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CDK-Dependent Potentiation of MPS1 Kinase Activity Is Essential to the Mitotic Checkpoint

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Summary

Accurate chromosome segregation relies upon a mitotic checkpoint that monitors kinetochore attachment toward opposite spindle poles before enabling chromosome disjunction [1]. The MPS1/TTK protein kinase is a core component of the mitotic checkpoint that lies upstream of MAD2 and BubR1 both at the kinetochore and in the cytoplasm [2, 3]. To gain insight into the mechanisms underlying the regulation of MPS1 kinase, we undertook the identification of *Xenopus* MPS1 phosphorylation sites by mass spectrometry. We mapped several phosphorylation sites onto MPS1 and we show that phosphorylation of S283 in the noncatalytic region of MPS1 is required for full kinase activity. This phosphorylation potentiates MPS1 catalytic efficiency without impairing its affinity for the substrates. By using *Xenopus* egg extracts depleted of endogenous MPS1 and reconstituted with single point mutants, we show that phosphorylation of S283 is essential to activate the mitotic checkpoint. This phosphorylation does not regulate the localization of MPS1 to the kinetochore but is required for the recruitment of MAD1/MAD2, demonstrating its role at the kinetochore. Constitutive phosphorylation of S283 lowers the number of kinetochores required to hold the checkpoint, which suggests that CDK-dependent phosphorylation of MPS1 is essential to sustain the mitotic checkpoint when few kinetochores remain unattached.

Results and Discussion

Phosphorylation of S283 Is Essential for Full MPS1 Kinase Activity

Analysis of *Xenopus* MPS1 electrophoretic mobility reveals a slower migration in mitotic compared to interphase extracts. Phosphatase treatment abolishes this mobility shift, showing that MPS1 is strongly phosphorylated during mitosis and to a lesser extent during interphase (Figure 1A). In the presence of ATP, recombinant wild-type (WT) but not kinase-dead (KD) MPS1 also showed retarded electrophoretic mobility and autophosphorylation onto tyrosine and threonine (Figure 1B), suggesting that MPS1 autophosphorylation activity contributes to its phosphorylation pattern. We sought to identify MPS1 phosphorylation sites by mass spectrometry. In total, we could count up to 27 phosphorylation

sites, out of which six were unambiguously identified (see Table S1 available online). Along with the latter ones, we selected five more sites on the basis of their conservation between human and *Xenopus* MPS1 to be individually mutagenized into nonphosphorylatable alanines (Figure 1C). Single-point mutants were translated into *Xenopus* egg extracts depleted from endogenous MPS1, immunoprecipitated, and tested for kinase activity in vitro by using MPS1 or myelin basic protein (MBP) as substrates (Figure 1D). Three of these mutants (S283A, T697A, and T707A) were found deficient to phosphorylate MBP, similarly to a kinase-dead (KD) version of MPS1 (D685A). We individually mutagenized each of these three phospho-sites into phosphorylation-mimicking amino acids (aspartate or glutamate). Remarkably, whereas both S283D and S283E mutations restored full MPS1 kinase activity, T697D, T697E, T707D, and T707E did not. This demonstrates that the defect observed in S283A mutant truly reflects a lack of phosphorylation. Although the intensity of MPS1-S283A autophosphorylation signal is weaker than that of WT-MPS1, the retarded electrophoretic mobility is very close to WT-MPS1. It is therefore likely that the efficiency of MPS1-S283A autophosphorylation activity is diminished, but not abolished (compare migration delay for autophosphorylation of S283A, T697A, T707A, and KD mutant). Indeed, quantification of MPS1 kinase activities (Figure 1E) indicates that although MPS1-S283A retains less than 10% of its activity toward the exogenous substrate MBP, roughly 60% of its autophosphorylation activity is maintained.

We then compared the kinetic parameters of recombinant MPS1 kinase toward two substrates: one short peptide that we designed after determining a MPS1 substrate consensus sequence (unpublished data), and the C-terminal domain of Cenp-E (tail) that we characterized as being heavily phosphorylated by MPS1 in vitro (Figure 1F) [4]. As shown in Figure 1G, the K_m parameter was not significantly different between wild-type and S283A or S283D MPS1 mutants, with either type of substrate. However, for both types of substrates, the maximal velocity of phosphorylation by S283A-MPS1 was diminished 2- to 3-fold, when compared to WT or S283D-MPS1 (Figure 1F). Calculation of the k_{cat}/K_m parameter confirmed that the enzyme efficiency was diminished 2- to 3-fold in S283A-MPS1 versus S283D or WT-MPS1 (Figure 1G). This shows that the phosphorylation of MPS1 at S283 does not modulate the affinity of MPS1 for these substrates, but increases its catalytic efficiency.

Because it was proposed that the dimerization of MPS1 could increase its kinase activity [5], we tested whether mutations at S283 affected dimerization of recombinant MPS1. To this end, we immunoprecipitated full-length MPS1 with an antibody directed against its C-terminal peptide and tested its interaction with the noncatalytic N-terminal domain (aa 1–542). As shown in Figure 1H, all full-length MPS1 proteins (wild-type, kinase-dead, S283A, or S283D mutants) were capable of interacting with the N-terminal domain of MPS1 in this assay. This suggests that the phosphorylation of MPS1 at S283 does not interfere with MPS1 dimerization to increase MPS1 catalytic efficiency.

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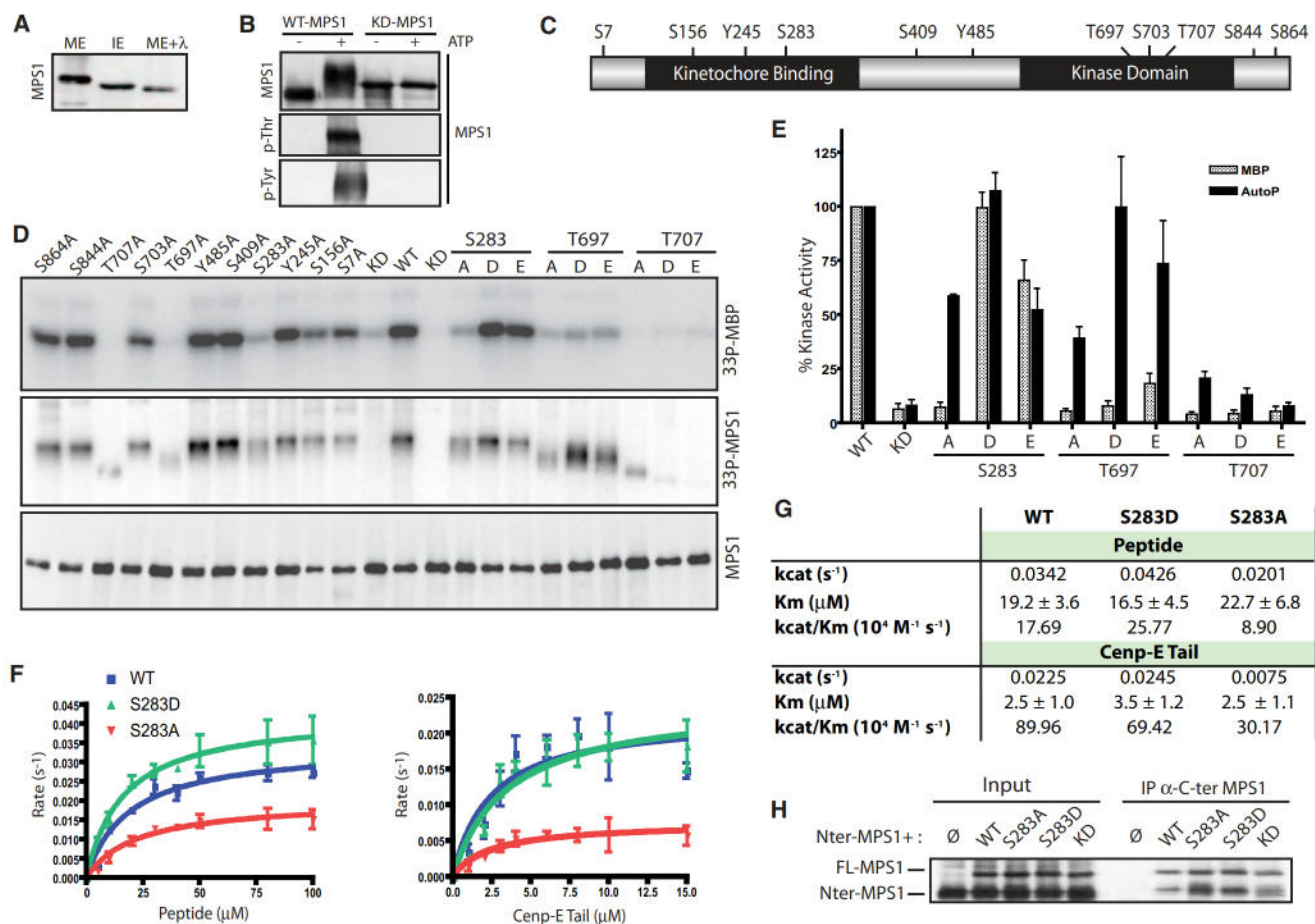


Figure 1. Phosphorylation of MPS1 at S283 Potentiates MPS1 Catalytic Efficiency

(A) Retarded mobility of MPS1 from mitotic extract (ME) or interphase extract (IE) compared to mitotic extracts treated with lambda phosphatase (ME+λ) detected by protein immunoblot with the anti-MPS1-DNT serum.

(B) WT-MPS1 but not KD-MPS1 autophosphorylates in the presence of ATP on both tyrosine and threonine, which promotes retarded in-gel mobility, as detected by protein immunoblot.

(C) Scheme of the phosphorylation sites mutated in this study.

(D) MPS1 single-point mutants were translated in mitotic extracts depleted from endogenous MPS1 and immunoprecipitated to measure their kinase activity with MBP or MPS1 autophosphorylation as substrates, in the presence of γ-[³³P]ATP. Samples were then analyzed by SDS-PAGE and autoradiography. The amount of immunoprecipitated MPS1 is evaluated by protein immunoblot after lambda phosphatase treatment to prevent retarded in-gel mobility of the phosphorylated MPS1.

(E) Quantification of MPS1 kinase activity of S283, T696, and T686 mutants bearing alanine, aspartate, or glutamate substitutions, toward MBP or MPS1 autophosphorylation, as described in (D). The error bars represent the deviation from the mean from three independent experiments.

(F) The kinetic parameters of MPS1 WT, S283A, or S283D mutants were measured against varying concentrations of a 13-mer peptide (0, 5, 10, 20, 30, 40, 50, 80, 100 μM) or the polypeptide Cenp-E Tail (0, 1, 2, 3, 4, 6, 8, 10, 15 μM), in the presence of 50 nM of recombinant MPS1 enzymes. The data from three independent experiments were plotted in Graph Pad Prism with the Michaelis-Menten equation to extract the affinity (Km) and the maximal velocity (Vmax) of the enzyme.

(G) Values of Km are expressed as the mean ± the standard error of the mean (SEM). The kcat (Vmax/[MPS1]) and kcat/Km values are also indicated.

(H) Recombinant WT, S283A, S283D, or KD-MPS1 were incubated with N-ter MPS1 for 20 min in XB buffer. The mixture was immunoprecipitated with MPS1 C-ter antibody, treated with lambda phosphatase, washed with XB, and loaded onto SDS-PAGE for visualization of MPS1 interaction by protein immunoblot with the antibody directed against MPS1 N-terminal domain.

See also Table S1.

Phosphorylation of MPS1 at S283 Is Essential to Recruit MAD1 and MAD2 at the Kinetochoe and to Activate the Mitotic Checkpoint

To determine whether these phosphorylations play a role during checkpoint signaling, we used a checkpoint activity assay in *Xenopus* egg extracts. In this system, the spindle checkpoint can be activated by the depolymerization of microtubules by nocodazole in the presence of a chromosome concentration (9,000 nuclei/μl), which mimics the nucleocytoplasmic ratio of somatic cells [6]. Under these conditions,

kinetochoe-dependent signaling prevents APC/C^{CDC20} activation, thereby maintaining high CDK1 activity, as assessed by measuring histone H1 phosphorylation. Because MPS1 kinase activity is essential to the mitotic checkpoint, its depletion prevents the maintenance of the mitotic state, which is restored if the extract is reconstituted with wild-type, but not kinase-dead, MPS1 [2] (Figures 2A and 2B). With this assay, we showed that all MPS1 mutants with impaired kinase activity (S283A, T697A, T697D, T697E, T707A, T707D, and T707E) are defective for checkpoint signaling, whereas

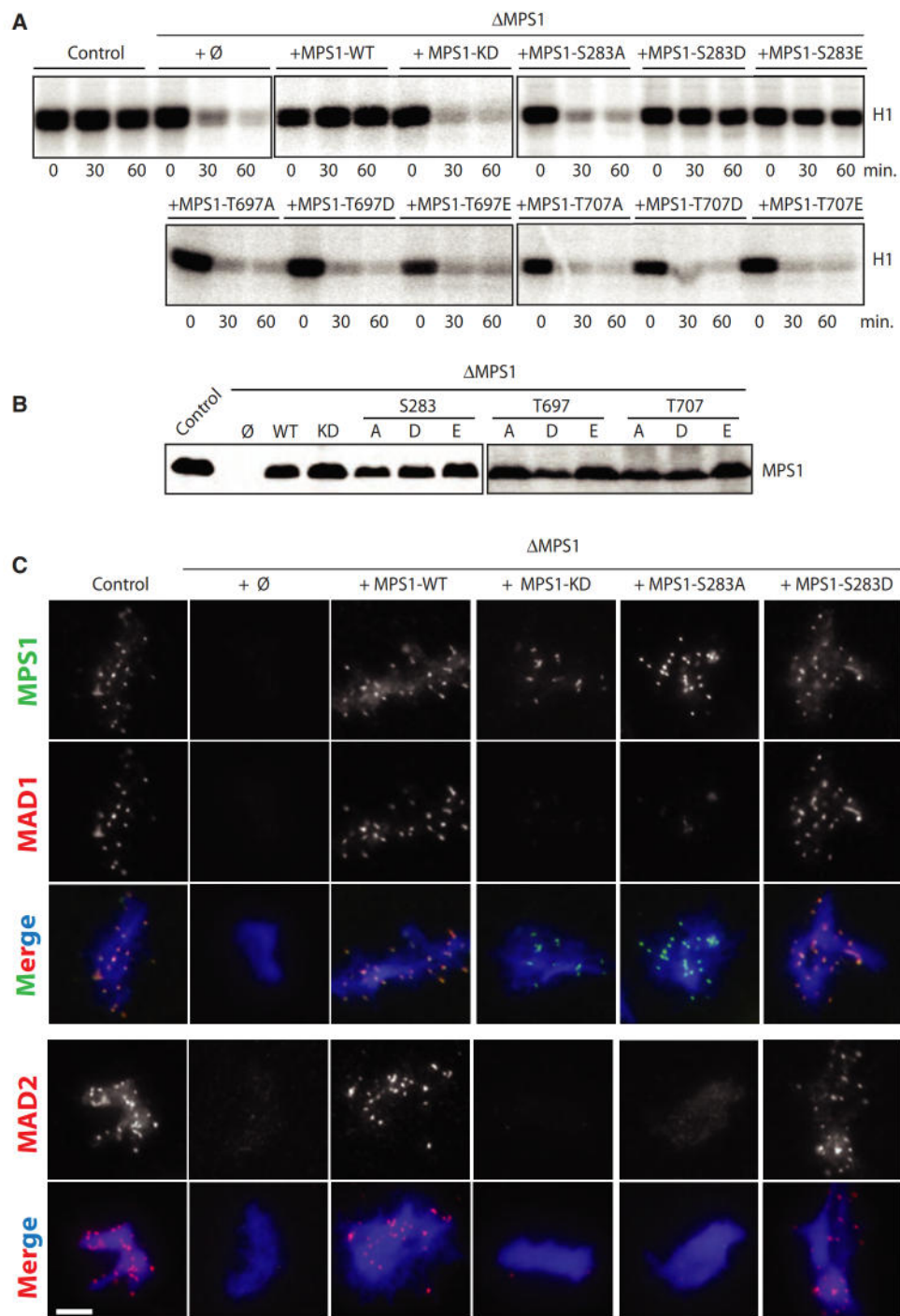


Figure 2. S283 Phosphorylation of MPS1 Is Essential to Recruit MAD2 at the Kinetochore and to Activate the Mitotic Checkpoint

(A) Control or MPS1-depleted extracts were reconstituted with buffer (\emptyset), in-extracto-translated WT, KD, S283A, S283D, S283E, T697A, T697D, T697E, T707A, T707D, or T707E-MPS1. Purified sperm heads (9,000/ μ l) and nocodazole are added to *Xenopus* egg extracts to activate the mitotic checkpoint. Activation of the checkpoint is scored by measuring CDK1 activity at 0, 30, or 60 min (min.) after release of the CSF arrest upon addition of 0.5 mM CaCl_2 . CDK1 activity is scored in the presence of purified histone H1 and γ -[^{32}P]ATP. Reaction products were analyzed by SDS-PAGE and autoradiography.

(B) The amount of endogenous and mutated MPS1 in the corresponding extracts is evaluated by protein immunoblot with the anti-MPS1-DNT serum.

(C) Control or MPS1-depleted extracts were reconstituted with buffer (\emptyset), recombinant WT, KD, S283A, or S283E-MPS1. Thirty minutes after activating the checkpoint with purified sperm heads (9,000/ μ l) and nocodazole, the extracts were assayed for MPS1, MAD1, or MAD2 immunofluorescence. Chromatin visualized with DAPI and MPS1/MAD1 costaining or MAD2 staining were merged.

Scale bar represents 5 μ m. See also Figures S1 and S2.

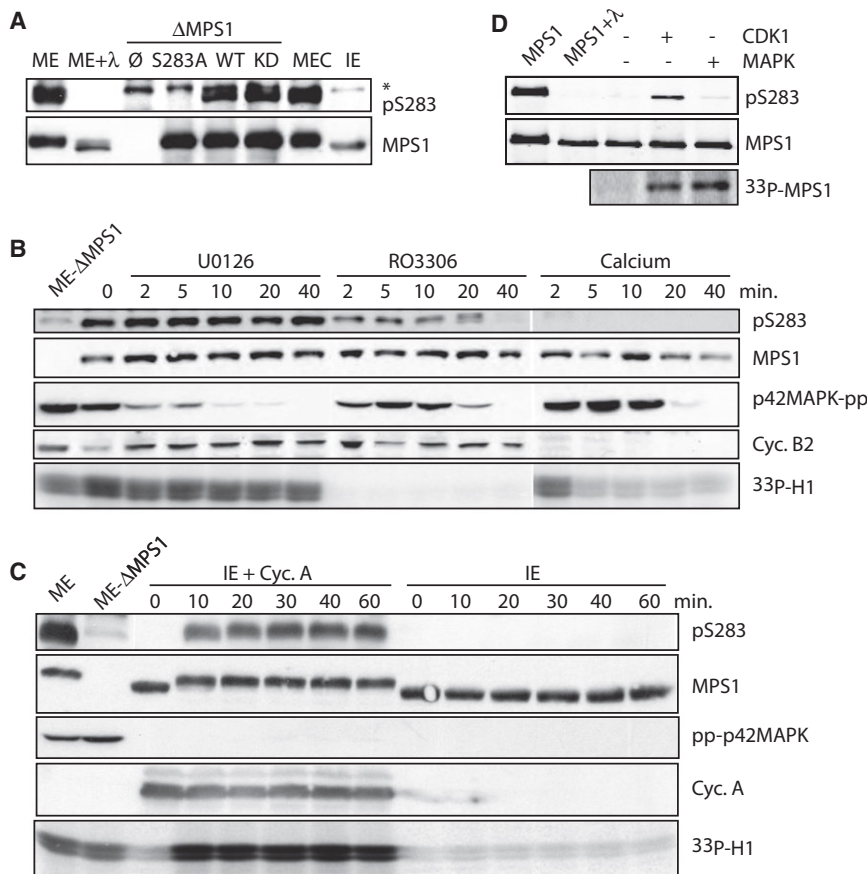


Figure 3. CDK1 Directly Phosphorylates MPS1 at S283

(A) Phosphospecific antibody raised against S283 (pS283) detects MPS1 in protein immunoblot from mitotic extracts (ME), MPS1-depleted extracts reconstituted with in-extracto-translated WT or KD-MPS1 and mitotic extracts in which the checkpoint is activated (MEC). On the contrary, pS283 antibody does not detect MPS1 from mitotic extracts treated with lambda phosphatase (ME+λ), interphase extracts (IE), or extracts depleted from MPS1 (Ø), even if reconstituted with in-extracto-translated S283A-MPS1. The total amount of MPS1 is detected by protein immunoblot with MPS1 antibody. The asterisk indicates a contaminant.

(B) Time course of mitotic extract treated with U0126 (0.4 mM) to inhibit MAPK kinase, RO3306 (0.4 mM) to inhibit CDK1 or CaCl₂ (0.5 mM) to promote exit from metaphase. Samples were evaluated by protein immunoblot at the indicated time points in minutes (min.) with the indicated antibodies and by autoradiography to score for CDK1 activity with histone H1 as a substrate in the presence of γ-[³³P]ATP.

(C) Time course of interphase extracts treated with cyclin A in the presence of cycloheximide to promote CDK1, but not MAPK activity, and analyzed as in (B).

(D) S283 phosphorylation onto recombinant KD-MPS1 is lost upon treatment with lambda phosphatase (MPS1+λ). After this treatment, KD-MPS1 was immunoprecipitated to wash out the phosphatase and incubated with ATP alone or in the presence of CDK1 or MAPK. Both kinases can phosphorylate recombinant KD-MPS1 as shown by the incorporation of γ-[³³P], but only CDK1 promotes phosphorylation of MPS1 at S283, as detected by the phosphospecific antibody.

both phospho-mimicking catalytically active mutants (S283D and S283E) restore MPS1 mitotic checkpoint function. As expected, kinase-activity-proficient phosphorylation mutants (S7A, S156A, Y245A, S409A, Y485A, S703A, and S864A) were all competent to reconstitute checkpoint signaling (Figure S1), except for the S844A mutant, which was previously shown to be defective in kinetochore recruitment [7]. We conclude that phosphorylation of S283 is essential for MPS1 kinase activity and function during checkpoint signaling.

To determine the mechanism by which MPS1 mutants compromised the spindle checkpoint, we examined by immunofluorescence the localization of MPS1 and recruitment of checkpoint proteins MAD1 and MAD2 to the kinetochore. We confirmed that in mock-depleted extracts, endogenous MPS1, MAD1, and MAD2 are recruited to kinetochores, whereas MAD1 and MAD2 are absent from kinetochores depleted from MPS1 (Figure 2C). Adding back recombinant WT-MPS1 restored MPS1, MAD1, and MAD2 colocalization at the kinetochores. The KD-MPS1 mutant was found at kinetochore but did not allow the recruitment of MAD1 and MAD2, confirming that MPS1 kinase activity is required to recruit MAD1 and MAD2. We found that lack of MPS1 phosphorylation at S283 did not prevent its own targeting to the kinetochore, but did impair MAD1 and MAD2 recruitment (Figure 2C). On the contrary, phosphomimicking mutation S283D promoted MAD1 and MAD2 recruitment. Phosphorylation of MPS1 at S283 is therefore essential for the correct targeting

of MAD1 and MAD2 at the kinetochore, a prerequisite to checkpoint signaling.

To further show that the checkpoint proficiency of MPS1-S283D was acting upstream of MAD2, we challenged the checkpoint either by depletion of MAD2 or by addition of polyclonal antibodies directed against MAD2. In both cases, perturbation of MAD2 prevented MPS1-S283D from sustaining the checkpoint (Figure S2). Addition of an excess of MAD2 is known to overcome the loss of kinetochore-dependent signaling [8]. We now show that MAD2 overexpression is still able to sustain the checkpoint despite the presence of MPS1-S283A (Figure S2). Altogether, these results show that phosphorylation of MPS1 at S283 is acting upstream of MAD2.

CDK1 Phosphorylates MPS1 at S283

To further investigate the regulation of MPS1 by phosphorylation, we produced an antibody specific for phospho-S283. As shown in Figure 3A, this antibody detects a protein in mitotic extracts (ME), but not in interphase extracts (IE), or in mitotic extracts treated with lambda phosphatase (ME+λ) or depleted from MPS1 (Ø), showing that it recognizes phosphorylated MPS1 in mitosis. This antibody detects WT-MPS1 but not S283A-MPS1, showing that it specifically recognizes the phosphorylated S283 onto MPS1. Interestingly, both WT and KD-MPS1 are recognized by this phospho-specific antibody, which demonstrates that S283 is not an autophosphorylation site. Although specifically occurring in mitosis, phosphorylation at S283 is not dependent upon kinetochores, because

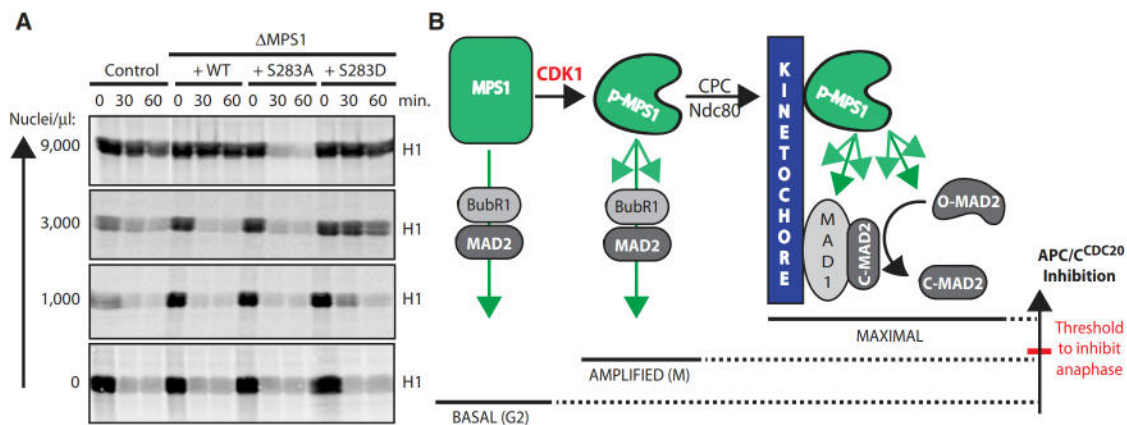


Figure 4. Constitutive Phosphorylation of S283 Sustains the Checkpoint despite an Insufficient Number of Kinetochore

(A) Extracts were complemented with the indicated number of nuclei (9,000; 3,000; 1,000 or 0 per μ l) in the presence of nocodazole. Timing of mitotic exit upon calcium addition (0.5 mM) at the indicated time points was assessed in control or MPS1-depleted extracts reconstituted with WT, S283A, or S283D-MPS1, by following CDK1 activity with histone H1 as a substrate in the presence of γ -[32 P]ATP. Samples were then analyzed by SDS-PAGE and autoradiography.

(B) Model: Maximal APC/C^{CDC20} inhibition as a three-step process. (1) BubR1- and MAD2-dependent basal inhibition of the APC/C^{CDC20} is achieved from the G2 phase onward. (2) Upon entry into mitosis, CDK1-dependent phosphorylation of MPS1 onto S283 potentiates its catalytic efficiency. This amplifies the inhibition of the APC/C^{CDC20}, also via a BubR1 and MAD2 dependent mechanism. (3) Part of hyperactive MPS1 is recruited to the kinetochore via the CPC and Ndc80 complexes, up to saturation of the kinetochore binding sites. This promotes the recruitment of MAD1-C-MAD2 to the kinetochore and the subsequent conformational change of cytoplasmic O-MAD2 into soluble C-MAD2 (see text for more details).

the antibody signal is as strong in chromosome-free mitotic extracts (ME) as in checkpoint competent extracts containing a high chromosome number and nocodazole (MEC) (Figure 3A). Because S283 is followed by a proline, we sought to test whether the two proline-directed kinases active in mitosis (CDK1 and MAPK) could be responsible for the phosphorylation of that site. We thus monitored the disappearance of S283 phosphorylation in mitotic extracts subjected to the MAPK kinase inhibitor U0126 or the CDK1 inhibitor RO3306. As shown in Figure 3B, although CDK1 inhibition rapidly abolishes phosphorylation of S283, MAPK inhibition does not. S283 dephosphorylation occurs rapidly during mitotic exit, concomitantly with CDK1-cyclin inactivation, but before MAPK dephosphorylation (Figure 3B, right: mitosis exit induced by calcium addition).

Reciprocally, reactivation of CDK1 activity in interphase extracts upon addition of cyclin A promotes phosphorylation of MPS1 at S283, despite the lack of MAPK activity (Figure 3C). These experiments (Figures 3B and 3C) were performed in extracts that were not complemented with chromosomes, showing again that phosphorylation at S283 occurs in the cytoplasm.

Finally, whereas both CDK1 and MAPK phosphorylate MPS1 *in vitro* as shown by incorporation of radioactive γ -phosphate onto KD-MPS1, only CDK1 promotes phosphorylation of the S283 of MPS1 (Figure 3D). Altogether, these results show that phosphorylation of MPS1 at S283 by CDK1 in the cytoplasm during mitosis is essential for checkpoint signaling.

Constitutive Phosphorylation of S283 Lowers the Number of Kinetochore Needed to Hold the Checkpoint

In *Xenopus* egg extracts, the minimal number of unattached kinetochores required to keep the metaphase-to-anaphase transition on hold corresponds to 9,000 nuclei per μ l. As shown in Figure 4A, lowering this number of kinetochores 3-fold prevents activation of the checkpoint in control or MPS1-depleted extracts reconstituted with WT or S283A-MPS1. On

the contrary, reconstitution of MPS1-depleted extracts with S283D-MPS1 sustains the checkpoint despite dilution of the normal kinetochore complement by three and to a lesser extent by nine (Figure 4A). However, constitutive phosphorylation of MPS1 is not sufficient to halt the metaphase-to-anaphase transition in the absence of kinetochore-dependent signaling. This further shows that phosphorylation of MPS1 at S283 potentiates checkpoint signaling.

In light of our findings, we thus propose that MPS1-dependent inhibition of APC/C^{CDC20} is a three-step process (modeled in Figure 4B). First, MAD2- and BubR1/BUB1B-dependent basal inhibition of APC/C^{CDC20} is achieved during G2 [3, 9]. Second, upon mitotic entry, CDK1-cyclin becomes active and phosphorylates MPS1, thus raising its kinase activity by at least 3-fold. We showed that this phosphorylation occurs in the cytoplasm where it is also known that MPS1 promotes the association of MAD2 and BubR1 with CDC20 [3]. Third, upon assembly of the CPC complex and the core kinetochore components from the Ndc80 complex, phosphorylated MPS1 reaches the kinetochore [10–13]. At the kinetochore, MPS1 promotes at least two key events: (1) recruitment of MAD1-C-MAD2 and (2) recruitment of cytosolic O-MAD2 that will be converted into C-MAD2 upon binding onto MAD1-C-MAD2 [14, 15].

S283 residue is located in MPS1 N-terminal noncatalytic domain, whose role in MPS1 structure and function is poorly understood. Previous studies have shown that the N-terminal domain (aa 1–301) targets MPS1 to kinetochores and that phosphorylation of residues T12 and S15 is required for the recruitment of MPS1 to the kinetochore [16–18]. However, our immunofluorescence assay reveals that lack of phosphorylation at residue S283 does not significantly impair MPS1 localization to kinetochores. We suggest that phosphorylation at S283 is not likely to be a major MPS1-kinetochore docking epitope but is required to amplify MPS1 kinase activity. In the present study, we analyzed the lack of MPS1 phosphorylation at residues T697 and T707 that were

previously shown to be autophosphorylation sites required for MPS1 full kinase activity in human cells [5, 18–21]. We also found that T697A and T707A mutants lack kinase activity. Phosphorylation-mimicking mutations at T697 partially restored MPS1 autophosphorylation activity but not its kinase activity toward MBP. On the contrary, T707D and T707E mutants remained inactive toward both types of substrates. Furthermore, all T697 and T707 mutants compromised the spindle checkpoint, in agreement with previous studies [5, 20].

Previous reports have shown that CDK1 activity is required to restrain APC/C activity during mitotic checkpoint in *S. cerevisiae* and vertebrates [22, 23]. In the present study, we uncovered one main target of CDK1 during checkpoint signaling. Phosphoproteomic studies of the human kinome also found S281 of MPS1 (equivalent to *Xenopus* S283) to be phosphorylated in vivo [24]. Interestingly, phosphorylation at S281 was found to be upregulated upon mitotic entry, remained stable during mitosis, and declined upon mitotic exit [25]. Neither the kinase responsible for S281 phosphorylation nor the significance of this phosphorylation was described. These observations are consistent with our findings of *Xenopus* MPS1, suggesting that the control of MPS1 by CDK1 is conserved among vertebrates. However, MPS1 might not be the only target of CDK1 during checkpoint signaling. Indeed, in *S. pombe*, Cdc2p was shown to act upstream of Bub1p in the mitotic checkpoint [26]. In vertebrates, CDK1 phosphorylates CDC20, which might be required to sustain the checkpoint [27], and CDK1-dependent phosphorylation of Borealin was shown to be essential to recruit the CPC at the kinetochore [28].

We favor a model in which inhibition of the APC/C^{CDC20} is amplified upon entry into mitosis via CDK1-dependent phosphorylation of MPS1 (and possibly other substrates) via kinetochore- and cytoplasmic-dependent mechanisms. This amplification contributes to the maintenance of a threshold of APC/C^{CDC20} inhibition capable of coping with progressive attachment and biorientation of the chromosomes and concomitant diminution of inhibitory signal emanating from the kinetochores. This model agrees with the proposal that the rate of MAD2 cycling on and off the kinetochore is not consistent with inhibition of the whole cellular pool of the APC/C^{CDC20} [29]. When all the kinetochores are being attached, the level of cytoplasmic signal becomes insufficient to inhibit the whole cellular pool of the APC/C^{CDC20}, thus allowing cyclin B degradation and lowering CDK activity. Reversion of the CDK-dependent mechanism that we uncovered could thus promote a rapid switch toward APC/C^{CDC20} full activation, allowing the exquisite synchrony of sister chromatid separation upon checkpoint silencing.

Experimental Procedures

Protein Expression

MPS1 phosphorylation-site mutations were introduced by PCR in pCS2-MPS1 plasmid with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The corresponding mRNAs were transcribed with the mMessage mMachine SP6 kit (Ambion) and translated in extracto. For the production of N-terminal-6His-tagged *Xenopus* MPS1 proteins via baculovirus expression system, MPS1-WT, KD, S283A, and S283D cDNAs were subcloned into pFastBac-Htb plasmid (Invitrogen). Recombinant 6His-MPS1 proteins were expressed in Sf9 cells according to the manufacturer's instruction given in the Bac-to-Bac Baculovirus Expression System (Invitrogen). In brief, MPS1 proteins were expressed for 72 hr at 28°C. Cells were collected by centrifugation at 500 × g, washed with PBS, disrupted by French press in lysis buffer, and centrifugated at 40,000 rpm for 30 min. Supernatants were incubated with Ni-Sepharose beads (GE Healthcare) for 1 hr at 4°C. After washes, 6His-MPS1 proteins were eluted with step gradient of

imidazole and dialyzed into Storage buffer (50 mM K-HEPES [pH 7.4], 200 mM NaCl, 2 mM MgCl₂, 10% sucrose, 20% glycerol). 6His-MAD2 was produced as described [8]. N-ter-MPS1 (aa 1–542) was subcloned into a pRSET vector, expressed in *E. coli* BL21, and purified with His-tag affinity. GST-WT-MPS1 and GST-KD-MPS1 were produced and purified as described [2]. Recombinant human cyclin A was produced as described [30]. Cenp-E Tail was produced as described [4].

Antibodies

Anti-MPS1-DNT (domain N-terminal) and C-ter serums were generated as described [2]. The anti-MPS1-DNT serum was used for protein immunoblot, and the anti-C-ter-purified antibody was used to immunoprecipitate active MPS1 kinase. For immunofluorescence, the purified anti-MPS1-DNT was biotinylated as described [2]. MPS1-pS283 antibody was generated against a phosphorylated peptide C-PVQPAT-pS-PDTRTR (residues 277–289). Anti-Human cyclin A and anti-*Xenopus* cyclin B2 antibodies were prepared as described [30]. The antibodies directed against MAD1 and MAD2 were produced as described [11]. p42 MAPK phosphorylation was analyzed by anti-phospho-p44/42-MAPK mouse monoclonal antibody (9106, Cell Signaling). Phosphothreonine (71–8200) and phosphotyrosine (sc-7020) antibodies were purchased from Zymed (Invitrogen) and Santa Cruz, respectively.

Phosphorylation-Site Mapping by Mass Spectrometry

Recombinant GST-MPS1-WT (4–30 µg) expressed via the baculovirus system was incubated in MPS1-depleted *Xenopus* egg extracts and then pulled down onto Glutathione-Sepharose beads. In parallel, endogenous MPS1 was immunoprecipitated from *Xenopus* CSF extracts. Samples were separated on a denaturing gel and colored with colloidal blue. Digested peptides were identified by mass spectrometry after enrichment of phosphopeptides on TiO₂ microcolumns, as described elsewhere [31].

Xenopus Egg Extracts and Immunofluorescence

Xenopus were handled according to French legislation. Mitotic extracts were prepared as described [8]. Interphase extracts were prepared from unfertilized *Xenopus* laid eggs and activated by adding ionophore A23187 (Sigma-Aldrich) to a final concentration of 2 ng/ml for 3–4 min and washed with XB buffer in the presence of cycloheximide at a final concentration of 0.1 µg/ml. To activate the mitotic checkpoint, CSF-arrested egg extracts were incubated with sperm nuclei (9,000/µl extracts) for 10 min and incubated for additional 20–30 min with 10 µg/ml of nocodazole at room temperature. To monitor activation of the mitotic checkpoint, the extracts were driven into interphase by the addition of calcium chloride to a final concentration of 0.5 mM, and samples of 1 µl of extracts were taken at the indicated time points for histone H1 kinase assays. Immunodepletion was performed for 30 min at room temperature with magnetic dynabeads-protein A coupled to the corresponding purified antibodies. For reconstitution experiments, in extracto translation of mRNA was performed in CSF-arrested *Xenopus* egg extracts immunodepleted from endogenous MPS1. In brief, extracts were supplemented with 1/100 volume of 0.1 M DTT, 1/100 volume of RNA Guard inhibitor (GE Healthcare) and incubated for 10 min at 10°C. tRNA was added to a final concentration of 0.1 mg/ml. For 100 µl of treated extracts, 10 µl of reticulocyte lysate (Promega), 2 µl of 0.5 M creatine phosphate, and 1 µl of 0.1 M spermidine were added. Aliquots of the translations were frozen in liquid nitrogen and stored at –80°C, thus allowing us to determine the protein yield by comparing endogenous and in-extracto-translated MPS1 by protein immunoblot. Fresh extracts depleted from endogenous MPS1 were then reconstituted with roughly equal amounts of in-extracto-translated MPS1. Immunofluorescence was performed as described [2]. Images were captured with a Zeiss AxioimagerZ1 motorized microscope and analyzed with the Metamorph software (Molecular Devices).

Kinase Assays

To test the kinase activity of in-extracto-translated MPS1 mutants, extracts were incubated with Dynabeads-Protein A coupled to MPS1-Cter antibody. Beads were washed and resuspended in 50 mM K-HEPES (pH 7.6). 10% of immunoprecipitated MPS1 was used to evaluate the yield by protein immunoblot, whereas 45% was used to measure its autophosphorylation activity, and the remaining 45% was used to measure its kinase activity in the presence of 0.5 mg/ml MBP. Phosphorylation experiments were performed in the presence of phosphorylation buffer (50 mM K-HEPES [pH 7.6], 5 mM MgCl₂, 100 µM ATP, and 0.1 µCi/µl γ-[³²P]ATP) during 15 min at room temperature and stopped by adding Laemmli buffer. Samples were analyzed by SDS-PAGE and autoradiography. For protein immunoblotting, the beads were incubated with 40 units of lambda protein phosphatase (NEB) for 1 hr shaking at 30°C. Samples were transferred onto nitrocellulose

membrane and blotted with anti-MPS1-DNT serum. The kinetic parameters of MPS1 were determined by using the indicated concentrations of peptide (YDDHTVWLWKKK) or Cenp-E tail (aa 2717–2954) substrates in the phosphorylation mix for 10 min. The experiment was stopped by loading the samples onto P81 paper and washed for 1 hr in 0.8% phosphoric acid. The cpm on dried P81 papers were measured with a scintillation counter (Perkin Elmer). MPS1 autophosphorylation was determined by measuring the phosphorylation in the absence of exogenous substrate and subtracted from the experiments in the presence of added substrates. Data were fit to the Michaelis-Menten equation to derive apparent V_{max} and K_m with the Graph Pad Prism software. For histone H1 kinase assays, 1 μ l of extract was frozen in liquid nitrogen at the indicated time points. Extract samples were thawed by the addition of H1 mix (0.15 mg/ml histone H1, 1 mM beta-glycerophosphate, 10 mM $MgCl_2$, 20 mM EGTA, 100 μ M ATP, 0.1 μ Ci/ μ l γ -[^{32}P]ATP) and incubated for 10 min at room temperature. Reactions were stopped by adding Laemmli buffer, loaded onto SDS-PAGE, and analyzed by autoradiography. To test for direct phosphorylation of MPS1, we immunoprecipitated recombinant GST-KD-MPS1 with MPS1-Cter antibody. One sample was taken for protein immunoblot, and the bulk was dephosphorylated with lambda phosphatase for 1 hr at 30°C. Beads were washed with 50 mM Tris-HCl (pH 7.4). Immunoprecipitated and dephosphorylated MPS1 was then incubated with phosphorylation buffers in the presence of cyclin B1/CDK1 (NEB-P6020) or p42 MAPK (NEB-P6080). After 30 min of incubation at room temperature, reactions were loaded onto a denaturing gel and analyzed by autoradiography or protein immunoblot.

Supplemental Information

Supplemental Information includes two figures and one table and can be found with this article online at [doi:10.1016/j.cub.2011.12.048](https://doi.org/10.1016/j.cub.2011.12.048).

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