The Polo-like kinase Plx1 interacts with and inhibits Myt1 after fertilization of *Xenopus* eggs

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During the meiotic cell cycle in *Xenopus* oocytes, p90^{rsk}, the downstream kinase of the Mos–MAPK pathway, interacts with and inhibits the Cdc2 inhibitory kinase Myt1. However, p90^{rsk} is inactivated after fertilization due to the degradation of Mos. Here we show that the Polo-like kinase Plx1, instead of p90^{rsk}, interacts with and inhibits Myt1 after fertilization of *Xenopus* eggs. At the M phase of the embryonic cell cycle, Cdc2 phosphorylates Myt1 on Thr478 and thereby creates a docking site for Plx1. Plx1 can phosphorylate Myt1 and inhibit its kinase activity both *in vitro* and *in vivo*. The interaction between Myt1 and Plx1 is required, at least in part, for normal embryonic cell divisions. Finally, and interestingly, Myt1 is phosphorylated on Thr478 even during the meiotic cell cycle, but its interaction with Plx1 is largely inhibited by p90^{rsk}-mediated phosphorylation. These results indicate a switchover in the Myt1 inhibition mechanism at fertilization of *Xenopus* eggs, and strongly suggest that Plx1 acts as a direct inhibitory kinase of Myt1 in the mitotic cell cycles in *Xenopus*.

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Introduction

In most eukaryotic cells, the mitotic cell cycle consists of two alternating S and M phases with intervening G1 and G2 phases. The G2/M transition is a crucial point for progression through the cell cycle and is controlled by the Cdc2–cyclin B complex (reviewed by Nurse, 1990; Morgan, 1995). The Wee1 family of protein kinases phosphorylates and inhibits Cdc2 during interphase of the cell cycle, while the Cdc25 phosphatase dephosphorylates and activates it at entry into the M phase (reviewed by Coleman and Dunphy, 1994). The Wee1 family consists of Wee1 (present in all eukaryotes), Mik1 (in fission yeast), and Myt1 (in metazoans). While Wee1 and Mik1, nuclear kinases, phosphorylate Cdc2 exclusively on Tyr15 (Featherstone and Russell, 1991; Mueller *et al.*, 1995a), Myt1, a membrane-associated kinase, phosphorylates it on

both Thr14 and Tyr15 (Mueller *et al.*, 1995b; Liu *et al.*, 1997). In addition to being a negative regulator of Cdc2, Myt1 may also function to sequester Cdc2–cyclin B to the Golgi apparatus (Liu *et al.*, 1999).

Immature animal oocytes are generally arrested at the first meiotic prophase (prophase-I), a stage equivalent to the G2 phase of the mitotic cell cycle (reviewed by Sagata, 1996; Nebreda and Ferby, 2000). In several known species, Myt1, but not Wee1, is present in immature oocytes and functions to inhibit Cdc2, thereby causing prophase-I arrest (Nakajo *et al.*, 2000; Okumura *et al.*, 2002). Upon oocyte maturation induced by hormonal stimulation, however, Myt1 becomes phosphorylated and inhibited by Akt/PKB kinase in starfish (Okumura *et al.*, 2002) and by p90^{rsk}, the downstream kinase of the Mos–MAPK pathway (reviewed by Sagata, 1997; Ferrell, 1999), in *Xenopus* (Palmer *et al.*, 1998). However, p90^{rsk} is inactivated on fertilization of mature metaphase-II-arrested oocytes because of the degradation of Mos (Watanabe *et al.*, 1991; Bhatt and Ferrell, 1999)—nevertheless, Myt1 activity can be downregulated by phosphorylation in fertilized eggs (Mueller *et al.*, 1995b). Therefore, there must be another inhibitory kinase for Myt1 after fertilization or during embryonic mitosis in *Xenopus*. Myt1 is also hyperphosphorylated and inhibited at M phase of the somatic cell cycle in humans (Booher *et al.*, 1997; Wells *et al.*, 1999), but, even in this case, the responsible inhibitory kinase(s) is not known.

Polo-like kinases (Plks) play key roles during multiple steps of mitosis, including prophase, metaphase, anaphase, and cytokinesis (reviewed by Barr *et al.*, 2004), and recent studies demonstrate the essential role of their C-terminal Polo-box domain (PBD) in the phospho-dependent recognition of target proteins (Elia *et al.*, 2003a,b). In human cells, Plk1 is expressed primarily during late G2 and M phases (Golsteyn *et al.*, 1995) and directly phosphorylates Cdc25C, perhaps to facilitate nuclear activation of Cdc2–cyclin B (Toyoshima-Morimoto *et al.*, 2002). Moreover, Plx1, a *Xenopus* homolog of mammalian Plk1, can phosphorylate and activate Cdc25C *in vitro* (Kumagai and Dunphy, 1996) and is required for activation of Cdc2 during both oocyte maturation and embryonic mitosis (Abrieu *et al.*, 1998; Qian *et al.*, 1998; Karaïskou *et al.*, 1999; Liu *et al.*, 2004). As Myt1 is inactivated by phosphorylation at the M phase (Mueller *et al.*, 1995b; Booher *et al.*, 1997), it might also be regulated by Plk1/Plx1 at the M phase. In some support of this possibility, human Plk1 can phosphorylate Myt1 *in vitro* (Nakajima *et al.*, 2003), and starfish Plk1 has recently been suggested to function somewhere upstream of Myt1 in eggs (Okano-Uchida *et al.*, 2003). So far, however, there is no direct evidence that Plk1 interacts with and inhibits Myt1 directly.

In this study, we have investigated the possible interaction between Myt1 and Plx1 during oocyte maturation and embryonic mitosis in *Xenopus*. We show strikingly that Myt1 forms a stable complex with Plx1 (but not with p90^{rsk}) during embryonic mitosis, but not during oocyte maturation. Myt1 interacts with Plx1 via its Cdc2-phosphorylated Plx1-docking

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motif, and phosphorylation by Plx1 can inhibit Myt1 activity both *in vivo* and *in vitro*. Furthermore, although Myt1 is phosphorylated on the Plx1-docking motif during oocyte maturation, its interaction with Plx1 is inhibited by p90^{rsk}-mediated phosphorylation. These results indicate that the mechanism of Myt1 inhibition switches over at fertilization of *Xenopus* eggs, and strongly suggest that Plk1/Plx1 is a direct inhibitory kinase of Myt1 during the mitotic cell cycle. In addition, our data implicate that recognition of target proteins by Plk1/Plx1 can be reversibly regulated by their phosphorylation status other than that at the Plk1-docking site.

Results

Physical interaction between Myt1 and Plx1 after egg activation (or fertilization)

Myt1 undergoes a large electrophoretic mobility upshift (due to hyperphosphorylation) during progesterone (PG)-induced *Xenopus* oocyte maturation, while p90^{rsk} and Plx1 undergo a small mobility shift (Figure 1A; see also Qian *et al.*, 1998; Gross *et al.*, 2000; Peter *et al.*, 2002). p90^{rsk} interacts with and phosphorylates Myt1 and thereby inhibits its kinase activity during oocyte maturation (Palmer *et al.*, 1998). First we examined whether endogenous Plx1 could physically interact with Myt1 during oocyte maturation or after calcium ionophore-induced egg activation (which mimics fertilization; Watanabe *et al.*, 1991). The kinase activity of Plx1 paralleled that of Cdc2–cyclin B, peaking at M phase both during oocyte

maturation and after egg activation (Figure 1B; see also Qian *et al.*, 1998). When first immunoprecipitated with anti-Myt1 antibody and then immunoblotted with anti-p90^{rsk} antibody, Myt1 was shown to be bound to p90^{rsk} in mature metaphase-II-arrested oocytes (MII oocytes, also called eggs), but not in activated eggs (Figure 1C). When the immunoprecipitates were blotted with anti-Plx1 antibody, however, Myt1 was found to be associated with Plx1 80 min after egg activation or at the first mitotic M phase (M1; see Figure 1B), but not appreciably in immature (IMO) or mature MII oocytes (Figure 1C). The interaction between Myt1 and Plx1 in M1 eggs, but not in MII oocytes, was also evident when it was analyzed by reciprocal immunoprecipitation (with anti-Plx1 antibody) and immunoblotting (IB; with anti-Myt1 antibody) (Figure 1D). Moreover, the Myt1–Plx1 interaction was also observed in normally fertilized eggs or embryos (see Figure 5). Thus, very interestingly, Plx1, instead of p90^{rsk}, interacted with Myt1 at the mitotic M phase after egg activation or fertilization.

Inhibition of Myt1 activity by Plx1 *in vitro*

Human Plk1 can phosphorylate Myt1 *in vitro* (Nakajima *et al.*, 2003), but it is not known whether this phosphorylation can regulate Myt1 activity. Given the (physical) interaction between Plx1 and Myt1 (Figure 1), however, Plx1 might naturally function to regulate Myt1 by phosphorylation. Recombinant wild-type Plx1 protein, but not its kinase-dead version (K73M), was able to phosphorylate Myt1 and cause a large mobility shift of it *in vitro* (Figure 2A). When first

Figure 1 Interaction of Plx1 and Myt1 at the mitotic but not meiotic M phase. **(A)** Immature oocytes (IMO, arrested at prophase-I) were treated with PG to induce maturation, and about 7 h later, fully mature oocytes (also called eggs) (MII, arrested at metaphase-II) were treated with calcium ionophore to induce activation (which mimics fertilization). The activated eggs were further incubated until the first mitotic M phase (M1; see (B)). (A similar *in vitro* maturation/activation system was employed below and in other experiments in this study.) Oocytes at the indicated stages and eggs at the indicated times (min after activation) were analyzed by IB for the indicated endogenous proteins. **(B)** At the indicated stages, kinase activities of endogenous Cdc2 and Plx1 were assayed *in vitro* by using histone H1 and α -casein as substrates, respectively. For details, see Materials and methods. **(C)** At the indicated stages, endogenous Myt1 was immunoprecipitated (IP) with anti-Myt1 antibody and then immunoblotted with either anti-Myt1, anti-p90^{rsk} or anti-Plx1 antibodies. In control IP, anti-Myt1 antibody was pre-incubated with antigen peptides (+ pep). Input, one oocyte or egg; IP, 10 oocytes or eggs. **(D)** IP and subsequent IB as in (C) were performed reciprocally by using anti-Plx1 and anti-Myt1 antibodies, respectively. Cont., immunoprecipitation with normal rabbit IgG.

Figure 2 Inhibition of Myt1 activity by Plx1 *in vitro*. (A) Recombinant Myt1 protein was incubated with either wild-type (wt) or kinase-dead (K73M) Plx1 protein in the presence or absence of [γ - 32 P]ATP and analyzed by SDS-PAGE followed by autoradiography (top) or by IB (bottom). The arrowhead (on the top) denotes autophosphorylated Myt1. (B) Myt1 protein incubated with Plx1 as in (A) was analyzed by IB (top) or further incubated with KR Cdc2-cyclin B, which was then analyzed by IB for phospho-Thr14/Tyr15 (bottom). (C) Wild-type Myt1 and two consensus Plx1 phosphorylation site mutants of Myt1, S424A and 5A (S424A/S433A/S487A/T508A/T546A), were incubated with either wt or K73M Plx1, and their activities to phosphorylate kinase-dead Cdc2 were analyzed as in (B). For detailed methods in (A–C), see Materials and methods.

incubated with kinase-dead Plx1 and then with (kinase-dead) Cdc2-cyclin B, Myt1 was able to normally catalyze inhibitory phosphorylation of the Cdc2 protein on Thr14/Tyr15; notably, when preincubated with wild-type Plx1, however, Myt1 failed to phosphorylate Cdc2 (Figure 2B). Thus, these results demonstrate, for the first time, that Plk1/Plx1 can inhibit Myt1 activity *in vitro*.

Human Myt1 can be phosphorylated at Ser426 by Plk1 *in vitro* (Nakajima *et al.*, 2003). We also independently identified Ser424 (equivalent to Ser426 of human Myt1) as a major Plx1 phosphorylation site of *Xenopus* Myt1 (data not shown, but see Figure 4A). However, a Ser424→Ala mutant (S424A) of Myt1, like wild-type Myt1, lost its kinase activity towards (kinase-dead) Cdc2-cyclin B after incubation with wild-type (but not kinase-dead) Plx1 (Figure 2C). Somewhat

surprisingly, even a Myt1 mutant (5A)—in which four other serine or threonine residues lying in the consensus Plk1 phosphorylation motif (Asp/Glu-X-Ser/Thr; Nakajima *et al.*, 2003) were also mutated to Ala—lost its activity towards Cdc2 after incubation with Plx1 (Figure 2C). Thus, although Myt1 could be phosphorylated and inhibited by Plx1, the inhibitory phosphorylation site(s) was not likely to reside in the proposed Plk1 phosphorylation motif, suggesting the occurrence of yet another Plx1 phosphorylation site(s) in Myt1. Consistent with this idea, the 5A (as well as S424A) mutant still underwent phosphorylation after treatment with Plx1 (see Supplementary Figure 1).

In vivo, efficient phosphorylation/inhibition of Myt1 by Plx1 seems to require a Cdc2-dependent docking of the two proteins (see below). In the above *in vitro* experiments (involving no active Cdc2), however, Plx1 probably phosphorylated (and inhibited) Myt1 directly without such a docking, similar to *in vitro* phosphorylation of Myt1 by human Plk1 (Nakajima *et al.*, 2003).

Phosphorylation of the potential Plx1-docking site Thr478 by Cdc2

Given the recent identification of a phospho-Plk1-docking motif (Ser-pSer/pThr-Pro) in a certain Plk1 target protein (Elia *et al.*, 2003a), the interaction between Plx1 and Myt1 (observed after egg activation) might occur via a similar docking motif present in Myt1 protein. We noticed that Thr478 of *Xenopus* Myt1 lies in the Plk1-docking motif (STP) and is well conserved in Myt1 kinases from both vertebrate and invertebrate species (Figure 3A). Therefore, we first determined whether Thr478 of Myt1 would undergo any phosphorylation during oocyte maturation or after egg activation, by using an anti-phospho-Thr478-peptide antibody. By direct IB, this antibody could not appreciably detect endogenous Myt1 protein from a single egg (although it could detect this protein after immunoprecipitation from 20 eggs; see Supplementary Figure 2). Therefore, to facilitate the detection, we ectopically expressed (a kinase-dead (KD; N231A) form of) Myt1 in oocytes or eggs (so that it would not affect cell cycle progression; see Figure 5). The anti-phospho-Thr478 antibody was now able to recognize the Myt1-KD protein (but not its T478A mutant) expressed in activated eggs, in a Myt1-phosphorylation-dependent manner (Figure 3B). When monitored during oocyte maturation and after egg activation, Thr478 phosphorylation of Myt1-KD was found to occur in maturing (MI) and mature (MII) oocytes, to substantially decrease shortly after egg activation (or during interphase), and then to reincrease at the first mitotic M phase (M1); this pattern of Thr478 phosphorylation closely paralleled that of Cdc2-cyclin B activity during development (Figure 3C). These results, together with the fact that Thr478 also lies in the consensus Cdc2 phosphorylation motif (S/TP; Morgan, 1995), raise the possibility that Thr478 may be phosphorylated by Cdc2. Consistent with this possibility, inhibition of endogenous Cdc2 activity by the Cdc2 inhibitor roscovitine abrogated phosphorylation of Myt1-KD on Thr478 both during oocyte maturation (Figure 3D) and after egg activation (Figure 3E). Moreover, a constitutively active (AF; T14A/Y15F), but not kinase-dead (KR), form of recombinant Cdc2-cyclin B was able to strongly phosphorylate Myt1-KD on Thr478 *in vitro* (Figure 3F). Endogenous Plx1 was not likely to be significantly involved in Thr478

Figure 3 Cdc2-mediated phosphorylation of Myt1 on Thr478 at M phase. (A) Conservation of a Plx1-docking motif (SS/TP) in Myt1 kinases from various species. Potential phosphorylation sites are shown in red. In the following figures (B–E), maturing oocytes and activated eggs were derived from immature oocytes that had been injected with 1 ng of mRNA encoding Myt1-KD or its mutant (T478A/KD). (B) Either Myt1-KD protein or T478A/KD protein immunoprecipitated from five activated eggs (80 min after activation) was treated or not with λ-phosphatase (λ-PPase), and analyzed by IB with anti-Myt1 or anti-phospho-Thr478 antibodies. (C) Extracts from the indicated stages of oocytes or eggs (expressing Myt1-KD) were either immunoblotted as in (B) or assayed for Cdc2 activity. h, time after PG treatment; min, time after egg activation. MII, 7 h after PG treatment or the time of egg activation. (D, E) Immature oocytes (1 h before PG treatment) (D) or eggs 30 min after activation (E), both expressing Myt1-KD, were treated (+ Ros) or not (–Ros) with roscovitine and, at the indicated times after PG treatment (D) or after egg activation (E), subjected to IB as in (B). (F) Recombinant Myt1-KD protein was incubated with either constitutively active (AF) or kinase-dead (KR) Cdc2–cyclin B complexes and then immunoblotted as in (B). For details, see Materials and methods.

phosphorylation, since endogenous-level expression of a constitutively active form of Plx1 in oocytes or eggs could not appreciably restore Thr478 phosphorylation in the presence of roscovitine (data not shown). Thus, these results strongly suggest that Cdc2 phosphorylates Myt1 on Thr478 at M phase both during oocyte maturation and after egg activation (or fertilization).

Phospho-Thr478-mediated interaction between Myt1 and Plx1 after egg activation

We then addressed whether Thr478 phosphorylation was required for Myt1 to interact with Plx1 after egg activation. Before addressing this question, we examined the (putative) Plx1 phosphorylation status of Myt1-KD and its T478A mutant during oocyte maturation and after egg activation. Compared to Myt1-KD, the T478A/KD mutant showed a similar (if not identical) mobility upshift during oocyte maturation, but a considerably larger downshift after egg activation (Figure 4A, top), suggesting that the T478A/KD mutant might have failed to be phosphorylated by Plx1 after

egg activation. Indeed, when analyzed with a phospho-specific antibody and compared to Myt1-KD, phosphorylation of Ser424, a major Plx1 phosphorylation site of Myt1 (see Figure 2C), was found to be severely impaired in the T478A/KD mutant after egg activation (Figure 4A, bottom). Somewhat surprisingly, Ser424 phosphorylation occurred even during oocyte maturation, but this phosphorylation was little affected in the T478A/KD mutant, suggesting that it was performed perhaps by another kinase during this period.

These results would support the idea that Thr478 phosphorylation is required for Myt1 to interact with (and, thereby, to be phosphorylated by) Plx1 after egg activation. Consistently, co-immunoprecipitation analysis revealed that the T478A/KD mutant, unlike Myt1-KD, was unable to interact with (endogenous) Plx1 in activated M1 eggs (Figure 4B, left), whereas it was able to normally interact with p90^{rsk} in mature MII oocytes (Figure 4B, right). Given these results (and the fact that Thr478 lies in the consensus Plx1/Plx1-docking motif), Thr478 phosphorylation may directly

Figure 4 Identification of phosphorylated Thr478 as the Plx1-docking site of Myt1. (A) Extracts from the indicated stages of oocytes or eggs expressing either Myt1-KD or the T478A/KD mutant as in Figure 3 were immunoblotted with anti-Myt1 or anti-phospho-Ser424 antibodies. Note that Ser424 phosphorylation of the T478A/KD mutant is undetectable at 80 min after egg activation. MI, maturing oocytes undergoing GVBD. (B) Extracts from MII oocytes or M1 eggs (expressing Myt1 constructs as in (A)) were first immunoprecipitated with either anti-Plx1 antibody (left) or anti-p90^{rsk} antibody (right) and then immunoblotted with anti-Myt1 antibody (top). Input, one oocyte or egg; IP, 10 oocytes or eggs. (C) Either phospho-Thr478-containing peptides (pT478) or unphosphorylated peptides (T478), coupled to agarose beads, were incubated with either interphase (I, 30 min after egg activation) or M1 egg extracts, pulled down (PD), and immunoblotted with anti-Plx1 antibody. Input, one egg; PD, 10 eggs. For details, see Materials and methods.

mediate the Myt1-Plx1 interaction in activated eggs. Consistent with this idea, short synthetic peptides (residues 470–481) containing phosphorylated Thr478, but not unphosphorylated peptides, could bind Plx1 in extracts from not only M1 but also interphase (I) eggs (Figure 4C). Thus, these results strongly suggest that Myt1 interacts with Plx1 via its Plx1-docking motif containing phospho-Thr478 after egg activation. These results, however, raise the question of why Myt1, although phosphorylated on Thr478 (Figure 3C), cannot interact with Plx1 during oocyte maturation, an issue that will be addressed below.

Inhibition of Myt1 by Plx1 during the embryonic cell cycle

We examined whether Thr478 phosphorylation (and, hence, Plx1 interaction) of Myt1 was required for progression through oocyte maturation or the embryonic cell cycle. For this, we expressed either the wild-type or T478A mutant forms of Myt1 in oocytes or in just fertilized eggs. The expression of the T478A mutant (about 20-fold over endogenous levels) decreased the rate of germinal vesicle breakdown (GVBD, a hallmark of oocyte maturation) of PG-treated oocytes, but essentially to the same extent as did the expression of wild-type Myt1 (Figure 5A). This result would be consistent with the undetectable physical interaction of Myt1

and Plx1 during oocyte maturation (Figures 1C, D and 4B). In contrast, the expression of the T478A mutant (about 15-fold over endogenous levels; see Figure 5C) in fertilized eggs caused an appreciable (or 2–3 cycles) delay in cell division in more than 65% of the stage 8 blastula embryos, whereas that of the wild-type Myt1 did so in less than 15% of the embryos (Figure 5B) (see Figure 5B legend for determination of the cleavage delay). Consistent with this result, the inhibitory Thr14/Tyr15 phosphorylation of endogenous Cdc2 was significantly stronger in the embryos expressing the T478A mutant than in the embryos expressing wild-type Myt1 (Figure 5C). Moreover, in these experiments, the T478A mutant, unlike wild-type Myt1, failed to interact with (endogenous) Plx1 (Figure 5D) and underwent a mobility downshift (Figures 5C and D), as expected. Thus, it seems clear that Plx1 interaction/phosphorylation via phosphorylated Thr478 can prevent Myt1 from phosphorylating and inhibiting Cdc2 in early embryos (consistent with Plx1 being capable of phosphorylating and inhibiting Myt1 *in vitro*; Figure 2). Indeed, in *in vitro* kinase assays, the T478A mutant from activated (M1) eggs had a significantly stronger kinase activity than wild-type Myt1 (see Supplementary Figure 3). Thus, these results show that inhibition of Myt1 by Plx1 plays a role, at least in part, in the mitotic cell cycle of early embryos.

Figure 5 Inhibition of embryonic cell division by ectopic expression of the T478A mutant. **(A)** A total of 50 immature oocytes left uninjected (none) or injected with 2 ng of mRNA encoding either wild-type Myt1 or the T478A mutant were incubated for 12 h, treated with PG, and then cultured and scored for the percentage GVBD. Inset, immunoblot analysis of the Myt1 proteins (just before PG treatment) with anti-Myt1 antibody. **(B)** In all, 50 two-cell embryos were uninjected (none) or injected (at their one blastomere) with 1 ng of mRNA encoding either wild-type or T478A Myt1, cultured, and analyzed for the external morphology at stage 8 (top; mRNA injected at the right side of the embryos) and for the percentage of embryos that showed a cleavage delay (by 2–3 cycles, determined by counting the number of cells per a fixed area) at stage 8 (bottom; data from three independent experiments with means $\pm$ s.e.m.). **(C)** One-cell embryos injected with 2 ng of the above-described mRNAs were cultured and, at the indicated stages, subjected to IB with either anti-Myt1 or anti-Cdc2 pT14/pY15 antibodies. **(D)** Extracts from stage 8 embryos prepared as in (C) were first immunoprecipitated with anti-Plx1 antibody and then immunoblotted with anti-Myt1 antibody. Input, one embryo; IP, 10 embryos.

Inhibition of the Plx1–Myt1 interaction by the Mos–MAPK pathway

Intriguingly, during oocyte maturation, Myt1 could not appreciably interact with Plx1 (Figures 1C, D and 4B), even though it was phosphorylated on Thr478 most likely by Cdc2 (Figures 3C and D). We suspected that the Mos–MAPK pathway, which plays key roles specifically in oocyte maturation (Sagata, 1997; Nebreda and Ferby, 2000), might act to inhibit the Myt1–Plx1 interaction during oocyte maturation. To test this possibility, first we ectopically expressed Mos in activated eggs (in which endogenous Mos was degraded) to reactivate the MAPK pathway (Figure 6A). As seen in Figure 6B, (ectopically expressed) Myt1-KD could not bind to Plx1 in the eggs expressing Mos, whereas it could do so in control eggs expressing no Mos. (The control eggs, about 2.5 h after egg activation, were in an M-like phase as determined by their Cdc2 activity; data not shown.) We next expressed a constitutively active (CA) form of $p90^{\text{rsk}}$, the direct downstream kinase of MAPK (which itself can interact with Myt1 during oocyte maturation), in activated eggs (Figure 6A). Even in this case, Myt1-KD could not appreciably bind to Plx1 (Figure 6B). In contrast to these, Myt1-KD could bind to $p90^{\text{rsk}}$ (whether endogenous or exogenous) in both Mos- and $p90^{\text{rsk}}$ -CA-expressing eggs, but not in control eggs (Figure 6C). Thus, even in activated eggs, the presence of

either Mos or activated $p90^{\text{rsk}}$ was able to reproduce the interaction of Myt1 with $p90^{\text{rsk}}$, but not with Plx1, as in mature MII oocytes (Figures 6B and C). To determine whether Mos could inhibit the Myt1–Plx1 interaction independently of $p90^{\text{rsk}}$ (Mos can bind to Myt1 during oocyte maturation; Peter *et al*, 2002), we also coexpressed Mos and MKP-1, a specific inhibitory phosphatase of MAPK (Sun *et al*, 1993), in activated eggs. In this case, Myt1-KD could normally bind to Plx1 as in untreated control eggs (Figure 6D), indicating the dependence of Mos-inhibited Myt1–Plx1 interactions on $p90^{\text{rsk}}$. These results suggest that $p90^{\text{rsk}}$, the most downstream kinase of the Mos–MAPK pathway, inhibits either directly or indirectly the interaction between Myt1 and Plx1 during oocyte maturation. Consistent with this, the inhibition of MAPK during oocyte maturation allowed the Myt1–Plx1 interaction to occur at GVBD (see Supplementary Figure 4).

Direct inhibition of the Plx1–Myt1 interaction by $p90^{\text{rsk}}$

Given the above results, $p90^{\text{rsk}}$ might cause some modification (e.g., phosphorylation) of either Myt1 or Plx1 to inhibit their interactions during oocyte maturation. To determine which of the two proteins was modified not to form a stable complex during oocyte maturation, we isolated ectopically expressed His-tagged Myt1 (KD or T478A/KD) and glutathione S-transferase (GST)-fused Plx1 from either mature

Figure 6 Inhibition of the Plx1-Myt1 interaction by the Mos-MAPK pathway. Activated eggs expressing Myt1-KD were uninjected (Cont.), injected with either 4 ng of Mos mRNA or 40 ng of Myc-p90^{rsk}-CA mRNA (A–C), or coinjected with 4 ng of Mos mRNA and 4 ng of MKP-1 mRNA (D), after 40 min of egg activation; 90 min later, they were immunoprecipitated with anti-Plx1 (B, D) or anti-p90^{rsk} antibodies (C) and then immunoblotted with anti-Myt1 antibody (B–D). As another control, mature MII oocytes (expressing only Myt-KD as ectopic protein) were also used. In (A) and (D), pMAPK denotes phosphorylated MAPK. Input, one oocyte or egg; IP, 10 oocytes or eggs.

MII oocytes or activated M1 eggs, mixed and incubated them (GST-Plx1 had been immobilized to glutathione-beads), and then tested for their binding by pulldown assays. GST-Plx1 isolated from either MII oocytes or M1 eggs was able to bind Myt1-KD (but not the T478A/KD mutant) isolated from M1 eggs (Figure 7A, lanes M1), indicating that Plx1 from MII oocytes had the ability to interact with Thr478-phosphorylated Myt1 (isolated from M1 eggs). In contrast, neither the T478A/KD mutant nor Myt1-KD isolated from MII oocytes could bind to GST-Plx1 isolated from either MII oocytes or M1 eggs (Figure 7A, lanes MII). Apparently, this was not due to the binding of (endogenous) p90^{rsk} to the ectopic Myt1 proteins, which was relatively poor (see Figure 6C) and had been eliminated by stringent washing of the Myt1 proteins (see Materials and methods). Thus, these results suggest strongly that, during oocyte maturation or in MII oocytes, Myt1 but not Plx1 is somehow modified so that they cannot form a stable complex. We infer that the Myt1 modification (if any) probably occurred somewhere outside the Plx1-docking motif, because we did observe that short phospho-Thr478-containing peptides (see Figure 4C) could bind efficiently to Plx1 even in MII oocyte extracts (Figure 7B).

p90^{rsk} can interact with and phosphorylate Myt1 (and, thereby, inhibits its activity) during oocyte maturation (Palmer *et al.*, 1998; see also Figures 1C, 4B and 6C). Therefore, given our results (Figures 6B, D and 7A), p90^{rsk} could also directly phosphorylate Myt1 and inhibit it from interacting with Plx1. To test this possibility, we isolated overexpressed His-tagged Myt1(-KD) from M1 eggs, incubated it with either the CA (see Figure 6A) or kinase-dead (KR) forms of recombinant p90^{rsk} protein, and then further

incubated it with GST-Plx1 (isolated from MII oocytes and immobilized to glutathione beads). Myt1 protein incubated with p90^{rsk}-CA underwent a mobility upshift (due to phosphorylation) and failed to bind to GST-Plx1, whereas that incubated with p90^{rsk}-KR could bind to GST-Plx1 as efficiently as the untreated control Myt1 protein (Figure 7C). The failure of p90^{rsk}-CA-phosphorylated Myt1 to interact with GST-Plx1 was not due to a preferential, inhibitory binding of p90^{rsk}-CA to Myt1, because, under the present conditions, p90^{rsk}-CA and p90^{rsk}-KR bound to Myt1 with an equal efficiency and only very weakly (Figure 7D). All together, these results strongly suggest that p90^{rsk} directly phosphorylates Myt1 and inhibits it from interacting with Plx1 during oocyte maturation.

Discussion

Myt1 undergoes hyperphosphorylation and inactivation during M phase of the mitotic cell cycle (Mueller *et al.*, 1995b; Booher *et al.*, 1997). Cdc2 can phosphorylate Myt1, but this phosphorylation itself is not likely to inhibit Myt1 activity (Booher *et al.*, 1997; Wells *et al.*, 1999). Circumstantial evidence suggests that Plk1 might target Myt1 (Nakajima *et al.*, 2003; Okano-Uchida *et al.*, 2003), but there is no direct evidence that Plk1 targets and inhibits Myt1 directly. Our results show that Plx1, a *Xenopus* homolog of Plk1, physically interacts with Myt1 *in vivo* and can phosphorylate it and inhibit its activity both *in vivo* and *in vitro*; the inhibitory phosphorylation site(s) of Myt1 is not likely to be the major Plx1 phosphorylation site or Ser424. Intriguingly, the (physical) interaction between Plx1 and Myt1 occurred during the

Figure 7 Direct inhibition of the Plx1-Myt1 interaction by p90^{rsk}. (A) Either His₆-Myt1 (KD or T478A/KD) or GST-Plx1 protein, each pre-expressed in immature oocytes by injection of 1 ng of their mRNA, was pulled down from either mature MII oocytes or activated M1 eggs using nickel- or glutathione-agarose beads (only His₆-Myt1 was eluted from the beads). The glutathione-bead-bound GST-Plx1 from MII oocytes (GT beads (MII-Plx1)) or from M1 eggs (GT beads (M1-Plx1)) was incubated with the His₆-Myt1 protein (from either MII oocytes or M1 eggs), pulled down, washed, and immunoblotted with anti-Myt1 or anti-GST antibodies. Input, one oocyte or egg; PD, 10 oocytes or eggs. (B) The experiment was performed as in Figure 4C, but using MII oocyte and M1 egg extracts. (C) Myt1 protein isolated from M1 eggs as in (A) was dialyzed, incubated with either the CA or kinase-dead (KR) forms of recombinant p90^{rsk}, and further incubated with GT-bead-bound GST-Plx1 (isolated from MII oocytes as in (A)). The GT beads were then washed and immunoblotted with anti-Myt1 or anti-GST antibodies. For details, see Materials and methods. (D) Myt1 protein incubated with p90^{rsk} as in (C) was immunoprecipitated with anti-Myt1 antibody and then immunoblotted with anti-p90^{rsk} antibody. Cont., normal rabbit IgG.

embryonic (or mitotic) cell cycle, but not appreciably during the meiotic cell cycle. Furthermore, ectopic expression of a Myt1 mutant incapable of interacting with Plx1 appreciably affected progression through the mitotic but not meiotic cell cycles. Thus, our results strongly suggest that Plx1/Plx1 acts as a direct inhibitory kinase of Myt1 at least in the mitotic cell cycle (Figure 8).

Recent studies have demonstrated the essential role of the C-terminal domain of Plks, the PBD, in their phospho-dependent recognition of target proteins (Elia *et al*, 2003a, b). Thus far, only a few physiological substrates have been shown to bind to the PBD of Plk1 via their phospho-Ser/Thr-binding

module (in the sequence motif S-pS/pT-P or its derivatives): they are the Wee1/Myt1-antagonizing phosphatase Cdc25C (Elia *et al*, 2003a), the peripheral Golgi protein Nir2 (Litvak *et al*, 2004), and the checkpoint protein Claspin (Yoo *et al*, 2004). We show here that, like Cdc25C (Elia *et al*, 2003a), Myt1 can be phosphorylated on the Plk1/Plx1-docking motif by Cdc2 both *in vivo* and *in vitro*, and that the resulting Plx1 interaction/phosphorylation can inhibit Myt1 from phosphorylating and inhibiting Cdc2. These results indicate that both Cdc2 and Plx1 are involved in the inhibition of Myt1 at M phase, and suggest that the previously undefined function of Cdc2-mediated phosphorylation of Myt1 (Booher *et al*, 1997;

Figure 8 Model for the inhibition of Myt1 in the meiotic and mitotic cell cycles in *Xenopus*. In the meiotic cell cycle, p90^{rsk}, the downstream kinase of the Mos–MAPK pathway, interacts with and phosphorylates Myt1, thereby inhibiting its activity. During this period, Myt1 is also phosphorylated on the Plx1-docking site (Thr478) by Cdc2, but its interaction with Plx1 is largely inhibited by p90^{rsk}-mediated phosphorylation. After fertilization, however, p90^{rsk} is inactivated due to the degradation of Mos, allowing Myt1 to interact with Plx1 via the Cdc2-phosphorylated Thr478 residue. Consequently, in embryonic mitosis, Plx1 can phosphorylate Myt1 (at as-yet-unidentified sites) and thereby inhibit its activity. The inhibition of the Plx1–Myt1 interaction in the meiotic cell cycle may allow a preferential binding of Plx1 to Cdc25C, resulting in strong activation and maintenance of Cdc2 activity during oocyte maturation. In the mitotic cell cycles, however, Plx1 may play a key role in the amplification loop of Cdc2 activation through both Cdc25C activation and Myt1 inhibition. KD, kinase domain; PBD, Polo-box domain; (P) phosphorylation. See also Discussion.

Wells *et al*, 1999) may be to prime the binding of Plx1 to Myt1. These results would also imply that Plx1 functions as a component of the so-called Cdc2 amplification loop at the G2/M transition (Morgan, 1995). Indeed, Plx1 has been suggested to function in the Cdc2 amplification loop, in part, by activating Cdc25C (Abrieu *et al*, 1998; Karaïskou *et al*, 1999). However, Plx1 might also function, at least in part, as a trigger kinase of the G2/M transition (see Qian *et al*, 1998; Toyoshima-Morimoto *et al*, 2002), as Plx1 itself can phosphorylate Myt1, albeit only weakly, on the docking site (Thr478) *in vitro* (our unpublished data).

Although the interruption of the Plx1–Myt1 interaction by T478A mutation largely impaired the Plx1 phosphorylation and inhibition of Myt1, it seemed to have a relatively small (although significant) effect on early embryonic cell divisions. This was presumably due, however, to the dramatic increase in levels of the Myt1/Wee1-antagonizing Cdc25A phosphatase during this period (Shimuta *et al*, 2002). In somatic cells, however, the level of Cdc25A is considerably lower (Shimuta *et al*, 2002; Zhao *et al*, 2002); therefore, the interruption of the Plx1–Myt1 interaction in somatic cells might have a significantly larger effect on cell divisions. In human somatic cells, Myt1 localizes to the endoplasmic reticulum and Golgi complex (Liu *et al*, 1997). Therefore, the binding of Plx1/Plx1 or the Myt1 substrate Cdc2–cyclin B to Myt1 could also be responsible, at least in part, for their known localization and function at the Golgi complex (Colanzi *et al*, 2003). Indeed, in *Drosophila* embryos, Myt1 has been implicated in Golgi fragmentation (Cornwell *et al*, 2002), a mitotic event involving both Cdc2 and Plx1 (Lin *et al*, 2000; Sutterlin *et al*, 2001).

Quite interestingly, we found that Myt1 is not appreciably complexed with Plx1 during oocyte maturation, despite its Plx1-docking site being fully phosphorylated as in activated eggs. We provide strong evidence that p90^{rsk} phosphorylates Myt1 and thereby inhibits it from interacting with Plx1, by

showing that a constitutively active form of p90^{rsk} can prevent (Thr478-phosphorylated) Myt1 from binding Plx1 both in activated eggs and *in vitro*. p90^{rsk} itself was able to bind to Myt1 (to inhibit its activity by phosphorylation; Palmer *et al*, 1998) during oocyte maturation, but this binding itself was not likely to be responsible for the inhibition of the Myt1–Plx1 interaction. Thus, it seems that p90^{rsk} phosphorylates Myt1 for at least two purposes during oocyte maturation, that is, to inhibit Myt1 activity and to inhibit its interaction with Plx1. Unlike full-length Myt1 protein, a short synthetic Myt1 peptide (containing phospho-Thr478) was able to strongly bind to Plx1 even in mature oocyte extracts. Therefore, it seems likely that phosphorylation by p90^{rsk} causes conformational changes of Myt1 so that this kinase cannot stably interact with Plx1 via its phospho-Thr478 (Figure 8). (Despite phosphorylation by p90^{rsk}, Myt1 might be able to interact with Plx1 only transiently or weakly (as could be inferred, in part, from a slight mobility downshift of a small fraction of T478A Myt1 during oocyte maturation; see Figure 4A), although such interaction, even if present, is not likely to significantly contribute to Myt1 inhibition (which is already performed by p90^{rsk}) (see Figure 5A).) Upon egg activation or fertilization, however, p90^{rsk} is inactivated due to the degradation of the upstream kinase Mos (Watanabe *et al*, 1991; Bhatt and Ferrell, 1999), apparently allowing Myt1 to normally interact with Plx1 in the embryonic cell cycles. Thus, our study indicates a switchover in inhibitory interaction for Myt1 from p90^{rsk} to Plx1 at fertilization of *Xenopus* eggs. Moreover, and importantly, our data imply that recognition of target proteins by Plx1/Plx1 can be reversibly regulated by their phosphorylation status other than that at the Plx1-docking site.

Apart from its mechanism, the biological significance of inhibition of the Myt1–Plx1 interaction during oocyte maturation is currently unknown. However, this inhibition might well be related to meiotic progression and arrest (at

metaphase-II) of oocytes. For example, as Cdc25C and Myt1 share the same Plx1-docking motif, Cdc25C could preferentially interact with Plx1 in the absence of the Myt1-Plx1 interaction, thereby causing strong activation and maintenance of Cdc2 activity during meiotic progression and arrest (Figure 8). Consistent with this idea, the interaction of Plx1 with Cdc25C can readily be detected during oocyte maturation (our unpublished data), whereas that with Myt1 cannot. Thus, inhibition of the Myt1-Plx1 interaction might have a rather positive role during oocyte maturation. In this context, it is noteworthy that the Mos-MAPK-p90^{rsk} pathway, which itself plays key roles (including Myt1 inhibition) in oocyte maturation (Sagata, 1997; Nebreda and Ferby, 2000), acts to inhibit the interaction between Myt1 and Plx1.

In summary, our results show that Plk1/Plx1 is a direct inhibitory kinase of Myt1 in *Xenopus* eggs. The Plx1-Myt1 interaction occurs after fertilization or during the mitotic cell cycles, but is largely inhibited most certainly by p90^{rsk} during oocyte maturation or the meiotic cell cycle. As the Plk1-docking motif is conserved in Myt1 kinases from both vertebrate and invertebrate species (Figure 3A), the direct regulation of Myt1 by Plk1 could universally occur in the mitotic cell cycles of all metazoans.

Materials and methods

Oocytes, eggs, and embryos

Oocytes, eggs, and embryos were prepared, cultured, and micro-injected as described (Nakajo *et al.*, 2000; Shimuta *et al.*, 2002). Oocytes were treated with PG (5 µg/ml; SIGMA) to induce maturation. Fully mature oocytes (i.e., eggs), usually 4 h after GVBD or 7 h after PG treatment, were treated with the calcium ionophore A23187 (1 µg/ml; SIGMA) to induce activation (called egg activation), which mimics fertilization. In some experiments, immature oocytes and activated eggs were treated with 100 and 400 µM roscovitine (SIGMA), respectively.

cDNAs and in vitro transcription

A cDNA encoding *Xenopus* Myt1 was described (Nakajo *et al.*, 2000), and a cDNA encoding *Xenopus* Plx1 was obtained by PCR of a *Xenopus* oocyte cDNA library. These cDNAs were subcloned into the His₆- or GST-tagged pT7G (UKII+) transcription vector (Shimuta *et al.*, 2002). A cDNA encoding *Xenopus* p90^{rsk} (p90^{rsk2}), obtained commercially (Invitrogen, IMAGE clone; ID 3401402), was subcloned into the N-terminally Myc-tagged pT7G (UKII+) transcription vector. A constitutively active form of p90^{rsk} (p90^{rsk-CA}) was generated essentially as described (Gross *et al.*, 2001). A cDNA encoding MKP-1 (or CL100) was a gift from SM Keyse. *In vitro* mutagenesis and transcription of the cDNAs were performed as described (Shimuta *et al.*, 2002).

Construction and purification of baculoviral recombinant proteins

All procedures for cultivation of Sf9 insect cells and generation of baculoviruses were performed according to the manufacturer's instruction (Invitrogen). His₆-tagged protein constructs, including Myt1, Plx1, p90^{rsk}, and their mutants, were produced in Sf9 cells by using the BAC-TO-BAC baculoviral expression system (Gibco-BRL) and the pFastHTa/HTb vectors (Invitrogen); to produce active Plx1, Sf9 cells were treated with okadaic acid for 2 h. Viruses encoding His₆-Myc-Cdc2AF, His₆-Myc-Cdc2KR, or ΔN85-GST-cyclin B2 were a kind gift from T Kishimoto. All the His₆-tagged proteins were purified by using nickel-agarose and eluted with imidazole by standard methods. Purified proteins were stored in aliquots at -80°C.

Antibodies and IB

Anti-*Xenopus* Myt1 phospho-Thr478 and phospho-Ser424 antibodies were raised in rabbits against the phosphorylated peptides RPSLGSTSpTPRN and NHLGESpSFSSD, respectively, and affinity-

purified by standard methods. Anti-*Xenopus* Myt1 antibody was raised against the C-terminal peptide (PRNLLGMFDDATEQ) in rabbits and purified. Routinely, proteins equivalent to one oocyte, egg, or embryo were analyzed by IB using antibodies against phospho-Thr478, phospho-Ser424, Myt1, *Xenopus* Plx1 (gift from EA Nigg), Cdc2 phospho-Thr14/Tyr15 (BIOSOURCE), p90^{rsk2} (Santa Cruz), *Xenopus* Mos (Watanabe *et al.*, 1991), or phospho-MAPK (Cell Signaling), essentially as described (Shimuta *et al.*, 2002).

Immunoprecipitation and pulldown

To detect endogenous Myt1-Plx1 or Myt1-p90^{rsk} interactions, 50 oocytes or eggs were homogenized in 250 µl of RIPA buffer (10 mM NaH₂PO₄/Na₂HPO₄ at pH 7.5, 1 mM EGTA, 1% Triton X-100, 80 mM β-glycerophosphate, 50 mM NaF, and one tablet of protease inhibitor cocktail (Roche)/25 ml RIPA) and centrifuged briefly; the supernatant obtained was subjected to immunoprecipitation and then IB using appropriate antibodies. To detect interactions of overexpressed proteins, five oocytes or eggs were homogenized in 50 µl of RIPA buffer and analyzed similarly. Phospho-Thr478-containing peptides (CRPSLGSTSpTPRN) were covalently linked to SulfoLink coupling agarose gel (Pierce), incubated with oocyte or egg extracts (equivalent to twenty oocytes or eggs), and pulled down for IB, as described (Uto *et al.*, 2004). His₆-Myt1 and GST-Plx1 proteins expressed in five oocytes or eggs were pulled down with nickel-agarose (QIAGEN) or glutathione-Sepharose beads in RIPA buffer, as described (Uto *et al.*, 2004). The pulled-down His₆-Myt1 protein was washed three times with 250 µl of RIPA buffer supplemented with 0.5% Tween-20, 300 mM NaCl and 10 mM EGTA (to remove any associated p90^{rsk} proteins), and then eluted with 100 µl of Hepes-buffered saline (HBS) containing 200 mM imidazole.

In vitro kinase assays and phosphatase treatment

Unless stated elsewhere, *in vitro* kinase assays of different kinases were performed using the same kinase buffer (50 mM Tris-HCl at pH 7.5, 10 mM MgCl₂, and 1 mM DTT) supplemented with 100 µM ATP with or without 2 µCi [γ-³²P]ATP. The kinase assay of endogenous Plx1 protein (immunoprecipitated from 10 oocytes or eggs) was performed in 40 µl of the kinase buffer containing 4 µg of α-casein (SIGMA) for 30 min at 30°C. The kinase activity of endogenous Cdc2 was assayed by using histone H1 as substrate, as described (Nakajo *et al.*, 2000). For phosphorylation of Myt1 by Cdc2 *in vitro*, 100 ng of His₆-Myt1 protein was incubated with 10 ng of Cdc2-cyclin B2 (immobilized to glutathione-beads) in 40 µl of the kinase buffer for 20 min at 23°C. For phosphorylation of Myt1 by Plx1, 10 ng of His₆-Myt1 protein was incubated with 100 ng of His₆-Plx1 protein in 40 µl of the kinase buffer for 30 min at 23°C. When the kinase buffer was supplemented with [γ-³²P]ATP, the phosphorylated proteins were analyzed by SDS-PAGE followed by autoradiography.

The kinase activity of Plx1-phosphorylated Myt1 was measured by *in vitro* phosphorylation of Cdc2-cyclin B2 on Thr14/Tyr15, as follows. His₆-Myt1 protein was first incubated with His₆-Plx1 protein as described above, except that the kinase buffer was supplemented with 0.1% bovine serum albumin (BSA) and 300 µM ATP. The reaction mixture was then incubated with 10 ng of kinase-dead recombinant Cdc2-cyclin B2 for another 20 min at 23°C and subjected to IB for phospho-Thr14/Tyr15.

In vitro phosphatase treatment of His₆-Myt1 protein (from five activated eggs expressing it) was performed in 50 µl of a phosphatase buffer containing 800 U of λ-phosphatase (NEB) for 40 min at 30°C.

Plx1-Myt1-binding assays

For *in vitro* binding assays of Myt1 and Plx1, His₆-Myt1 protein and glutathione-bead-immobilized GST-Plx1 protein, each from five oocytes or eggs, were mixed and incubated in 200 µl of RIPA buffer for 1 h at 4°C with constant agitation; the glutathione beads were then washed and subjected to IB. When the His₆-Myt1 protein was phosphorylated by p90^{rsk} before the binding assay, it was first dialyzed against HBS buffer for 1 h twice, then incubated with 100 ng of recombinant His₆-p90^{rsk} protein in 50 µl of the kinase buffer containing 500 µM ATP and 0.1% BSA for 30 min at 23°C, and after addition of 100 µl RIPA, subjected to the Plx-binding assay.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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