MPF) by dephosphorylation of the inhibitory Thr14/Tyr15 sites of Cdc2. However, underlying mechanisms vary. In *Xenopus* oocytes, deciphering the intervening chain of events has been hampered by a sensitive amplification loop involving Cdc2-Cyclin B, the inhibitory kinase Myt1 and the activating phosphatase Cdc25. In this study we provide evidence that the critical event in meiotic resumption is a change in the balance between inhibitory Myt1 activity and Cyclin B neosynthesis. First, we show that in fully grown oocytes Myt1 is essential for maintaining prophase I arrest. Second, we demonstrate that, upon upregulation of Cyclin B synthesis in response to progesterone, rapid inactivating phosphorylation of Myt1 occurs, mediated by Cdc2 and without any significant contribution of Mos/MAPK or Plx1. We propose a model in which the appearance of active MPF complexes following increased Cyclin B synthesis causes Myt1 inhibition, upstream of the MPF/Cdc25 amplification loop.

KEY WORDS: Cdc2, Cyclin B, Meiosis, Myt1, Oocyte, *Xenopus*

INTRODUCTION

In vertebrate oocytes, arrest of the meiotic cell cycle at the first prophase (prophase I) during oocyte growth can last many years. The hormonal signals that reawaken the meiotic cycle lead to rapid entry into the first meiotic M phase, mediated by activation of the universal M phase-promoting factor (MPF). Although the main regulators of the MPF complex (Cdc2 kinase-Cyclin B) are well known, the exact series of events that lead to MPF activation are still not completely resolved.

In *Xenopus*, fully grown oocytes contain a stockpile of MPF in an inactive state (pre-MPF). This accumulates throughout oogenesis, as free Cdc2 binds slowly synthesized Cyclin B and becomes phosphorylated and inactivated on two inhibitory sites, Thr14 and Tyr15, by the Wee1/Myt1 kinase (Coleman and Dunphy, 1994; Karaiskou et al., 2004; Morgan, 1995). During meiosis resumption, the inhibitory phosphorylations on Cdc2 are reversed due both to inhibition of Wee1/Myt1 and activation of the counteracting phosphatase Cdc25 (Karaiskou et al., 2004). The regulation of Cdc25 and Wee1/Myt1 is not completely understood, although both undergo phosphorylation upon M-phase entry (Perry and Kornbluth, 2007). MPF itself contributes to the phosphorylation of these key regulators, creating an amplification loop that ensures rapid and complete conversion of pre-MPF into active MPF. To initiate the positive amplification loop, a small 'starter' population of active MPF must first be generated (Masui and Markert, 1971). Potential candidates in this triggering process are Mos kinase (upstream of the MAPK pathway) and Cyclin B, the early synthesis of which initiates parallel, redundant and independent pathways for MPF activation (Amiel et al., 2009; Haccard and Jessus, 2006). One hypothesis is that the starter population of active MPF is generated by binding of newly synthesized Cyclin B to free Cdc2 to form a complex that somehow evades inactivation by phosphorylation. In line with this hypothesis, the Cyclin B1 accumulation that is observed in response to progesterone occurs independently of the MPF auto-amplification loop (Frank-Vaillant et al., 1999).

In this study we have focused on the role and regulation of Myt1, the member of the Wee1 family of inhibitory kinases that is expressed in *Xenopus* prophase I oocytes. The Wee1 family of kinases comprises Wee1, which is present in all eukaryotes, and Myt1, which is restricted to metazoans (Coleman and Dunphy, 1994). Wee1 is a nuclear kinase, phosphorylating Cdc2 on Tyr15, whereas Myt1 possesses a dual Thr14/Tyr15 Cdc2-phosphorylating activity (Liu et al., 1997; Mueller et al., 1995) and associates with cell membranes (Booher et al., 1997; Jin et al., 2005; Liu et al., 1997; Mueller et al., 1995; Nakajo et al., 2000; Okumura et al., 2002). In *Xenopus* oocytes only Myt1 is detectable at the protein level during the last stages of growth and the early steps of meiosis reinitiation. The two forms of *Xenopus* Wee1 (Wee1A and Wee1B) are not expressed in the prophase I-arrested oocytes (Nakajo et al., 2000). Wee1A becomes detectable only after completion of the first meiotic division (Murakami and Vande Woude, 1998; Nakajo et al., 2000) and Wee1B is expressed after the gastrula stage (Okamoto et al., 2002). Myt1 kinase has been implicated in both male and female gametogenesis in various animals, either alone, as is the case in *C. elegans*, *Drosophila*, starfish and *Xenopus* (Burrows et al., 2006; Jin et al., 2005; Nakajo et al., 2000), or in concert with Wee1, as in mouse oocytes (Han et al., 2005; Oh et al., 2010). In starfish, Myt1 has been clearly demonstrated to be involved both in the G2 arrest of oocytes and in meiosis re-entry, which involves downregulation of Myt1 activity by Akt phosphorylation (Okumura et al., 2002). Whether Myt1 also plays an essential role in *Xenopus* oocyte meiotic resumption remains unclear.

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The main aim of our study was to determine the contribution of Myt1 to the prophase arrest of fully grown *Xenopus* oocytes and to meiosis re-entry. A crucial issue was to determine which kinases are responsible for the initial phosphorylation and downregulation of Myt1 activity following progesterone treatment. As well as Cdc2, candidate kinases activated at around the same time include Plx1 (the *Xenopus* homolog of *Drosophila* Polo kinase) and members of the MAPK cascade, including Mos and $p90^{Rsk}$ kinase (Palmer et al., 1998; Peter et al., 2002; Inoue and Sagata, 2005).

We found that Myt1 function is required to maintain prophase I arrest in fully grown oocytes, such that experimental Myt1 inhibition promoted meiosis re-entry. At the onset of maturation, the inactivating phosphorylation of Myt1 was found to precede the activating phosphorylation of Cdc25 and to be mediated principally by Cdc2 itself in association with newly synthesized Cyclins, rather than by the Mos/MAPK cascade or Plx1. We propose a model in which the significant upregulation of Cyclin B synthesis following progesterone stimulation produces a small population of active Cdc2-Cyclin B, which is responsible for early Myt1 phosphorylation and inhibition, ahead of full Cdc25 activation and thus entry into the auto-amplification loop. In *Xenopus*, the change in the balance between Cyclin B synthesis and Myt1 activity following hormone stimulation is therefore a key feature of meiotic re-entry.

MATERIALS AND METHODS

Materials

Xenopus laevis adult females (*Xenopus* Express, France) were bred and maintained under laboratory conditions. Mos-MBP, $p21^{Cip1}$-GST, Myt1-GST, Cyclin B-GST and Cyclin A-GST proteins were purified as described previously (Haccard and Jessus, 2006; Hochegger et al., 2001; Karaiskou et al., 1999). Oligonucleotides and morpholinos were designed as described (Dupre et al., 2002) and synthesized by MWG and Gene Tools, respectively. mRNAs from pBSRNP3-myc plasmids containing XePlx1T201D and XePlx1N172A [a kind gift from E. Nigg (Descombes and Nigg, 1998)], pGEM plasmids containing *Xenopus* Cyclin B1 and Cyclin B2, pSpE3 plasmids containing Cdc2wt and Cdc2AF were synthesized using the mMessage mMachine kit (Ambion) and purified on Qiagen RNA Cleanup columns (Qiagen). BI2536 Plk1 inhibitor was from Axon Medchem, PD0166285 Wee1/Myt1 inhibitor from Tocris and the Wee1/Myt1 kinase assay kit from Cyclex. Other reagents were from Sigma.

Xenopus oocyte treatments

Xenopus oocytes were isolated, prepared and maintained at 16°C as described previously (Jessus et al., 1990). Oocytes were microinjected with antisense, sense or morpholino oligonucleotides (25-75 ng), with recombinant proteins (0.25-0.75 mg/ml) or mRNAs (0.25-1 mg/ml). Oocytes were incubated with 1 μM progesterone to trigger maturation or with 0.01 μM progesterone, which does not trigger meiosis. In some experiments oocytes were preincubated in 3 mM 6-DMAP, 50 μM U0126 (Promega), 5 μM PD0166285 (Tocris), 5 μM BI2536 (Axon) or 100 μg/ml cycloheximide. Oocyte maturation was monitored by the appearance of a white spot at the animal pole and groups of at least 40 oocytes were scored for each type of treatment. In some cases, germinal vesicle breakdown (GVBD) was confirmed by dissection of oocytes after fixation in 5% trichloroacetic acid for 20 minutes.

Xenopus oocyte extracts for western blot analysis

Oocytes were homogenized at 4°C in 10 μl extraction buffer (EB; 80 mM β-glycerophosphate pH 7.3, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol, 1 μM okadaic acid, protease inhibitor cocktail) per oocyte. Lysates were centrifuged at 15,000 ***g*** at 4°C for 1 minute and supernatants were collected and analyzed.

In order to perform lambda phosphatase treatment (New England Biolabs), prophase I and GVBD oocyte extracts were supplemented with 200 U lambda phosphatase or EB and incubated for 30 minutes at 30°C. Extracts were submitted to SDS-PAGE and analyzed with the anti-Myt1 antibody.

GST pull-downs were performed as follows: extracts from prophase I-arrested oocytes were incubated (or not) with bacterially produced Myt1-GST recombinant protein (250 ng for 20 μl of extract) for 1 hour, and then 10 μl of glutathione beads (Sigma) were added. The protein content of the beads was analyzed by anti-Cdc2 western blotting.

Myt1 kinase assay

Single oocytes were collected at different times after progesterone addition. Extracts were prepared and Myt1 kinase activity assayed using a Wee1/Myt1 kinase activity kit (Cyclex), an immunoassay based on plates pre-coated with recombinant Cdc2 and incubation with an antibody against the Tyr15-phosphorylated form of Cdc2. Horseradish peroxidase on the secondary antibody catalyzes the conversion of colorless tetramethylbenzidine (TMB) to a colored solution, the intensity of which was quantified by spectrophotometry. All assays were performed in duplicate in extracts of single oocytes and the activity measured in prophase I-arrested oocytes was taken as 100%.

Western blotting

Samples (one oocyte lysed and loaded per lane, a consistent 30 μg protein load) were electrophoresed by 10% or 12% SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes (Schleicher and Schuell). Transfer quality and protein loading were monitored by Ponceau Red staining. The following antibodies were used: anti-Myt1, raised against the C-terminal peptide (NLLGMFDDATEQ) in rabbits (Eurogentec) and affinity purified; polyclonal rabbit anti-*Xenopus* Plx1 (a kind gift of Dr T. Lorca, CRBM-CNRS, France) and mouse anti-phospho-T210-Plk1 (BD Pharmingen); polyclonal rabbit anti-*Xenopus* Cdc25 (a kind gift from Dr J. Maller, HHMI, Denver, USA) and monoclonal rabbit anti-phospho-Ser216-Cdc25 (Cell Signaling); goat anti-*Xenopus* Cyclin B2 (De Smedt et al., 1995); mouse monoclonal anti-phospho-MAPK and polyclonal rabbit anti-phospho-Tyr15-Cdc2 (Cell Signaling); polyclonal rabbit anti-Mos and polyclonal goat anti-$p90^{Rsk2}$ (Santa Cruz); mouse monoclonal anti-Cdc2 (3E1 clone, a kind gift of Dr T. Hunt, South Mimms, UK); and goat polyclonal anti-importin (Santa Cruz). Secondary HRP-conjugated antibodies were from Jackson ImmunoResearch and were detected by chemiluminescence (Perkin-Elmer). In cases where signal quantification was needed, 'Gel Eval' software was used (version 1.22, FrogDance Software) and the importin signal served as a loading control.

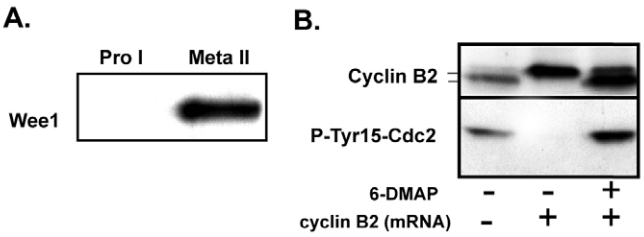
RESULTS

Myt1 kinase maintains prophase arrest in fully grown *Xenopus* oocytes

Myt1 kinase is the only detectable Cdc2 kinase expressed in *Xenopus* fully grown prophase I-arrested oocytes (Nakajo et al., 2000) (Fig. 1A and see Fig. S1 in the supplementary material), but its activity at this stage has never been addressed directly. To this end, we injected oocytes with *Cyclin B* mRNA. As oocytes contain an excess of free Cdc2, this treatment provided fresh substrate to Myt1. MPF activation and subsequent feedback loop-dependent Myt1 inhibition were prevented by treatment with 6-DMAP, which selectively inhibits Cdc2 (Jessus et al., 1991). Under these conditions, oocytes showed a net increase in the phospho-Tyr15-Cdc2 level (Fig. 1B), demonstrating that Myt1 is active in fully grown prophase I-arrested oocytes.

As a second approach to assay Myt1 kinase activity we used an in vitro immunoassay kit using recombinant Cdc2 as an Myt1 substrate (see Materials and methods). Myt1 kinase activity was consistently detected in extracts from single oocytes in prophase I, but not after M-phase entry (GVBD or metaphase II oocytes) (Fig. 1C).

The contribution of Myt1 to maintaining G2 arrest was then investigated by incubating oocytes with the chemical Myt1 inhibitor PD0166285. PD0166285 has been shown to inhibit phospho-Tyr15-Cdc2 activity in vitro and in cultured mammalian cells (Hashimoto et al., 2006; Panek et al., 1997; Potapova et al.,



C.

	5% GVBD			
	Prophase I	no GVBD	GVBD	Metaphase II
female 1	0.27	0.02	0.01	0.02
female 2	0.13	0.01	0.02	0.01
female 3	0.21	0.02	0.01	0.00

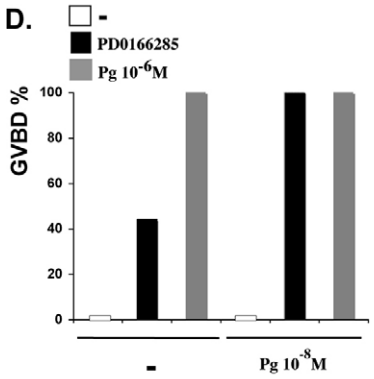


Fig. 1. Activity and localization of Myt1 kinase in prophase I-arrested oocytes. (**A**) Extracts from prophase I (Pro I)-arrested or metaphase II (Meta II)-arrested oocytes were immunoblotted with an anti-*Xenopus* Wee1A antibody. (**B**) Prophase I-arrested oocytes were incubated overnight in the presence (or not) of 3 mM 6-DMAP, and then microinjected (or not) with *Cyclin B2* mRNA. Samples were collected when germinal vesicle breakdown (GVBD) was observed in all *Cyclin B2*-microinjected oocytes. Extracts were immunoblotted with antibodies against Cyclin B2 and phospho-Tyr15-Cdc2. (**C**) Myt1 kinase activity was assayed using an immunoassay kit in single-oocyte extracts. Oocytes were collected at four different times during meiosis re-entry: prophase I, just before GVBD ('no GVBD'; oocytes without a white spot in a population where 5% GVBD was observed, knowing that 100% GVBD occurs 1 hour later), GVBD and metaphase II (2 hours after GVBD). Results from three representative females are shown. Values presented correspond to the average of duplicate optical density measurements after subtraction of the negative control value. (**D**) Prophase I-arrested oocytes were incubated overnight in the absence (–) or presence of 5 μM PD0166285 or 1 μM progesterone (Pg 10^{-6} M) and in the presence (Pg 10^{-8} M) or absence (–) of 0.01 μM progesterone. After 18 hours GVBD was scored.

2011). Overnight incubation with PD0166285 was sufficient to promote meiotic entry in the absence of progesterone stimulation, as judged by GVBD (45% GVBD in 16 hours, Fig. 1D). In the presence of 0.01 μM progesterone, a concentration that is insufficient to trigger meiotic maturation, the Myt1 inhibitor triggered GVBD in 100% of oocytes (Fig. 1D). These results indicate that *Xenopus* oocytes fail to maintain prophase I arrest when Myt1 kinase activity is inhibited, demonstrating the important inhibitory role of Myt1 in preventing MPF activation during the meiotic G2–M transition.

Myt1 kinase preferentially targets new Cdc2-Cyclin B complexes

As an independent approach to assess the role of Myt1 in G2 arrest, we microinjected mRNA encoding a double 'AF' mutant form (T14A-Y15F) of Cdc2 that cannot be phosphorylated by Myt1. This was sufficient to trigger M-phase entry without progesterone stimulation (Fig. 2A), albeit with slow kinetics, as previously reported (Pickham et al., 1992). Under the same conditions, injection of a wild-type form of Cdc2 had no effect (Fig. 2A). In prophase oocytes Cyclin B is synthesized at a slow rate and associates with Cdc2, forming the pre-MPF (Rime et al., 1994; Thibier et al., 1997). Our results suggest that excess Cdc2 binds this slowly accumulating Cyclin B and the new complexes are immediately subjected to inhibitory phosphorylation of Cdc2. When this fails, as for the AF mutant, the newly formed complexes remain active and fire the MPF auto-amplification loop. To test this hypothesis, Cyclin B synthesis was prevented with a mix of antisense oligonucleotides targeted against mRNA encoding the different B Cyclins of the *Xenopus* oocyte (B1, B2, B4 and B5) (Hochegger et al., 2001) (see Fig. S2 in the supplementary material) and different forms of Cdc2 were then expressed in these oocytes. Control 'sense' oligonucleotides were also injected and had no impact on progesterone-induced meiotic maturation, as previously reported (data not shown) (Haccard and Jessus, 2006; Hochegger et al., 2001). In the antisense-microinjected oocytes, the Cdc2AF mutant was not able to trigger meiosis re-entry (Fig. 2A), implying that this requires both Cyclin B synthesis and the absence of inhibitory phosphorylation on Cdc2. We conclude that Myt1 activity in prophase I oocytes is essential for inhibiting the activity of the slowly accumulating Cdc2-Cyclin B complexes, thereby accounting for its crucial role in maintaining prophase arrest.

To further assess Myt1 function during meiosis re-entry, oocytes were microinjected with an mRNA encoding Myt1 and then stimulated with progesterone. Overexpression of Myt1 protein in the oocytes delayed GVBD (Fig. 2B) in a dose-dependent manner (data not shown). Analysis of phospho-Tyr15-Cdc2 levels during meiosis entry in the presence of overexpressed Myt1 revealed persistent Tyr15 phosphorylation of Cdc2 at the time of GVBD, as compared with control oocytes, although both sets of oocytes exhibited Cdc2 kinase activity (Fig. 2C). This implies that Myt1-overexpressing oocytes contain a mix of active Cdc2 (non-phosphorylated on Tyr15) and inactive Cdc2 (phosphorylated on Tyr15) at GVBD. This latter population could correspond to newly synthesized Cyclin B associating with Cdc2. To address the hypothesis that Myt1 kinase targets new Cdc2-Cyclin B complexes, oocytes were co-injected with *Myt1* mRNA and the Cyclin B antisense oligonucleotide mix. Re-entry of these oocytes into meiosis following progesterone addition was significantly delayed and Cdc2 was found to be fully dephosphorylated at GVBD (Fig. 2B,C). These observations demonstrate that Myt1 does not contribute to Cdc2 phosphorylation in the absence of Cyclin B synthesis and thus that Myt1 preferentially targets newly formed Cdc2-Cyclin B complexes.

Myt1 phosphorylation is an early event in oocyte meiotic maturation

The experiments described above indicate that Myt1 activity must be downregulated to allow M-phase entry in response to progesterone. We thus focused on Myt1 phosphorylation, which correlates with inhibition of its kinase activity (Palmer et al., 1998).

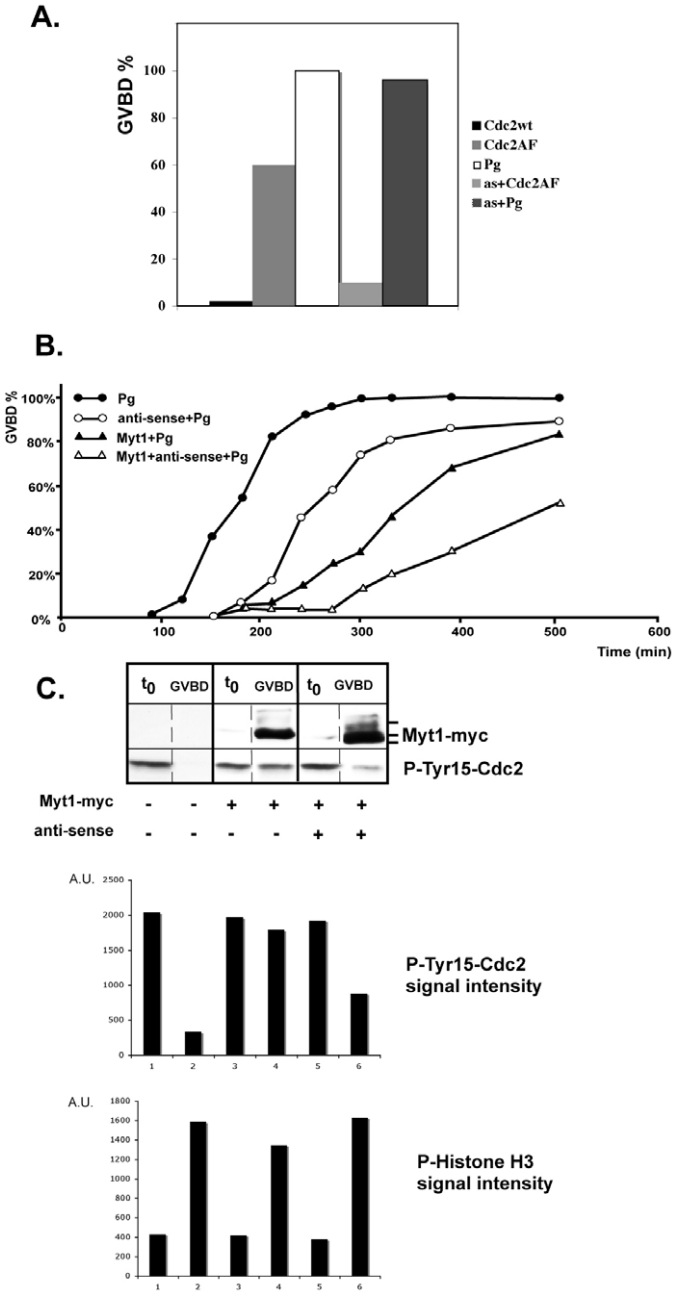


Fig. 2. Myt1 kinase preferentially phosphorylates new Cdc2-Cyclin B. (**A**) Prophase I-arrested oocytes were microinjected (or not) with a mix of antisense oligonucleotides (as) directed against *Cyclin B* mRNAs. Two hours later they were microinjected with mRNAs encoding the wild-type form of *Xenopus* Cdc2 (Cdc2wt) or the AF mutant version (Cdc2AF). Twelve hours later some oocytes were stimulated with progesterone (Pg). GVBD was scored 12 hours after progesterone addition. (**B**,**C**) Prophase I-arrested oocytes were microinjected (or not) with a mix of antisense oligonucleotides directed against *Cyclin B* mRNAs (antisense). After overnight incubation, oocytes were microinjected (or not) with mRNA encoding a myc-tagged version of *Xenopus* Myt1. Control oocytes were treated with progesterone (Pg). (B) GVBD timecourse. (C) Extracts immunoblotted using antibodies against the myc tag (Myt1-myc), phospho-Tyr15-Cdc2 and anti-phospho-Histone H3 (data not shown), at the time of progesterone addition (t_0), and at GVBD. The two lower panels show quantification of the phospho-Tyr15-Cdc2 signal and the phospho-Histone H3 signal (raw data not presented). A.U., from GelEval Software.

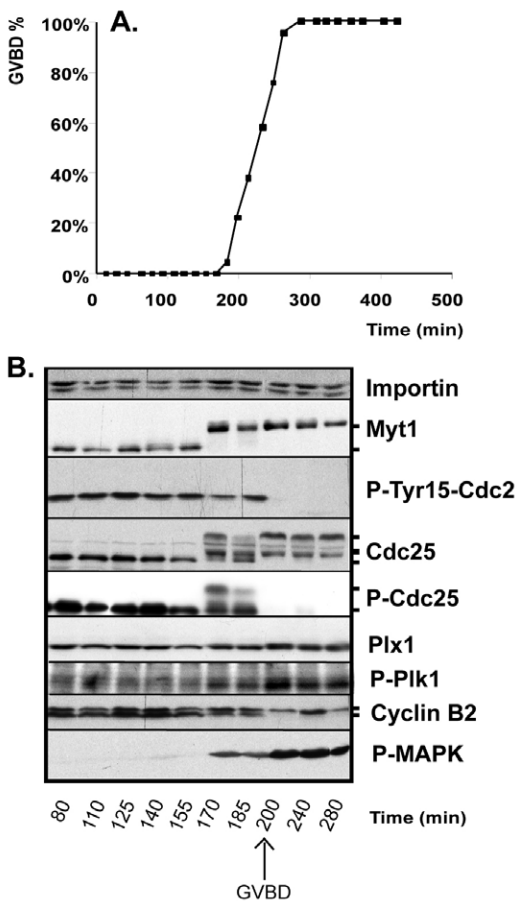


Fig. 3. Myt1 phosphorylation during progesterone-triggered M-phase entry. Prophase I-arrested *Xenopus* oocytes were treated with progesterone and single oocytes were frozen at different times post-hormone treatment as indicated. (**A**) GVBD was scored and (**B**) oocyte extracts were immunoblotted with the antibodies indicated. This is a representative experiment that was reproduced using oocytes from eight females.

We raised a rabbit polyclonal antibody to characterize the Myt1 electrophoretic migration profile, which reflects its phosphorylation level as previously reported (Mueller et al., 1995). The effect of phosphorylation on the migration profile was confirmed by phosphatase treatment (see Fig. S3 in the supplementary material). We monitored the Myt1 phosphorylation profile during progesterone-induced meiotic progression and compared it with the activation of Cdc25, Plx1, the Mos/MAPK/p90^{Rsk} cascade and MPF. Extracts derived from single oocytes were loaded in each lane, ensuring uniform protein loading and analysis of phosphorylation levels in a single cell. In the representative experiment documented in Fig. 3, GVBD started at 200 minutes after progesterone stimulation. Myt1 remained in its 'prophase I' underphosphorylated state up to 155 minutes and then shifted directly to its fully phosphorylated form at 170 minutes, before GVBD completion (Fig. 3B). This shift correlates with the sharp drop in Myt1 activity measured in oocyte extracts (Fig. 1C). Myt1 kinase activity was not detectable in the oocytes collected just before GVBD (Fig. 1C) (oocytes without a GVBD spot were collected at the time at which only 5% oocytes displayed GVBD), supporting the hypothesis that phosphorylation causes Myt1 kinase inhibition prior to GVBD.

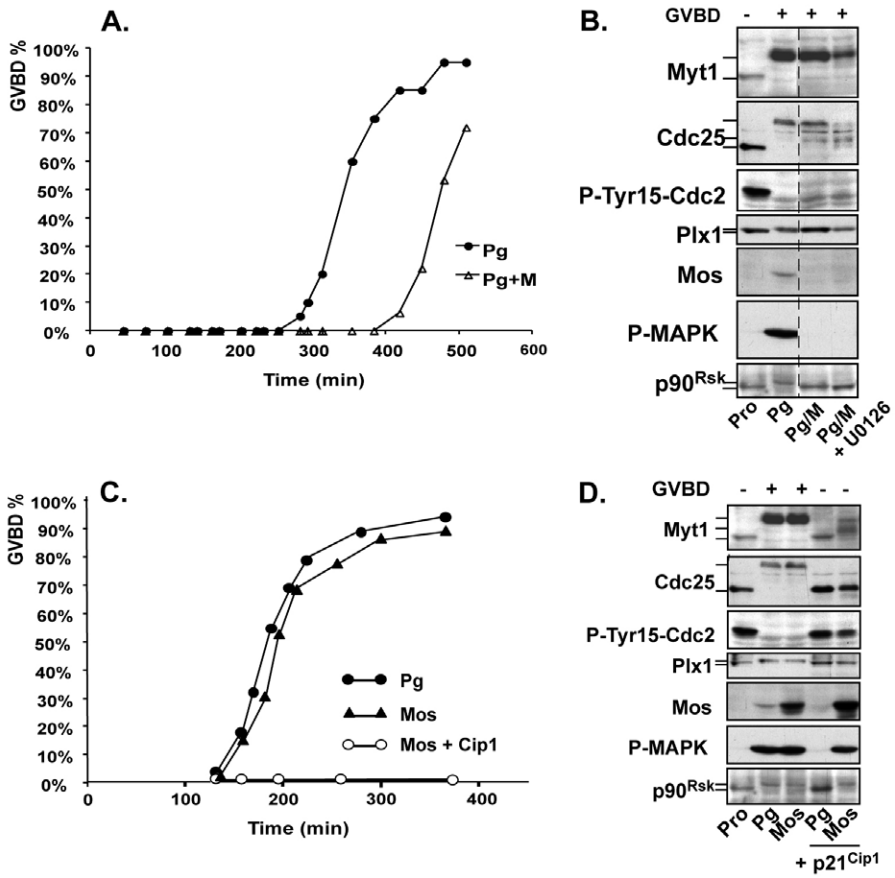


Fig. 4. Contribution of the Mos/MEK/MAPK/p90^{Rsk} pathway to Myt1 phosphorylation. (**A**,**B**) *Xenopus* prophase oocytes (Pro) were microinjected with Mos morpholinos (M) and after overnight incubation 50 μM U0126 was added (or not). Two hours later, oocytes were stimulated with progesterone (Pg). (A) GVBD timecourse. (B) Oocytes were frozen at the time of GVBD in progesterone-treated oocytes, and extracts immunoblotted with the antibodies indicated. (**C**,**D**) Prophase oocytes were injected (or not) with p21^{Cip1} recombinant protein. Twelve hours later, oocytes were stimulated with progesterone or injected with Mos protein. (C) GVBD timecourse. (D) Oocytes homogenized at the time of GVBD in progesterone-treated oocytes and immunoblotted with the antibodies indicated.

Cdc25 activation was followed using two antibodies: a polyclonal (Izumi et al., 1992) and a commercial anti-phospho-Ser216-Cdc25. The first antibody was used to reveal the electrophoretic mobility of Cdc25, reflecting its phosphorylation level (Izumi et al., 1992); the second, which was specifically raised against the inhibitory phosphorylation site of Cdc25 (Ser216 in human, Ser287 in *Xenopus*), recognizes inactive Cdc25 (Kumagai et al., 1998). Three different phosphorylation states of Cdc25 were detected during meiosis entry, perfectly reflecting Tyr15 phosphatase activity, as revealed by monitoring phospho-Tyr15-Cdc2 levels in the same oocytes. Prior to 155 minutes post-progesterone, Cdc25 showed a rapidly migrating electrophoretic profile and was still fully phosphorylated on Ser287, correlating with high phospho-Tyr15-Cdc2 levels. From 170 to 185 minutes, Cdc25 was only partially activated, as reflected by the partial shift visualized with the anti-Cdc25 antibody, a decrease in the anti-phospho-Ser216-Cdc25 signal and the persistence of the phospho-Tyr15-Cdc2 form (Fig. 3B). Full Cdc25 activation was observed only at 200 minutes, corresponding to the time of GVBD.

The activating phosphorylation of Plx1, as monitored by its characteristic mobility shift and a phospho-specific antibody, was found to be partial between 170 and 185 minutes and complete 200 minutes after progesterone addition. MAPK activation, which was monitored by the detection of its phosphorylated active form by a specific anti-phospho-MAPK antibody, was initiated at 170 minutes and completed at 200 minutes (Fig. 3B). As expected, phosphorylation of Cdc2 Tyr15 became undetectable at GVBD (200 minutes), the time of full Cdc25 activation. This also correlated with the upshift of Cyclin B2, which migrates as a doublet when associated with inactive Tyr15-phosphorylated Cdc2 and as a single upper band when associated with active Cdc2.

In summary, just before GVBD we observed full Myt1 phosphorylation that correlated with full inhibition of its kinase activity, as shown by its electrophoretic retardation and by direct kinase measurements, but only partial Cdc25 phosphorylation and activation as reflected in its electrophoretic shift, its phospho-Ser287 status and the phospho-Tyr15-Cdc2 results. Full phosphorylation of Plx1 and MAPK and the Tyr15 dephosphorylation of Cdc2 are attained at the time of GVBD. These observations suggest an early Myt1 phosphorylation-based regulatory mechanism involving a kinase that is active slightly before GVBD.

The Mos/MAPK/p90^{Rsk} pathway is neither necessary nor sufficient for in vivo Myt1 phosphorylation

It has been proposed that p90^{Rsk} and/or Mos could be major Myt1 regulators (Fisher et al., 1999; Inoue and Sagata, 2005; Palmer et al., 1998; Peter et al., 2002). This hypothesis is not easy to reconcile, however, with the observation that progesterone can trigger MPF activation when the MAPK pathway is inhibited (Dupre et al., 2002; Gross et al., 2000). In order to test the involvement of the MAPK pathway in Myt1 inhibition, we monitored Myt1 phosphorylation while Mos synthesis was inhibited by morpholino oligonucleotides. As expected, the injection of Mos morpholinos did not prevent GVBD induced by progesterone, but delayed the maturation timecourse (Fig. 4A). Mos accumulation and MAPK phosphorylation were effectively inhibited (Fig. 4B). Nevertheless,

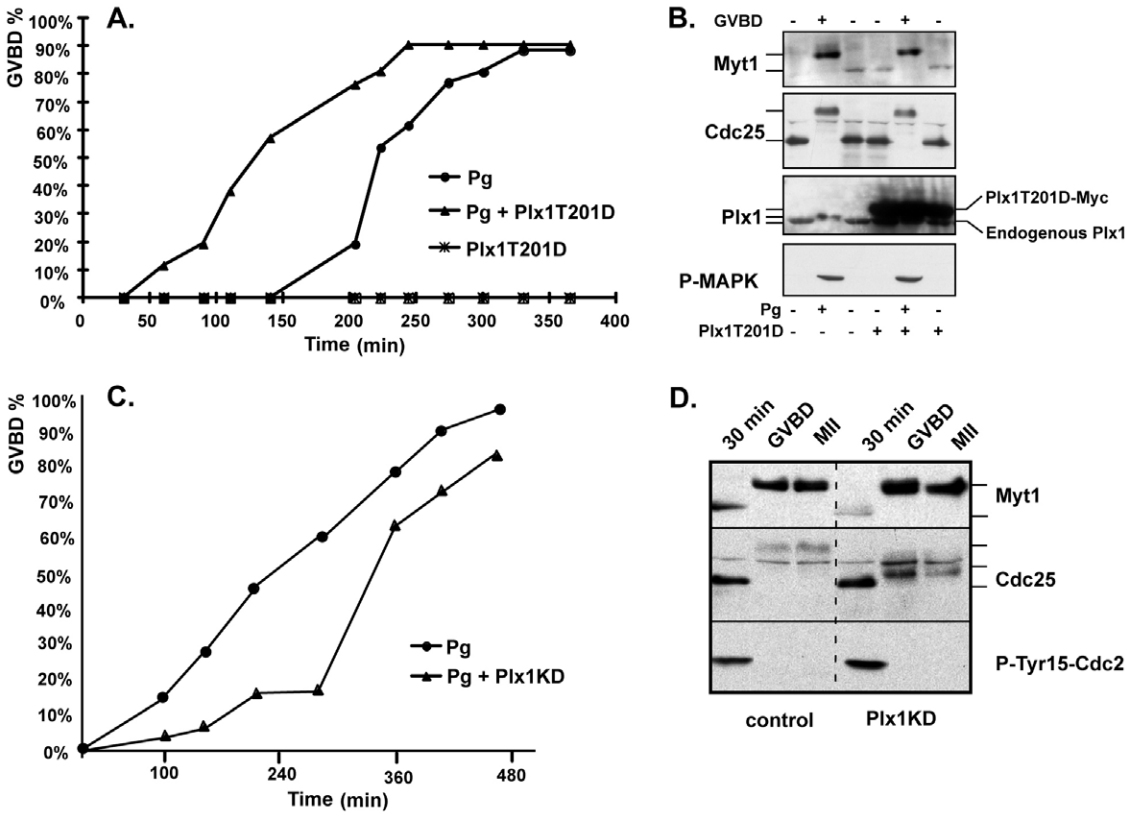


Fig. 5. Contribution of Plx1 to Myt1 phosphorylation. (**A**,**B**) Prophase I-arrested *Xenopus* oocytes were injected (or not) with mRNA encoding Plx1T201D. Twelve hours later, oocytes were injected (or not) with $p21^{Cip1}$ recombinant protein and then incubated (or not) in progesterone (Pg). (A) GVBD timecourse. (B) Oocytes were homogenized at the time of GVBD or at the end of the experiment in cases where GVBD did not occur. Extracts were immunoblotted with the antibodies indicated. (**C**,**D**) Prophase I-arrested oocytes were microinjected (or not) with mRNA encoding the kinase-dead version of Plk1 (Plx1KD). After overnight incubation, progesterone was added. (C) GVBD timecourse. (D) Oocytes were collected either 30 minutes after progesterone stimulation or at the time of GVBD or 2 hours post-GVBD (metaphase II stage, MII) and immunoblotted with the antibodies indicated.

Cdc2 kinase was activated (as monitored by Tyr15 dephosphorylation), as were Cdc25 and Plx1 (Fig. 4B). Note that the Cdc25 mobility shift was only partial in the absence of Mos, indicating that the MAPK pathway is involved in the full activation of Cdc25. Importantly for this study, hyperphosphorylation of Myt1 was unaffected by Mos/MAP kinase inhibition (Fig. 4B). It has been reported that $p90^{Rsk}$ kinase can be partially activated in oocytes injected with Mos morpholinos (Dupre et al., 2002). We showed that $p90^{Rsk}$ is not responsible for Myt1 phosphorylation in Mos morpholino-injected oocytes, as additional treatment with the MEK inhibitor U0126 did not affect Myt1 hyperphosphorylation (Fig. 4B). This indicates that the MAPK pathway is not required for Myt1 phosphorylation in response to progesterone, but does contribute to full Cdc25 activation.

We then investigated the impact of MAPK activity on Myt1 phosphorylation in the absence of active Cdc2 kinase. Oocytes were first injected with the CDK inhibitor $p21^{Cip1}$ and then with the recombinant Mos protein to activate the MAPK pathway. As expected (Frank-Vaillant et al., 1999), $p21^{Cip1}$ prevented progesterone-induced maturation; GVBD did not occur and the activation of Cdc2, Cdc25 and Plx1 did not take place (Fig. 4C,D). Injection of Mos protein led to the full activation of both MAPK and $p90^{Rsk}$ (Fig. 4D). In the absence of active Cdc2, the MAPK/$p90^{Rsk}$ pathway induced only a partial mobility shift of Myt1. We demonstrated that this partially phosphorylated Myt1 still possessed kinase activity by providing newly formed Cdc2-Cyclin complexes as a substrate for Myt1 kinase in oocytes injected with Mos and $p21^{Cip1}$ and subsequently with *Cyclin A* mRNAs. Under these conditions, Cyclin A expression led to an increase in the Tyr15 phosphorylation level of Cdc2 (see Fig. S4 in the supplementary material, compare lanes 4 and 5), indicating that the new Cdc2-Cyclin complexes were phosphorylated by Myt1. Thus, in the absence of active Cdc2, the partial phosphorylation of Myt1 induced by MAPK/$p90^{Rsk}$ is insufficient for its full inhibition.

Plx1 activity is neither sufficient nor necessary for Myt1 phosphorylation in vivo

According to published work, probable candidates for phosphorylation of Myt1 in the absence of an active Mos/MEK/MAPK/$p90^{Rsk}$ pathway are the Cdc2 and/or Plx1 kinases. To test whether Plx1 can exert a direct effect on Myt1, we used a Plx1 mutant carrying a phosphomimic mutation on Thr201 (Plx1T201D), which has been shown to increase Plx1 kinase activity several fold (Qian et al., 1999). Oocytes were injected with mRNA encoding Plx1T201D, leading to expression at a high level (Fig. 5B). This strongly accelerated progesterone-induced oocyte maturation, showing that the protein is active (Fig. 5A). Despite the high expression level, the mutant protein was not able to trigger meiotic maturation alone, nor to regulate the phosphorylation level of Cdc2, Cdc25 or Myt1 (Fig. 5A,B).

In order to monitor the contribution of Plx1 activity to Myt1 inhibition, oocytes were injected with mRNA encoding a kinase-dead version of Plx1 [Plx1N172A or Plx1KD (Qian et al., 1999)] and progesterone was added. Progesterone-induced meiosis entry was delayed by Plx1KD expression, showing that the mutant exerts a dominant-negative effect (Fig. 5C); however, at the time of GVBD, Myt1 phosphorylation was comparable with that of control oocytes (Fig. 5D). By contrast, Cdc25 displayed only partial phosphorylation in the Plx1KD-injected oocytes (Fig. 5D). This result clearly indicates that phosphorylation by Plx1 is dispensable for full Myt1 phosphorylation, in contrast to Cdc25 phosphorylation which is partially dependent on Plx1. Plx1 inhibition was also achieved by treatment with the Plk1 inhibitor BI2536 (Steegmaier et al., 2007). Incubation of oocytes in 5 μM BI2536 gave similar results to those obtained by Plx1KD injection (see Fig. S5 in the supplementary material).

Cdc2-Cyclin B activity alone is sufficient and necessary for early Myt1 phosphorylation

The experiments described above show that Plx1 and the MAPK pathway, as proposed Myt1 regulators, are neither necessary nor sufficient for Myt1 phosphorylation, an early event in meiosis resumption preceding the MPF auto-amplification loop. We then investigated whether active Cdc2-Cyclin B complexes resulting from the upregulation of Cyclin B synthesis in response to progesterone could be responsible for Myt1 regulation.

We tested this by generating Cdc2-Cyclin B activity in oocytes in the absence of MAPK pathway activation, Plx1 activity and any newly synthesized protein. Oocytes were treated with the protein synthesis inhibitor cycloheximide and then microinjected with Cyclin B protein, which is sufficient to induce GVBD in *Xenopus* oocytes (Karaiskou et al., 2001). Myt1 phosphorylation reached its full level 1 hour after Cyclin B injection, even in oocytes where GVBD was not yet completed (Fig. 6A), in the absence of MAPK and Plx1 activity (Fig. 6A). By contrast, in the presence of cycloheximide, the Cdc25 phosphorylation level induced by Cyclin B injection was only partial. These experiments indicate that, in contrast to Cdc25, full Myt1 phosphorylation can be triggered by Cdc2-Cyclin B kinase independently of Plx1, the Mos/MAPK pathway and any other newly synthesized protein.

Finally, we demonstrated that the progesterone-induced newly formed Cdc2-Cyclin B complexes play a major role in Myt1 regulation by injecting prophase I-arrested oocytes with the mix of antisense oligonucleotides against *Cyclin B* mRNAs. In control progesterone-treated oocytes, Myt1 underwent a phosphorylation shift before GVBD, whereas Cdc2 was still phosphorylated on Tyr15 (Fig. 6B). In the antisense-injected oocytes, Myt1 phosphorylation was not complete before GVBD and was only achieved between 4 and 5 hours, at the time of GVBD, Cdc2 activation and Cdc25 upregulation (Fig. 6B). Therefore, the phosphorylation timecourse of Myt1 depends on the formation of new Cdc2-Cyclin B complexes. When Cyclin B synthesis is inhibited, Myt1 phosphorylation no longer precedes Cdc2 and Cdc25 full activation, but occurs only at the time of GVBD, probably owing to active MPF generated from the pre-MPF. This strongly argues for a major role of the newly formed Cdc2-Cyclin B complexes in Myt1 phosphorylation.

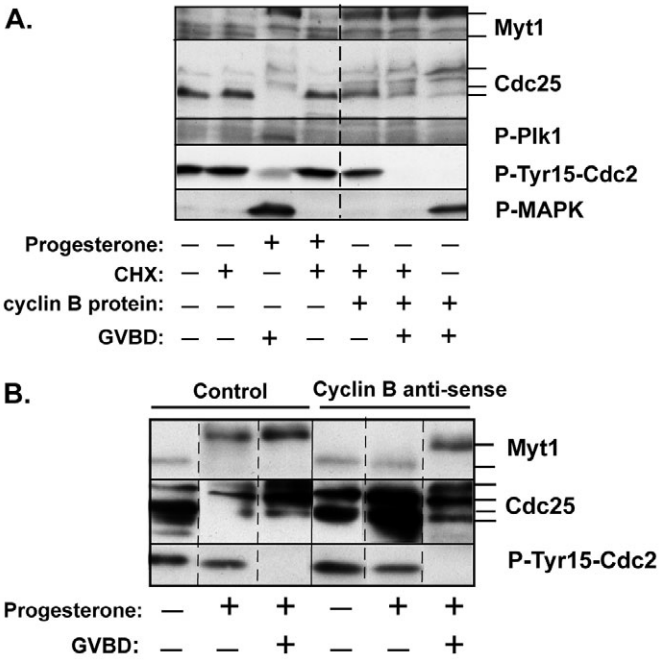


Fig. 6. Cdc2-Cyclin B implication in Myt1 phosphorylation. (**A**) Prophase I-arrested *Xenopus* oocytes were incubated (or not) in the presence of 100 μg/ml cycloheximide (CHX). Oocytes were then microinjected with Cyclin B protein or treated with progesterone. Cyclin B-injected oocytes were collected 1 hour post-injection, independently of GVBD. Non-injected oocytes were collected at the time of GVBD in progesterone-treated oocytes. Extracts were analyzed by immunoblotting using the antibodies indicated. (**B**) Prophase I oocytes were microinjected (or not) with a mix of antisense oligonucleotides directed against *Cyclin B* mRNAs. After overnight incubation, oocytes were stimulated with progesterone (or not) and GVBD kinetics was followed. 50% GVBD was attained at 3 hours 30 minutes post-progesterone addition for control oocytes and at 5 hours for oligonucleotides-injected oocytes. Oocyte extracts were analyzed by immunoblotting with the antibodies indicated, 30 minutes prior to GVBD or at the time of GVBD.

DISCUSSION

In this study we provide in vivo evidence that Myt1, the Wee1 family member expressed specifically in *Xenopus* prophase I-arrested oocytes, is a key regulator both of prophase I arrest and of the initial triggering of the MPF amplification loop. Furthermore, we were able to dissociate Myt1 and Cdc25 regulation ahead of GVBD. We propose a model in which the critical event at the transition between prophase arrest and meiotic entry is the balance between Cyclin B synthesis and Myt1 activity (Fig. 7). Before hormonal stimulation, Cyclin B accumulation is slow and newly formed Cdc2-Cyclin B is immediately inactivated by Myt1. Following progesterone treatment, stimulation of Cyclin B translation leads to abundant newly formed Cdc2-Cyclin B complexes that escape Myt1 inhibition and cause Myt1 downregulation, triggering initiation of the MPF activation loop.

Myt1 maintains prophase arrest in *Xenopus* oocytes

In G2-arrested fully grown *Xenopus* oocytes, the pre-MPF stock is in place and its direct activator, Cdc25, is present and inactive. A simple scenario for progesterone-induced MPF activation could rely solely on the activation of Cdc25 converting pre-MPF into active MPF, without the involvement of Myt1. The role of Myt1 in the maintenance of G2 arrest and the necessity for Myt1 inhibition for meiosis entry in *Xenopus* oocytes had not been explicitly addressed.

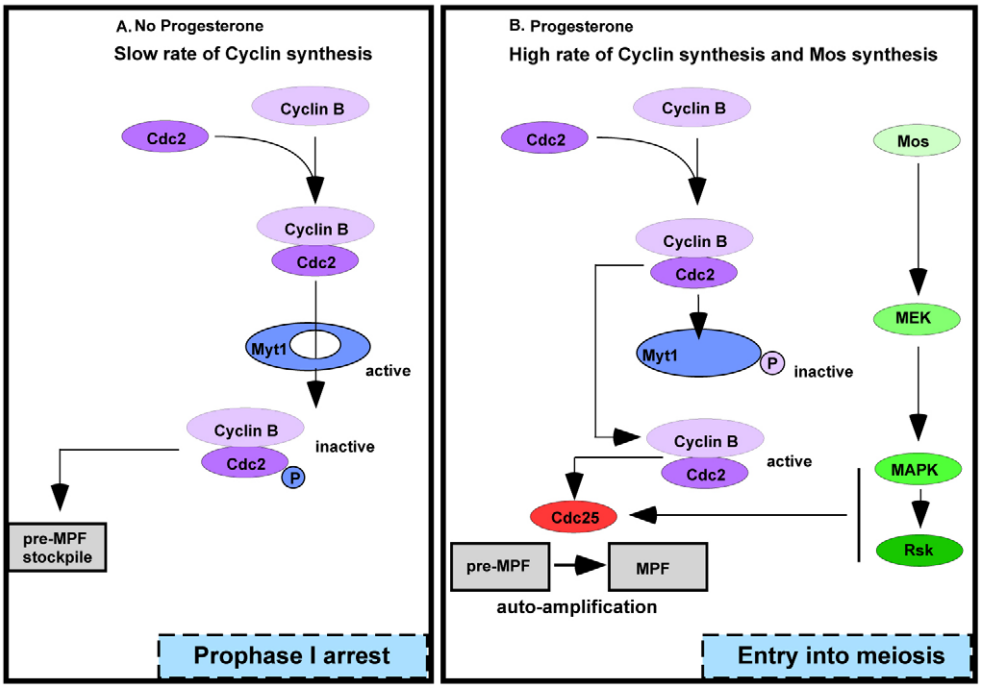


Fig. 7. Revised model for meiosis resumption. (**A**) Prophase I-arrested fully grown *Xenopus* oocytes contain a large pool of pre-MPF. Cdc2-Cyclin B complexes continue to accumulate slowly but are immediately subject to phosphorylation and inhibition by colocalized and fully active Myt1 kinase, thereby maintaining prophase I arrest. (**B**) In response to progesterone, the Cyclin B synthesis rate increases significantly and Mos starts to accumulate. Myt1 kinase activity is insufficient to maintain inhibition of new Cdc2-Cyclin B complexes, which phosphorylate and thereby inhibit their own inhibitor, Myt1. The active newly formed Cdc2-Cyclin B complexes thus participate, along with the MAPK pathway, in the full activation of Cdc25, firing the MPF auto-amplification loop. MPF, M phase-promoting factor.

We have demonstrated for the first time the presence of active Myt1 in fully grown prophase I-arrested oocytes by observing an increase in the phospho-Tyr15-Cdc2 level under experimental conditions in which the initiation of the MPF positive-feedback loop was prevented and also by a direct in vitro assay of Myt1 activity. We found that Myt1 overexpression delays meiosis resumption induced by progesterone and, conversely, that its experimental inhibition is sufficient to trigger entry into meiotic M phase without progesterone. These results indicate that regulation of Myt1 activity is a key element for the meiotic G2–M transition. Furthermore, overexpression of a non-phosphorylatable form of Cdc2 (T14A, Y15F) was sufficient to induce meiosis entry in a Cyclin B synthesis-dependent manner, whereas injected Myt1 preferentially phosphorylated newly formed Cdc2-Cyclin B complexes. We thus conclude that new Cdc2-Cyclin B complexes, which are continuously formed in fully grown *Xenopus* oocytes, are immediately inactivated by Myt1. If this fails, meiotic resumption starts, demonstrating the essential role of Myt1 in prophase I arrest.

To summarize, we have shown that fully grown oocytes sustain continuous formation of new Cdc2-Cyclin B complexes, the activity of which are actively inhibited by Myt1. We propose that this kinase plays a major role in maintaining the prophase I arrest of fully grown oocytes and that its downregulation is crucial to allow M-phase entry. Interestingly, this model is not specific to *Xenopus*, as Myt1 and Wee1 are both required to control meiotic entry in mouse oocytes (Han et al., 2005; Oh et al., 2010).

Phosphorylation and regulation of Cdc25 and Myt1 are dissociable prior to GVBD

If Myt1 maintains prophase arrest, how is its action circumvented following hormone stimulation? Our detailed comparison of the timing of inhibitory phosphorylation of Myt1, activating phosphorylation of Cdc25 and the regulatory phosphorylations of other key MPF regulators following progesterone treatment revealed that Myt1 phosphorylation is the first event to be completed, before full Cdc25 activating phosphorylation, indicating that the regulatory mechanisms of these two opposing enzymes are uncoupled under physiological conditions of prophase I release. In addition to Cdc25 phosphorylation, Plx1 and MAPK phosphorylation and the Tyr15 dephosphorylation of Cdc2 are all completed later than Myt1 phosphorylation. Moreover, measurement of endogenous Myt1 kinase activity showed for the first time that Myt1 activity is downregulated in response to progesterone, and that it occurs just before GVBD, at the same time as Myt1 phosphorylation. Overall, these observations suggest that Myt1 kinase is subject to inhibitory phosphorylation just before GVBD.

This uncoupling of Myt1 inhibition and Cdc25 activation in ovo following progesterone stimulation can be explained by differences in the identity of their kinases. Importantly, we found that Myt1 phosphorylation and inhibition can be solely mediated by Cdc2, whereas the Mos-MAPK pathway and Plx1 make a significant contribution to the phosphorylation of Cdc25.

Human and *Xenopus* Myt1 have been shown to be directly phosphorylated by Cdc2 in vitro (Inoue and Sagata, 2005), whereas Cdc2 activation by RINGO/Speedy injection in *Xenopus* oocytes leads to phosphorylation of microinjected Myt1 (Ruiz et al., 2008; Ruiz et al., 2010). As Cyclin B synthesis occurs early in response to progesterone, independently of the MPF auto-amplification loop, and Myt1 activation is the earliest detectable phosphorylation among the MPF regulators, we propose that Myt1 full phosphorylation is achieved by the rapidly accumulating Cdc2-Cyclin B complexes post-progesterone. This is supported by our demonstration that activating Cdc2-Cyclin B alone, in the absence of MAPK and Plx1 activities, can fully account for Myt1 phosphorylation. These results strongly argue for a crucial role of newly formed Cdc2-Cyclin B complexes in Myt1 inhibitory phosphorylation, which occurs in advance of the MPF auto-amplification loop and can be considered a meiosis trigger.

Roles for the Mos/MAPK and Plx1 pathways

Mos and $p90^{Rsk}$ have been proposed as important negative regulators of Myt1 (Inoue and Sagata, 2005; Palmer et al., 1998; Peter et al., 2002). Nevertheless, MPF activation in response to progesterone can occur in the absence of the MAPK pathway (Dupre et al., 2002). We show that progesterone can induce full Myt1 phosphorylation in the absence of both Mos synthesis and the activity of its downstream kinases, i.e. MEK, MAPK and $p90^{Rsk}$. This indicates for the first time that the MAPK pathway is dispensable for full phosphorylation and thus inhibition of Myt1. Furthermore, Myt1 was found to be only partially phosphorylated and was not inhibited under conditions in which the MAPK pathway was activated but Cdc2 and Plx1 inhibited. Thus, the MAPK pathway cannot be the sole Myt1 regulator.

It is important to note that, unlike Myt1 phosphorylation, Cdc25 phosphorylation was reproducibly impaired when the MAPK cascade was blocked. This strengthens the hypothesis of a role for the MAPK pathway in Cdc25 phosphorylation and could explain how the upregulation of the synthesis of Mos, an important early event in meiosis re-entry, contributes to MPF activation.

Another plausible candidate for the initial Myt1-inactivating kinase during meiosis resumption is Plx1. Polo kinases have been reported to phosphorylate Myt1 in HeLa cells, in starfish oocytes and in *Xenopus* fertilized eggs (Inoue and Sagata, 2005). Gain- or loss-of-function approaches allowed us to establish unambiguously that Plx1 kinase is neither sufficient nor necessary for Myt1 phosphorylation upon meiosis resumption. This result was not entirely unexpected as it was already established that Plx1 kinase activation depends on Cdc2 kinase (Abrieu et al., 1998; Karaiskou et al., 1998). Furthermore, Plx1 kinase functions by binding to its substrates via a Polo-binding domain (PBD) that requires prior phosphorylation by Cdks (Elia et al., 2003).

A new model for meiosis reinitiation

Taken together, our observations lead us to propose the following sequence of events in response to progesterone in *Xenopus* oocytes (Fig. 7). In fully grown prophase I oocytes, a large stock of pre-MPF is present, but more is continuously generated by slow Cyclin B synthesis and the formation of new Cdc2-Cyclin B complexes (Rime et al., 1994; Thibier et al., 1997), which are phosphorylated and inhibited by Myt1, ensuring prophase I arrest (Fig. 7A). In response to progesterone, the Cyclin B synthesis rate rises significantly (Frank-Vaillant et al., 1999; Kobayashi et al., 1991) and the new Cdc2-Cyclin B complexes escape Myt1 inhibitory phosphorylation and are able to inhibit Myt1. The 'escape' could be explained by the titration of Myt1 kinase by increasing quantities of new Cdc2-Cyclin B complexes and/or because the new complexes are biochemically different (Cyclin B1 and B4 and RINGO/Speedy are expressed in response to progesterone) (Ferby et al., 1999; Gutierrez et al., 2006) or differently localized. The kinase activity of these new Cdc2 complexes is sufficient to inhibit their own inhibitor, Myt1, thus creating the initial trigger for MPF amplification and meiosis entry.

Our study revealed an unexpected asymmetry between the two antagonistic regulators of Cdc2, i.e. Cdc25 and Myt1, in promoting meiotic entry. Intriguingly, new data suggest that the Cyclin synthesis-Wee1/Myt1 activity balance could serve as a trigger for the entry of somatic cells into mitotic M phase, with active Cdc2-Cyclin A accumulation being a key Wee1 and M-phase entry regulator (Deibler and Kirschner, 2010; Fung et al., 2007; Lorca et al., 2010). It will be fascinating to uncover more about how this critical balance is controlled in meiosis and mitosis.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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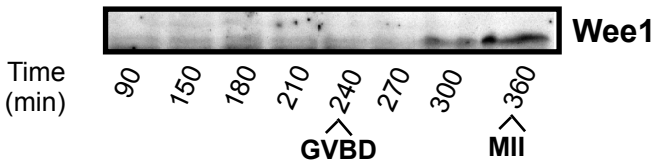
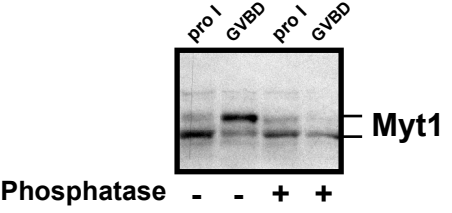
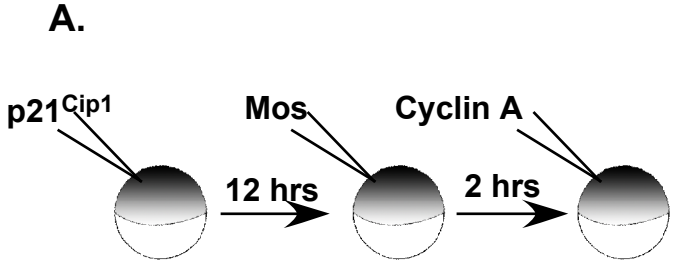

Wee1
Time
(min)
90
150
180
210
240
270
300
360
GVBD
MII

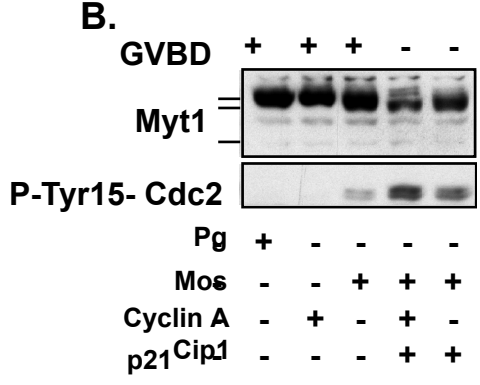
cyclin B1

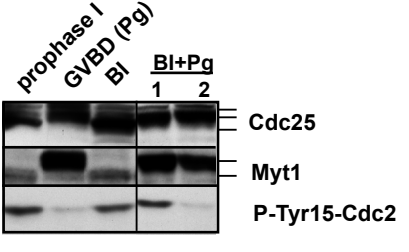
anti-sens oligos: - + - +

Prophase GVBD


pro I
GVBD
pro I
GVBD
Myt1
Phosphatase - - + +


A.
p21Cip1
Mos
Cyclin A
12 hrs
2 hrs


B.
GVBD + + + - -
Myt1
P-Tyr15- Cdc2
Pg + - - - -
Mos - - + + +
Cyclin A - + - + -
p21Cip1 - - - + +


prophase I
GVBD (Pg)
BI
BI+Pg
1
2
Cdc25
Myt1
P-Tyr15-Cdc2