

**Mammalian cell overexpression and siRNA depletion**

Yip3 was cloned into the mammalian vector pcDNA3.1. HEK293 cells were transfected with pcDNA3.1 human Yip3 or pcDNA3.1, collected 42 h later, lysed by osmotic shock and shearing, and membranes were purified by ultracentrifugation. Membrane recruitment was as published<sup>6,7</sup> in buffer plus 100 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 100 μM GTPγS, 0.1 mg ml<sup>-1</sup> BSA, protease inhibitor cocktail (0.34 units ml<sup>-1</sup> aprotinin, 0.01 mg ml<sup>-1</sup> leupeptin, 1 μM pepstatin A), 0.2 mM DTT and ATP regeneration system<sup>6,7</sup>. Rab9-GDI complex (300 ng, 4.1 pmol) and membranes were mixed in 250 μl for 40 min at 37 °C. Ice-cold buffer (64 mM HEPES-KOH, pH 8.0, 100 mM NaCl, 8 mM MgCl<sub>2</sub>, 2 mM EDTA; 0.5 ml) was added and each reaction was then layered over 0.4 M sucrose + ice-cold buffer and spun for 10 min at 342,000g, 2 °C in a TLA-100.2 rotor (Beckman). Pellets were analysed by 12.5% SDS-PAGE and anti-Rab9 immunoblot.

HeLa S-3 cells were transfected with 0.13 μM siRNA duplex (sense 5'-(GCUUGUGUCUUUGGCCGA)d(TT)-3'; Dharmacon) using Oligofectamine (Invitrogen). After 77 h, cells were lysed in 25 mM HEPES pH 7.3 with protease inhibitors by 10 passages through a 25G needle. Extracts were spun 30s at 1,000 g; supernatants were centrifuged 15 min, 321,000g in a TLA 120.1 rotor (Beckman). The membrane pellet was washed in PBS.

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**Targets of the cyclin-dependent kinase Cdk1**

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The events of cell reproduction are governed by oscillations in the activities of cyclin-dependent kinases (Cdks)<sup>1</sup>. Cdks control the cell cycle by catalysing the transfer of phosphate from ATP to specific protein substrates. Despite their importance in cell-cycle control, few Cdk substrates have been identified<sup>2</sup>. Here, we screened a budding yeast proteomic library for proteins that are directly phosphorylated by Cdk1 in whole-cell extracts. We identified about 200 Cdk1 substrates, several of which are phosphorylated *in vivo* in a Cdk1-dependent manner. The identities of these substrates reveal that Cdk1 employs a global regulatory strategy involving phosphorylation of other regulatory molecules as well as phosphorylation of the molecular machines that drive cell-cycle events. Detailed analysis of these substrates is likely to yield important insights into cell-cycle regulation.

We developed methods for the systematic identification of Cdk substrates in the budding yeast *Saccharomyces cerevisiae*, a simple eukaryote in which the cell cycle is controlled by a single Cdk, Cdk1 (or Cdc28). We used a recently developed approach that allows the specific labelling of the substrates of a single kinase in a cell extract<sup>3,4</sup>. This method involves the mutation of a conserved bulky residue in the ATP-binding pocket to a glycine or alanine. The resulting analogue-sensitive (as) kinase is able to bind bulky ATP analogues that fit into the mutant-binding pocket but cannot bind to wild-type kinases. Importantly, the mutation is deep in the ATP-binding pocket, far from the protein substrate binding site, and is not expected to alter the protein substrate specificity of the protein kinase<sup>5</sup>. The addition of radiolabelled ATP analogue to a

Table 1 Nucleotide specificity of Cdk1-as1

Kinase	Nucleotide	K <sub>m</sub> (μM)	k <sub>cat</sub> (min <sup>-1</sup> )	k <sub>cat</sub> /K <sub>m</sub> (μM <sup>-1</sup> min <sup>-1</sup> )
Cdk1-Clb2	ATP	35	132	3.73
Cdk1-as1-Clb2	ATP	322	21.3	0.07
Cdk1-Clb2	N <sup>6</sup> -(benzyl) ATP	>1,000	NM	NM
Cdk1-as1-Clb2	N <sup>6</sup> -(benzyl) ATP	1.5	13.6	9.07

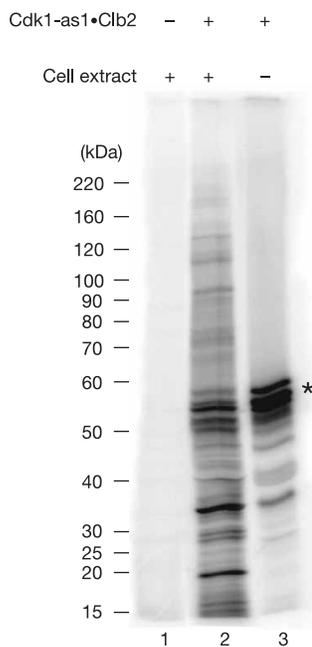
Active cyclin-Cdk complexes were formed by the addition of excess MBP-Clb2 to purified Cdk1-His<sub>6</sub> or Cdk1-as1-His<sub>6</sub>, and their ability to phosphorylate histone H1 at different N<sup>6</sup>-(benzyl) ATP or ATP concentrations was measured to generate K<sub>m</sub> and k<sub>cat</sub> values. NM, V<sub>max</sub> not measurable at 1 mM nucleotide.

cell extract containing a single as-kinase therefore leads to the specific labelling of the direct substrates of that kinase.

In previous work, we constructed an analogue-sensitive version of yeast Cdk1, Cdk1-as1, by replacing phenylalanine 88 with glycine<sup>6</sup>. We showed that Cdk1-as1 is functional *in vivo* and is uniquely sensitive to a bulky chemical inhibitor that interacts with the enlarged ATP-binding site. In the present work, we measured Cdk1-as1 activity with the bulky ATP analogue N<sup>6</sup>-(benzyl) ATP. As predicted, Cdk1-as1 (in a complex with the mitotic cyclin Clb2) exhibited a high affinity for the ATP analogue, whereas wild-type Cdk1 had no measurable activity with this substrate (Table 1). Cdk1-as1 was approximately 130-fold more active towards N<sup>6</sup>-(benzyl) ATP than ATP, that is,  $(k_{cat}/K_m)^{N6-(benzyl)ATP} / (k_{cat}/K_m)^{ATP} = 130$  (Table 1). Thus, the substitution of a glycine for a phenylalanine in the active site of Cdk1 results in a mutant that displays high affinity and selectivity for N<sup>6</sup>-(benzyl) ATP.

Concentrated cell extracts, prepared by gentle detergent lysis of yeast spheroplasts, were incubated with radiolabelled N<sup>6</sup>-(benzyl) ATP. Few proteins were labelled, indicating that wild-type kinases in the extract were not using the ATP analogue at a significant rate (Fig. 1, lane 1). We then added purified Cdk1-as1·Clb2 complexes at a concentration (7 nM) that resulted in levels of activity similar to those in mitotic cell extracts. Many radiolabelled proteins were generated (Fig. 1, lane 2). Incubation of purified Cdk1-as1·Clb2 with ATP analogue in the absence of cell extract resulted in autophosphorylation of Clb2 and other contaminants (Fig. 1, lane 3), and revealed that most of the proteins labelled in lane 2 represent direct Cdk1 substrates in the crude cell extract.

We next developed a simple, rapid and highly sensitive approach to scan the yeast proteome for proteins phosphorylated by Cdk1-as1·Clb2. We used a library of 6,144 yeast strains, each expressing a unique open reading frame (ORF) with an amino-terminal fusion



**Figure 1** Addition of Cdk1-as1 and radiolabelled N<sup>6</sup>-(benzyl) ATP to a cell extract results in the appearance of many specific phosphoproteins. 5  $\mu$ Ci  $\gamma$ -<sup>32</sup>P-N<sup>6</sup>-(benzyl) ATP was added to 30  $\mu$ g whole yeast extract (lanes 1, 2) or buffer (lane 3) in the presence (lanes 2, 3) or absence (lane 1) of 7 nM purified Cdk1-as1·Clb2. After incubation for 30 min at room temperature (23 °C), reactions were analysed by SDS-PAGE on a 5–15% gradient gel, and autoradiographed. Asterisk indicates autophosphorylated Clb2 bands.

to glutathione-S-transferase (GST)<sup>7</sup>. We reasoned that we could perform kinase reactions with Cdk1-as1 in lysates of strains from this library, and then purify the GST-tagged protein to determine if it was phosphorylated.

Pilot studies revealed that tagged substrate phosphorylation was not readily detected if reactions were performed with extracts made from a pool of multiple GST-ORF yeast strains. It was therefore necessary to perform separate reactions for each GST-tagged protein. Rather than performing over six thousand reactions, we focused on several hundred candidate ORFs that seemed likely to encode Cdk targets. As most known Cdk substrates contain multiple Cdk consensus phosphorylation sites (S/T\*-P-x-K/R, where x = any amino acid), we first tested the 385 ORFs encoding proteins with two or more of these sites. In addition, because cell-cycle regulators are often controlled by transcriptional mechanisms, we screened the 137 ORFs encoding proteins with a single Cdk consensus site and whose transcripts are cell-cycle regulated<sup>8</sup>. Finally, in an attempt to estimate the number of Cdk targets in the entire proteome, we tested 198 randomly chosen ORFs. These candidates encompass a total of 695 unique ORFs (due to 25 overlaps between the random 198 and the 522 candidates), which represent about 11% of the yeast proteome.

Results from twelve representative reactions are shown in Fig. 2a. To rank the substrates, we measured the phosphorylation of each tagged protein (and fragments) and divided by the amount of tagged protein present in the reaction. The amounts of phosphate incorporated per nanogram of protein ranged over seven orders of magnitude. The logarithms of these values were calculated and defined as the ‘P-score’ for each tagged protein.

Of the 695 tested proteins, 360 were detectably phosphorylated (Fig. 2b). Of these 360 proteins, 181 had P-scores of 2.0 or higher and represent the most interesting group of substrates because they were less-abundant proteins with high levels of phosphorylation. As shown in Fig. 2c, 179 of the 181 top substrates were present in our reactions at very low concentrations (<50 pM to 50 nM; see Fig. 2c legend), suggesting that phosphorylation of these proteins was not an artefact of high substrate concentrations. Forty of the top 181 substrates are listed in Table 2 (a complete list of results with all tested proteins is available as Supplementary Information).

Approximately 12 proteins containing full Cdk consensus sites have been identified previously as likely Cdk targets in budding yeast (Table 2, marked in bold: Swi5, Sic1, Cln2, Cdh1, Far1, Gin4, Swi1, Cdc6, Orc2, Orc6, Sld2 and Pds1)<sup>9–20</sup>. Ten of these proteins had P-scores greater than 2 in our screen. Phosphorylation of the remaining two, Cdc6 and Cln2, was apparently undetectable as a result of the limitations of the tagged protein library (see Methods). Thus, essentially all known Cdk targets with full consensus sites are among the top 181 substrates we identified, which strongly supports the validity of our approach.

Our results provide insights into mechanisms that govern substrate recognition by Cdks. A small number of Cdk targets in yeast and other species do not contain the full Cdk consensus site (S/T\*-P-x-K/R) but are instead phosphorylated at a minimal consensus site containing a serine or threonine residue followed by proline (S/T\*-P)<sup>21</sup>. Of the 198 random proteins we tested, 16 were phosphorylated with P-scores of greater than two. Thirteen of these proteins contain the full consensus sequence, and the remaining three contain multiple copies of the minimal consensus site. We therefore suspect that the great majority of Cdk targets contain the full consensus site. However, the presence of a full consensus site is not sufficient for Cdk phosphorylation: we found 123 proteins with this site that were not phosphorylated, despite being expressed at detectable levels.

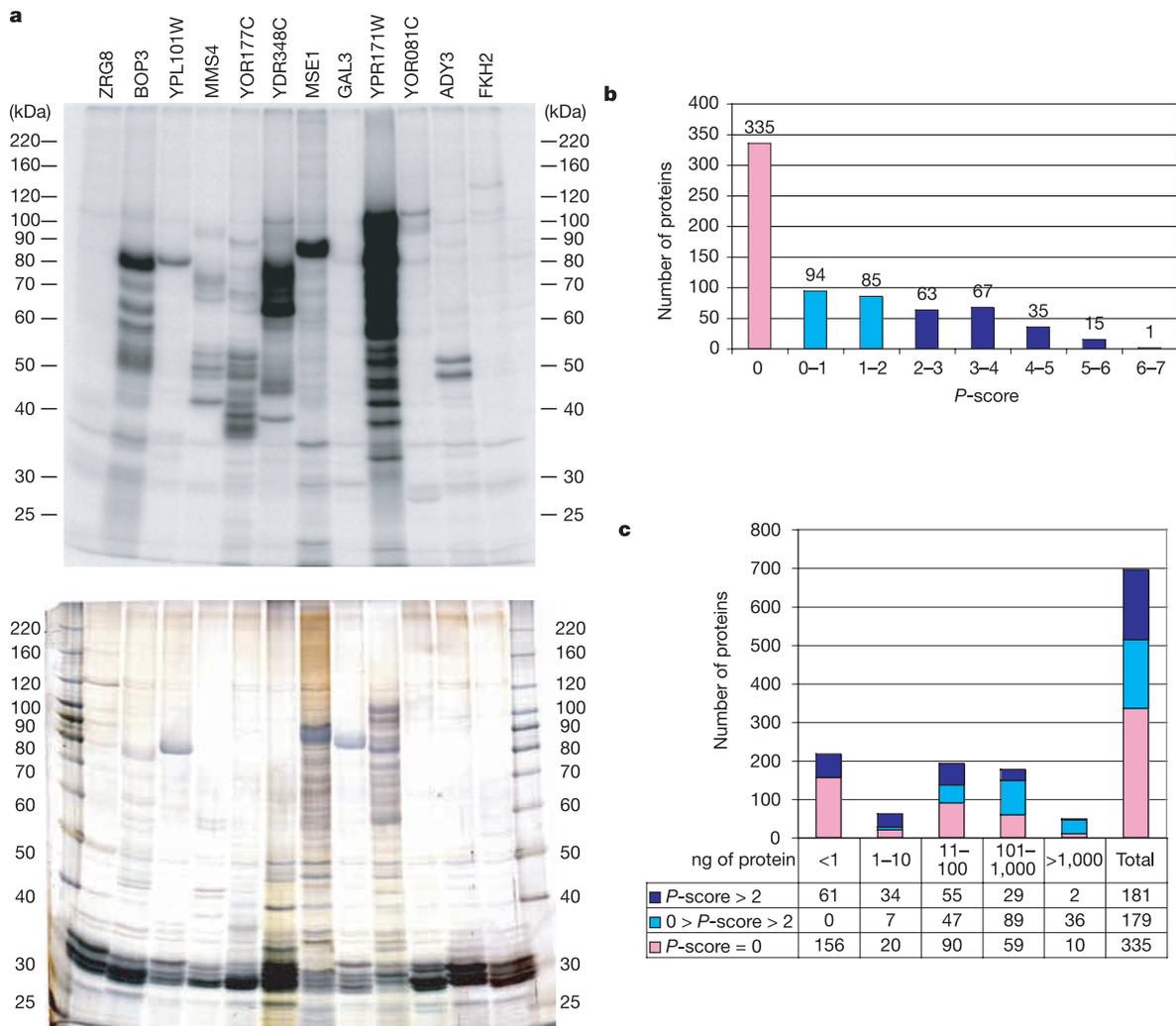
Substrate recognition by Cdks is influenced by the associated cyclin subunit<sup>22,23</sup>. Although our screen was carried out with the mitotic Cdk1-as1·Clb2 complex, we found that several substrates involved in DNA replication (including Orc6, Mcm3 and Sld2) are

phosphorylated tenfold more rapidly by the S-phase Cdk1-Clb5 complex (M. Loog and D.O.M., unpublished results). We therefore believe that our screen has led to the identification of a broad range of Cdk targets involved in multiple cell-cycle stages.

To confirm that we have identified proteins that are substrates of Cdk1 in the cell, it is important to show that phosphorylation of these proteins *in vivo* is dependent on Cdk1 activity. Traditionally, temperature-sensitive *cdk1* mutants have been used to demonstrate that phosphorylation is Cdk1-dependent *in vivo*. However, inactivation of these mutants takes 2 to 3 h at the restrictive temperature and results in a cell-cycle block (typically in G1), making it difficult to rule out indirect effects caused by the shift in cell-cycle position. These problems can be circumvented with the *cdk1-as1* strain, in which it is possible to inhibit much of the cell's Cdk1 activity within 5–10 min by addition of high concentrations (5–25  $\mu$ M) of 1-NM-PP1, a small molecule that inhibits the analogue-sensitive mutant Cdk1 without affecting other kinases in the cell<sup>6</sup>. The speed of inhibition reduces the likelihood of indirect effects.

To rapidly assess the phosphorylation state of a subset of substrates *in vivo*, we identified candidates that migrate on poly-

acrylamide gels as multiple bands (such mobility shifts can be a useful indication of phosphorylation). We prepared or obtained strains expressing epitope-tagged versions of 171 of the 181 candidate substrates expressed at their endogenous loci. A hundred proteins were detectable on western blots, and approximately 35 of these proteins displayed reproducible mobility shifts. We initially focused on Slk19, a non-essential regulator of spindle assembly and stability. Slk19 is a substrate of separase (Esp1)<sup>24</sup> and migrates on gels as a pair of band clusters, in which the lower cluster is the carboxy-terminal separase-cleavage product. As reported previously<sup>24</sup>, the reduced mobility of the upper forms in each cluster is caused by phosphorylation and is maximal in cells arrested in S phase or mitosis (Fig. 3a), consistent with phosphorylation by Cdk1. Treatment of *cdk1-as1* cells with 1-NM-PP1 caused dephosphorylation of the upper Slk19 band within five minutes, arguing that phosphorylation *in vivo* is Cdk1-dependent (Fig. 3b). After 30 min of treatment, the drop in mitotic cyclin levels presumably reflects activation of the Cdh1-dependent APC, which is normally inhibited by Cdk1<sup>12,13</sup>. Phosphorylation of the separase-cleavage product was not reversed by Cdk1 inhibition, perhaps indicating



**Figure 2** 181 proteins are efficiently phosphorylated by Cdk1-as1-Clb2. **a**, Autoradiograph (top) and silver-stained gel (bottom) of reactions with twelve GST-fusion proteins. 7 nM Cdk1-as1-Clb2 and 5  $\mu$ M  $\gamma$ -<sup>32</sup>P-N<sup>6</sup>-(benzyl) ATP were added to whole yeast extracts expressing a single GST-tagged protein and incubated for 30 min at room temperature, after which the GST-tagged protein was purified and analysed by SDS-PAGE. **b**, Histogram of the distribution of *P*-scores for the 695 GST-tagged proteins

reveals 181 efficiently phosphorylated Cdk1 substrates (*P*-score > 2, dark blue). **c**, Most of the best Cdk1 substrates are present at low concentrations in our reactions. GST-tagged proteins were grouped by their estimated protein amounts (nanograms in a 200  $\mu$ l reaction volume). If we assume a relative molecular mass of 100,000, then 1,000 ng tagged protein represents a concentration of 50 nM. The number of proteins with *P*-scores above 2, between 0 and 2, or equal to zero, are shown for each group.

that this fragment is less susceptible to phosphatases or is phosphorylated by other kinases.

Slk19 contains three full Cdk consensus sites (S/T\*-P-x-K/R) and three minimal Cdk consensus sites (S/T\*-P). Changing all six of these residues to alanine abolished phosphorylation of the protein *in vivo* (data not shown), further arguing that Slk19 is phosphorylated by Cdk1 *in vivo*. Replacement of the endogenous *SLK19* gene with a mutant gene encoding the non-phosphorylatable mutant resulted in a strain that appeared wild-type in all respects (cell-cycle timing, spindle structure and sporulation), indicating that Slk19 phosphorylation at these sites is not essential for cell-cycle progression under routine laboratory conditions.

We next analysed the effects of Cdk1 inhibition on other candidates with detectable gel mobility shifts. A fifteen-minute treatment of asynchronous *cdk1-as1* cells with 1-NM-PP1 caused a complete or partial collapse in the mobility of 11 novel Cdk1 substrates in addition to Slk19, arguing that phosphorylation of these proteins *in vivo* is Cdk1-dependent (Fig. 3c).

We also analysed two previously identified Cdk1 substrates, Orc6<sup>18</sup> and Pds1<sup>20</sup>. These proteins, as well as some of our candidates, were only partially dephosphorylated upon inhibitor addition (Fig. 3c). An underlying assumption in all approaches involving kinase inhibition *in vivo* is that phosphates on proteins are rapidly turned over in the cell. The steady-state phosphate content at any

phosphorylation site is determined by the relative rates of the protein kinase(s) and protein phosphatase(s) acting on that site. For some substrates, it is conceivable that kinase activity is present in 100- or 1,000-fold excess over phosphatase activity, in which case 90 or 99% inhibition of the kinase might not lead to a detectable drop in the phosphate content of the substrate. Thus, this method will work only when the relevant phosphatase activity is present in significant amounts.

Our results suggest that a significant fraction of the 181 candidate substrates are Cdk1 targets *in vivo*. Because of limitations in our analysis, however, it is not yet possible to accurately estimate the total number of bona fide Cdk1 targets on our list. New methods will be required to allow more rapid analysis of the phosphorylation state *in vivo* of large numbers of proteins in the presence and absence of Cdk1 activity. Recently, for example, Ficarro *et al.*<sup>25</sup> used mass spectrometry to sequence large numbers of phosphopeptides from yeast extracts. Peptides from three of our 181 best substrates (Mcm3, Mob1 and YDL113C) were found to be phosphorylated at Cdk consensus sites in their analysis. Similar studies with lysates of *cdk1-as1* cells, with and without 1-NM-PP1, could provide a global approach to validating Cdk1 substrate candidates.

About one-third of the 181 best substrates, like those listed in Table 2, are involved in processes that are regulated during the cell cycle. Many of these processes are governed by Cdk1, but the

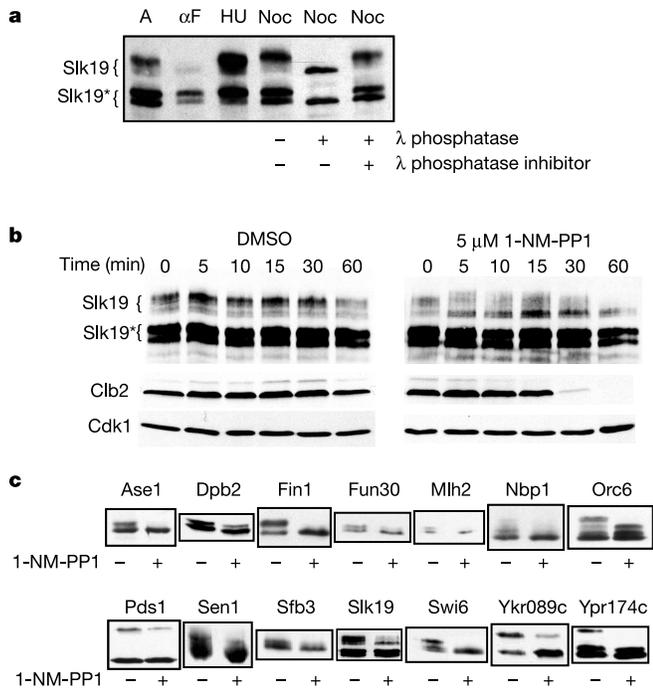
Table 2 **Forty selected Cdk1 substrates**

Protein*	Peak†	Full Cdk consensus sites	Estimated protein level (ng)‡	Phosphorylation (arbitrary units)	P-score	Biological process
<b>Cdk1 regulation</b>						
<b>Cdh1</b>		6	25	304,756	4.1	APC activating factor in M/G1
<b>Cln2</b>	G1	1	30	0	0.0	G1-specific cyclin controlling events at START
<b>Far1</b>	G2/M	4	100	65,166	2.8	Mating-specific Cdk1 inhibitor
Mih1		2	5	35,512	3.9	Phosphatase that dephosphorylates Y19 on Cdk1
<b>Sic1</b>	M/G1	3	200	996,630	3.7	Clb-specific Cdk1 inhibitor
<b>Swe1</b>	G1	2	2	87,408	4.6	Protein kinase that phosphorylates Y19 on Cdk1
<b>Swi5</b>	G2/M	8	0.1	5,206	4.7	G1-specific transcription factor
<b>DNA replication</b>						
<b>Cdc6</b>	M/G1	5	0.1	0	0.0	Required for pre-replicative complex formation
Dbf4		2	0.1	5,310	4.7	Regulatory subunit of the protein kinase Cdc7
Eco1	G1	1	30	127,694	3.6	Required for establishment of sister chromatid cohesion
Mcm3	M/G1	5	300	372,637	3.1	Required for initiation of DNA replication
<b>Orc2</b>		6	200	730,607	3.6	Required for pre-replicative complex formation
<b>Orc6</b>		4	50	588,875	4.1	Required for pre-replicative complex formation
<b>Sld2</b>	G1	5	50	51,144	3.0	Required for initiation of DNA replication
Smc4		5	0.1	1,373	4.1	Subunit of the condensin protein complex
<b>Mitosis</b>						
Cdc20	G2/M	1	2.5	6,138	3.4	APC-activating factor in anaphase
Cdc5	G2/M	1	5	7,404	3.2	Polo-like kinase, promotes anaphase and mitotic exit
Dbf2	G2/M	2	50	80,255	3.2	Protein kinase required for mitotic exit
Dbf20	S/G2	1	20	31,795	3.2	Protein kinase required for mitotic exit
Fkh2		3	0.1	2,735	4.4	Transcription factor regulating G2/M gene expression
Lte1		8	0.1	3,749	4.6	GDP exchange factor involved in mitotic exit
Mob1	G2/M	2	50	305,565	3.8	Regulatory subunit of Dbf2, required for mitotic exit
Ndd1	G1	4	10	24,327	3.4	Interacts with Fkh2 to control G2/M gene expression
Net1		3	0.1	4,452	4.6	Regulator of the phosphatase Cdc14
<b>Pds1</b>	G1	3	25	32,213	3.1	Regulator of Esp1 (separase)
<b>Spindle assembly</b>						
Ase1	G2/M	7	0.1	555	3.7	Microtubule binding protein required for spindle stability
Kar3	G1	2	75	182,904	3.4	Kinesin-like protein required for proper spindle assembly
Kip2	S/G2	2	150	91,993	2.8	Kinesin-like protein required for proper spindle assembly
Kip3	S/G2	1	50	7,592	2.2	Kinesin-like protein required for proper spindle assembly
Slk19	G1	3	0.1	17,857	5.3	Kinetochores protein required for spindle assembly
Stu2	S	1	50	5,178	2.0	SPB component regulating microtubule dynamics
<b>Actin polarization</b>						
Bem1	G2/M	2	100	44,466	2.6	Protein required for proper cell polarization
Bem3		5	0.1	596	3.8	GTPase-activating protein for Rho1 & Cdc42
Bni1		3	0.1	15,168	5.2	Protein involved in Rho protein signal transduction
<b>Other process</b>						
Ash1	M/G1	7	5	2,013	2.6	Daughter-cell-localized transcription factor
Bbp1	G1	1	10	13,328	3.1	Spindle-pole-body-associated protein
Chs2	G2/M	4	50	2,744,790	4.7	Chitin synthase II required for proper septum formation
<b>Gin4</b>	G1	2	0.1	781	3.9	Protein kinase required for septin organization
Rad9		9	0.1	604	3.8	DNA damage checkpoint protein
Rts1		2	0.1	2,281	4.4	PP2A regulatory subunit

\* *Saccharomyces* Genome Database name (<http://www.yeastgenome.org/>). Protein names in bold encode previously identified Cdk1 substrates (see main text).

† Cell-cycle-dependent mRNA transcription peak<sup>8</sup>.

‡ In those cases where protein was not detectable, an arbitrary value of 0.1 ng was assigned.



**Figure 3** Validation of yeast Cdk1 substrates *in vivo*. **a**, Slk19 is a phosphoprotein with maximal phosphorylation in mitosis. Slk19-Myc<sub>13</sub> was immunoprecipitated from a lysate of asynchronous cells (A) or cells arrested in G1 with alpha factor ( $\alpha$ F), in S phase with hydroxyurea (HU), or in mitosis with nocodazole (Noc), and immunoblotted with anti-Myc antibodies. Immunoprecipitates in the last two lanes were treated with lambda phosphatase, with or without phosphatase inhibitors. Asterisk indicates separate cleavage products. **b**, Inhibition of Cdk1 results in the rapid dephosphorylation of Slk19. Asynchronous *cdk1-as1 SLK19-MYC<sub>13</sub>* cells were treated with DMSO or 5  $\mu$ M 1-NM-PP1 for the indicated time, lysed in urea buffer, analysed by SDS-PAGE, and immunoblotted for Slk19-Myc<sub>13</sub>, Clb2 and Cdk1. **c**, Cdk1 inactivation causes a complete or partial collapse in the mobility of 12 new Cdk1 substrates. Asynchronous yeast cultures containing the indicated epitope-tagged protein were grown to mid-log phase, treated with DMSO (–) or 20  $\mu$ M 1-NM-PP1 (+) for 15 min at 30 °C, lysed in urea lysis buffer, and analysed by immunoblotting.

molecular targets of Cdk1 have remained unidentified. Mitotic spindle assembly, for example, involves a broad range of molecular motors and other microtubule-associated proteins, but little is known about how these components are regulated. Several of these proteins were identified as Cdk1 substrates in our screen, and it seems likely that the analysis of their regulation by Cdk1 will lead to insights into the control of spindle assembly and stability during mitosis.

The majority of the substrates, including about 50 that are encoded by ORFs with no known function, have not been implicated previously in cell-cycle-dependent events. Studies of these substrates may lead to the discovery of unforeseen regulatory connections in cell-cycle control.

It is now possible to estimate the total number of Cdk1 targets in the proteome. Of the random 198 proteins we tested, sixteen (8.1%) had *P*-scores of 2.0 or greater. If this fraction is representative of the entire yeast proteome, then there may be about 500 Cdk1 substrates in yeast. A number of this magnitude does not seem unreasonable, given that we did not test ~900 ORFs that have a single full Cdk consensus site and thousands more that contain multiple copies of the minimal consensus site. Identification of all Cdk1 substrates will require scanning the entire proteome, either by automated methods with proteomic libraries or by the identification of endogenous Cdk1-as1 targets in cell lysates.

It is possible to generate analogue-sensitive mutant forms of almost any protein kinase, although in some cases multiple mutations are required to produce a mutant that retains normal enzymatic activity<sup>26,27</sup>. Our method can therefore be used for the systematic identification of substrates for any protein kinase. Such comprehensive analyses of protein kinase targets are clearly an important step towards extracting useful functional information from genome sequence. □

## Methods

### Yeast methods

All strains were derivatives of W303 or S288C, and were grown at 30 °C unless otherwise noted. Construction of epitope-tagged strains was performed as described<sup>28</sup>. Ase1 and Slk19 were Myc<sub>13</sub>-tagged, Nbp1 was TAP-tagged, and Fin1, Orc6 and Pds1 were HA<sub>3</sub>-tagged. C-terminally TAP-tagged versions of 169 of the 181 candidate substrates, expressed at their endogenous loci, were obtained from a proteomic library kindly provided by E. K. O’Shea and J. S. Weissman. For western blotting, protein extracts were prepared by bead beating in urea lysis buffer (20 mM Tris-HCl pH 7.4, 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 50 mM NaF, 80 mM beta-glycero-phosphate, 1 mM sodium orthovanadate, 1 mM PMSE). Immunoprecipitation and phosphatase assays were performed as described<sup>13</sup>.

### Kinase assays in cell extracts

Cdk1-His<sub>6</sub>, Cdk1-as1-His<sub>6</sub>, and MBP-Clb2 were purified as described<sup>6</sup>. Active Cdk1-as1-Clb2-TAP was purified from *cdk1-as1 sic1Δ pGAL-CLB2-TAP* yeast cells as described<sup>29</sup>, except that cell lysates were prepared by bead beating. GST-tagged protein expression was performed as described<sup>7</sup>. Spheroplasting was performed using Zymolyase-100T (Seikagaku America Inc.) in 1.5-ml deep-well 96-well plates. Cells were disrupted by hypotonic lysis with detergent (25 mM Hepes-NaOH pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSE, 1  $\mu$ g ml<sup>-1</sup> aprotinin, leupeptin, pepstatin A) and proteins were solubilized by the addition of 300 mM NaCl for 10 min (resulting in efficient extraction of nuclear proteins). Phosphatase inhibitors were not present during lysate preparation, which generally results in dephosphorylation of proteins in the lysate. Lysates were clarified by centrifugation. 500  $\mu$ g of whole-cell extract was mixed with 7 nM Cdk1-as1-MBP-Clb2 or 7 nM Cdk1-as1-Clb2-TAP, a creatine-kinase-based 1 mM ATP-regenerating system, 10 × kinase reaction buffer (200 mM Hepes-NaOH pH 7.4, 100 mM NaCl, 10 mM MgCl<sub>2</sub>), 10 × phosphatase inhibitors (500 mM NaF, 800 mM  $\beta$ -glycerophosphate, 10 mM Na<sub>3</sub>VO<sub>4</sub>), and 5  $\mu$ Ci  $\gamma$ -<sup>32</sup>P-N<sup>6</sup>-(benzyl) ATP<sup>30</sup> and brought to a final volume of 200  $\mu$ l. After a 30-min reaction at room temperature, GST-tagged proteins were purified as described previously<sup>7</sup>. Glutathione eluates were loaded onto 9% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels, silver-stained, and exposed to a PhosphorImager cassette (Molecular Dynamics) for 72 h.

A key advantage of our approach is that the kinase reaction is performed in a whole-cell extract, which approximates the normal cellular environment better than typical kinase reactions performed with purified substrates. Protein kinases in crude extracts are exposed to a wide variety of potential protein substrates. If kinase and substrate concentrations are minimized, as in our studies, then only targets with high affinity for the kinase will be phosphorylated, while non-specific phosphorylation events will be reduced.

In the absence of the ATP-regenerating system, the addition of radiolabelled N<sup>6</sup>-(benzyl) ATP leads to the phosphorylation of several proteins in the extract, indicating that some protein kinases are able to use the bulky analogue at a low but appreciable rate (data not shown). This background phosphorylation is presumably lost in the presence of high ATP concentrations (Fig. 1, lane 1) because wild-type kinases are more selective for regular ATP. By contrast, the Cdk1-as1 mutant is much more selective for the radiolabelled bulky analogue, so that its activity with the analogue remains high even in the presence of abundant ATP. It is likely that the addition of ATP also protects the radiolabelled ATP analogue from hydrolysis by the abundant ATPases in the cell extract.

### Quantification of phosphorylation, protein levels and *P*-score

For each GST-tagged protein, phosphorylation levels were measured using Imagequant (Molecular Dynamics). Many of the tagged proteins were accompanied by multiple degradation products, which were present before cell lysis, as they were present even when cells were lysed in boiling SDS-PAGE sample buffer (data not shown). For proteins with multiple degradation products, the phosphorylation level represents the sum of the phosphorylation of every degradation product. Phosphorylation levels were normalized between gels and radiolabelled analogue batches by measuring background radioactivity levels for each gel. Protein levels were estimated by comparing protein band intensity, size and shape to BSA standards. *P*-scores were calculated by taking the logarithmic value of the normalized phosphorylation divided by the estimated protein level.

For simplicity, we assumed in these measurements that all silver-stained protein bands beneath the full-length tagged protein are fragments of that protein. However, some of these bands may represent associated proteins. It is remotely possible that these proteins were phosphorylated, resulting in an artefactual increase in the apparent *P*-score. Similarly, fragmentation of tagged proteins could lead to false positives due to exposure of phosphorylation sites that are normally inaccessible.

We suspect that several proteins were not phosphorylated in our screen but are actually Cdk1 substrates. One possible cause of false negatives is that degradation of tagged proteins can result in the loss of phosphorylation sites. Cln2, for example, was detected only as short N-terminal fragments lacking the C-terminal region that contains the Cdk consensus sites. Another source of false negatives is that many of the tagged proteins are

not present in cell lysates in appreciable amounts. Cdc6, for example, was not detectable in cell lysates by silver stain or western blot, suggesting that it is either not expressed in the library or that it is insoluble in our lysis conditions. Because of the method by which the GST-ORF library was constructed, it is likely that each yeast strain contains a mixture of cells expressing either GST alone or the desired GST-tagged protein. As there is no selection for the GST-ORF-containing cells, long-term culture could result in cell lysates containing very low levels of GST-tagged protein and high levels of GST (as illustrated in Fig. 2a, bottom panel). This may explain the very low levels of tagged protein expression we observed (Fig. 2c), and suggests that there are probably more Cdk substrates among the 156 proteins that were not detectable by silver stain and apparently not phosphorylated.

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## addendum

# An expressed pseudogene regulates the messenger-RNA stability of its homologous coding gene

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In this Letter, it is shown using transgene insertion mouse mutants that the *Makorin1-p1* pseudogene regulates the expression of its related coding gene. An example has been drawn to our attention of another transcribed pseudogene that regulates the expression of its related coding gene, but by a different mechanism, in the mollusc *Lymnaea stagnalis*<sup>1</sup>. □

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