Regulation of Cdc25C by ERK-MAP Kinases during the G$_2$/M Transition

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SUMMARY

Induction of G$_2$/M phase transition in mitotic and meiotic cell cycles requires activation by phosphorylation of the protein phosphatase Cdc25. Although Cdc2/cyclin B and polo-like kinase (PLK) can phosphorylate and activate Cdc25 in vitro, phosphorylation by these two kinases is insufficient to account for Cdc25 activation during M phase induction. Here we demonstrate that p42 MAP kinase (MAPK), the *Xenopus* ortholog of ERK2, is a major Cdc25 phosphorylating kinase in extracts of M phase-arrested *Xenopus* eggs. In *Xenopus* oocytes, p42 MAPK interacts with hypophosphorylated Cdc25 before meiotic induction. During meiotic induction, p42 MAPK phosphorylates Cdc25 at T48, T138, and S205, increasing Cdc25's phosphatase activity. In a mammalian cell line, ERK1/2 interacts with Cdc25C in interphase and phosphorylates Cdc25C at T48 in mitosis. Inhibition of ERK activation partially inhibits T48 phosphorylation, Cdc25C activation, and mitotic induction. These findings demonstrate that ERK-MAP kinases are directly involved in activating Cdc25 during the G$_2$/M transition.

INTRODUCTION

Induction of mitosis and meiosis in the eukaryotic cell cycle requires the coordinated activation of multiple M phase-inducing protein kinases. Central in this process is activation of Cdc2/cyclin B, which results from dephosphorylation of Cdc2 by the protein phosphatase Cdc25. For this rate-limiting event to occur, Cdc25 needs to be activated in an initiation step prior to Cdc2 activation and then is further activated in an amplification step after

Cdc2 activation. Previous studies demonstrated that Cdc2/cyclin B phosphorylates the N-terminal regulatory domain of Cdc25 at specific S/T-P motifs, thereby increasing Cdc25's phosphatase activity (Izumi and Maller, 1993; Hoffman et al., 1993). These findings led to the hypothesis that Cdc2 kinase forms a direct positive feedback loop with Cdc25, thereby amplifying Cdc25 activation. Previous studies also established that a polo-like kinase (PLK), which is activated downstream of Cdc2 kinase (Karaiskou et al., 1998, 1999; Okano-Uchida et al., 2003), binds the N-terminal regulatory domain of Cdc25 and phosphorylates it at a D/E-X-S/T-Ø-X-D/E motif (Kumagai and Dunphy, 1996; Nakajima et al., 2003; Toyoshima-Morimoto et al., 2002). Since PLK interaction with Cdc25 is promoted by Cdc2-catalyzed phosphorylation of Cdc25 (Elia et al., 2003a; Elia et al., 2003b; Sillje and Nigg, 2003), PLK is likely to be also involved in the amplification step of Cdc25 activation (Glover et al., 1998). However, Cdc2/cyclin B and PLK do not phosphorylate recombinant Cdc25 at all sites that are phosphorylated in M phase cell lysates (Kumagai and Dunphy, 1996; Margolis et al., 2006). In addition, in interphase cell lysates, phosphorylation of Cdc25 by both Cdc2/cyclin B and PLK does not recapitulate the Cdc25 phosphorylation and activation observed in M phase cell lysates (Karaiskou et al., 1998). In the absence of Cdc2 activity, Cdc25 can still be phosphorylated and activated by inhibiting okadaic acid (OA)-sensitive phosphatases (Izumi et al., 1992). These unexplained observations predict that one or multiple additional kinases are involved in Cdc25 activation during the G$_2$/M transition.

To search for the putative additional kinase(s), we analyzed the Cdc25 phosphorylating activity in M phase-arrested *Xenopus* egg extracts (MEE) by protein fractionation. MEE is an excellent model system, as it contains hyperphosphorylated and activated Cdc25, and incubation of recombinant Cdc25 with MEE recapitulates the M phase-associated phosphorylation and activation of Cdc25 (Izumi et al., 1992; Kumagai and Dunphy, 1992). Thus, most, if not all, of the putative additional kinases

involved in Cdc25 activation should be present in active forms in MEE. By biochemical fractionation, we discovered that p42 MAP kinases (MAPK), the *Xenopus* ortholog of ERK2, accounts for a major portion of the Cdc25-phosphorylating activity in MEE, leading us to hypothesize that ERK-MAP kinases are crucial components in the Cdc25 activating system. To test this hypothesis, we examined phosphorylation and activation of Cdc25 by p42 MAPK and mammalian ERKs both in vitro and during M phase induction. Our results not only demonstrate crucial involvement of p42 MAPK in Cdc25 activation during meiotic maturation of *Xenopus* oocytes but also show that ERK-MAP kinases are involved in activating Cdc25C during mitotic induction of mammalian cells.

RESULTS

MEE Contains Novel Cdc25-Phosphorylating Activities

To analyze the Cdc25-phosphorylating activity in MEE, we initially determined the contribution of Cdc2 kinase and/or *Xenopus* PLK ortholog 1 (Plx1) to the total Cdc25-phosphorylating activity by three independent approaches. MEE was prepared with the classic MPF extraction buffer (Wu and Gerhart, 1980) that was supplemented with ATP-γ-S and the protein phosphatase inhibitor OA, and 1:4 to 1:64 diluted MEE phosphorylated GST-tagged Cdc25 (GST-Cdc25) in a dose-dependent manner (Figure 1A). We observed that depletion of Cdc2 with p13-sepharose and Plx1 with anti-Plx1 antibodies from 1:10-diluted MEE removed 90% of the histone H1 kinase activity but <20% of the Cdc25-phosphorylating activity (Figure 1B). Partially purified Cdc2/cyclin B from MEE containing 90% of the H1 kinase activity in 1:10-diluted MEE catalyzed 10% of the Cdc25 phosphorylation by the MEE sample (Figure 1C). Further, we fractionated the 40% ammonium sulfate precipitate of MEE by consecutive gel filtration and Q-sepharose chromatography and followed Cdc25-phosphorylating activity by a GST-Cdc25 gel mobility shift assay. One broad peak of Cdc25-phosphorylating activity was recovered (Figure 1D), indicative of recovery of multiple Cdc25-phosphorylating kinases. Most importantly, the Q-sepharose chromatography separated the Cdc25-phosphorylating activity into a minor peak (QF) and a major peak (QE1) (Figure 1E). The minor activity in QF coincided with most of the recovered H1 kinase activity and could be depleted with the Cdc2 affinity resin p13-sepharose, whereas the major activity in QE1 contained little H1 kinase activity and could not be depleted with p13-sepharose (Figure 1F). Further, QE1 contained little Plx1-dependent Cdc25-phosphorylating activity (Figure S1). Together, these results indicate that *Xenopus* egg extracts contain previously unrecognized Cdc25-phosphorylating kinases.

p42 MAPK Is One of the Novel Cdc25-Phosphorylating Kinases in *Xenopus* Egg Extracts

To identify the novel Cdc25-phosphorylating kinases in MEE, we fractionated QE1 further by consecutive sucrose gradient sedimentation, hydroxyapatite chromatography, Affi-Gel blue chromatography, and Superose 6 gel filtration and followed the Cdc25-phosphorylating activity by the gel mobility assay. One peak of Cdc25-phosphorylating activity was recovered in each step, and the activity recovered in the last step coeluted with the 44 kDa standard protein (Figure S2E). Since *Xenopus* p42 MAPK, the *Xenopus* ortholog of ERK2, is both activated during *Xenopus* oocyte maturation and important for Cdc2 activation (Gotoh et al., 1995; Huang and Ferrell, 1996; Kosako et al., 1994; Palmer and Nebreda, 2000; Shibuya et al., 1992), we determined whether the purified activity from QE1 was due to p42 MAPK. We observed that in the last step of the purification, the Cdc25-phosphorylating activity copurified with the phosphorylating activity toward the MAPK substrate myelin basic protein (MP) (Figure 2A). The activity peak fractions contained a 42 kDa polypeptide (Figure 2B, upper panel) that was recognized by anti-MAPK antibodies (Figure 2B, lower panel). Moreover, immunodepletion of MAPK from the peak fractions removed 95% of the Cdc25-phosphorylating activity and 80% of the MP-phosphorylating activity (Figure 2C). Immunodepletion of MAPK from unfractionated QE1 removed 50% of the Cdc25-phosphorylating activity (Figure 2D). These results demonstrate that the purified activity from QE1 was due to p42 MAPK. To evaluate the contribution of p42 MAPK to the total Cdc25-phosphorylating activity in MEE, we used both depletion and reconstitution approaches. Depletion of p42 MAPK from 1:10-diluted MEE removed 50% of the MP-phosphorylating activity and 60% of the Cdc25-phosphorylating activity (Figure 2E). Purified MAPK that contained 50% of the MP phosphorylating activity in 1:10-diluted MEE phosphorylated GST-Cdc25 at 40% of the efficiency of the MEE sample (Figure 2F). These results demonstrate that MAPK is one of the major Cdc25-phosphorylating kinases in MEE.

p42 MAPK Phosphorylates Cdc25 at T48, T138, and S205 In Vitro

Cdc25 is known to be phosphorylated at five conserved S/T-P motifs in *Xenopus* egg extracts (Izumi and Maller, 1993), including T48, T67, T138, S205, and S285 (Figure 3A). To identify the MAPK phosphorylation sites in Cdc25, we first constructed five GST-tagged Cdc25 fragments (F1–F5) that contained residues 9–129, 128–213, 251–353, 371–500, and 499–550 of Cdc25, respectively (Figure S3A), and phosphorylated these fragments with MEE, p42 MAPK, or Cdc2 kinase. MEE phosphorylated F1, F2, and F3; Cdc2 kinase phosphorylated F2 and F3; and MAPK phosphorylated F1 and F2 (Figure S3B). We then mutated each of the potential phosphorylation sites in F1, F2, and F3 and determined the effect on phosphorylation by the relevant kinase(s). For Cdc2 kinase, the T138V mutation completely eliminated phosphorylation of F2, whereas the S205A mutation had little effect (Figure 3B, left panel). Elimination of most of the F3 phosphorylation required both the S285A and T308V mutations
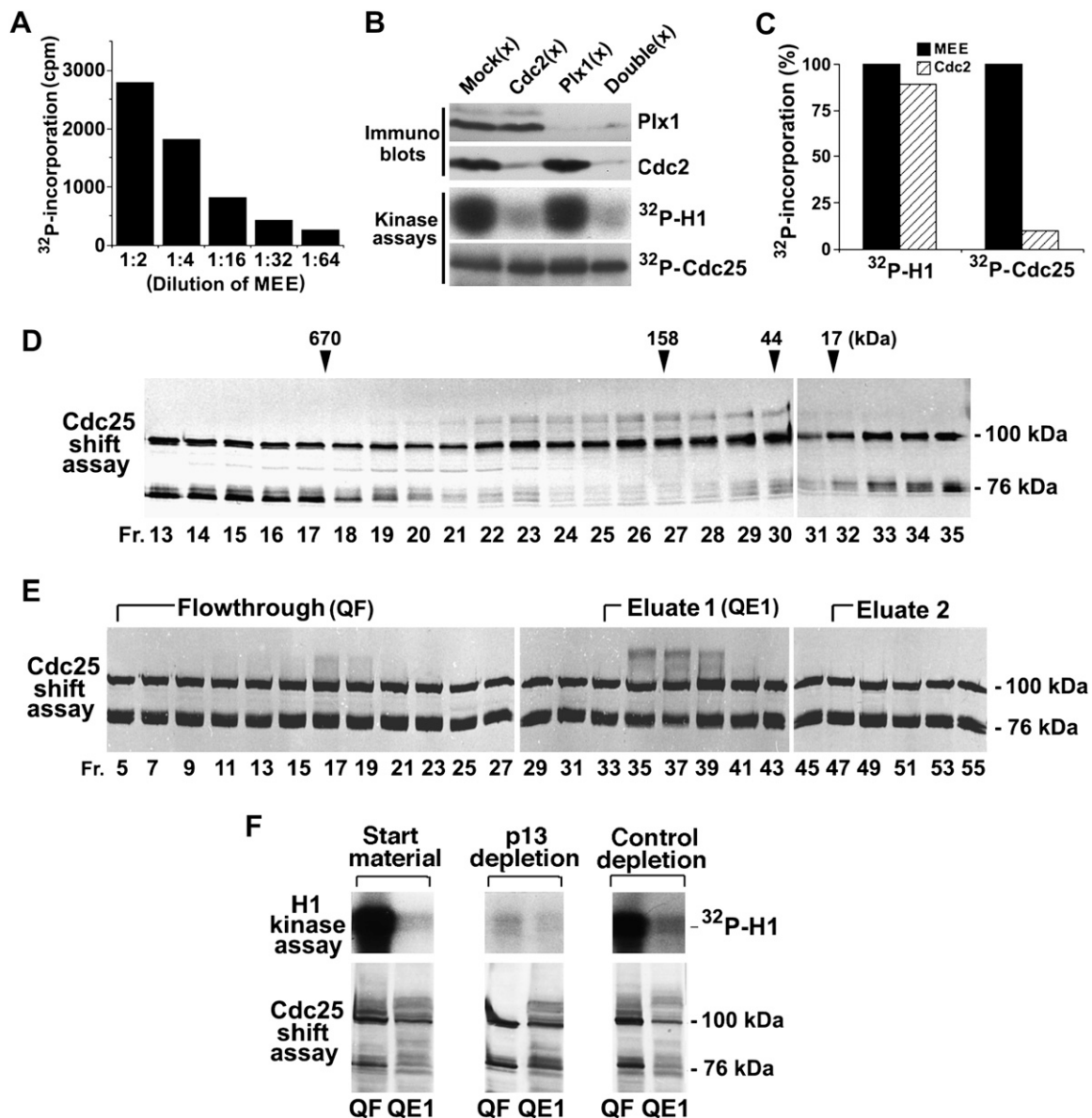
Figure 1. MEE Contains Novel Cdc25-Phosphorylating Activities

(A) Quantification of GST-Cdc25 phosphorylation catalyzed by 1:4 to 1:64 diluted MEE.

(B) After 1:10-diluted MEE was mock treated or depleted of Cdc2, Plx1, or both, samples were immunoblotted for each of the indicated proteins and assayed for phosphorylation of histone H1 or GST-Cdc25 by ^{32}P incorporation.

(C) MEE that is 1:10-diluted and partially purified Cdc2/cyclin B that contained 90% of the H1 kinase activity in the MEE sample were assayed for GST-Cdc25 phosphorylation by ^{32}P incorporation.

(D) Fractions from Ultrogel AcA 34 gel filtration of the 40% ammonium-sulfate precipitate of MEE were measured for inducing gel mobility shifts in the full-length (100 kDa) and truncated (76 kDa) GST-Cdc25.

(E) Fractions from the consecutive Q-sepharose chromatography after the Ultrogel AcA 34 gel filtration were assayed for inducing the GST-Cdc25 shift.

(F) After QF and QE1 were absorbed with p13 or control sepharose, the starting material and unbound proteins were assayed for phosphorylating histone H1 and inducing the GST-Cdc25 shift.

(Figure 3B, right panel). These results indicate that Cdc2 kinase selectively phosphorylates T138, S285, and T308 in Cdc25. For p42 MAPK, the T48V mutation eliminated 80% of the phosphorylation of F1 (Figure 3C, left panel), and the additional T67V mutation abolished the residual

phosphorylation of F1 (data not shown). The T138V mutation completely eliminated phosphorylation of a cleaved product of but not of the full-length F2 (Figure 3C, middle panel). The additional S205A mutation removed 70% of the phosphorylation of the full-length F2 (Figure 3C, right
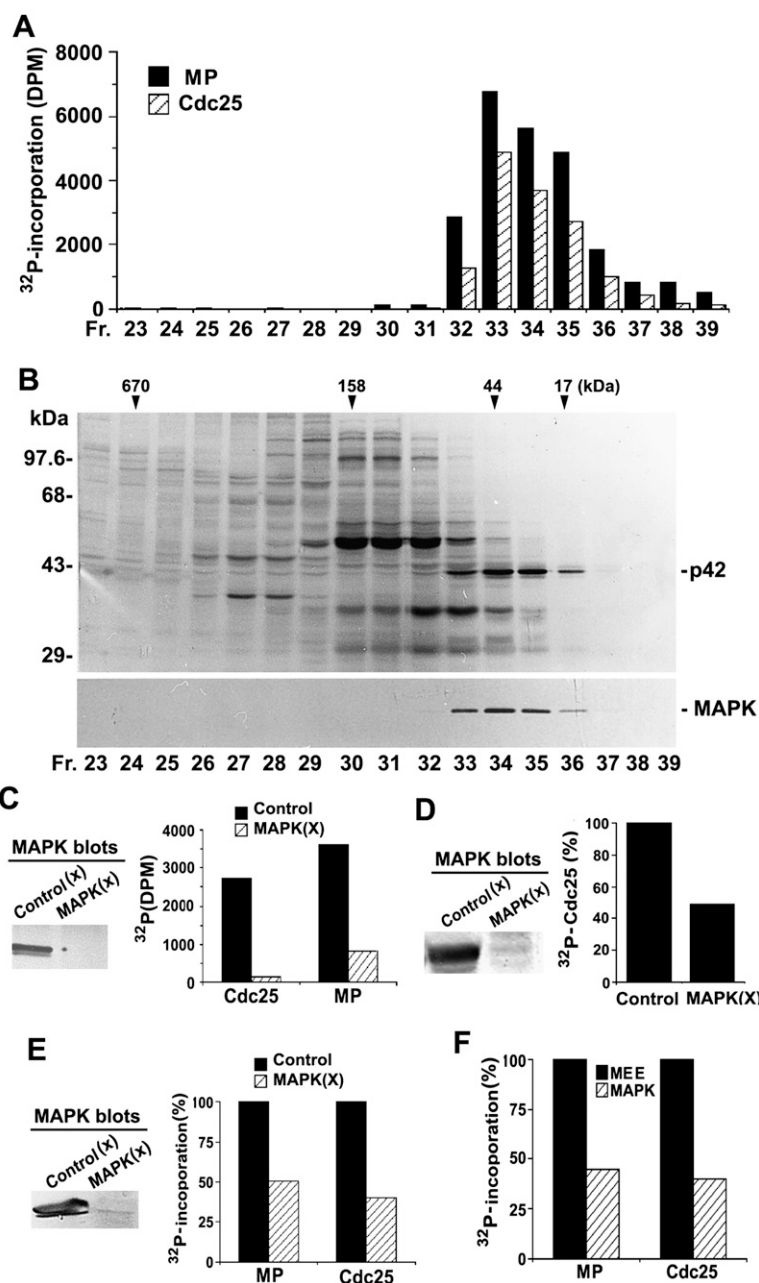
Figure 2. Identification of p42 MAPK as a Major Cdc25-Phosphorylating Kinase in MEE

(A) Indicated fractions from the last step of the consecutive chromatography of QE1, Superose 6 gel filtration, were quantified for phosphorylation of MP and GST-Cdc25.

(B) SDS-PAGE-separated proteins in the indicated fractions from the Superose 6 gel filtration were stained with silver (upper panel) and immunoblotted with anti-MAPK antibodies (lower panel).

(C) Control or MAPK-depleted peak fractions of the Cdc25-phosphorylating activity from the Superose 6 gel filtration were quantified for phosphorylation of MP and GST-Cdc25.

(D) Control or MAPK-depleted QE1 was quantified for relative phosphorylation of GST-Cdc25.

(E) Control or MAPK-depleted 1:10 diluted MEE was quantified for relative phosphorylation of MP and GST-Cdc25.

(F) MEE that is 1:10-diluted and purified MAPK from QE1 that contained 50% of the MB phosphorylating activity in the MEE sample were quantified for relative phosphorylation of MP and GST-Cdc25.

panel). These results indicate that MAPK mainly phosphorylates T48, T138, and S205 in Cdc25. In support of this conclusion, MAPK readily phosphorylated the 9–205 but not the 204–550 fragment of Cdc25 (Figure S3C). The single (T48V) and triple (T48V, T138V, and S205A) mutations of the full-length Cdc25 reduced the MAPK-catalyzed phosphorylation of Cdc25 by 50% and 80%–90%, respectively (Figure 3D). Moreover, antibodies that specifically recognize T48-phosphorylated Cdc25 (Figures S4A and S4B) preferentially detected GST-Cdc25 that had been phosphorylated by MAPK (Figure 3E). Depletion of MAPK, but not Cdc2 and Plx1, from MEE greatly reduced

the T48 phosphorylation (Figure 3F). Consistent with Cdc2 and MAPK phosphorylating different sites in Cdc25, MAPK and Cdc2 kinase phosphorylated different cleavage products of GST-Cdc25 (Figure S3D) and produced different two-dimensional phosphopeptide maps from GST-Cdc25 (Figure S3E).

p42 MAPK Phosphorylates Cdc25 at T48 and T138 in *Xenopus* Oocytes

To determine whether p42 MAPK phosphorylates Cdc25 in *Xenopus* oocytes, we first examined whether MAPK can phosphorylate Cdc25 at T48 and T138 in the absence
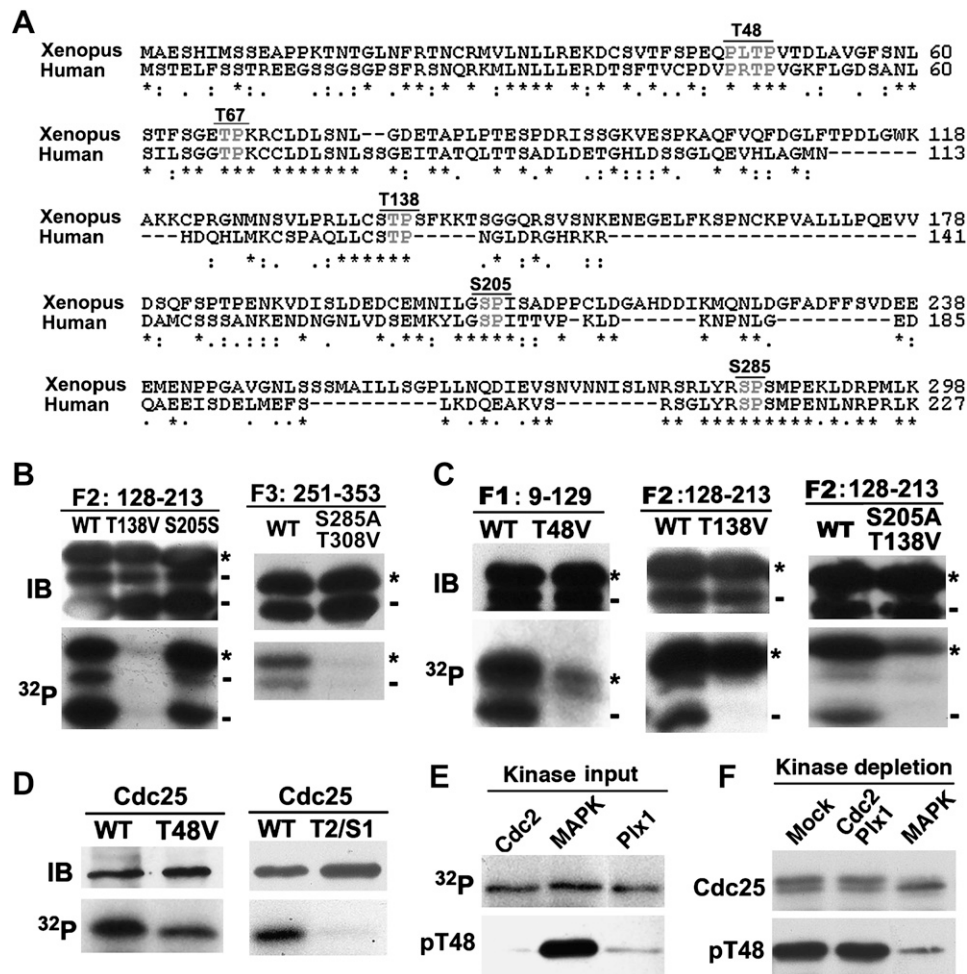
Figure 3. Identification of Three In Vitro MAPK Phosphorylation Sites in Cdc25

(A) Sequence alignment of the N-terminal regulatory domains of *Xenopus* Cdc25 and human Cdc25C with five conserved T/S-P motifs highlighted in red.

(B) Immunoblots (IB) and phosphorylation (^{32}P) of wild-type and the indicated mutant forms of F2 and F3 by semirecombinant Cdc2/cyclin B.

(C) Immunoblots (IB) and phosphorylation (^{32}P) of wild-type and the indicated mutant forms of F1 and F2 by activated recombinant MAPK. Asterisks and bar symbols indicate the full-length and truncated substrates, respectively.

(D) Immunoblots (IB) and phosphorylation (^{32}P) of wild-type and the indicated mutant forms of GST-Cdc25 by activated recombinant MAPK.

(E) Immunoblots of GST-Cdc25 that was phosphorylated to a similar extent by Cdc2 kinase, MAPK, and Plx1 with anti-pT48 antibodies.

(F) Immunoblots of GST-Cdc25 that was phosphorylated by 1:4-diluted MEE that was mock treated or depleted of Cdc2 plus Plx1 or MAPK alone with anti-GST or anti-pT48 antibodies.

of Cdc2 activation. To this end, *Xenopus* oocytes were injected with a constitutively activated MEK1 (CA-MEK1) plus either Wee1 or a control molecule, Xp95 (Che et al., 1999), and activation of MAPK, phosphorylation of Cdc25, and dephosphorylation of Cdc2 were followed by immunoblotting. Both coinjections synchronously induced MAPK activation and phosphorylation of T48 and T138 within 2.5 hr in the absence of Cdc2 dephosphorylation (Figure 4A). The coinjected Wee1 was functional, since it effectively blocked the eventual dephosphorylation of Cdc2 and the gel mobility shift of Cdc25 (Figure 4B) as well as increases in histone H1 kinase activity and mitotic phosphoproteins recognized by MPM-2 monoclonal

antibody (Davis et al., 1983) (Figure S5A). *Xenopus* oocytes were also injected with Xp95 or Wee1 alone and then stimulated with progesterone. In Wee1-injected oocytes, progesterone induced low levels of both MAPK activation and Cdc25 phosphorylation at T48 in the absence of Cdc2 activation (Figure S5B). These results demonstrate that MAPK can phosphorylate Cdc25 independently from Cdc2 activation. Next, we determined whether MAPK phosphorylates Cdc25 in the presence of Cdc2 activation. To this end, *Xenopus* oocytes were injected with a constitutively activated Cdc2 (Cdc2-AF), and MAPK activation, Cdc25 phosphorylation, and Cdc2 dephosphorylation were followed by immunoblotting.
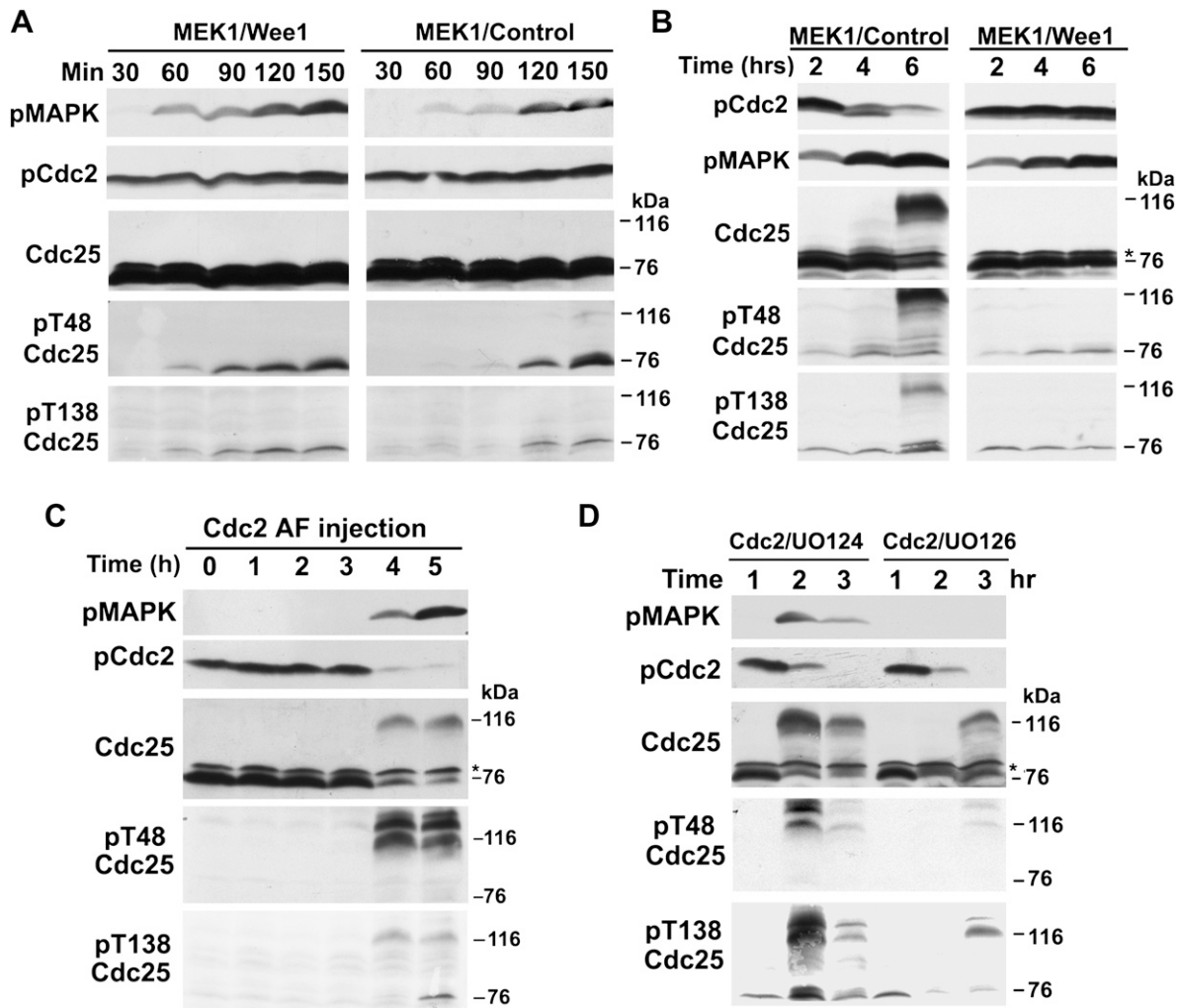
Figure 4. p42 MAPK Phosphorylates Cdc25 at T48 and T138 in *Xenopus* Oocytes

(A) Extracts of oocytes collected at the indicated minutes after coinjection of CA-MEK1 and Wee1 (MEK1/Wee1) or CA-MEK1 and Xp95 (MEK1/Control) were immunoblotted with antibodies that recognize phosphorylated/activated MAPK (pMAPK), phosphorylated/inactivated Cdc2 (pCdc2), Cdc25, T48-phosphorylated Cdc25 (pT48-Cdc25), and T138-phosphorylated Cdc25 (pT138-Cdc25).

(B) Extracts of oocytes collected at the indicated hours after coinjection of CA-MEK1 and Xp95 mRNAs (MEK1/control) or CA-MEK1 and Wee1 mRNAs (MEK1/Wee1) were immunoblotted as described for (A).

(C) Extracts of oocytes collected at the indicated times after injection of Cdc2AF were immunoblotted as described for (A).

(D) Extracts of oocytes collected at the indicated times after injection of Cdc2AF and culture in the presence of UO124 or UO126 were immunoblotted as described for (A). The asterisk indicates a nonspecific band recognized by anti-Cdc25 antibodies.

Cdc2-AF concurrently induced activation of MAPK, phosphorylation of T48 and T138, the Cdc25 gel mobility shift, and dephosphorylation of Cdc2 (Figure 4C). While inhibition of MAPK activation by the MEK1 inhibitor UO126 (Favata et al., 1998) did not affect the kinetics of Cdc2 dephosphorylation, this treatment significantly delayed and greatly reduced the phosphorylation of T48 and T138 as well as the Cdc25 gel mobility shift (Figure 4D). These results demonstrate that in the presence of activated Cdc2 kinase (and potentially other mitotic kinases), MAPK still plays a significant role in Cdc25 phosphorylation.

p42 MAPK Interacts with Cdc25

Since MAPK typically interacts with its substrates (Biondi and Nebreda, 2003; Pearson et al., 2001; Tanoue and Nishida, 2003), we next examined whether MAPK interacts with Cdc25. By GST pull-down assays, GST-Cdc25 interacted with maltose binding protein (MBP)-tagged MAPK (MBP-MAPK) but not MBP alone (Figure 5A). MBP-MAPK bound two C-terminal fragments of Cdc25 containing residues 375–550 and 204–550, respectively, but not the three N-terminal fragments (Figure 5B). Further deletion of the C-terminal 50 residues did not reduce
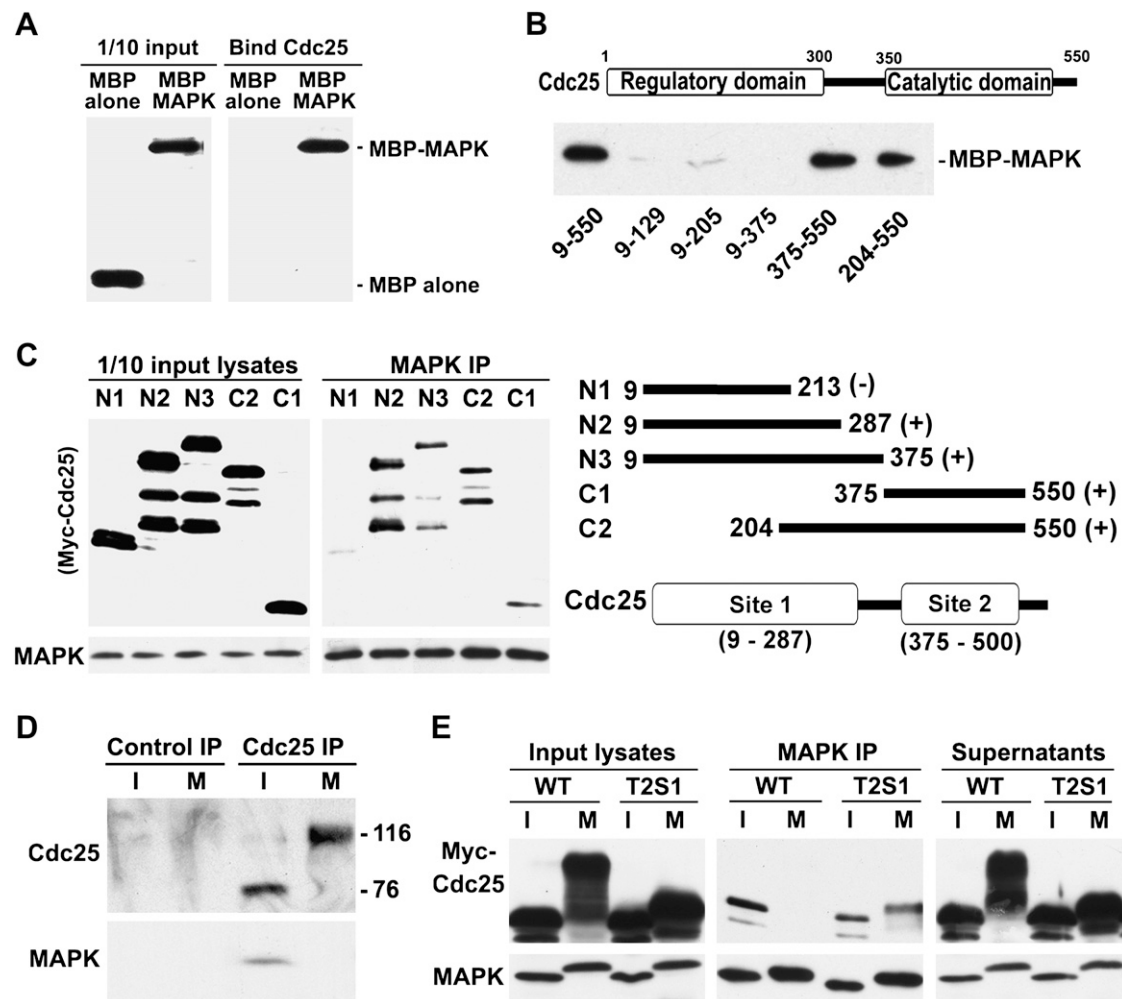
Figure 5. p42 MAPK Interacts with Hypophosphorylated Cdc25

(A) MBP and MBP-MAPK were incubated with GST-Cdc25, and proteins bound to GST-Cdc25 (right panel) were immunoblotted in parallel with 1/10 of the input protein (left panel) with anti-MBP antibodies.

(B) MBP-MAPK was incubated with GST or GST-tagged Cdc25 fragments, and proteins bound to each of the GST-Cdc25 products were immunoblotted with anti-MBP antibodies.

(C) Extracts of oocytes collected at 6 hr after injection with myc-tagged Cdc25 fragments were immunoprecipitated with anti-MAPK antibodies. The MAPK immunocomplexes were immunoblotted in parallel with 1/10 of the input oocyte lysates with anti-myc and anti-MAPK antibodies (left panel). The results and two deduced MAPK binding regions in Cdc25 are illustrated (right panel).

(D) After extracts of immature (I) or mature (M) oocyte extracts were immunoprecipitated with mouse IgG or an anti-Cdc25 monoclonal antibody, immunocomplexes were immunoblotted with polyclonal anti-Cdc25 and anti-MAPK antibodies.

(E) After extracts of immature (I) and mature (M) oocytes that ectopically expressed the control (wt) or the T2S1 mutant form of myc-CI-Cdc25 were immunoprecipitated with anti-MAPK antibodies, the input oocyte extracts, immunoprecipitated proteins, and supernatant after the immunoprecipitation were immunoblotted with anti-myc and anti-MAPK antibodies.

GST-Cdc25 interaction with MBP-MAPK (data not shown). These results show that the C-terminal catalytic domain of Cdc25 contains a docking site for MAPK. By coimmunoprecipitation approach, a myc epitope-tagged catalytically inactive Cdc25 (myc-CI-Cdc25), which was ectopically expressed in *Xenopus* oocytes, coimmunoprecipitated with endogenous MAPK (data not shown). Among the truncation products of myc-CI-Cdc25 tested, the fragments N2 (9–287), N3 (9–375), C1 (375–550), and C2 (204–550), but not the fragment N1 (9–213), coimmu-

noprecipitated with MAPK (Figure 5C, left panel). However, the 213–375 fragment of Cdc25 did not coimmunoprecipitate with MAPK (data not shown). These results indicate that MAPK interacts with both the regulatory and catalytic domains of Cdc25 in *Xenopus* oocytes (Figure 5C, right panel) and strengthen the conclusion that Cdc25 is a physiological substrate of MAPK.

Since MAPK interacts with latent p90rsk in immature oocytes but not MAPK-phosphorylated p90rsk in mature oocytes (Hsiao et al., 1994; Palmer et al., 1998), we further
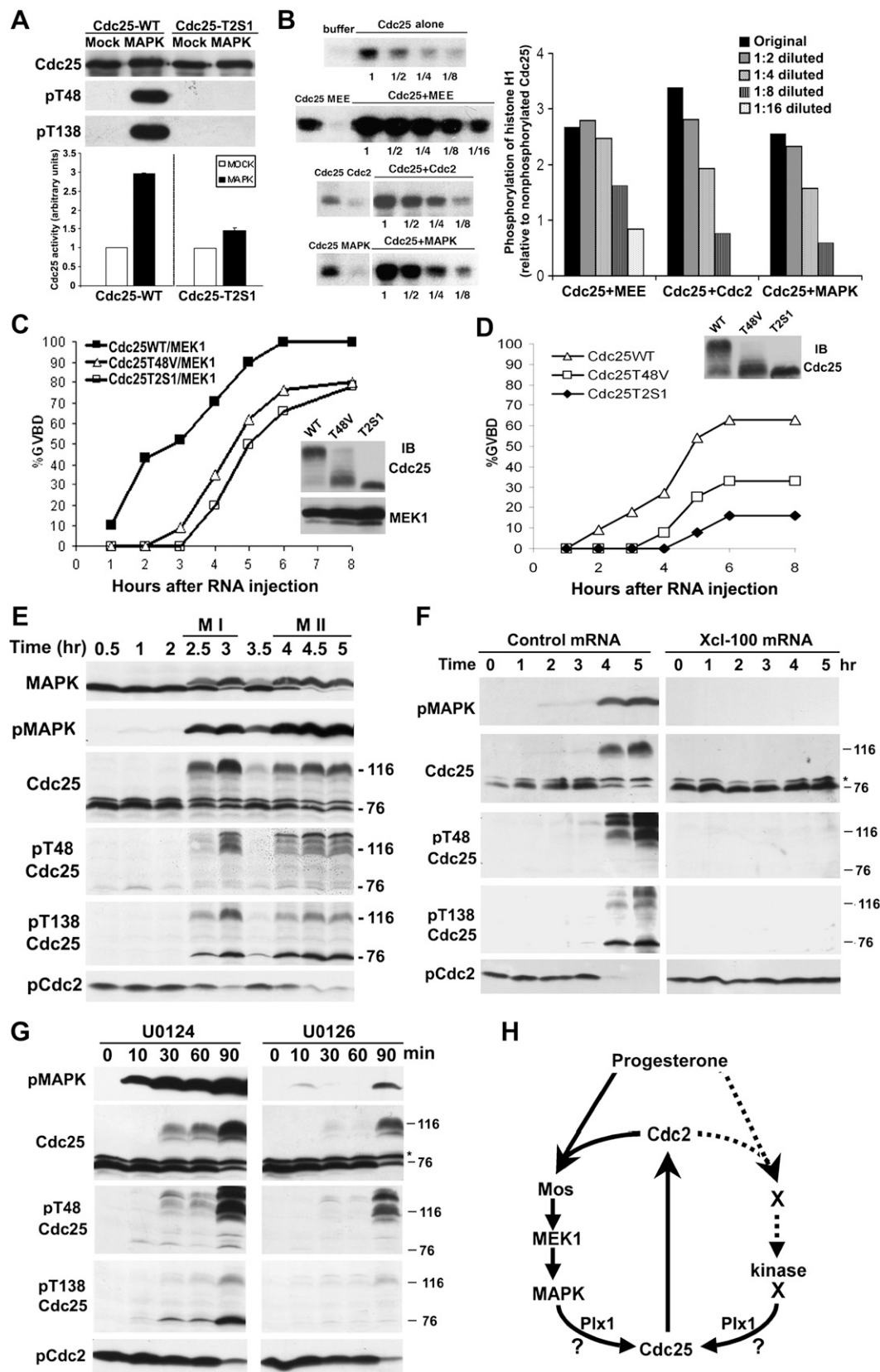
determined whether phosphorylation of Cdc25 by MAPK inhibits the Cdc25-MAPK interaction. Endogenous Cdc25 coimmunoprecipitated with MAPK in immature but not mature oocyte extracts (Figure 5D). Although myc-CI-Cdc25 behaved similarly as endogenous Cdc25, the T2S1 mutant form of myc-CI-Cdc25 coimmunoprecipitated with MAPK in both extracts (Figure 5E). These results indicate that phosphorylation of Cdc25 by MAPK has negative effects on the Cdc25-MAPK interaction.

p42 MAPK Activates Cdc25 In Vitro

To determine whether phosphorylation of Cdc25 by MAPK activates Cdc25 in vitro, wild-type and the T2S1 mutant form of His-Cdc25 were phosphorylated with activated recombinant MAPK, and phosphorylated and nonphosphorylated proteins were measured in parallel for the ability to dephosphorylate 3-O-methylfluorescein phosphate (OMFP) (Gottlin et al., 1996). Phosphorylation increased the phosphatase activity of the wild-type His-Cdc25 several times but had much lesser effects on the mutant His-Cdc25 (Figure 6A). Since MAPK also phosphorylates T67 and possibly other sites, the residual activation of the mutant His-Cdc25 could be due to phosphorylation of these sites. In addition to this direct approach, selected preparations of GST-Cdc25 that had low basal levels of the Cdc2-activating activity were phosphorylated either with 1:4-diluted MEE that had been immunodepleted of endogenous Cdc25 or with an amount of purified MAPK or Cdc2 kinase that contained similar levels of the GST-Cdc25-phosphorylating activity. The phosphorylated Cdc25 by each kinase sample was then assayed in parallel with nonphosphorylated Cdc25 for the ability to activate latent Cdc2/cyclin B. As shown in Figure 6B, nonphosphorylated GST-Cdc25 activated Cdc2/cyclin B in a dose-dependent manner. While MEE-phosphorylated GST-Cdc25 required a 16-fold dilution to match the activity of the nonphosphorylated GST-Cdc25, Cdc2- and MAPK-phosphorylated GST-Cdc25 each reached the activity of nonphosphorylated Cdc25 after a 4- to 8-fold dilution. These results indicate that both MAPK and Cdc2 kinase partially activate Cdc25. We should note that phosphorylation of GST-Cdc25 by both Cdc2 and MAPK

did not further increase the endpoint dilution (data not shown), indicating that additional factors are required for complete activation of Cdc25.

Phosphorylation of Cdc25 by MAPK Enhances Cdc25's Maturation-Inducing Activity

To determine if MAPK is involved in Cdc25 activation in *Xenopus* oocytes, we used multiple indirect approaches. First, *Xenopus* oocytes were injected with CA-MEK1 plus the wild-type or T48V/T2S1 mutant form of myc-Cdc25, and the effect of the MAPK-site mutations on the combined ability of CA-MEK1 and Cdc25 to induce oocyte maturation was examined. The T48V and the T2S1 mutations retarded Cdc25-induced oocyte maturation by 2 and 2.5 hr, respectively (Figure 6C), indicating that phosphorylation of Cdc25 by MAPK is positively involved in MAPK-induced oocyte maturation. Second, *Xenopus* oocytes were injected with the wild-type or one of the mutant forms of myc-Cdc25 alone, and the effect of the MAPK-site mutations on Cdc25's ability to induce *Xenopus* oocyte maturation was examined. The T48V and the T2S1 mutations reduced the ability of Cdc25 to induce oocyte maturation by 2- and 3-fold, respectively, and delayed oocyte maturation by 1 and 2 hr, respectively (Figure 6D), indicating that phosphorylation of Cdc25 by MAPK is involved in Cdc2-induced oocyte maturation. Further, oocyte maturation was induced by progesterone stimulation or MEE injection, and the relationship between phosphorylation of T48 and T138 and the dramatic gel mobility shift of Cdc25, indicative of its M phase-associated activation, was examined. In progesterone-stimulated oocytes, MAPK activation and T48/T138 phosphorylation correlated with the Cdc25 gel mobility shift at the onset of both meiosis I and II (Figure 6E). Delaying MAPK activation by a brief treatment of oocytes with UO126 before progesterone stimulation delayed both T48/T138 phosphorylation and the Cdc25 gel mobility shift (Figure S6A). When activation of MAPK was completely inhibited by injection of the MAPK phosphatase XCL-100 (Lewis et al., 1995; Sohaskey and Ferrell, 2002) or incubation with UO126, both T48/T138 phosphorylation and the Cdc25 gel mobility shift were inhibited (Figures 6F

Figure 6. p42 MAPK Activates Cdc25 In Vitro and In Vivo

(A) After wild-type or the T2S1 mutant form of His-Cdc25 was mock treated or phosphorylated by recombinant MAPK, the end products were immunoblotted with anti-Cdc25 or anti-phospho-Cdc25 antibodies or assayed for dephosphorylation of OMFP. Error bars represent the standard deviation from triplicate experiments.

(B) Latent Cdc2/cyclin B was treated with each of the indicated samples and then measured for phosphorylating histone H1.

(C) Oocytes were coinjected with CA-MEK1 and the wt or each of the indicated mutant forms of myc-Cdc25, and percentage of germinal vesicle breakdown (GVBD) was scored after the indicated times. Oocyte extracts were made at the last timepoint and immunoblotted with anti-myc and anti-HA antibodies.

(D) Oocytes were injected with wt or each of the indicated mutant forms of myc-Cdc25, and percentage of GVBD was scored after the indicated times. Oocyte extracts were then made and immunoblotted with anti-myc antibodies.

(E) At the indicated times after progesterone stimulation, oocyte extracts were made and immunoblotted as described for Figure 4A.

(F) Oocytes were injected with Xp95 (control) or XCL-100 mRNA and stimulated with progesterone after 3 hr. Extracts of oocytes collected at the indicated times after the progesterone stimulation were immunoblotted as described for Figure 4A.

(G) Oocytes that had been cultured in the presence of UO124 or UO126 for 1 hr were injected with MEE in the same culture medium, and extracts of oocytes after the indicated hours were immunoblotted as described for Figure 4A.

(H) A schematic illustration of potential involvement of MAPK and another yet-to-be-identified Cdc25-phosphorylating kinase in Cdc25 activation during progesterone-induced *Xenopus* oocyte maturation.

and S6B). In MEE-injected oocytes, MAPK activation and T48/T138 phosphorylation also correlated with the Cdc25 gel mobility shift. Inhibition of MAPK activation by UO126 delayed and reduced both T48/T138 phosphorylation and the Cdc25 gel mobility shift (Figure 6G). These results strongly indicate that MAPK is involved in both the initiation and amplification steps of Cdc25 activation during *Xenopus* oocyte maturation.

Mammalian ERKs Interact with and Phosphorylate Cdc25C in Somatic Cell Cycles

To determine if ERK-MAP kinases in mammalian somatic cells are involved in regulating Cdc25C, the closest relative of *Xenopus* Cdc25 (Kumagai and Dunphy, 1992), we first determined whether ERK2 interacts with Cdc25C (hCdc25C) in a cell-cycle-dependent manner. By coimmunoprecipitation, myc-hCdc25C ectopically expressed in ovarian cancer A2780 cells interacted with ERK2 much more significantly in interphase cells than in mitotically arrested cells (Figure 7A). Similar results were obtained when interaction between endogenous hCdc25C and ERK2 was examined (Figure S7A). In contrast, interaction of ERK2 with hCdc25A or hCdc25B was not observed (Figure S7B). Second, since hCdc25C is similarly phosphorylated in mitosis as *Xenopus* Cdc25 in meiosis (Strausfeld et al., 1994), we determined whether ERK2 phosphorylates hCdc25C at T48 in mitotic cells. Immunoblotting with T48 phosphospecific antibodies showed that T48 phosphorylation is greatly elevated in mitotically arrested relative to asynchronous A2780 cells (Figure 7B). Immunodepletion of ERK1/2 from mitotic cell lysates greatly reduced the T48 phosphorylating activity (Figure 7C). Examination of in vitro phosphorylation of GST-tagged hCdc25A, B, and C showed that while p42 MAPK phosphorylated all three forms of hCdc25, the phosphorylation was most robust with hCdc25C (Figure S7C). Third, we treated A2780 cells with the mitotic blocker nocodazole, either alone or together with the MEK inhibitor, UO126, and determined the effect of inhibiting ERK activation on T48 phosphorylation, gel mobility shift of hCdc25C, and accumulation of MPM-2 reactive phosphoproteins. UO126 reduced T48 phosphorylation and the ratio of shifted to nonshifted hCdc25C by 50% and decreased the level of MPM-2 reactive proteins by 30%–40% (Figures 7D–7F). These results concurred with the previous observations demonstrating that inhibition of the ERK activation pathway by a variety of approaches, including mitogen starvation, siRNA-mediated knockdown, or dominant-negative forms of essential components in the ERK-activation pathway or specific pharmacological inhibitors, causes a delay or partial inhibition in mitotic entry (Foijer et al., 2005; Liu et al., 2004; Roberts et al., 2002; Wright et al., 1999). These results demonstrate that, although not specifically activated during mitotic induction, mammalian ERKs are part of the regulatory system that activates Cdc25C during mitotic induction. These results also imply that mammalian cells probably possess a negative control mechanism

that inhibits Cdc25C phosphorylation by ERKs or alternatively, a cell-cycle-regulated positive control mechanism that makes ERKs efficiently phosphorylate Cdc25C during the G_2/M transition.

DISCUSSION

The molecular mechanism by which Cdc25 becomes activated during M phase induction is one of the key unresolved problems in the eukaryotic cell-cycle control. For the ultimate goal of resolving this problem, we utilized *Xenopus* oocytes/eggs, model systems highly conducive in studying the regulation of the G_2/M transition biochemical approaches, to search for previously unrecognized kinases involved in Cdc25 activation, and sought confirmation of our findings in mammalian cells. Our results show that in both systems, the ERK-MAPK kinase interacts with the C-type Cdc25 (Cdc25C) in interphase and phosphorylates and activates Cdc25C during M phase induction. These findings have uncovered novel and important roles of ERK-MAP kinases in the M phase-associated Cdc25C-activation system.

Full activation of Cdc25 during *Xenopus* oocyte maturation requires both initiation and amplification steps (King et al., 1994; Perdiguero and Nebreda, 2004). In principle, kinases that are involved in the initiation step can be activated prior to Cdc2 activation, leading to a threshold level of Cdc25 activity. Our results show that in progesterone-stimulated *Xenopus* oocytes, p42 MAPK can be activated at low levels prior to and independent of Cdc2 activation (Figures 6E and S5B), as previously reported by Fisher et al. (1999). These low levels of MAPK activation correlated with low levels of Cdc25 phosphorylation (Figure S5B), and inhibiting MAPK activation inhibited Cdc25 phosphorylation and activation (Figure 6F). Thus, p42 MAPK is an excellent candidate for one of the kinases involved in initiating Cdc25 activation during *Xenopus* oocyte maturation. Theoretically, the kinases that are involved in the amplification step of Cdc25 activation can both be activated by Cdc2 and activate Cdc25. Previous studies demonstrated that activated Cdc2 kinase can induce MAPK activation through both mRNA stabilization and posttranslational modification of Mos (Castro et al., 2001; Gotoh et al., 1991; Nebreda et al., 1995; Yue and Ferrell, 2004). Our results show that MAPK activation is critically involved in, although not absolutely required for, Cdc2- or MEE-induced phosphorylation and activation of Cdc25 (Figures 4D and 6G). Thus, p42 MAPK is involved in the amplification step of Cdc25 activation during *Xenopus* oocyte maturation through a positive feedback loop consisting of Cdc2, Mos, MEK, MAPK, and Cdc25 (Figure 6H). This positive feedback loop may explain the long-established requirement of Cdc2 activation for robust MAPK activation and the frequently observed synchrony of these two events during progesterone-induced *Xenopus* oocyte maturation (Ferrell, 1999; Gotoh and Nishida, 1995; Kosako et al., 1994; Palmer and Nebreda, 2000). The involvement of p42 MAPK in both the initiation and
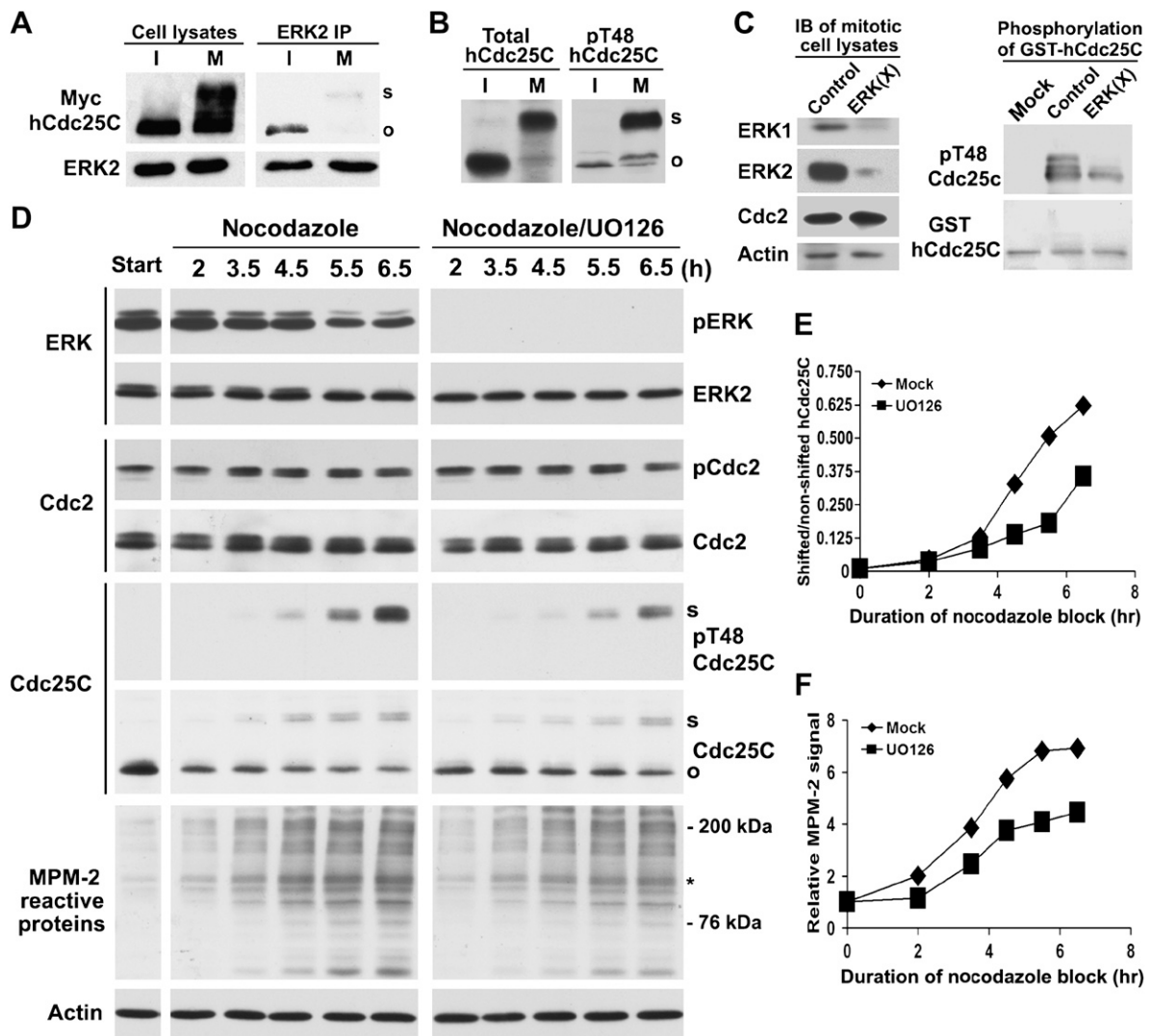
Figure 7. Mammalian ERK2 Regulates Cdc25C in Somatic Cell Cycles

(A) After ERK2 was immunoprecipitated from interphase (I) or mitotic (M) extracts of A2780 cells that ectopically expressed myc-hCdc25C, input cell lysates and the ERK2 immunoprecipitates were immunoblotted with anti-myc and anti-ERK antibodies.

(B) Interphase (I) and mitotic (M) extracts of A2780 cells were immunoblotted with anti-hCdc25C and anti-pT48-hCdc25C antibodies.

(C) Control or ERK1/2-immunodepleted lysates of mitotic A2780 cells were immunoblotted with antibodies for each of the indicated proteins (left panel) or incubated with GST-hCdc25C, which was then immunoblotted with anti-pT48-hCdc25C and anti-GST antibodies (right panel).

(D) Crude lysates of A2780 cells that had been blocked with nocodazole either alone or together with U0126 for the indicated times were immuno-blotted with antibodies for each of the indicated proteins. The "s" and "o" indicate nonshifted hCdc25C in interphase cells and shifted hCdc25C in mitotic cells, respectively.

(E) Relative levels of the shifted and nonshifted Cdc25C in (D) were determined, and the ratio calculated was plotted.

(F) The MPM-2 reactive protein indicated by asterisk in (D) was quantified, and the normalized level was plotted.

amplification steps of Cdc25 activation may also explain the requirement of active MAPK for Mos-induced Cdc2 activation in *Xenopus* oocyte extracts in the absence of protein synthesis (Huang and Ferrell, 1996), and the inhib-itory effect of an N-terminally truncated p90rsk mutant (named D2) that constitutively interacts with MAPK during *Xenopus* oocyte maturation (Gavin et al., 1999). In addition to these direct roles of p42 MAPK in Cdc25 activation, the MAPK phosphorylation site T138 in *Xenopus* Cdc25 is the

equivalent site in hCdc25C, whose phosphorylation promotes PLK binding (Sillje and Nigg, 2003), raising the possibility that ERK-MAP kinases also promote PLK-dependent Cdc25 activation.

Having established the role of ERK-MAP kinases in the Cdc25C-activation system, the next question is whether MAPK is the only previously unrecognized kinase involved in Cdc25C activation during M phase induction. Although phosphorylation of Cdc25 by p42 MAPK accounts for

Mos-induced Cdc2 activation in *Xenopus* oocyte extracts, this finding does not explain how the phosphatase inhibitor OA, which can bypass the requirement for progesterone to induce *Xenopus* oocyte maturation (Rime et al., 1990; Goris et al., 1989), induces Cdc2 activation without activation of p42 MAPK (Huang and Ferrell, 1996). In some of the previous studies, inhibition of MAPK activation delays, but does not block, progesterone-induced *Xenopus* oocyte maturation (Dupre et al., 2002; Gross et al., 2000; Fisher et al., 1999). In contrast to the natural stimulus progesterone, forced activation of MAPK does not always induce Cdc2 activation in *Xenopus* oocytes or cell-free systems (Shibuya et al., 1992). When it does, there is a significant delay between robust MAPK activation and Cdc2 dephosphorylation (Figure 4B) (Huang and Ferrell, 1996; Nebreda and Hunt, 1993). These multiple unexplained observations indicate that progesterone stimulation of *Xenopus* oocytes activates at least one additional kinase that is involved in Cdc25 activation. While sufficient activation of p42 MAPK is probably able to initiate and amplify Cdc25 activation in cooperation with Cdc2 kinase and Plx1, composite roles of p42 MAPK and this additional kinase may cause quicker and more robust activation of Cdc25 and Cdc2/cyclin B during *Xenopus* oocyte maturation (Figure 6H). In agreement with this hypothesis, our gel filtration of MEE resulted in recovery of a much broader peak of Cdc25-phosphorylating activity than can be accounted for by p42 MAPK and/or Cdc2 activities (Figure 1D). Depletion of MAPK from QE1, which contained most of the unaccounted-for Cdc25 phosphorylating activity in *Xenopus* egg extracts, removed only ~50% of the Cdc25-phosphorylating activity (Figure 2D). The remaining activity can be stabilized by OA and thiophosphorylation and is almost certainly due to a kinase complex of >200 kDa that does not contain Cdc2, Plx1, or p42 MAPK (unpublished data). Thus, we hypothesize that this yet-to-be-identified Cdc25-phosphorylating activity in MEE represents the additional kinase or one of the additional kinases involved in Cdc25 activation during *Xenopus* oocyte maturation. The presence of alternative regulators in Cdc25 activation may explain why studies by different investigators have yielded differing results with respect to the requirement of MAPK for Cdc2 activation during *Xenopus* oocyte maturation.

EXPERIMENTAL PROCEDURES

Preparation of *Xenopus* Egg Extracts, Immunoblotting, Kinase Depletion, Kinase Activity Assays, and Chromatography

Preparation of *Xenopus* egg extracts, immunoblotting, p13-based Cdc2 absorption, immunodepletion, and histone H1 kinase assay were performed as previously described (He et al., 2005; Kuang and Ashorn, 1993; Kuang et al., 1991). Antibodies used in this study and protocols for the consecutive chromatography of *Xenopus* egg extracts are described in Supplemental Experimental Procedures.

Phosphorylation of Recombinant Cdc25 and GST Pull-Down

Preparation of GST-tagged Cdc25 or Cdc25 fragments and phosphorylating kinases are described in Supplemental Experimental Procedures. Measurement of phosphorylation of these proteins by ^{32}P incorporation was carried out by incubation of 2.0 μg of GST-Cdc25 bound to 5 μl of glutathione agarose (Sigma-Aldrich) with 15 μl of the kinase sample in EB (80 mM sodium β-glycerol phosphate, 20 mM EGTA, and 15 mM MgCl$_2$) and 1 mM DTT supplemented with 0.2 μCi/μl [γ -^{32}P]-ATP, 0.2 mM ATP, and 1 μM cAMP-dependent protein kinase inhibitor (Sigma) at room temperature for 30 min. After the beads were washed as done for immunoprecipitation, bound proteins were eluted with SDS-PAGE sample buffer and separated by SDS-PAGE. The ^{32}P incorporation into GST-Cdc25 was revealed by autoradiography and quantified by scintillation counting of dissected bands from dried gels. GST-Cdc25 gel mobility shift assay was carried out by incubation of MEE or chromatography fractions with 0.2–0.5 μg of GST-Cdc25 at 22°C for 45 min in a final volume of 50 μl containing 20 mM NaF, 2 mM ATP, 5 mM DTT, and 1.5 μM OA followed by immunoblotting of end products with anti-Cdc25 antibodies.

For GST pull down, 5 μg of GST-tagged proteins were affinity absorbed onto 15 μl glutathione agarose preblocked with 5% milk at 4°C for 1 hr and equilibrated in TBS (150 mM NaCl in 50 mM Tris-HCl [pH 7.4]). After incubation at 4°C for 2 hr with 100 ng of MBP-tagged proteins dissolved in 50 μl TBS, beads were washed three times with 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% Triton X-100, 0.5% Tween-20, and 1 mM DTT, and eluted proteins were immunoblotted with anti-MBP antibodies.

Cdc25 Phosphatase Activity Assays

One-half micrograms of His-Cdc25 immobilized onto 5 μl of Ni-NTA beads was mock treated or phosphorylated by recombinant MAPK and washed. Its phosphatase activity was measured by incubation with 250 μl of 0.5 M 3-O-methylfluorescein phosphate (OMFP) in the reactive buffer containing 100 mM Tris-HCl (pH 8.2), 40 mM NaCl, 1 mM DTT, 10 mM glutathione, and 20% glycerol or the reaction buffer alone at 37°C for 15 min followed by measurement of absorbance at 477 nm (Gottlin et al., 1996). GST-Cdc25 was mock treated or phosphorylated to similar extents by 1:4-diluted MEE or partially purified Cdc2/cyclin B from QF or purified MAPK from QE1, and the end products were measured for Cdc2-activating activity as described in Supplemental Experimental Procedures.

Injection and Maturation of *Xenopus* Oocytes

Stage VI *Xenopus* oocytes were obtained, microinjected, matured with progesterone (Sigma), observed for GVBD, and extracted as previously described (Che et al., 1999). The MEK1 inhibitor U0126 and its negative control analog U0124 (Calbiochem) were added to cultures of stage VI oocytes at 50 μM. RNAs for oocyte injection were produced by in vitro transcription of the linearized plasmids described in Supplemental Experimental Procedures using mMESSAGE SP6 Large Scale In Vitro Transcription kit for Capped RNAs (Ambion) and purified by LiCl precipitation.

Cell Culture, cDNA Transfection, Preparation of Cell Lysates, and Immunoprecipitation

Ovarian cancer A2780 cells were cultured in RPMI 1640 medium (Mediatech) that was supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Atlanta Biologicals) and transfected with 8 μg cDNA/60 mm dish using Lipofectamine 2000 (Invitrogen) according to the manufacturer instruction. Nocodazole was added to the cell-culture medium at 400 ng/ml for 18 hr, and mitotically arrested cells were selectively detached by manual tapping of culture dishes. Cell lysates were prepared, immunoblotted, and immunoprecipitated as previously described (He et al., 2005). Signals on immunoblots were semiquantified with NIH image 1.62 software as previously described (Wu et al., 2001).

Supplemental Data

Supplemental Data include seven figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/128/6/1119/DC1/.

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