

Report

Mad3/BubR1 Phosphorylation during Spindle Checkpoint Activation Depends on both Polo and Aurora Kinases in Budding Yeast

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Received 04/26/05; Accepted 05/09/05

Previously published online as a *Cell Cycle* E-publication:
<http://www.landesbioscience.com/journals/cc/abstract.php?id=1829>

KEY WORDS

Mad3/BubR1, mitosis, spindle assembly checkpoint, Polo kinase, Aurora kinase

ABBREVIATIONS

APC	anaphase promoting complex
Cdk	cyclin-dependent kinase
FACS	fluorescence activated cell sorter
HU	hydroxyurea
MEN	mitotic exit network
SAC	spindle assembly checkpoint

ACKNOWLEDGEMENTS

We wish to thank A. Amon, S. Biggins, S. Elledge, R. Fraschini, T. Hyman, K. Nasmyth, F. Severin and W. Zachariae for strains and plasmids; M. Stark and K. Hardwick for useful discussions and for communicating data prior to publication; M.P. Longhese for critical reading of the manuscript. This work has been supported by grants from Associazione Italiana Ricerca sul Cancro to S.P., Cofinanziamento 2003 MURST-Università di Milano-Bicocca, Fondo per gli investimenti della Ricerca di Base (FIRB) and Progetto Strategico MIUR-Legge 449/97 to G.L.

ABSTRACT

During mitosis the spindle assembly checkpoint (SAC) delays the onset of anaphase and mitotic exit until all chromosomes are bipolarly attached to spindle fibers. Both lack of attachment due to spindle/kinetochore defects and lack of tension across kinetochores generate the "wait anaphase" signal transmitted by the SAC, which involves the evolutionarily conserved Mad1, Mad2, Mad3/BubR1, Bub1, Bub3 and Mps1 proteins, and inhibits the activity of the ubiquitin ligase Cdc20/APC, that promotes both sister chromatid dissociation in anaphase and mitotic exit. In particular, Mad3/BubR1 is directly implicated, together with Mad2, in Cdc20 inactivation in both human and yeast cells, suggesting that its activity is likely finely regulated. We show that budding yeast Mad3, like its human orthologue BubR1, is a phosphoprotein that is hyperphosphorylated during mitosis and when SAC activation is triggered by microtubule depolymerizing agents, kinetochore defects or lack of kinetochore tension. In vivo Mad3 phosphorylation depends on the Polo kinase Cdc5 and, to a minor extent, the Aurora B kinase Ipl1. Accordingly, replacing with alanines five serine residues belonging to Polo kinase-dependent putative phosphorylation sites dramatically reduces Mad3 phosphorylation, suggesting that Mad3 is likely an in vivo target of Cdc5.

INTRODUCTION

Mitosis is the most vulnerable window of the cell cycle: errors arising during the process of chromosome segregation can lead to mutated or unbalanced genetic material and eventually be fatal if left unrepaired. To avoid this risk, eukaryotic cells have surveillance mechanisms called checkpoints that delay the onset of anaphase until DNA damage or errors in spindle formation and cell polarity have been corrected. In particular, the spindle assembly checkpoint (SAC) monitors the presence of kinetochores that are either unattached to spindle fibers or mono-oriented with respect to spindle poles (syntelic attachment), and delays the separation of sister chromatids until a correct bipolar or amphitelic attachment is achieved.^{1,2} The final goal of the SAC is to prevent chromosomal dissociation of the cohesin complex, which normally tethers sister chromatids together until anaphase, when the separase Esp1 cleaves its Scc1 component, thus triggering anaphase onset.³ Esp1 activation is in turn switched on by the proteolysis of securin, which keeps separase in a locked inactive state.^{4,5} Securin destruction is driven by the Anaphase Promoting Complex (APC),^{6,7} a cell cycle-regulated ubiquitin ligase that targets specific substrates to degradation by appending poly-ubiquitin chains to specific lysine residues.⁸ The SAC directly inhibits Cdc20, an indispensable APC activator at the metaphase to anaphase transition, thus leading to securin accumulation and separase inhibition, thereby preventing cohesin cleavage and subsequent sister chromatid separation.^{1,2} Besides preventing the onset of anaphase, the SAC inhibits also mitotic exit and cytokinesis by causing the accumulation of cyclin B-dependent CDKs, whose cyclin subunits are ubiquitinated and thereby targeted to degradation by Cdc20/APC, similarly to securin.⁸ Most SAC factors, like Mps1, Mad1, Mad2, Mad3/BubR1, Bub1 and Bub3, are conserved throughout evolution, and localize to either unattached or syntelically attached kinetochores in most eukaryotic organisms. Their recruitment to kinetochores seems to correlate, at least in some experimental conditions, with checkpoint signalling.¹ A protein complex formed by Bub3, Mad2, Mad3/BubR1 and Cdc20 has been found both in yeast and HeLa cells.⁹⁻¹¹ Its formation in budding yeast depends upon Mad1,¹¹ which is required for Mad2 recruitment to kinetochores,¹² while Bub1 is needed for that of Mad2, BubR1 and Bub3 in *Xenopus*,¹³ suggesting that Mad1, and perhaps Bub1, act upstream to the other checkpoint

components. Both Mad2 and Mad3/BubR1 seem to contribute to Cdc20 inhibition in a synergistic manner,^{9,14} and BubR1 appears to be a more potent inhibitor of Cdc20/APC ubiquitin ligase activity than Mad2.¹⁴ In addition to the above proteins, the Aurora B/Ipl1 protein kinase has been implicated in SAC response to the absence of tension across kinetochores, like during syntelic attachment, while it seems to be dispensable for SAC activation by the lack of kinetochore attachment to spindle fibers.¹⁵⁻¹⁷ This evolutionarily conserved kinase has been proposed to directly correct syntelic attachment by destabilizing kinetochore-microtubule connections until stable bipolar attachments are achieved.^{18,19}

Due to the central role of Mad3/BubR1 in Cdc20/APC inhibition, we have been trying to correlate possible post-translational modifications of budding yeast Mad3 with checkpoint functions. We show here that Mad3 is phosphorylated in mitosis and its phosphorylated isoforms are maintained at high levels during checkpoint activation by lack of kinetochore attachment or tension. We find that Mad3 phosphorylation largely depends on the Polo kinase Cdc5 and, to a minor extent, on the Aurora B/Ipl1 kinase. Accordingly, replacing by alanine five Mad3 serine residues that belong to consensus sites for phosphorylation by Polo kinases^{20,21} dramatically reduced Mad3 phosphorylation both during a normal cell cycle and upon treatment with microtubule depolymerizing drugs.

MATERIALS AND METHODS

Strains, media and reagents. All yeast strains were derivatives of or were backcrossed at least three times to W303 (*ade2-1, trp1-1, leu2-3, 112, his3-11, 15, ura3, ssd1*). Strains used for this work are listed in Table 1. Cells were grown in YEP medium (1% yeast extract, 2% bacto-peptone, 50 mg/l adenine) supplemented with 2% glucose (YEPD) or 2% raffinose (YEPR). Galactose was eventually added to YEPR at 1% to make YEPRG. *MET3-CDC5* cells were grown in medium lacking methionine (-Met, *MET3* promoter on). To shut-off expression from the *MET3* promoter cells were shifted to YEPD medium supplemented with 2mM methionine. Alpha factor was used at 2 µg/ml, nocodazole at 15 µg/ml and hydroxyurea at 100 mM, unless otherwise specified. All strains were normally grown at 25°C and eventually shifted to 37°C to inactivate the function of temperature sensitive alleles.

Plasmid constructions and genetic manipulations. Standard genetic techniques were used to manipulate yeast strains²² and standard protocols were used for genetic manipulations.²³ *MAD3* was tagged immediately before the stop codon by one-step gene tagging.²⁴ The *MET3-CDC5* fusion was integrated at the *LEU2* locus of a strain carrying a null allele of *CDC5* (*cdc5::S.p.ura4*). The *mad3-5A* allele was generated using the QuickChange MultiSite-Directed Mutagenesis kit (Stratagene): a yeast Yiplac128 integrative plasmid²⁵ carrying the coding region, 480 bp of promoter and 200 bp of terminator of *MAD3* (pSP292) was used as template for PCR with the following 5' phosphorylated primers: 5'-CAATATTCAGCTAGGAAATGA-AATAGCGATGGACTCATTAG-3'; 5'-CGTATTTGTAGATGGAGAA-GAGGCTGATGTAGAATTCGAAAC-3'; 5'-CTAAAGGTGGTAGA-CTAGAATTTGCGCTAGAAGAAGTCTTGGC-3'; 5'-CCAAAGGGAC-GAAGCACTGAGACGGCTGATACTACTTCAGCTGTC-3'; 5'-GAG-ATCATTTTCAGATGATGACAAGGCGAGTTCGTCTTTCATATGC-3'.

Protein extracts and analysis. For most experiments total protein extracts were prepared by TCA precipitation as previously described²⁶ and run on 12.5% acrylamide gels. For the phosphatase treatment in Figure 1B protein extracts were made in the following breaking buffer: 50 mM Tris-Cl pH 7.5, 0.1% SDS, 1% Triton, 1% Na-deoxycholate, 120 mM β-glycerophosphate, 1 mM Na-orthovanadate, 1 mM DTT supplemented with protease inhibitors (Complete Boehringer). Mad3-HA3 was immunoprecipitated from 1 µg of cleared extracts upon incubation with anti-HA antibodies crosslinked to protein A-sepharose for 1 hour at 4°C. The slurry was washed three times with PBS, resuspended in 60 µl of phosphatase buffer containing

Table 1 **Strains used in this study**

Name	Relevant genotype
ySP1577	MATa, <i>mad3::TRP1</i>
ySP2220	MATa, <i>MAD3-HA3::URA3</i>
ySP2249	MATa, <i>mad1::LEU2, MAD3-HA3::URA3</i>
ySP2251	MATa, <i>bub3::LEU2, MAD3-HA3::URA3</i>
ySP2259	MATa, <i>ndc10-1, MAD3-HA3::URA3</i>
ySP2261	MATa, <i>bub2::HIS3, MAD3-HA3::URA3</i>
ySP2263	MATa, <i>mps1-1, MAD3-HA3::URA3</i>
ySP2268	MATa, <i>cdc26::K.I.URA3, MAD3-HA3::URA3</i>
ySP2272	MATa, <i>mad2::TRP1, MAD3-HA3::URA3</i>
ySP2277	MATa, <i>bub1::S.p.HIS5, MAD3-HA3::URA3</i>
ySP2301	MATa, <i>clb1Δ, clb3::TRP1, clb4::HIS3, clb2-VI, MAD3-HA3::URA3</i>
ySP2368	MATa, <i>cdc15-2, MAD3-HA3::URA3</i>
ySP2374	MATa, <i>ipl1-321, MAD3-HA3::URA3</i>
ySP2380	MATa, <i>cdc5::S.p.URA4, leu2::LEU2::MET3-CDC5, MAD3-HA3::URA3</i>
ySP2384	MATa, <i>ura3::URA3::GAL1-MPS1, MAD3-HA3::URA3</i>
ySP2479	MATa, <i>ndc10-2, MAD3-HA3::URA3</i>
ySP2481	MATa, <i>cep3-10, MAD3-HA3::URA3</i>
ySP2510	MATa, <i>cdc6::hisG, ura3::URA3::GAL1-ubiR-CDC6, MAD3-HA3::URA3, PDS1-myc18::LEU2</i>
ySP2512	MATa, <i>scc1-73, MAD3-HA3::URA3</i>
ySP2656	MATa, <i>MAD3-HA3::URA3, PDS1-myc18::LEU2</i>
ySP2733	MATa, <i>cdc26::K.I.URA3, bub1::S.p.HIS5, MAD3-HA3::URA3</i>
ySP2735	MATa, <i>cdc26::K.I.URA3, mad1::LEU2, MAD3-HA3::URA3</i>
ySP2759	MATa, <i>mad2::TRP1, cdc6::hisG, ura3::URA3::GAL1-ubiR-CDC6, MAD3-HA3::URA3, PDS1-myc18::LEU2</i>
ySP2762	MATa, <i>cdc26::K.I.URA3, cdc5::S.p.URA4, leu2::LEU2::MET3-CDC5, MAD3-HA3::URA3</i>
ySP2870	MATa, <i>MAD3-HA3::URA3, bar1::kanMX, CEN-TRP1-GAL1-CDC5</i>
ySP3755	MATa, <i>clb5::HIS3, clb6::LEU2, MAD3-HA3::URA3</i>
ySP3852	MATa, <i>GAL1-CDC14::URA3 (3X), MAD3-HA3::URA3</i>
ySP4212	MATa, <i>ipl1-321, cdc5::S.p.URA4, leu2::LEU2::MET3-CDC5, MAD3-HA3::URA3</i>
ySP4213	MATa, <i>ipl1-321, cdc6::hisG, ura3::URA3::GAL1-ubiR-CDC6, MAD3-HA3::URA3</i>
ySP4214	MATa, <i>cdc15-2, cdc6::hisG, ura3::URA3::GAL1-ubiR-CDC6, MAD3-HA3::URA3</i>
ySP4215	MATa, <i>ipl1-321, cdc15-2, cdc6::hisG, ura3::URA3::GAL1-ubiR-CDC6, MAD3-HA3::URA3</i>
ySP4962	MATa, <i>mad3::KITRP1, leu2::LEU2::mad3-5A</i>
ySP4993	MATa, <i>mad3::KITRP1, leu2::LEU2::mad3-5A-HA3::URA3</i>

20 units of lambda phosphatase (Biolabs) and 2mM MnCl₂ and incubated 30 minutes at 30°C before loading. For the experiment shown in Figure 7B, the same breaking buffer as above was used, with the exception that it contained 2mM Na-orthovanadate, 15 mM para-nitro-phenyl-phosphate and 1 mM AEBSEF. Protein extract concentrations were measured with Bio-rad Protein Assay. For Western blot analysis proteins were transferred to Protran membranes (Schleicher and Schuell). Mad3-HA3 was detected with Mab 12CA5. Anti-Clb2 polyclonal antibodies were a kind gift from W. Zachariae.

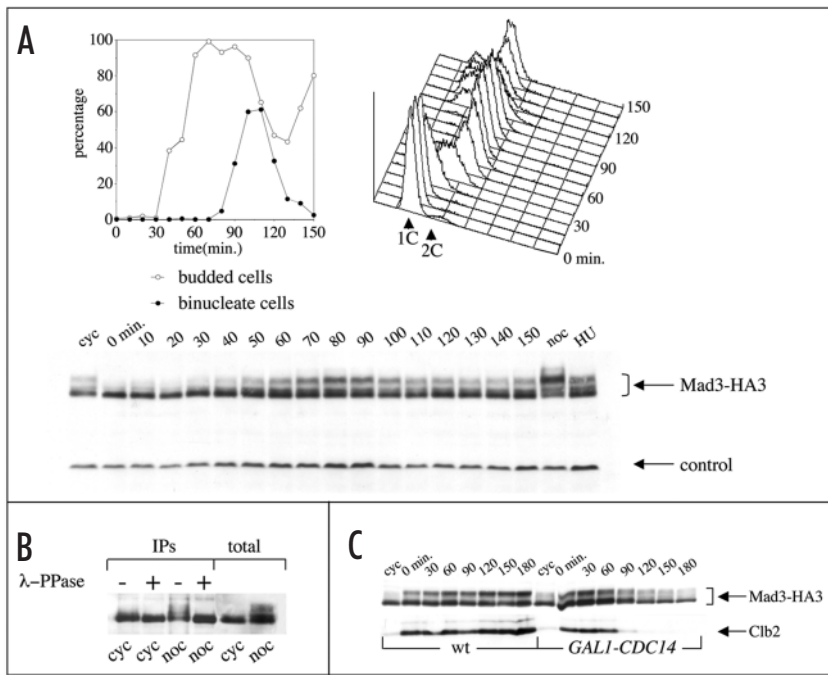


Figure 1. Budding yeast Mad3 is phosphorylated during mitosis and upon spindle checkpoint activation. (A) A cycling culture of *MAD3-HA3* cells (ySP2220) was arrested in G_1 by α factor and released into the cell cycle at 25°C. At the indicated time points cell samples were collected to measure kinetics of budding and nuclear division (top left), DNA contents by FACS analysis (top right) and Mad3-HA3 levels and electrophoretic mobility by Western blot analysis of protein extracts with anti-HA antibodies (bottom). Part of the culture was also treated with nocodazole (noc) or hydroxyurea (HU) for 3 hours and 3.5 hours, respectively, at 25°C. (B) Mad3-HA3 was immunoprecipitated with anti-HA antibodies (IPs) from protein extracts of logarithmically growing (cyc) or nocodazole-treated (noc) cells (ySP2220). Immunoprecipitates were incubated with λ phosphatase (+) or buffer alone (-). Total extracts before immunoprecipitation were also loaded as control. (C) Wild type (ySP2220, wt) and *GAL1-CDC14* (ySP3852) cells expressing Mad3-HA3 were grown in YEPR medium and arrested in metaphase with nocodazole followed by galactose addition (time = 0). Samples were withdrawn at the indicated time points for FACS analysis of DNA contents (not shown) and western blot analysis of protein extracts with anti-HA and anti-Clb2 antibodies. Cyc: cycling culture.

Secondary antibodies were purchased from Amersham and proteins were detected by an enhanced chemiluminescence system according to the manufacturer.

Other techniques. Flow cytometric DNA quantitation was determined according to Epstein and Cross (1992)²⁷ on a Becton-Dickinson FACScan. In situ immunofluorescence was performed as described in Fraschini et al, 1999.²⁸

RESULTS

Budding yeast Mad3 is periodically phosphorylated during the cell cycle. We analysed budding yeast Mad3 levels and possible post-translational modifications using haploid strains where the sole Mad3 form was expressed from the *MAD3* promoter as a Mad3-HA3 fusion, where three HA epitopes at the C-terminus made it recognizable by commercial antibodies. Mad3-HA3 is perfectly functional in terms of spindle checkpoint response, as it does not prevent cell cycle arrest in the presence of nocodazole and, unlike *MAD3* deletion,²⁹ does not cause lethality to *cin8* Δ cells (data not shown). Western blot analysis of protein extracts from synchronized cells revealed that Mad3-HA3 protein levels were fairly constant during the cell cycle (Fig. 1A). However, slowly migrating forms of Mad3-HA3 appeared at the onset of S phase (time = 40 minutes), increased in intensity during G_2 and M, and decreased at the end of mitosis (time = 100 minutes), right before cytokinesis. In addition, the Mad3 modified forms were present in cells arrested in S phase by hydroxyurea (HU), and accumulated as the predominant forms upon nocodazole treatment (Fig. 1A).

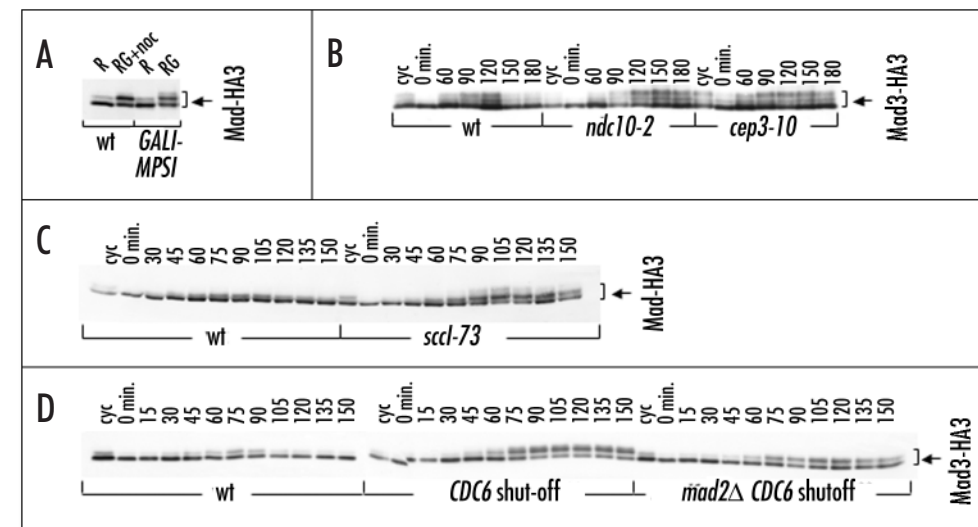


Figure 2. Mad3 phosphorylation correlates with spindle checkpoint activation. (A) *MAD3-HA3* (ySP2220, wt) and *MAD3-HA3 GAL1-MPS1* cells (ySP2384) growing in raffinose-containing medium (R) were induced by galactose (RG) for 3 hours at 25°C. Nocodazole was added at the time of galactose addition to the wild type culture only. (B) *MAD3-HA3* (ySP2220, wt), *MAD3-HA3 cep3-10* (ySP2481), *MAD3-HA3 ndc10-2* (ySP2479) cell cultures were synchronized in G_1 with α factor at 25°C and released at time zero (0) into fresh medium at 37°C. 10 μ g/ml of α factor was added back at 90' after the release. (C) *MAD3-HA3* (ySP2220, wt) and *MAD3-HA3 scc1-73* (ySP2512) cell cultures were synchronized in G_1 with α factor at 25°C and released (time = 0) into fresh medium at 37°C. 10 μ g/ml of α factor was added back at 90' after the release. (D) *MAD3-HA3* (ySP2656, wt), *MAD3-HA3 cdc6 Δ GAL1-ubiR-CDC6* (ySP2510) and *MAD3-HA3 cdc6 Δ GAL1-ubiR-CDC6 mad2 Δ* (ySP2759) cell cultures grown in YEPG were presynchronized in S-phase with HU. After 3,5 hrs, cells were released into YEPD medium (GAL1-ubiR-CDC6 off) containing α factor. G_1 arrested cells were then released into fresh YEPD medium (time = 0). 10 μ g/ml of α factor was added back at 75' after the release. (B-D) Samples were taken at the indicated time points for FACS analysis of DNA contents (not shown) and for western blot analysis of protein extracts with anti-HA antibodies. Cyc: exponentially growing cells.

Since human BubR1 has been shown to be a phosphoprotein,^{17,30} we verified whether the Mad3-HA3 mobility shift might be due to phosphorylation events. To this purpose, Mad3-HA3 was immunoprecipitated from logarithmically growing and nocodazole-treated cells, followed by incubation with either lambda-d-phosphatase or buffer alone. Indeed, the slowly migrating forms of Mad3-HA3 disappeared upon phosphatase treatment (Fig. 1B), indicating that they arise from phosphorylation events. Thus, budding yeast Mad3, like its vertebrate counterpart BubR1, is a phosphoprotein, whose phosphorylation is maximal during mitosis and spindle checkpoint activation. Since the amount of phosphorylated Mad3 decreases at the end of mitosis, when the Cdc14 phosphatase reverses different phosphorylation events,³¹ we asked whether overexpression of *CDC14* from the galactose-inducible *GAL1* promoter could induce Mad3 dephosphorylation in nocodazole treated cells. Wild type and *GAL1-CDC14* cells were therefore arrested in metaphase with nocodazole in raffinose containing medium and then *GAL1-CDC14* expression was induced by galactose addition. As previously shown,³¹ high levels of Cdc14 in these conditions caused ectopic degradation of the mitotic cyclin Clb2 (Fig. 1C) due to APC activation. In addition, *CDC14* overexpression caused a gradual disappearance of slowly migrating Mad3 forms, that were instead maintained at high levels in wild type cells (Fig. 1C), suggesting that activation of Cdc14 can counteract the kinases responsible for Mad3 phosphorylation. Thus, Cdc14 might directly or indirectly promote Mad3 dephosphorylation, resetting it in a G₁ state.

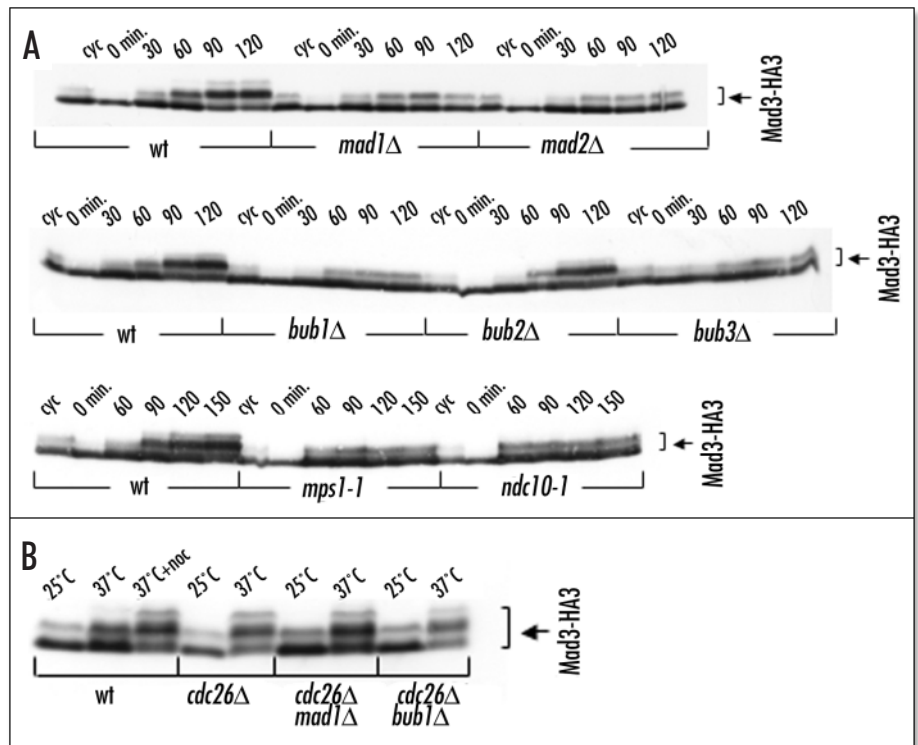
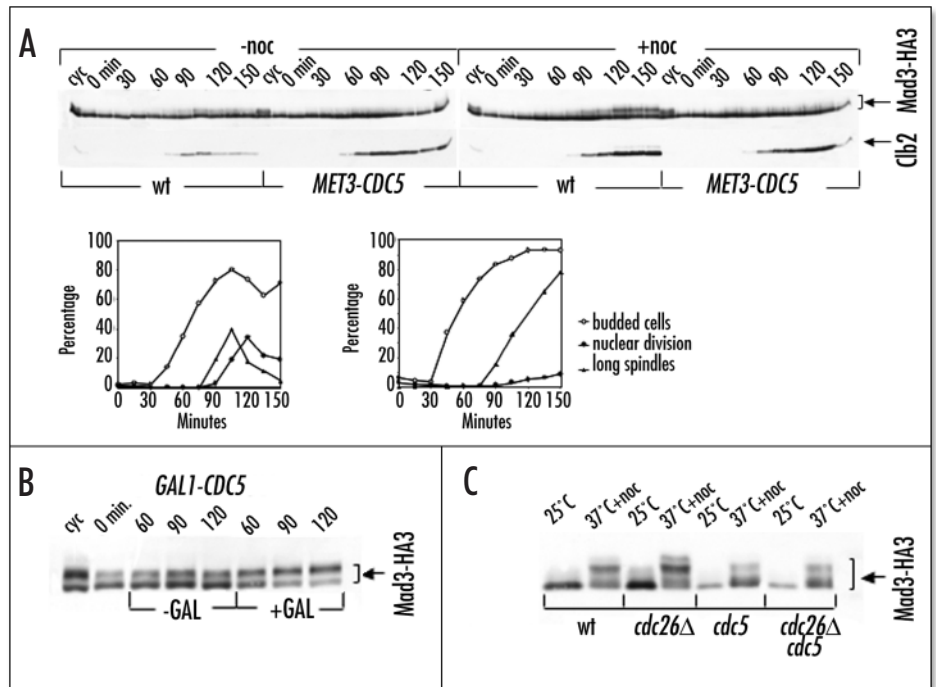


Figure 3. Accumulation of phosphorylated Mad3 in nocodazole depends on spindle checkpoint components. (A) Wild type (ySP2220, wt), *mad1Δ* (ySP2249), *mad2Δ* [ySP2272], *bub1Δ* (ySP2277), *bub2Δ* (ySP2261) and *bub3Δ* (ySP2251) cells, all expressing Mad3-HA3, were synchronized in G₁ with α factor at 25°C and released into fresh medium containing nocodazole at 25°C. Similarly treated *mps1-1* (ySP2263), *ndc10-1* (ySP2259) and wild type cells (ySP2220, wt) expressing Mad3-HA3, were released at 37°C. (B) Cycling cultures of wild type (ySP2220, wt), *cdc26Δ* (ySP2268), *cdc26Δ mad1Δ* (ySP2753) and *cdc26Δ bub1Δ* (ySP2733) strains, all carrying the *MAD3-HA3* gene and grown at 25°C, were shifted to 37°C for 3 hrs. Wild type cells were shifted to 37°C either in the absence or in the presence of nocodazole. (A and B) At indicated time points after the release (time = 0) cell samples were collected for FACS analysis of DNA contents (not shown) and for western blot analysis of protein extracts with anti-HA antibodies. Cyc: exponentially growing cells.

Figure 4. Mad3 phosphorylation requires the Polo kinase Cdc5. (A) *MAD3-HA3* (ySP2220, wt) and *MAD3-HA3 cdc5Δ MET3-CDC5* (ySP2380) cell cultures were grown in -Met medium (*MET3* promoter on), arrested in G₁ with α factor (time = 0) and released in the presence of methionine (*MET3* promoter off), either in the absence (-noc) or in the presence (+noc) of nocodazole. At the indicated time points cells were withdrawn for FACS analysis of DNA contents (not shown), western analysis of Mad3-HA3 and Clb2 (top), kinetics of budding, nuclear division and spindle elongation (bottom). (B) A *MAD3-HA3 CDC5 GAL1-CDC5* (ySP2870) cell culture logarithmically growing in YEPR (cyc) was arrested in S-phase with HU for 3.5 hrs. At time zero the culture was splitted in two and 1% of galactose (+ Gal) was added to half of the culture. (C) Wild type (ySP2220, wt), *cdc26Δ* (ySP2268), *cdc5Δ MET3-CDC5* (ySP2380, *cdc5*) and *cdc26Δ cdc5Δ MET3-CDC5* (ySP2762, *cdc5 cdc26Δ*) strains, all expressing Mad3-HA3, were grown at 25°C in -Met medium, arrested in G₁ with α factor and then released into YEPD containing nocodazole and 2 mM methionine at 37°C for 3 hrs.



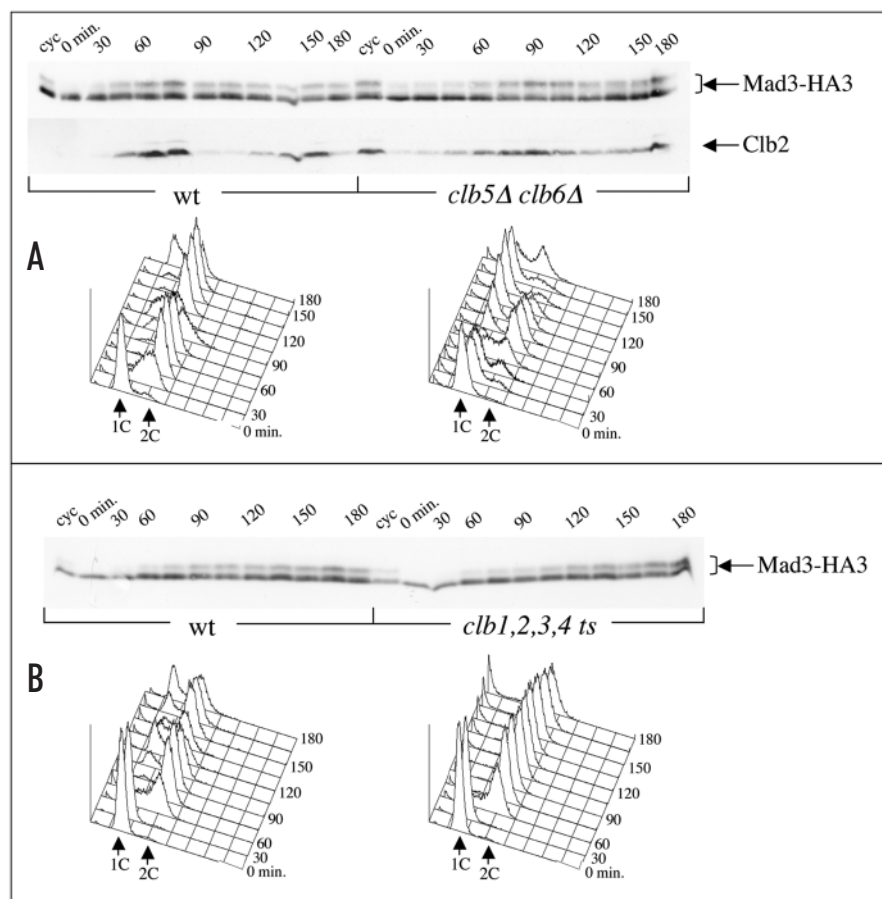


Figure 5. Cyclin B-dependent CDKs are not directly required for Mad3 phosphorylation. (A) *MAD3-HA3* (ySP2220, wt) and *MAD3-HA3 clb5Δ clb6Δ* (ySP3755) cell cultures were synchronized in G_1 with α factor and released into fresh medium at 25°C (time = 0). (B) *MAD3-HA3* (ySP2220, wt) and *MAD3-HA3 clb1Δ clb2-V1 clb3Δ clb4Δ* (ySP2301, *clb1,2,3,4 ts*) cell cultures were synchronized in G_1 with α factor at 25°C and released into fresh medium at 37°C (time = 0).

Mad3 phosphorylation correlates with spindle checkpoint activation. Since Mad3 is phosphorylated at higher levels upon nocodazole treatment than during an unperturbed mitosis, we asked whether Mad3 hyper-phosphorylation could be induced also by other conditions engaging the spindle checkpoint. Since *MPS1* overexpression is known to activate the checkpoint in the absence of spindle perturbations,³² we first verified if Mad3 can be hyperphosphorylated under these conditions. As shown in Figure 2A, *Mps1* overproduction led to Mad3-HA3 phosphorylation levels that were similar to those observed after nocodazole treatment. High Mad3 phosphorylation levels were also maintained at the restrictive temperature in mutants defective in kinetochore function, like *ndc10-2* and *cep3-10*, known to cause a G_2/M delay due to checkpoint activation.³³⁻³⁵ In fact, when wild type, *ndc10-2* and *cep3-10* cell cultures expressing Mad3-HA3 were synchronized in G_1 by α factor and then released into the cell cycle at 37°C, Mad3 phosphorylation raised and then was maintained at high levels until the end of the experiment in both *ndc10-2* and *cep3-10* mutant cells (Fig. 2B), reflecting their mitotic delay (not shown), whereas it increased during mitosis and decreased in telophase in wild type cells, as expected. This suggests that faulty kinetochore-microtubule attachments prevent Mad3 de-phosphorylation.

We then asked whether the lack of kinetochore tension, as opposed to the lack of kinetochore occupancy caused by nocodazole or kinetochore defects, could also lead to accumulation of high levels of phosphorylated Mad3. We therefore prevented kinetochore tension by either a temperature-sensitive mutation affecting the cohesin subunit *Scc1* (*scc1-73*) or depletion of the replication protein *Cdc6*. The lack of functional cohesin allows sister chromatids to precociously separate soon after DNA replication,³⁶ whereas cells do not duplicate their chromosomes in the absence of *Cdc6* but

nevertheless undergo a haploid mitosis with random chromosome segregation to the poles.³⁷ Both conditions are known to activate the spindle checkpoint.^{16,38,39} As shown in Figure 2C, *scc1-73* cells at the restrictive temperature accumulated more phosphorylated Mad3-HA3 than wild type cells under the same conditions and showed delayed Mad3 dephosphorylation, consistently with the cytokinetic delay caused by activation of the spindle checkpoint.³⁹ A yet stronger effect on Mad3-HA3 phosphorylation was obtained by depletion of *Cdc6* (Fig. 2D), indicating that indeed phosphorylation of Mad3-HA3 is stimulated by the lack of both kinetochore occupancy and tension. Interestingly, deletion of *MAD2* could partially decrease the amount of hyper-phosphorylated Mad3-HA3 and accelerate its dephosphorylation in *Cdc6*-depleted cells (Fig. 2D), in agreement with its ability to erase activation of the spindle checkpoint in these cells.^{38,39}

Full Mad3 phosphorylation upon nocodazole treatment requires SAC-induced cell cycle arrest. The correlation between spindle checkpoint activation and amount of Mad3 phosphorylation prompted us to verify whether and which known checkpoint genes were required for Mad3 hyper-phosphorylation. To this purpose, different checkpoint mutants expressing Mad3-HA3 were synchronized in G_1 by α factor and then released in the presence of nocodazole, followed by Mad3-HA3 western blot analysis at different time points. As shown in Figure 3A, phosphorylated Mad3-HA3 failed to accumulate to wild type levels in *mad1Δ*, *mad2Δ*, *bub1Δ*, *bub3Δ* and in the temperature-sensitive *mps1-1* and *ndc10-1* cells, that are all affected in the same spindle checkpoint pathway monitoring correct kinetochore-microtubule attachment.^{40,41} Conversely, full nocodazole-induced Mad3 phosphorylation did not require *Bub2* that is involved in a different branch

of the checkpoint responding to spindle misorientation.^{28,41-47}

Two possible explanations could account for the partial dependency of Mad3 hyper-phosphorylation upon spindle checkpoint proteins in the presence of nocodazole: (1) the kinase(s) involved is specifically upregulated when the spindle checkpoint is engaged; (2) the kinase(s) involved is cell cycle-regulated and accumulates during the cell cycle arrest caused by microtubule depolymerization. In the latter case, the low levels of phosphorylated Mad3 in spindle checkpoint mutants might be the consequence of their inability to sustain cell cycle arrest upon nocodazole treatment, while the checkpoint defect of *bub2Δ* cells allows to escape the nocodazole-induced cell cycle block later than the other *mad* and *bub* mutants. Therefore, we asked whether conditions blocking the cell cycle in metaphase other than spindle damage (i.e., conditions that satisfy the spindle assembly checkpoint) could also lead to accumulation of phosphorylated Mad3 to levels similar to those observed in the nocodazole-arrested cells. To this purpose, we used mutations affecting the APC, like *cdc26Δ*, that arrest cells at 37°C with short metaphase spindles and undivided nuclei, due to their failure to dissolve cohesion between sister chromatids.⁴⁸ As shown in Figure 3B, the levels of phosphorylated Mad3 in *cdc26Δ* cells at restrictive temperature were similar to those detected in nocodazole-treated wild type cells, independently of the presence of the spindle checkpoint protein *Mad1* and *Bub1*. Similar results were obtained with another APC mutant, *cdc23-1*, and by depletion of *Cdc20* (data not shown), leading us to conclude that cell-cycle regulated kinases likely phosphorylate Mad3, and that spindle checkpoint proteins contribute to maintain high levels of phosphorylated Mad3 by virtue of their ability to block cell cycle progression in the presence of kinetochore or spindle dysfunctions.

Mad3 phosphorylation requires the Polo kinase Cdc5. To find which kinase(s) could be involved in Mad3 phosphorylation, we analysed the levels of phosphorylated Mad3-HA3 upon nocodazole treatment in mutants defective in different mitotic kinases. This preliminary analysis indicated no requirement of the Wee1-like Cdk1 inhibitory kinase Swe1⁴⁹ or the Cdc15 and Dbf2 kinases involved in the Mitotic Exit Network (MEN)⁵⁰ for full Mad3 phosphorylation, that instead required the Polo kinase Cdc5 (data not shown). We therefore constructed a *MAD3-HA3* strain carrying a deletion of the endogenous *CDC5* gene and kept alive by a *MET3-CDC5* fusion, whose expression can be completely inhibited upon methionine addition. Wild type and *cdc5Δ* *MET3-CDC5* cells were synchronized in G₁ by α factor and then released in the presence of methionine and either in the absence or in the presence of nocodazole. Western blot analysis of protein extracts prepared from the above cultures at different times after release from α factor showed that Mad3 mostly failed to become phosphorylated in repressed *cdc5Δ* *MET3-CDC5* cells under both conditions (Fig. 4A), suggesting that indeed Cdc5 is involved in Mad3 phosphorylation. It should be noted, however, that some residual Mad3 phosphorylation could be observed, suggesting that either other kinases are involved or Cdc5 depletion was not complete.

Since it was shown that *CDC5* overexpression can enhance phosphorylation of Cdc5 targets,^{51,52} *MAD3-HA3* cells carrying an additional *CDC5* copy under the galactose-inducible *GAL1* promoter (*GAL1-CDC5*) were arrested in S phase with HU, where Mad3 is phosphorylated at low levels, followed by galactose addition to only half of the culture. As shown in Figure 4B, *CDC5* overexpression enhanced Mad3 phosphorylation, although it did not allow cells to escape the cell cycle block (data not shown), supporting the idea that Cdc5 might directly phosphorylate Mad3. Based on these data, accumulation of phosphorylated Mad3 in *apc* mutants at the restrictive temperature (Fig. 3B) could be attributed to their failure to destroy Cdc5, whose degradation in G₁ requires the APC.^{53,54} Indeed, switching off *MET3-CDC5* expression at 37°C in nocodazole-treated *cdc5Δ cdc26Δ* cells effectively decreased the amount of phosphorylated Mad3 compared to *cdc26Δ* cells under the same conditions (Fig. 4C). Thus, Cdc5 is likely required for the high levels of phosphorylated Mad3 found in *apc* mutants at restrictive temperature, and might phosphorylate Mad3 from S phase to the end of mitosis.

Cyclin B-dependent CDKs are not directly involved in Mad3 phosphorylation. Phosphorylation by Polo kinases is thought to be enhanced by cyclin B-dependent CDKs in different ways: (1) by activating Polo gene transcription,⁵⁵ (2) by promoting a positive feedback loop that results in Polo kinase activation,⁵⁶ (3) by directly phosphorylating Polo⁵⁷ and (4) by creating docking sites for Polo binding on its targets, thus stimulating their phosphorylation.⁵⁸ Therefore, we asked whether cyclin B-dependent kinases could be involved in Mad3 phosphorylation. As shown in Figure 5A, synchronized *clb5Δ clb6Δ* cells, lacking the S-phase B-type cyclins, entered S phase with 30–45 minutes delay compared to wild type, as previously reported.⁵⁹ Mad3 phosphorylation was delayed accordingly, but reached levels similar to wild type cells, indicating that the Clb5/6-Cdk1 kinases might play a role in the timing but not in the extent of Mad3 phosphorylation. In a similar experiment (Fig. 5B), we addressed the role in this process of the mitotic Clb1/2/3/4-Cdk1 kinases, that are necessary for the onset of mitosis.⁶⁰ Mad3 phosphorylation was only slightly delayed at non-permissive temperature in cells lacking *CLB1*, *CLB3*, *CLB4* and carrying the temperature sensitive

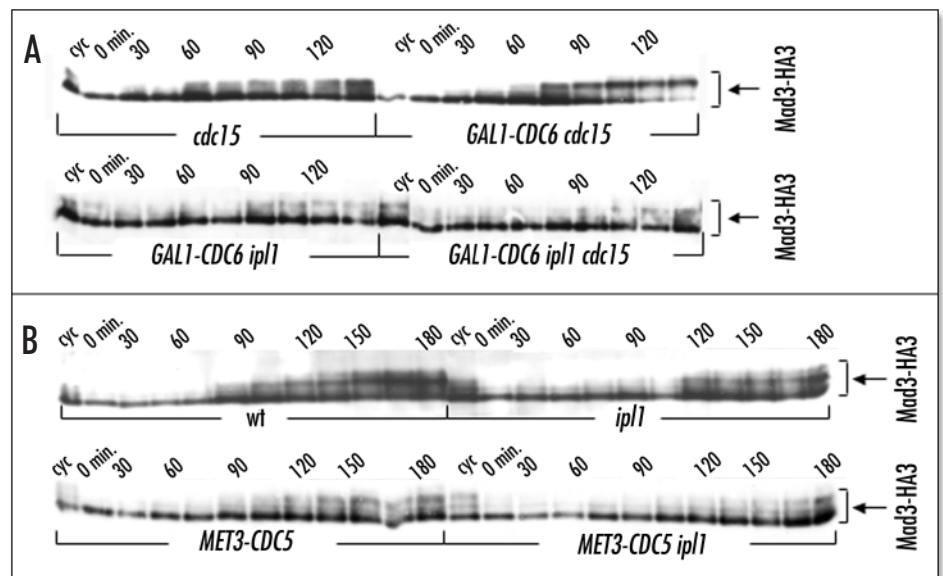


Figure 6. The Aurora kinase Ipl1 contributes to Mad3 phosphorylation. (A) *cdc15-2* (ySP2368), *cdc6Δ* *GAL1-ubiR-CDC6 cdc15-2* (ySP4214), *cdc6Δ* *GAL1-ubiR-CDC6 ipl1-321* (ySP4213) and *cdc6Δ* *GAL1-ubiR-CDC6 ipl1-321 cdc15-2* (ySP4215) strains, all carrying *MAD3-HA3*, were grown in YEPRG medium (*GAL1* promoter on) and presynchronized in S-phase with HU. After 3,5 hrs cells were released into YEPD medium (*GAL1* promoter off) containing a α factor and 2 hours later cells were released from the G₁ block into fresh YEPD medium at 37°C (time = 0). (B) Wild type (ySP2220, wt), *ipl1-321* (ySP2374), *cdc5Δ* *MET3-CDC5* (ySP2380) and *ipl1-321 cdc5Δ* *MET3-CDC5* (ySP4212) strains, all expressing *Mad3-HA3*, were grown in -Met medium, arrested in G₁ with α factor and released in the presence of methionine and nocodazole at 37°C (time = 0). Cyc: cycling cells.

clb2-VI allele, which caused them to arrest in G₂.⁶¹ Thus, cyclin B dependent CDKs do not seem to be directly implicated in Mad3 phosphorylation, while the delay observed in cells lacking them might be consistent with their proposed role in facilitating Polo activation or interaction with its targets.

Ipl1 participates to Mad3 phosphorylation upon lack of kinetochore tension and attachment. The Aurora B kinase has been found to be required for phosphorylation of BubR1, the human homologue of Mad3,¹⁷ and the budding yeast Aurora B-like kinase Ipl1 has been implicated in the checkpoint responding to the lack of kinetochore tension.¹⁶ We therefore asked whether Mad3 phosphorylation required Ipl1 under these conditions, which were obtained by Cdc6 depletion that prevents DNA replication³⁷ and thus induces monopolar kinetochore attachment (see Fig. 2D and related text). Since spindle checkpoint activation upon Cdc6 depletion can be bypassed by the temperature-sensitive *ipl1-321* mutation,¹⁶ we analysed Mad3 phosphorylation during a synchronous release from a G₁ arrest of Cdc6-depleted *ipl1-321* cells. In order to prevent mitotic exit, which would lead to Mad3 dephosphorylation and possible misinterpretation of the results, we blocked the cell cycle progression in telophase using the temperature-sensitive *cdc15-2* allele. Similarly treated *cdc15-2*, Cdc6-depleted and Cdc6-depleted *ipl1-321* cells were used as control. As shown in Figure 6A, Mad3 hyper-phosphorylation caused by the absence of kinetochore tension was largely dependent on Ipl1, because it mostly failed to take place in Cdc6 depleted cells carrying the temperature-sensitive *ipl1-321* allele.

We then asked whether Ipl1 might contribute, together with Cdc5, to Mad3 phosphorylation also upon nocodazole treatment. To this purpose, wild type, *ipl1-321*, *cdc5Δ* *MET3-CDC5* and *cdc5Δ* *MET3-CDC5 ipl1-321* strains were synchronized in G₁ and then released into the cell cycle in the presence of nocodazole and methionine, to shut-off *MET3-CDC5* transcription, at 37°C, to inactivate the Ipl1-321 protein. In these conditions Mad3 was promptly phosphorylated in wild type cells, whereas its phosphorylation was severely compromised in the absence of Cdc5 (Fig. 6B). It should be noted, however, that switching off *CDC5* transcription at 37°C allowed higher levels of Mad3 phosphorylation than at 25°C (compare Fig. 6B, with Fig. 4A), due to the less efficient Cdc5 depletion at this temperature (our

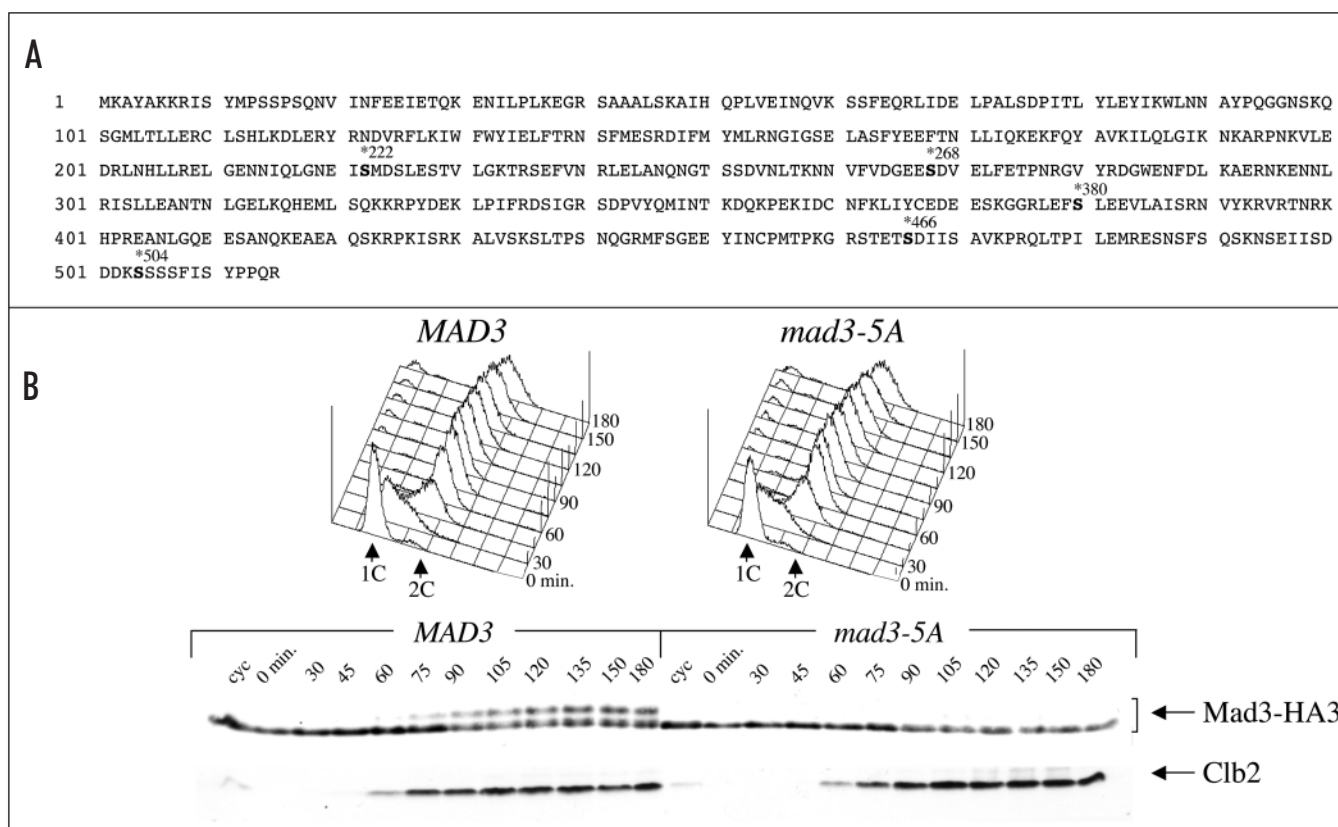


Figure 7. Serine to alanine substitutions in five putative phosphorylation sites for Cdc5 markedly affect Mad3 phosphorylation. (A) Mad3 primary sequence. Asterisks mark the five serines that have been changed to alanine. (B) Log phase cultures of wild type (ySP2220, *MAD3*) and *mad3-5A* cells (ySP4993), expressing Mad3-HA3 and Mad3-5A-HA3, respectively, were arrested in G₁ by α factor and released into fresh medium containing nocodazole. Samples were withdrawn at the indicated times for FACS analysis of DNA contents (top) and western blot analysis of protein extracts with anti-HA and anti-Clb2 antibodies (bottom).

unpublished observation). Ipl1 inactivation also delayed and decreased Mad3 phosphorylation and had an additive effect with the lack of Cdc5, since *cdc5Δ MET3-CDC5 ipl1-321* cells displayed lower levels of phosphorylated Mad3 than each single mutant (Fig. 6B). Thus, both Cdc5 and Ipl1 independently promote Mad3 hyper-phosphorylation, although Cdc5 seems to have a predominant role in this process when kinetochores are not bound to microtubules, as in nocodazole-treated cells.

Alanine substitutions of five Mad3 serine residues in putative Cdc5 phosphorylation sites dramatically reduce Mad3 phosphorylation. In order to gain insights into the role of Mad3 phosphorylation, we substituted with alanines five serine residues of Mad3 (Fig. 7A) that might be phosphorylated by Cdc5, based on consensus phosphorylation sites recently described for targets of human²¹ and *Xenopus*²⁰ Polo-like kinase 1 (Plk1). Mad3 carries one perfect match (surrounding S380, Fig. 7A) and two unperfect matches (surrounding S222 and S268, Fig. 7A) to the consensus E/D X S/T Φ X E/D for optimal Plk1-induced phosphorylation,²¹ where Φ is any aliphatic amino acid (V, L, I, M, P). In addition, Mad3 contains six amino acid sequences matching a looser Plk1 phosphorylation site E/D X S/T.²⁰ Out of these six S/T residues, S466 and S504 (Fig. 7A) were predicted to have high probability to be phosphorylated according to the NetPhos 2.0 server. We therefore changed the S222, S268, S380, S466 and S504 codons into alanine codons by site-directed mutagenesis. The deriving *mad3-5A* allele was then fused in frame to a three HA epitope coding sequence before the stop codon, in order to follow phosphorylation of the corresponding gene product by Western blot analysis. *MAD3-HA3* and *mad3-5A-HA3* cells were synchronized in G₁ by α factor and then released in the presence of nocodazole to activate the spindle checkpoint. Whereas Mad3-HA3 got promptly hyper-phosphorylated under these conditions, Mad3-5A-HA3 showed

dramatically reduced phosphorylation levels (Fig. 7B), suggesting that the mutated residues are likely phosphorylated in vivo. In addition, Mad3-5A-HA3 was poorly phosphorylated also during an unperturbed cell cycle (data not shown). We also produced two additional *mad3-2A-HA3* and *mad3-4A-HA3* mutant alleles, where the S222 and S380 or the S222, S380, S466 and S504 codons, respectively, were mutated to alanine codons. Whereas Mad3-2A-HA3 was phosphorylated to a similar extent to wild type Mad3 in the presence of nocodazole, Mad3-4A-HA3 showed reduced phosphorylation, though not as low as Mad3-5A-HA3 under the same conditions (data not shown), suggesting that S268 is a major phosphorylation site. The presence of the *mad3-5A* allele as sole Mad3 source caused a slightly increased sensitivity to the microtubule depolymerizing drug benomyl (data not shown), which did not seem to reflect defects in the spindle assembly checkpoint. In fact, *mad3-5A* cells remained arrested in nocodazole with replicated but unseparated chromatids, very similarly to wild type (data not shown). It is therefore possible that other redundant mechanisms contribute, together with phosphorylation, to Mad3 regulation.

DISCUSSION

Anaphase is the most dramatic mitotic event and, being an irreversible step, requires extreme care in order to guarantee that each daughter cell inherits faithfully a full set of chromosomes. The SAC, which surveys this process, is indeed active during each cell cycle while kinetochores capture microtubules, and shut off when all kinetochores are amphitelicly attached to spindle fibers.⁶² So far, how the SAC is switched on and off at the molecular level is not

known. Formation of the multiprotein complex containing Bub3, Mad2, Mad3/BubR1 and Cdc20, which is essential for checkpoint response, does not appear to be cell cycle regulated, since it takes place independently of kinetochores as soon as the Cdc20 protein appears in mitosis.⁹⁻¹¹ In addition, the above complex prepared from interphase cells has the same *in vitro* inhibitory activity towards the APC as that prepared from mitotic cells,¹⁰ suggesting that other cell cycle regulatory mechanisms might modulate either the recruitment to kinetochores or the activity of the complex *in vivo*. In fact, despite complex formation is independent of kinetochores, there is a tight correlation between SAC activation and localization of its components to kinetochores,^{1,2} arguing that the complex might require kinetochore recruitment in order to be active *in vivo*. Post-translational modification of checkpoint proteins could therefore play an important role in the transmission and/or in the switch-off of the “wait anaphase” signal. Several SAC components have been reported to be phosphorylated, like fission yeast Bub1 whose phosphorylation by the CDK Cdc2 is required for checkpoint activation.⁶³ In addition, Cdc20 phosphorylation by both Bub1⁶⁴ and mitogen-activated protein kinase⁶⁵ has been implicated in its checkpoint-mediated inhibition. Also Mad1 is phosphorylated upon checkpoint activation by the SAC kinase Mps1,⁶⁶ although the functional significance of this is currently unknown. Conversely, other phosphorylation events correlate with SAC inactivation, like phosphorylation of Mad2 that prevents it from interacting with Mad1 and the APC.⁶⁷ We have found that Mad3, like its human and *Xenopus* BubR1 counterparts,^{17,30} is hyperphosphorylated during mitosis and upon checkpoint activation. Phosphorylation of Mad3 is maintained at high levels in all conditions engaging the SAC, such as microtubule depolymerization, kinetochore defects and lack of kinetochore tension. In addition, SAC components are required to maintain high levels of phosphorylated Mad3, suggesting that phosphorylation might be implicated in activating, rather than inhibiting, Mad3 checkpoint function. Since we have not found any preference for binding to Cdc20 between the hyper- and the hypo-phosphorylated form of Mad3 (data not shown), we think that Mad3 phosphorylation might influence the activity, rather than the formation, of the Bub3/Mad2/Mad3/Cdc20 complex. Interestingly, Mad3 phosphorylation requires the Polo kinase Cdc5 and, to a minor extent, the Aurora B kinase Ipl1. Aurora B kinases have been previously implicated in checkpoint activation in response to lack of kinetochore tension.^{15-17,68,69} In contrast, whether Polo kinases are involved in the spindle checkpoint is presently unclear. Depletion of a human kinase of the Polo-like kinase family, Plk2/Snk, seems to prevent mitotic catastrophe in the presence of spindle poisons.⁷⁰ In addition, a hyperactive mutant version of human Plk1 has been recently shown to induce a Mad2- and BubR1-dependent mitotic delay, suggesting that inactivation of the spindle checkpoint is somewhat impaired.⁷¹ In spite of showing markedly reduced Mad3 phosphorylation, budding yeast *cdc5* mutants do not show any obvious checkpoint defect (our unpublished observations). It should be noted, however, that Cdc5 promotes a number of mitotic process, like sister chromatid separation⁵¹ and APC activation,⁵⁴ and this may mask its possible involvement in the checkpoint. The fact that Mad3 might be a common target of Cdc5 and Ipl1 suggests that these two kinases share overlapping functions in cell cycle progression. Accordingly, in several eukaryotic organisms, both Aurora B and Polo kinase contribute to bipolar spindle assembly and cohesin phosphorylation. Two lines of evidence suggest that Mad3 might be directly phosphorylated by Cdc5. First, Cdc5 overproduction drives

Mad3 hyperphosphorylation in HU-arrested cells. Second, replacement with alanine of five serines that are part of Polo kinase-dependent putative phosphorylation sites severely impairs Mad3 phosphorylation. The *mad3-5A* mutant allele, however, does not cause any detectable checkpoint defect in the presence of nocodazole and, unlike deletion of *MAD3*, is not lethal for *cin8* mutants (data not shown). Thus, Mad3 phosphorylation might be redundant with other regulatory mechanisms, as it is often the case for processes involving phosphorylation events, or regulate processes unrelated to the checkpoint. Alternatively, some residual Mad3-5A phosphorylation might still support its checkpoint functions. Interestingly, we have found that *mad3-5A* cells are slightly benomyl-sensitive, suggesting that the presence of the *mad3-5A* allele might cause subtle defects in other spindle/microtubule related functions. In this context, the human Mad3 homologue BubR1 has been implicated in chromosome alignment in the absence of spindle perturbations.^{17,72} In addition, BubR1 interacts with the kinesin CENP-E.^{73,74} The identification and mutation of all relevant Mad3 *in vivo* phosphorylation sites will be a challenge for the future and might contribute to shed light on Mad3 regulation and function.

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