Regulation of cell cycle-specific gene expression in fission yeast by the Cdc14p-like phosphatase Clp1p

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Summary

Regulated gene expression makes an important contribution to cell cycle control mechanisms. In fission yeast, a group of genes is coordinately expressed during a late stage of the cell cycle (M phase and cytokinesis) that is controlled by common *cis*-acting promoter motifs named pombe cell cycle boxes (PCBs), which are bound by a *trans*-acting transcription factor complex, PCB binding factor (PBF). PBF contains at least three transcription factors, a MADS box protein Mbx1p and two forkhead transcription factors, Sep1p and Fkh2p. Here we show that the fission yeast Cdc14p-like phosphatase Clp1p (Flp1p) controls M–G1 specific gene expression through PBF. Clp1p binds in vivo both to Mbx1p, a MADS box-like transcription factor, and to the promoters of genes transcribed at this cell cycle time. Because Clp1p dephosphorylates Mbx1p in vitro, and is required for Mbx1p cell cycle-specific dephosphorylation in vivo, our observations suggest that Clp1p controls cell cycle-specific gene expression through binding to and dephosphorylating Mbx1p.

Key words: Cdc14p, Clp1p, Fission yeast, Gene expression

Introduction

Gene expression at particular times during the mitotic cell division cycle is widespread amongst eukaryotes and makes an important contribution to this fundamental process (Spellman et al., 1998; Rustici et al., 2004). A major area of interest concerns understanding how this regulated gene expression is integrated into general cell cycle controls.

In fission yeast, at least four waves of gene expression have been described, and in each case the transcription factor complex that regulates their specific expression has been identified (McInerny, 2004; Bähler, 2005). One wave occurs at the M–G1 interval, with a number of genes transcribed at this cell cycle time that encode proteins required for cytokinesis and cell division (Anderson et al., 2002). The transcription factor complex that regulates this wave of gene expression has been identified and named PBF (pombe cell cycle box binding factor). PBF consists of at least three components: Mbx1p, a MADS box-like transcription factor, and two forkhead transcription factors, Sep1p and Fkh2p (Buck et al., 2004; Bulmer et al., 2004). It is thought that primary regulation of gene expression occurs through the forkhead transcription factors, with Sep1p activating and Fkh2p repressing gene expression. However, PBF is also regulated directly by the polo-like kinase Plo1p, which binds to and phosphorylates Mbx1p (Papadopoulou et al., 2008). Because both *fkh2*$^{+}$ and *plo1*$^{+}$ are themselves transcribed at M–G1 under PBF control, this suggests that feedback loops might operate to both turn on and turn off cell cycle gene expression at this cell cycle time. Related mechanisms have been identified in budding yeast and humans, showing that they are conserved and so play a significant part in cell cycle controls in all eukaryotic organisms (Darieva et al., 2006; Fu et al., 2008).

Cdc14p proteins form a class of phosphatases that have important and diverse roles in controlling cell cycle progression, through regulating a number of crucial target substrate proteins, to couple nuclear and cytoplasmic divisions (Simanis, 2003; Stegmeier and Amon, 2004; Vázquez-Novelle et al., 2005; Clemente-Blanco et al., 2006; Clifford et al., 2008a). In budding yeast, one function of Cdc14p is to control cell cycle-specific gene expression through regulating the nuclear localisation of transcription factors required for this process. These transcription factors include Swi5p, Ace2p and Swi6p, whose dephosphorylation permits their nuclear entry, and consequent activation of transcription of target genes at M–G1 and G1–S, respectively (Visintin et al., 1998; Geymonat et al., 2004).

In this paper we identify a new function for the fission yeast Cdc14p-like phosphatase, Clp1p (Flp1p). We show that Clp1p controls cell cycle-specific gene expression at the M–G1 interval through a novel mechanism, binding to and dephosphorylating Mbx1p on the promoters of genes transcribed at this cell cycle time. Thus it appears that this Cdc14p-like phosphatase has the ability to interact with gene promoters to directly regulate gene expression, a new property for this type of protein.

Results

Clp1p binds to and dephosphorylates Mbx1p

To identify proteins that bind to Clp1p, we purified tandem affinity purification (TAP) complexes from mitotically arrested cells expressing TAP-tagged Clp1p (Gould et al., 2004). Two-

dimensional liquid chromatography tandem mass spectrometry was used to characterise the protein composition of these complexes. One protein identified was Mbx1p, with 50 peptides recovered providing 29% sequence coverage. We confirmed that this interaction occurs both in vivo and in vitro by co-immunoprecipitation, pull-down and yeast two-hybrid experiments. Mbx1p–13myc from fission yeast extracts was detected in a pull-down assay with purified MBP–Clp1p (Fig. 1A). The interaction was direct because GST–Mbx1p purified from bacterial extracts pulled-down purified MBP–Clp1p (Fig. 1B). The interaction between Clp1p and Mbp1p was also shown using yeast two-hybrid (Fig. 2C, data not shown). Furthermore, the interaction was cell cycle-specific because Clp1p–3HA was only detected binding in vivo to Mbx1p–13myc during late M phase after release from an *nda3* M phase arrest (Fig. 1C).

Mbx1p undergoes cell cycle-dependent phosphorylation, which is directly regulated by the polo-like kinase, Plo1p (Papadopoulou et al., 2008). We mapped the amino acid residues in Mbx1p that are modified by phosphorylation using tandem mass spectrometry and identified four serine residues (Fig. 2A; supplementary material Fig. S1); a further serine was identified by database analysis. Of these, the amino acids around two (46 and 106) resembled the consensus sites for polo-kinases, whereas another two (183 and 372) resembled those for cyclin-dependent kinase 1 (Cdc2p) (Nakajima et al., 2003). We examined the significance of the phosphorylation of four of these serine residues by mutating them in pairs either to aspartic acid or alanine, mimicking permanently phosphorylated or unphosphorylatable forms. These phosphorylation sites are relevant to Mbx1p function because a combination of serine to alanine changes at residues 46 and 106 reduced the in vivo interactions of Mbx1 with both Clp1p and Plo1p; similarly, serine to alanine changes at residues 183 and 372 displayed reduced in vivo interaction with Plo1p, but not Clp1p (Fig. 2C). Perhaps most strikingly, serine to aspartic acid changes at residues 46 and 106 resulted in a defective cell morphology and cell separation phenotypes, with cell clumping reminiscent of *mbx1Δ* (Fig. 2D) (Buck et al., 2004). These mutations also allowed these cells to mate with both h^+ and h^- strains, as observed with *mbx1Δ* (Papadopoulou et al., 2008).

We also explored the ability of Clp1p to dephosphorylate Mbx1p. In separate in vitro kinase reactions, Mbx1p was exposed to Clp1p after pre-phosphorylation by either Cdk1 or Plo1p (Fig. 2B, data not shown). Under such conditions, Clp1p efficiently dephosphorylated Mbx1p phosphorylated by Cdk1, but not by Plo1p. Consistent with this function, we observed that a phosphatase-dead mutant (with cysteine 286 changed to serine) (Wolfe et al., 2006) affected Mbx1p cell cycle-specific phosphorylation in vivo: the presence of lower mobility, hyperphosphorylated forms of Mbx1p were detected in the mutant *clp1-C286S* compared with wild type (Fig. 1C).

Clp1p binds to PCB promoter DNAs in vivo

The observation that Clp1p binds to Mbx1p suggested that this phosphatase might regulate M–G1 gene expression by binding to promoter DNAs. We tested this using chromatin

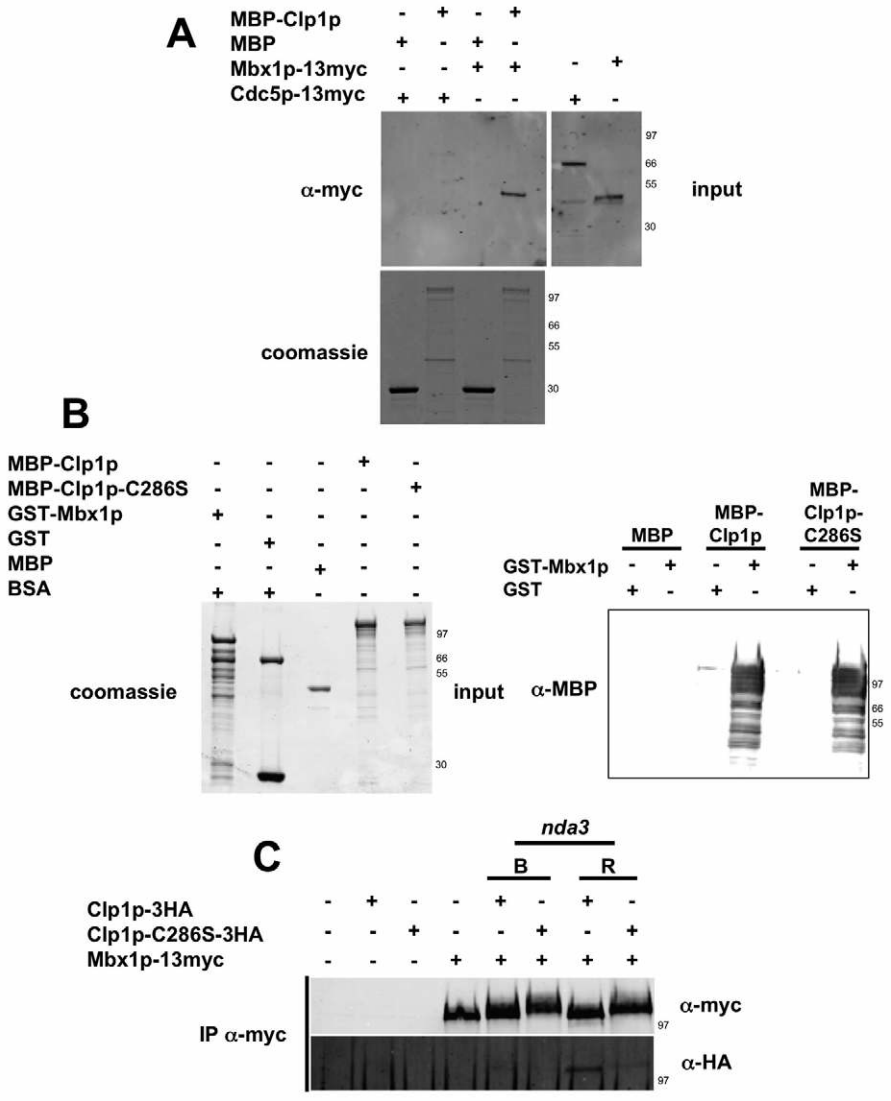

Fig. 1. Clp1p binds to Mbx1p in vivo. (**A**) Mbx1p is pulled-down with Clp1p from fission yeast extract. Bacterially expressed and purified MBP or MBP–Clp1p bound to amylose beads was mixed with soluble extracts of fission yeast expressing Mbx1p–13myc or Cdc5p–13myc from their endogenous promoters. Proteins bound to the beads after extensive washing were analysed by western blotting with antibodies against Myc. (**B**) Mbx1p binds to Clp1p in vitro. Bacterially expressed and purified GST and GST–Mbx1p bound to beads were incubated with bacterially produced and purified MBP, MBP–Clp1p or MBP–Clp1p-C286S (left panel). Proteins associated with beads following washes were analysed by western blotting with antibody against MBP (right panel). (**C**) Mbx1p is co-immunoprecipitated with Clp1p from fission yeast extract during late M phase. Immunoprecipitations with anti-Myc antibody from the strains indicated were divided in half and were analysed by western blotting with anti-Myc or anti-HA antibodies. For *nda3-km311*, cells were arrested by incubation at 19°C for 6 hours (B) or then released to 32°C for 30 minutes (R) prior to lysis.

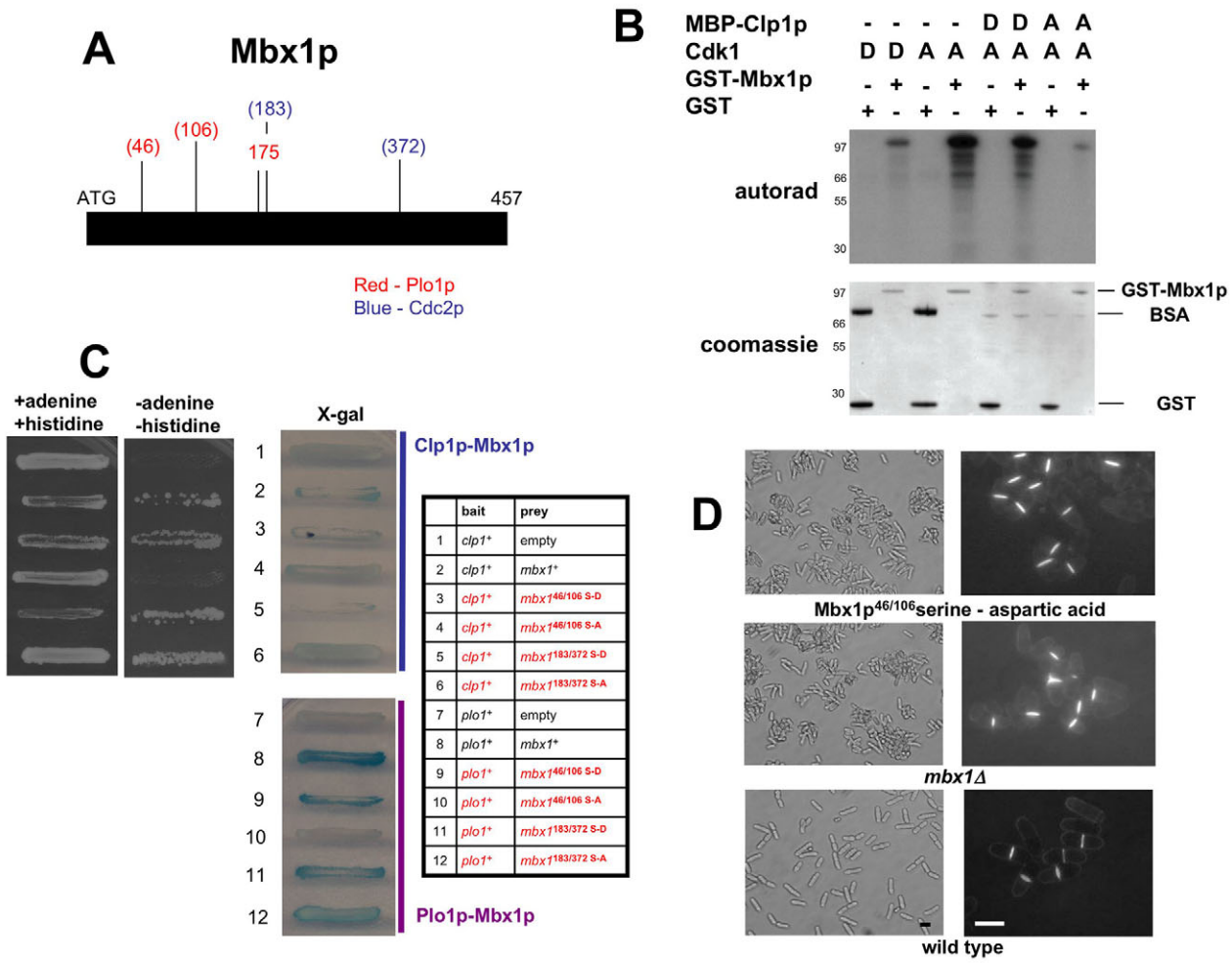

Fig. 2. Mbx1p is a phosphoprotein dephosphorylated by Clp1p. (**A**) Mbx1p amino acid residues phosphorylated by Cdc2p and Plo1p. Residues were mapped by LC-mass spectrometry from Mbx1p purified from Clp1p–TAP eluates arrested in M phase and released, and from in vitro kinase assays using MALDI-TOF. Serines phosphorylated by Plo1p (red) or Cdc2p (blue) are indicated; those resembling consensus sequences in brackets. (**B**) Mbx1p is phosphorylated by Cdk1 and dephosphorylated by Clp1p. GST and GST–Mbx1p were expressed and purified from bacteria, and incubated in appropriate kinase buffer with Cdk1, as indicated. The reactions were either stopped by boiling in SDS sample buffer (lanes 1–4) or were washed three times and further incubated with MBP–Clp1p or MBP–Clp1p-C286S (lanes 5–8). Reactions were analysed by SDS-PAGE, stained with Coomassie Blue and exposed for autoradiography. BSA was added to purified GST and MBP–Clp1 proteins, to counteract degradation. D, kinase- or phosphatase-dead; A, kinase- or phosphatase-active. (**C**) Effect of phosphorylation site mutations in Mbx1p on binding to Clp1p and Plo1p. Two-hybrid interaction between Clp1p, Plo1p and Mbx1p. Wild-type *clp1*$^{+}$ or *plo1*$^{+}$ in the two-hybrid bait vector was transformed into budding yeast, with the prey vector containing phosphorylation site mutations in *mbx1* (e.g. *mbx1*$^{46/106\ S\text{-}D}$ mutation of serines 406 and 106 to aspartic acid; *mbx1*$^{183/372\ S\text{-}A}$ mutation of serines 183 and 372 to alanine). Transcriptional readout was monitored by either adenine/histidine expression or by β-galactosidase assays with X-gal. (**D**) Cell morphology phenotype of an *mbx1* phosphorylation site mutant. Strains expressing either *mbx1*$^{46/106\ S\text{-}D}$, *mbx1*Δ or *mbx1*$^{+}$ were grown in liquid culture at 30°C and examined microscopically using differential interference contrast optics, or after calcofluor staining to reveal septa. Scale bars: 10 μm.

immunoprecipitation (ChIP) analysis of Clp1p, using a tagged version of the protein under the control of its native promoter to detect whether it binds in vivo to promoters of genes containing PCB motifs (to which PBF binds) expressed at M–G1 (Papadopoulou et al., 2008). Clp1p specifically bound to the promoters of PCB-regulated genes (Fig. 3A). Furthermore, binding was cell cycle-specific: minimum Clp1p binding occurred when target gene expression was maximum and vice versa (Fig. 3B), implying a repressive function for Clp1p in controlling gene expression. The ability of Clp1p to bind to PCB promoters is dependent on Mbx1p, because ChIP analysis in cells lacking Mbx1p revealed that Clp1p cannot bind to PCB promoters (Fig. 3C). Similarly, Plo1p binding to PCB promoters is also dependent on Mbx1p (Fig. 3C).

Clp1p is required for M–G1 specific gene expression in fission yeast

The observation that Clp1p binds to PCB promoters through Mbx1p suggests that it has a role in controlling M–G1 specific gene transcription. We therefore examined M–G1 gene expression in *clp1*$^{+}$ deletion mutant (*clp1Δ*) cells, synchronised either by centrifugal elutriation (Fig. 4A) or by *cdc25-22* transient block and release (Fig. 4B). *cdc15*$^{+}$ and *spo12*$^{+}$ mRNAs were present through more of the cell cycle, compared with the distinct peaks at M–G1 observed in wild-type cells. Consistent with this observation, Cdc15p protein levels are elevated in *clp1Δ* cells (Clifford et al., 2008b). Such increased gene expression outside the M–G1 interval in cells lacking Clp1p supports the idea that it has a repressive role

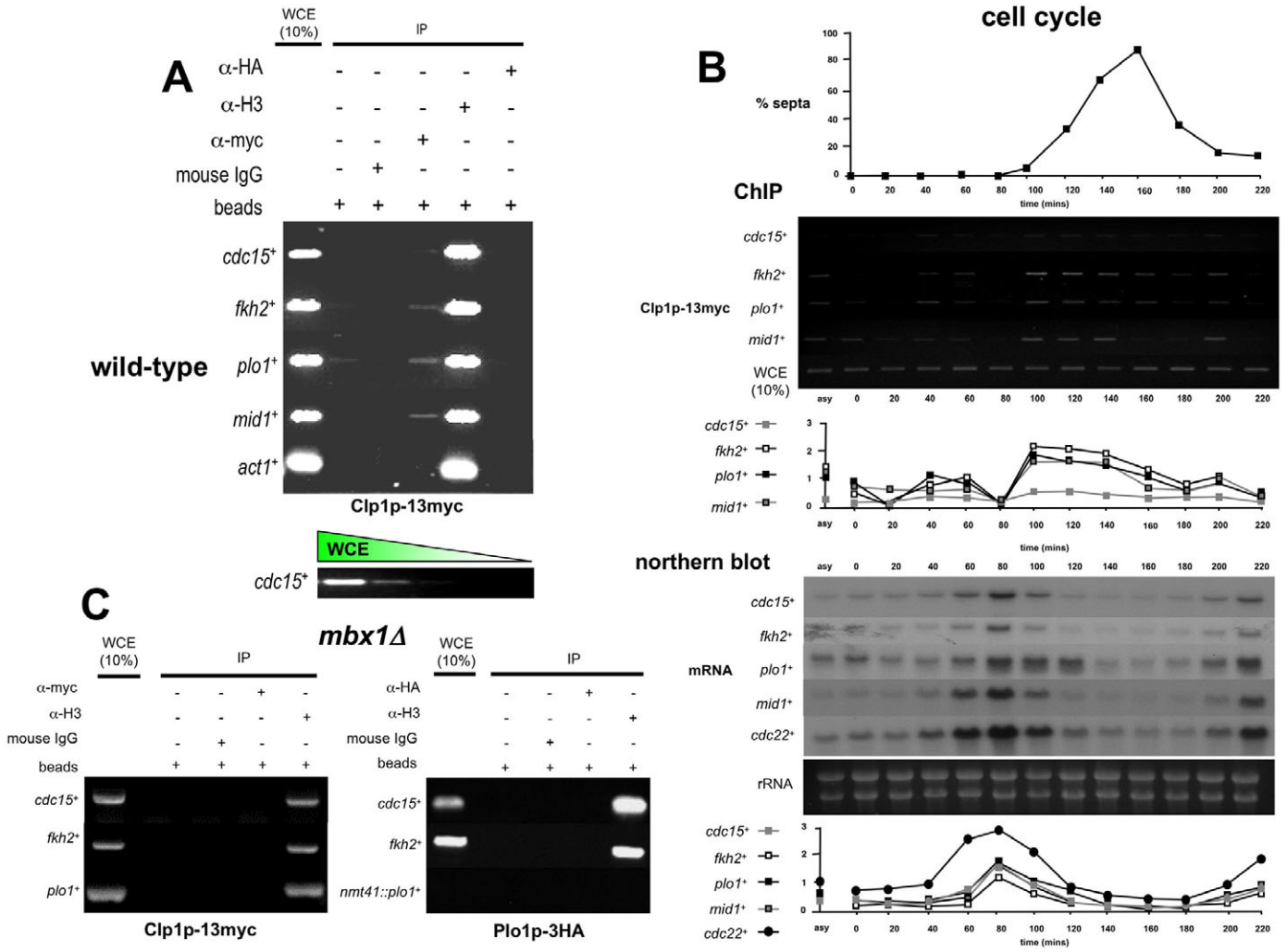

Fig. 3. Clp1p binds to PCB motifs in vivo. (**A**) ChIP experiments with a tagged version of Clp1p–13myc on the *cdc15*$^{+}$, *fkh2*$^{+}$, *plo1*$^{+}$ and *mid1*$^{+}$ promoters. WCE (whole cell extracts; non-immunoprecipitated sample) and IP (immunoprecipitates). Approximately ten times more of the precipitates were loaded than the WCE input DNA. As separate negative controls, beads alone, mouse IgG and anti-HA antibody were used for precipitations. DNA of the *act1*$^{+}$ coding sequence was used as substrate. A further negative control ChIP with anti-Myc antibody was done with a wild-type untagged strain (Papadopoulou et al., 2008). As a positive control, histone H3 was also analysed by ChIP onto the *cdc15*$^{+}$, *fkh2*$^{+}$, *plo1*$^{+}$ and *mid1*$^{+}$ promoters with an anti-H3 antibody. PCR amplification was judged to be within a linear range because PCR amplification with fivefold serially diluted WCE DNA resulted in a corresponding reduction in the observed signal. Promoter regions amplified during the ChIP procedure are shown in supplementary material Fig. S2. (**B**) Cell cycle-dependent binding of Clp1p to PCB promoter DNAs. A population of *cdc25-22 clp1-13myc* cells was synchronised by transient temperature arrest, and samples were taken every 20 minutes after return to the permissive temperature for ChIP and northern blot analysis. Crosslinked DNA was prepared from each sample and Clp1p–13myc analysed by ChIP using anti-Myc antibodies. Binding of Clp1p–13myc to the *cdc15*$^{+}$, *fkh2*$^{+}$, *plo1*$^{+}$ and *mid1*$^{+}$ promoters was detected by PCR. As a loading control, PCR was performed with 10% WCE containing input DNA and *cdc15*$^{+}$ oligonucleotides. Quantification of Clp1p binding to input DNA is shown. RNA was prepared from duplicate samples and *cdc15*$^{+}$, *fkh2*$^{+}$, *plo1*$^{+}$ and *mid1*$^{+}$ mRNA levels quantified by northern blot analysis; 'asy' indicates control RNA sample from asynchronous cells prior to synchronisation. mRNA levels of *cdc22*$^{+}$, a known G1–S expressed transcript independent of PBF–PCB control, were detected to confirm synchrony of the experimental culture. (**C**) Requirement of Mbx1p for Clp1p and Plo1p promoter binding in vivo. ChIP experiments with Clp1p–13myc and Plo1p–3HA from extracts of *mbx1Δ* cells. Crosslinked DNA complexes with Clp1p–13myc and Plo1p–3HA were prepared and analysed by ChIP using anti-Myc and anti-HA antibodies, and binding to PCB promoter fragments from *cdc15*$^{+}$, *fkh2*$^{+}$ and *plo1*$^{+}$ detected by PCR. WCE and IP. As negative controls, beads alone, and mouse IgG were used for precipitations. A further negative control ChIP with anti-HA antibody used extracts from a wild-type untagged strain (Papadopoulou et al., 2008). As a positive control, histone H3 was analysed by ChIP with an anti-H3 antibody. Both Clp1p and Plo1p DNA binding could be restored by placing plasmid-borne wild-type *mbx1*$^{+}$ in *mbx1Δ* cells. Equal levels of Clp1p–13myc and Plo1p–3HA protein in the strains used for ChIP were confirmed by western blotting (supplementary material Fig. S3).

in controlling gene transcription at these cell cycle times. This phenotype is exacerbated by combining *clp1Δ* with *mid1Δ* (Argarwal et al., 2010), suggesting that the Clp1p and Mid1p proteins have overlapping repressive roles in controlling M–G1 gene expression in fission yeast.

We also examined M–G1 gene expression and Clp1p binding in *clp1-3A* cells, a gain-of-function mutant that is not inhibited in phosphatase activity as effectively as the wild type (Wolfe et al., 2006). The *clp1-3A* mutation resulted in delayed PCB-regulated gene expression, with *cdc15*$^{+}$ mRNA levels peaking after *cdc22*$^{+}$,

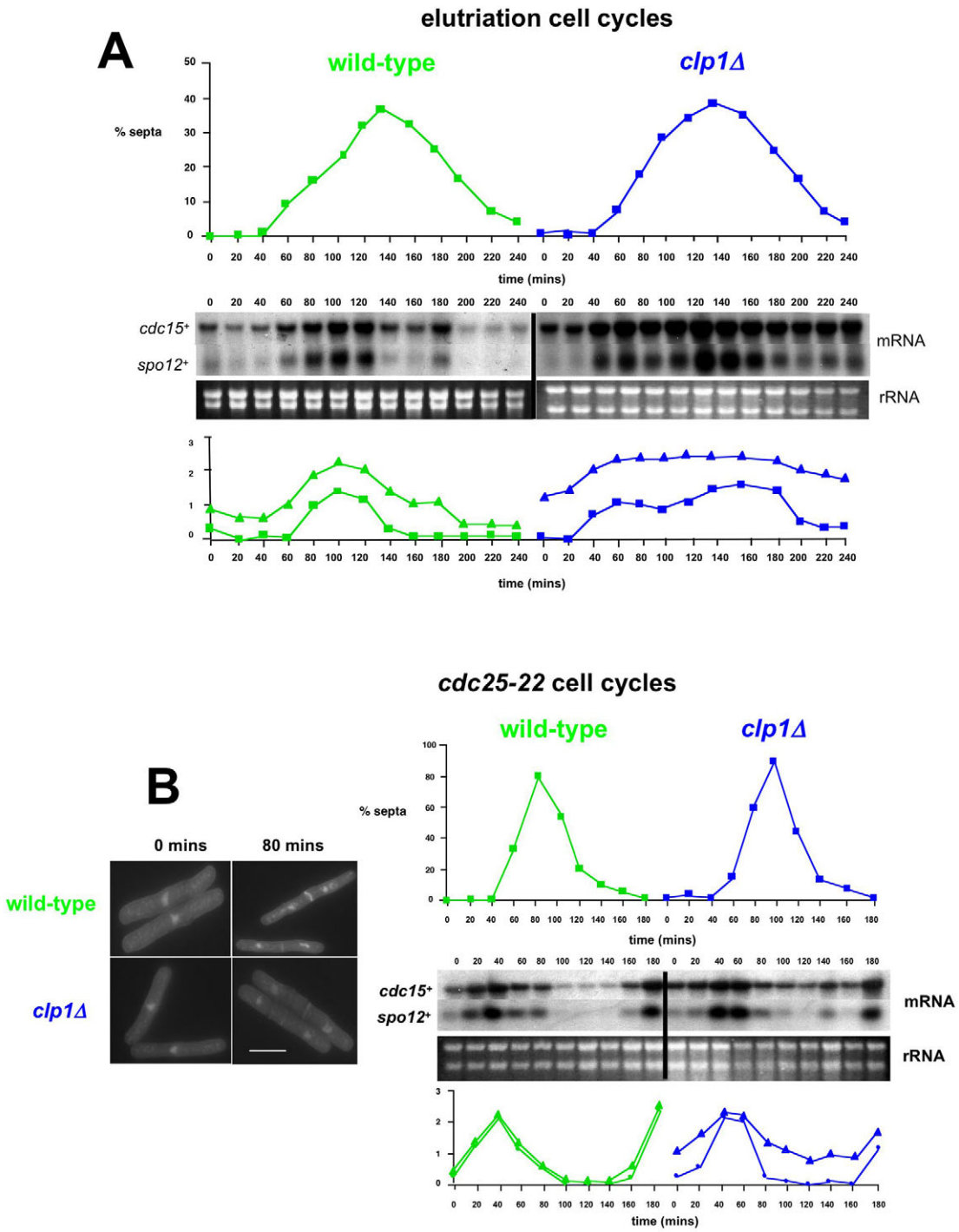

Fig. 4. M–G1 gene expression in *clp1Δ* cells. (**A**) Separate populations of wild-type and *clp1Δ* cells were synchronised by centrifugal elutriation and samples taken every 20 minutes. *cdc15*$^{+}$ and *spo12*$^{+}$ mRNA levels were detected and quantified by northern blot analysis. Both blots were exposed to identical radiolabelled probe, and are thus directly comparable. (**B**) Separate populations of *cdc25-22* (wild-type) and *cdc25-22 clp1Δ* cells were synchronised by transient temperature arrest, and samples taken every 20 minutes after return to the permissive temperature. *cdc15*$^{+}$ and *spo12*$^{+}$ mRNA levels were detected and quantified by northern blot analysis. Micrographs of representative cells stained with DAPI or calcofluor were taken at the times indicated. Scale bar: 5 μm.

an MBF-regulated gene that is expressed at G1–S (Fig. 5A). In wild-type cells, *cdc15*$^{+}$ mRNA levels peak before those of *cdc22*$^{+}$ mRNA (Fig. 5A) (Anderson et al., 2002). Furthermore, ChIP analysis revealed that Clp1p-3A was unable to contact PCB promoters in vivo (Fig. 5B).

clp1^{+} shows genetic interactions with *plo1*$^{+}$ and genes encoding components of PBF

To explore further the role of Clp1p in cell cycle regulation, we searched for synthetic phenotypes of mutations in *clp1* (*clp1Δ*, gain-of-function mutant *clp1-3A*, and phosphatase-dead mutant

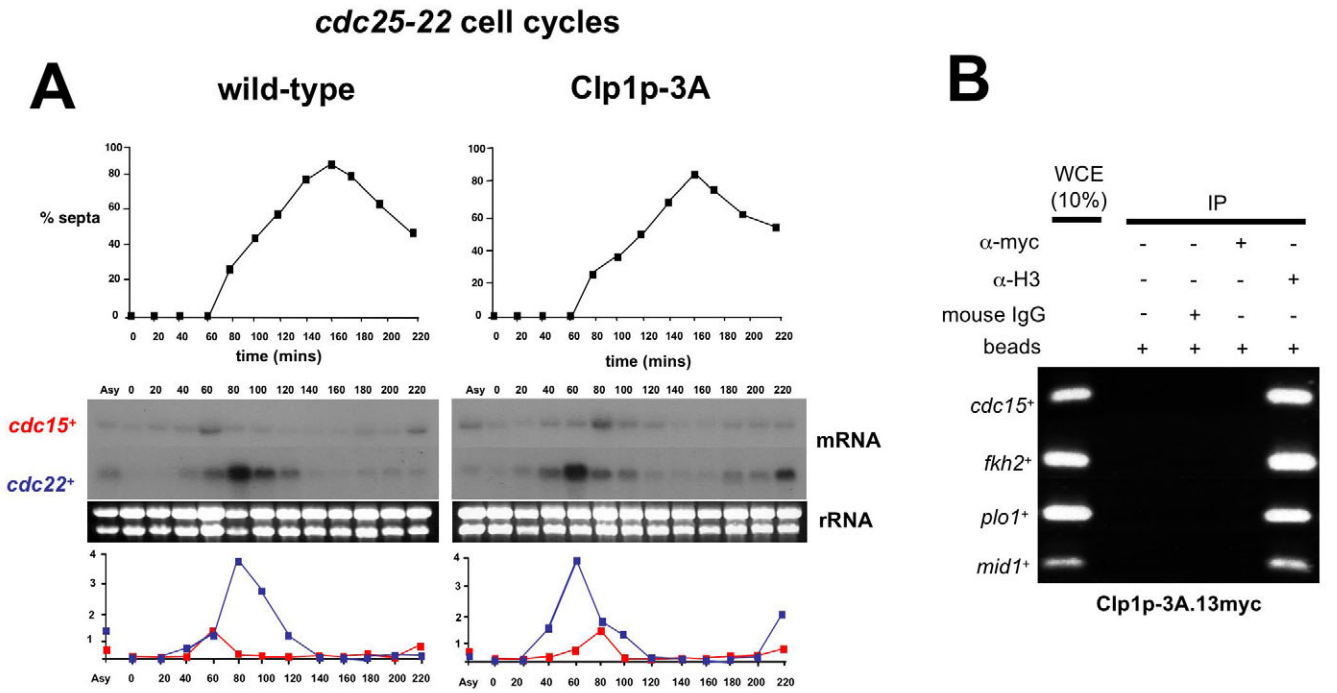

Fig. 5. M–G1 gene expression and Clp1p binding to PCB promoters in *clp1-3A* cells. (**A**) Separate populations of *cdc25-22* (wild-type) and *cdc25-22 clp1-3A* cells were synchronised by transient temperature arrest and samples taken every 20 minutes after return to the permissive temperature. *cdc15*$^+$ and *cdc22*$^+$ mRNA levels were detected and quantified by northern blot analysis. 'Asy' indicates control RNA sample from asynchronous cells. This experiment was repeated three times. (**B**) The ChIP experiment monitored Clp1p-3A.13myc binding on the *cdc15*$^+$, *fkh2*$^+$ *plo1*$^+$ and *mid1*$^+$ promoters. WCE (whole cell extracts; non-immunoprecipitated sample) and IP (immunoprecipitates). As negative controls, beads alone and mouse IgG were used for precipitations. As a positive control, histone H3 was analysed by ChIP with an anti-H3 antibody.

clp1-D257A) with mutations in *plo1*, *fkh2*, *sep1* or *mbx1*. A summary of the results of these various crosses is shown in Fig. 6A. Strikingly, a *clp1Δ fkh2Δ* double deletion mutant was found to be synthetically lethal. Because Fkh2p is thought to negatively regulate M–G1 gene expression, with deletion of *fkh2*$^+$ causing overexpression of target genes (Buck et al., 2004; Bulmer et al., 2004; Rustici et al., 2004), the effect of also deleting *clp1*$^+$, another repressor, might exacerbate de-regulated expression of target genes to lethal levels. The three mutants in *clp1* also showed synthetic phenotypes with *plo1*, reducing the restrictive temperature of the temperature-sensitive mutant *plo1-ts35* from >35°C to 30°C. The phenotype of *plo1-ts35 clp1-D257A* cells was particularly severe, with the cells exhibiting malformed shape and slow growth. Again, these synthetic phenotypes support the idea that *clp1*$^+$ and *plo1*$^+$ interact to control cellular events. Consistent with this suggestion, Clp1p cannot bind to PCB promoters in vivo in *plo1-ts35* cells (data not shown).

We also examined the effect of deletions of *mbx1*$^+$ and *clp1*$^+$ on the lethal phenotype observed when overexpressing *plo1*$^+$ (Ohkura et al., 1995). Deletions of either suppressed the lethal phenotype caused by overexpressing *plo1*$^+$ (Fig. 6B), although the cells did not return to a wild-type phenotype (data not shown), again consistent with the encoded proteins functioning together in vivo.

Discussion

Clp1p regulation of cell cycle-specific gene expression

The observations described here provide the first evidence for a new function for the fission yeast Cdc14p-like phosphatase Clp1p in controlling cell cycle-specific gene expression at M–G1. We reveal that Clp1p binds Mbx1p to directly regulate gene expression. Cdc14p in budding yeast has been shown to control cell cycle-specific gene expression, but in this organism the mechanism is more indirect, with the phosphatase controlling the nuclear location of transcriptions factors required for this process (Visintin et al., 1998; Geymonat et al., 2004). Dephosphorylation of Swi5p, Ace2p or Swi6p allows their entry into the nucleus to then bind to promoters and stimulate gene expression. Thus, in these cases Cdc14p has a positive, activating role in allowing gene expression to occur at the end of the cell cycle.

A repressive role for Clp1p in controlling gene expression

In fission yeast we propose a novel mechanism by which Clp1p controls cell cycle-specific gene expression. Here Clp1p more directly effects transcription by binding to a transcription factor and dephosphorylating it while it is bound to gene promoters (Fig. 7).

Another striking difference between Cdc14p and Clp1p is that the evidence points to Clp1p having a negative, repressive role in controlling cell cycle-specific gene expression. Removal of Clp1p from cells through chromosomal deletion of *clp1*$^+$ (*clp1Δ*) does not prevent M–G1 gene expression, as would be expected if Clp1p had a positive, activating role, but instead results in gene expression not restricted to M–G1. This phenotype suggests that Clp1p normally has a function to prevent gene expression outside the M–G1 interval. This conclusion is supported by other observations with Clp1p. ChIP analysis through the cell cycle reveals that this phosphatase only binds to promoters when genes are not being transcribed, and is present when their expression is inhibited. Furthermore, combining *clp1Δ* with *mid1Δ* results in higher overexpression (Argarwal et al.,

A

		clp1Δ	*clp1-3A*	*clp1-D257A*
plo1-ts35	growth/phenotype	OK	OK	slow/sick
	temperature sensitive	↓30°C	↓30°C	↓30°C
fkh2Δ	growth/phenotype	dead	OK	slow/sick
sep1Δ	growth/phenotype	OK	OK	OK
mbx1Δ	growth/phenotype	OK	OK	OK

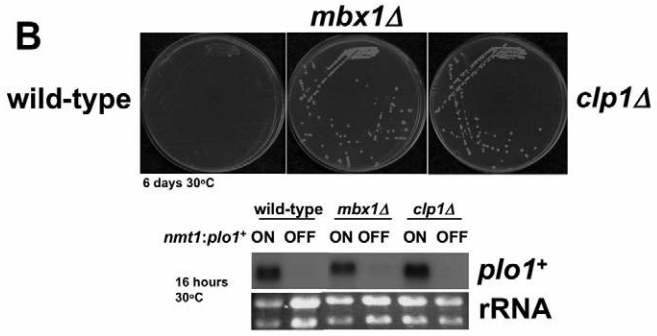

Fig. 6. Genetic interactions between *clp1*$^+$, *plo1*$^+$ and genes encoding components of PBF. (**A**) Summary of synthetic phenotypes revealed by double mutations in *clp1*, *plo1*, *fkh2*, *sep1* and *mbx1*. (**B**) Effect on cell growth of overexpression of *plo1*$^+$ in deletion mutants of *clp1* and *mbx1*. Cells containing pREP1: *plo1*$^+$ were grown on solid medium lacking thiamine for 6 days. To confirm overexpression of *plo1*$^+$, the same cells were grown to mid-exponential phase in liquid medium before the removal of thiamine to induce overexpression. After 16 hours, cells were removed for northern blot analysis of *plo1*$^+$ mRNA levels.

2010), suggesting that the two have overlapping repressive roles in controlling M–G1 gene expression.

Genetic experiments also support the hypothesis that Clp1p has a negative role in gene expression. A repressive function has been ascribed to the Fkh2p transcription factor, with *fkh2Δ* cells showing related phenotypes to *clp1Δ* (Buck et al., 2004; Bulmer et al., 2004; Papadopoulou et al., 2008). It is notable that the double deletion mutant *fkh2Δ clp1Δ* is synthetically lethal, which could be explained by the combinatorial removal of both repressive proteins to allow such high levels of overexpression of target genes that this is lethal to cells.

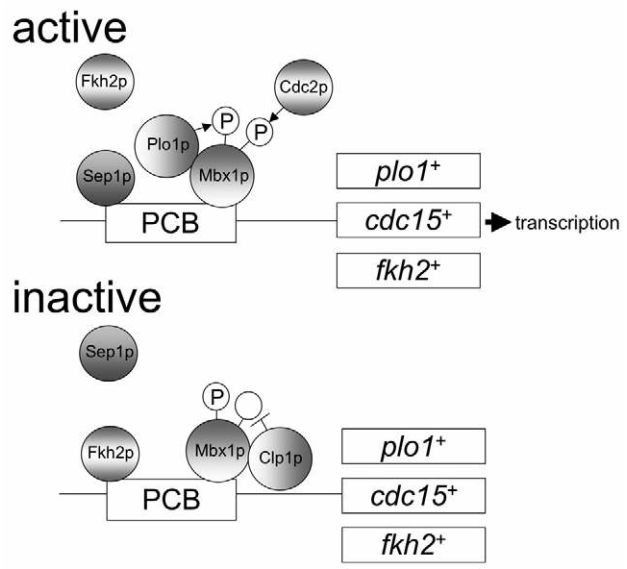

Fig. 7. Clp1p, Plo1p, Mbx1p, Fkh2p and Sep1p regulation of M–G1 gene expression in fission yeast. Gene expression during late M phase in fission yeast is controlled by a transcription factor complex containing at least three proteins, Fkh2p and Sep1p, both forkhead transcription factors, and Mbx1p, a MADS box protein. Fkh2p and Sep1p control cell cycle expression through cell cycle-specific binding to PCB sequences, with Fkh2p repressing and Sep1p activating gene expression. Activation of gene expression occurs through Plo1p binding to and phosphorylating Mbx1p, whereas Clp1p contributes to repression of gene expression by dephosphorylating Mbx1p, after pre-phosphorylation by Cdc2p.

Integration into general cell cycle control mechanisms

An important area of study in cell cycle-regulated transcription concerns understanding how each wave of gene expression is integrated into the wider context of the cell cycle. In budding yeast, it has been proposed that each wave of gene expression is controlled by the previous wave, thereby making a 'transcription cycle' within a cycle (Simon et al., 2001; Tyers, 2004). However, in fission yeast such continuous linked waves of gene expression are not complete, and thus some of the waves must be linked to the cell cycle by other, independent control mechanisms (McInerny, 2004; Bähler, 2005). Here, we show that Clp1p directly controls M–G1 gene expression. As Clp1p is an established cell cycle control protein that regulates many important events during M phase and cytokinesis, these experiments offer a direct way in which gene expression at this cell cycle time is linked to the processes of division and cell duplication.

Conservation of controls

The observations presented in this paper show that fission yeast Clp1p has a direct role in controlling cell cycle-specific gene expression, a new function for this phosphatase. A related function for Cdc14p has been identified in budding yeast in controlling at least two waves of cell cycle gene expression, although the mechanistic details are different (Visintin et al., 1998; Geymonat et al., 2004). Budding yeast Cdc14p has also been shown to reduce transcription by RNA polymerase I during anaphase (Clemente-Blanco et al., 2009). Thus, it appears that the regulation of gene expression during the cell cycle by Cdc14p phosphatases might be a widespread and general property of this class of proteins in yeasts. It will be fascinating to see if this property extends to higher eukaryotes, including humans.

Materials and Methods

Yeast strains and growth conditions

The general molecular procedures of Sambrook et al. (Sambrook et al., 1989) were performed, with the standard methodology and media used for the manipulation of *Schizosaccharomyces pombe* as described in Moreno et al. (Moreno et al., 1991). The strains used in this study are shown in supplementary material Table S1. *mbx1* phospho-mutants were cloned into plasmid pJK148 containing wild-type *mbx1*$^+$ promoter and terminator sequences, and integrated in single copy at the *leu1-32* locus into an *mbx1Δ* strain. Cells were routinely grown in complete yeast extract (YE) medium at 25°C or 30°C. Synchronous cultures of *cdc25-22* cells were prepared by transient temperature shifts to 36°C for 4 hours. Septation indices of such cultures were counted microscopically and plotted to indicate synchrony. Overexpression of *plo1*$^+$ was from pREP1 (Maundrell, 1993). Cells were grown in Eagle's modified medium (EMM) with 5 mg ml^{-1} thiamine (*nmt1* promoter 'off') to early exponential stage, washed three times in thiamine-free EMM and then grown for 15 hours in EMM without thiamine (*nmt1* promoter 'on').

DNA constructs and RNA manipulations

GST-mbx1$^+$, *MBP-clp1*$^+$ and *CDK1* constructs have been described (Clifford et al., 2008b; Papadopoulou et al., 2008). The *plo1*$^+$, *mbx1*$^+$ and *clp1*$^+$ two-hybrid constructs have been described (Clifford et al., 2008b; Papadopoulou et al., 2008). The *mbx1*$^+$ plasmid has been described (Buck et al., 2004). Site-directed mutagenesis was performed using the pALTER vector (Promega). *S. pombe* total RNA was prepared and analysed by northern blotting (Buck et al., 2004). Levels of individual transcripts were quantified by normalisation against levels of rRNA in each sample.

Two-hybrid analysis

Two-hybrid analysis using *plo1*$^+$ and *clp1*$^+$ as bait was carried out as described (Clifford et al., 2008b; Papadopoulou et al., 2008).

Chromatin immunoprecipitation

Chromatin immunoprecipitations were performed as described (Papadopoulou et al., 2008). Promoter regions amplified during the ChIP procedure are shown in supplementary material Fig. S2.

Immunoprecipitations, pull-downs and western blot analysis

Preparation of whole cell extracts, immunoprecipitations, pull-downs and western blot analysis were performed as described (Clifford et al., 2008b; Papadopoulou et al., 2008). Purification of Clp1p–TAP and subsequent identification of Clp1p and proteins by mass spectrometry was performed as described (Gould et al., 2004; Roberts-Galbraith et al., 2009). Mass spectrometry was used to identify the Mbx1p phosphorylation sites in Clp1p–TAP eluates, and in GST–Mbx1p phosphorylated in vitro by purified His–Plo1p (Papadopoulou et al., 2008).

Kinase and phosphatase assays

Bacterially produced recombinant GST–Mbx1p was purified and left on glutathione beads. Kinase assays were conducted as described (Wolfe et al., 2006) with the following modifications: Cdk kinase assay buffer consisted of 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM DTT; Plo1p kinase assay buffer was composed of 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 2 mM DTT. Clp1p phosphatase assays were as described (Clifford et al., 2008b).

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/24/4374/DC1

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