Supplemental Information

Dynamics of Cdk1 Substrate Specificity during the Cell Cycle

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(A) Phos-Tag SDS-PAGE western blotting experiments using constitutively expressed Sic1ΔC-(T2)T5/T33/T45/T76 constructs bearing the Clb2-specific P-X-T-P-X-K or the Cln2-specific P-X-T-P-K-X substrate consensus motifs, and the same constructs containing both vllpp and 1234rxl mutations. Cells were released from α-factor arrest and the phosphorylation-dependent mobility shifts were followed as the cells progressed through the cell cycle. Sic1ΔC-3HA with all the Cdk1 consensus sites mutated to alanines (Sic1ΔC-9A) was included as a control. The signals were scanned using the GelDoc (GE) and the indicated phospho-bands were quantified to generate the plots shown in Figure 5A and B.

(B) We confirmed that the changing fraction of the phosphorylation shifts was Cdk1-dependent, using a yeast strain with an analog-sensitive Cdk1 allele (as-Cdk1, (Bishop et al., 2000)). The specific inhibitor 1NM-PP1 was added at the indicated time points after the shifts had already formed. The phosphoshifts present in G1 were not removed by the inhibitor and are probably caused by some other kinase. These bands were also included in the calculations of the steady-state phosphorylation levels.

(C) A similar experiment as in (B), except that the inhibitor was added immediately after the release of cells from the α-factor arrest.

(D) Cln2- and Clb5-dependent phosphorylation in vitro of Sic1ΔC-(T2)T5/T33/T45/T76 constructs and their docking site mutants (vllpp or 1234rxl). Separation of the phosphorylated forms was performed using Phos-Tag SDS-PAGE followed by autoradiography.
Inhibitory phosphorylation specificity of Swe1 towards Cdk1 is cyclin-specific and correlates with changes in the optimal consensus site specificity of Cdk1.

(A) Phosphorylation of different cyclin-Cdk1 complexes by purified Swe1 compared with phosphorylation of Swe1 by the same kinase complexes.

(B) The relative $k_{cat}/K_M$ profile for Swe1-catalyzed phosphorylation of cyclin-Cdk1 complexes (open bars) compared with the $k_{cat}/K_M$ profile for the cyclin-Cdk1-catalyzed phosphorylation of Swe1 (black bars) and of the model substrate PKTPKKAKKL (grey bars). The values for different cyclin-Cdk1 complexes are normalized relatively to the values obtained for Clb2-Cdk1.
Table S1
Specificity constants ($k_{cat}/K_M$) determined for four representative cyclin-Cdk1 complexes and for the hydrophobic patch mutants (hpm) of the three B-type cyclin-Cdk1 complexes using forms of T33-Sic1ΔC with varied amino acids around the phosphorylation site T33.

| Substrate                      | $k_{cat}/K_M$, µM⁻¹min⁻¹ |
|-------------------------------|--------------------------|
|                              | Cln2 | Clb5 | Clb3 | Clb2 | Clb5hpm | Clb3hpm | Clb2hpm |
| QKTPQKPSQNL                   | 3.21 |  18  |  8.13 |  3.94 |    0.33  |    0.51  |    2.04  |
| QKTPQKPSQNL-1234rxl           | 0.70 |  0.61|   1.08|   1.71|    0.19  |    0.45  |    1.52  |
| QKTPQAPSQNL                   | 1.00 |  1.38|  0.27 |  0.26 |    0.02  |    0.04  |    0.16  |
| PKTPQKPSQNL                   | 4.89 |  13.8|  7.26 |  9.31 |    0.40  |    0.52  |    1.76  |
| PKTPQAPSQNL                   | 1.49 |  0.30|  0.20 |  0.35 |    0.03  |    0.04  |    0.09  |
| QKTPKAPSQNL                   | 1.73 |  0.28|  0.32 |  0.39 |    0.02  |    0.03  |    0.13  |
| QKTPRAPSQNL                   | 3.02 |  2.74|  0.73 |  0.39 |     -     |     -     |     -     |
| PKTPKAPSQNL                   | 4.14 |  1.29|  1.83 |  0.88 |    0.02  |    0.02  |    0.21  |
| QKTPKKPSQNL                   | 2.32 |  7.74|  4.57 |  5.02 |    0.19  |    0.36  |    1.67  |
| PKTPKKPSQNL                   | 2.28 |  13.4|  7.07 |  9.77 |    0.42  |    0.74  |    3.28  |
| PKTPQKKKKNL                   | 2.74 |  24.4|  31.7|  39.0 |    1.11  |    4.22  |    17.3  |
| PKTPQKKKKNL-1234rxl           | 0.80 |  1.11|  4.22 |  17.3 |     -     |     -     |     -     |
| PKTPKKAKKLL**                 | 4.97 |  50.0|  58.0|  24.1 |    1.35  |    4.85  |   12.35  |
| QKAPQKPSQNL                   | 0.02 |  0.04|  0.04 |  0.10 |    0.06  |    0.09  |    0.07  |
| QKTAQKPSQNL                   | 0.05 |  0.10|  0.16 |  0.19 |    0.02  |    0.02  |    0.04  |

* - Assay performed in the presence of LP peptide.
** - Cdk phosphorylation motif of Histone H1.
Table S2
Specificity constants ($k_{cat}/K_M$) for the phosphorylation of different substrates of Cdk1 determined with the four representative cyclin-Cdk1 complexes and the hydrophobic patch mutants (hpm) of the three B-type cyclin-Cdk1 complexes (relative values, not divided by [S]).

| Substrate | Cln2 | Clb5 | Clb3 | Clb2 | Clb5hpm | Clb3hpm | Clb2hpm |
|-----------|------|------|------|------|---------|---------|---------|
| Histone H1 | 1.394 | 0.605 | 2.237 | 4.900 | 0.427 | 1.957 | 4.287 |
| ACE2      | 0.025 | 0.004 | 0.029 | 0.069 | -       | -       | -       |
| ASH1      | 0.422 | 0.111 | 0.697 | 0.462 | 0.052 | 0.134 | 0.389 |
| BOP3      | 0.521 | 0.027 | 0.101 | 0.109 | -       | -       | -       |
| CDC6      | 0.084 | 2.898 | 2.268 | 0.078 | 0.138 | 0.350 | 1.688 |
| CDH1      | 0.016 | 0.025 | 0.024 | 0.035 | 0.006 | 0.008 | 0.023 |
| EXO84     | 0.084 | 0.019 | 0.031 | 0.074 | 0.006 | 0.014 | 0.062 |
| FAR1      | 0.042 | 0.115 | 0.031 | 0.029 | 0.003 | 0.008 | 0.033 |
| FIN1      | 1.800 | 23.02 | 10.96 | 12.43 | 3.651 | 7.488 | 14.08 |
| FIR1      | 0.042 | 0.032 | 0.223 | 0.836 | 0.008 | 0.017 | 0.111 |
| MSA1      | 0.157 | 0.024 | 0.043 | 0.034 | -       | -       | -       |
| NDD1      | 0.143 | 0.388 | 2.121 | 3.129 | 0.096 | 0.222 | 1.108 |
| ORC2      | 0.073 | 0.405 | 0.132 | 0.018 | 0.012 | 0.028 | 0.067 |
| ORC6      | 0.002 | 0.029 | 0.018 | 0.009 | 0.001 | 0.002 | 0.005 |
| PDS1      | 0.249 | 0.008 | 0.015 | 0.049 | 0.002 | 0.005 | 0.035 |
| PLM2      | 0.428 | 1.724 | 9.051 | 9.072 | 0.428 | 1.144 | 6.351 |
| PXL1      | 0.031 | 0.031 | 0.139 | 0.571 | 0.015 | 0.033 | 0.244 |
| RTT109    | 0.067 | 0.010 | 0.027 | 0.080 | -       | -       | -       |
| SLI15     | 0.422 | 0.025 | 0.141 | 0.109 | 0.004 | 0.032 | 0.060 |
| STB1      | 2.011 | 0.191 | 0.622 | 0.878 | -       | -       | -       |
| SWI5      | 0.178 | 0.475 | 1.686 | 2.961 | 0.172 | 0.207 | 1.387 |
| SWI6      | 0.004 | 0.118 | 0.105 | 0.412 | 0.058 | 0.075 | 0.481 |
| TOS4      | 0.708 | 0.502 | 8.484 | 2.373 | 0.593 | 2.397 | 2.136 |
| TOS8      | 2.830 | 0.069 | 0.276 | 0.254 | -       | -       | -       |
| WHI5      | 0.283 | 0.016 | 0.033 | 0.046 | 0.001 | 0.006 | 0.027 |
| XBP1      | 0.200 | 0.020 | 0.065 | 0.103 | 0.007 | 0.013 | 0.084 |
| YHP1      | 0.524 | 0.020 | 0.066 | 0.385 | 0.002 | 0.009 | 0.046 |
| YML119w   | 0.048 | 0.029 | 0.134 | 0.130 | -       | -       | -       |
| YOX1      | 0.584 | 0.244 | 2.094 | 4.536 | -       | -       | -       |
**Table S3**

Plasmid constructs used in this study.

| Plasmid                  | Description                                                                 |
|--------------------------|-----------------------------------------------------------------------------|
| pMK0001-0052             | Sic1AA-T33KTPQKPSQNL-ΔC-pET28a and mutated variants                         |
| pMK0063                  | ACE2-pET28a                                                                 |
| pMK0064                  | ASH1-pET28a                                                                 |
| pMK0065                  | BOP3-pET28a                                                                 |
| pMK0066                  | CDC6-pET28b                                                                 |
| pMK0067                  | EXO84-pET28a                                                                |
| pMK0068                  | FIN1-pET28a                                                                 |
| pMK0069                  | FIR1-pET28a                                                                 |
| pMK0070                  | NDD1-pET28a                                                                 |
| pMK0071                  | ORC2-pET28a                                                                 |
| pMK0072                  | ORC6-prSAB1234                                                              |
| pMK0073                  | PDS1-pGEX-4T-1                                                              |
| pMK0074                  | PLM2-pET28a                                                                 |
| pMK0075                  | PXL1-pET28a                                                                 |
| pMK0076                  | RTT109-pET28a                                                               |
| pMK0077                  | SLI15-pET28a                                                                |
| pMK0078                  | STB1-pET28a                                                                 |
| pMK0079                  | SWI5-pET28a                                                                 |
| pMK0080                  | SWI6-pET28a                                                                 |
| pMK0081                  | TOS4-pET28a                                                                 |
| pMK0082                  | TOS8-pET28a                                                                 |
| pMK0083                  | WHI5-pET28a                                                                 |
| pMK0084                  | XBP1-pET28a                                                                 |
| pMK0085                  | YML119W-pET28a                                                              |
| pMK0086                  | MSA1-pET28a                                                                 |
| pMK0087                  | YHP1-pET28a                                                                 |
| pMK0088                  | YOX1-pET28a                                                                 |
| pMK0089                  | YPR174C-pET28a                                                              |
| pMK0090                  | Sic1AA-T33KTPQKPSQNL-1234rxl-ΔC-pET28a                                      |
| pMK0091                  | Sic1AA-ΔC-pET28a                                                            |
| pMK0092-0097             | Sic1AP-T33-ΔC-pET28a and rxl variants                                       |
| pMK0098-0103             | Sic1AP-S76-ΔC-pET28a and rxl variants                                       |
| pMK0104                  | Sic1AA-T33KTPQAPSQNL-1234rxl-ΔC-pET28a                                      |
| pMK0105                  | Sic1AA-T33KTPKAPSQNL-1234rxl-ΔC-pET28a                                      |
| pMK0106                  | Sic1AA-T33KTPKAPSQNL-1234rxl-ΔC-pET28a                                      |
| pMK0107                  | Sic1AA-T33KTPQKKNKNL-1234rxl-ΔC-pET28a                                      |
| pMK0114-0119             | Sic1AA-T5-ΔC-pET28a and rxl variants                                       |
| pMK0125                  | Sic1AP-T45-1234rxl-ΔC-pET28a                                               |
| pMK0131                  | Sic1AP-S69-1234rxl-ΔC-pET28a                                               |
| pMK0137                  | Sic1AP-S80-1234rxl-ΔC-pET28a                                               |
| pMK0143                  | Sic1AP-T173-1234rxl-ΔC-pET28a                                              |
| pMK0149                  | Sic1AP-S191-1234rxl-ΔC-pET28a                                              |
| pMK0150                  | Sic1wt-ΔC-pET28a                                                            |
| pMK0184-0190             | Sic1wt-T2(1-85)-pET28a and other truncated variants                         |
| pMK0191                  | Sic1wt-v/lpp(136AAAAA140)-ΔC-pET28a                                        |
| pMK0192                  | Sic1AP-PXTPKΔC-pET28a                                                       |
| pMK0193                  | Sic1AP-PXTPKA-1234rxl-ΔC-pET28a                                            |
| pMK0194                  | Sic1AP-PXTPKA-v/lpp-ΔC-pET28a                                              |
| pMK0195                  | Sic1AP-PXTPAK-ΔC-pET28a                                                     |
| pMK0196                  | Sic1AP-PXTPAK-1234rxl-ΔC-pET28a                                            |
| pMK0197                  | Sic1AP-PXTPAK-v/lpp-ΔC-pET28a                                              |
| pMK0500 | Sic1wt-3XHA-pRS413 |
|---------|-------------------|
| pMK0502 | Sic1wt-123rxl-3XHA-pRS413 |
| pMK0504 | Sic1wt-23rxl-3XHA-pRS413 |
| pMK0505 | Sic1wt-14rxl-3XHA-pRS413 |
| pMK0525 | Sic1wt-2rxl-3XHA-pRS413 |
| pMK0526 | Sic1wt-3rxl-3XHA-pRS413 |
| pMK0527 | Sic1wt-vllpp-3XHA-pRS413 |
| pMK0528 | Sic1wt-2rxl-vllpp-3XHA-pRS413 |
| pMK0529 | Sic1wt-3rxl-vllpp-3XHA-pRS413 |
| pMK0530 | Sic1AP-PXTPKA-ΔC-3XHA-pRS315 |
| pMK0531 | Sic1AP-PXTPKA-1234rxl-vllpp-ΔC-3XHA-pRS315 |
| pMK0532 | Sic1AP-PXTPAK-ΔC-3XHA-pRS315 |
| pMK0533 | Sic1AP-PXTPAK-1234rxl-vllpp-ΔC-3XHA-pRS315 |
| pMK0534 | Sic1AP-ΔC-3XHA-pRS315 |
| pMK0545 | Sic1wt-1234rxl-vllpp-3XHA-pRS413 |
### Table S4

Yeast strains used in this study.

| Strain   | Description                                                                 |
|----------|----------------------------------------------------------------------------|
| DOM0949  | CLN2-3HA:HISMX6 bar1:hisG                                                  |
| DOM0962  | CLB5-3HA:HISMX6 bar1:hisG                                                  |
| DOM0950  | CLB3-3HA:HISMX6 bar1:hisG                                                  |
| DOM0948  | CLB2-3HA:HISMX6 bar1:hisG                                                  |
| DOM0976  | gal-CLB5-TAP pRSAB1234-URA3 bar1:HisG sic1d::LEU2                         |
| DOM0977  | gal-CLB2-TAP pRSAB1234-URA3 bar1:HisG sic1d::LEU2                         |
| DOM0957  | gal-CLB3-TAP pRSAB1234-URA3 bar1:HisG sic1d::LEU2                         |
| DOM0963  | gal-CLB5hpm-TAP pRSAB1234-URA3 bar1:HisG sic1d::LEU2                       |
| DOM0964  | gal-CLB2hpm-TAP pRSAB1234-URA3 bar1:HisG sic1d::LEU2                       |
| DOM0958  | gal-SWE1-TAP pRSAB1234-URA3                                               |
| MK0168   | gal-CLB3hpm-TAP pRSAB1234-URA3 bar1:HisG sic1d::LEU2                       |
| MK0169   | gal-ORC6-TAP pRSAB1234-URA3 bar1:HisG                                      |
| DMY305   | gal-CLN2-3HA                                                                |
| DOM0030  | cdc28::cdc28 as1 bar1:HisG                                                 |
| DOM0090  | bar1:HisG                                                                  |
| MK0260   | DOM0030 [Sic1AP-PXTPAK-ΔC-3XHA-pRS315]                                      |
| MK0261   | DOM0030 [Sic1AP-ΔC-3XHA-pRS315]                                             |
| MK0262   | DOM0090 [Sic1AP-PXTPKA-ΔC-3XHA-pRS315]                                      |
| MK0263   | DOM0090 [Sic1AP-1234rxl-vllpp-PXTPKA-ΔC-3XHA-pRS315]                        |
| MK0264   | DOM0090 [Sic1AP-PXTPAK-ΔC-3XHA-pRS315]                                      |
| MK0265   | DOM0090 [Sic1AP-1234rxl-vllpp-PXTPAK-ΔC-3XHA-pRS315]                        |
| MK0266   | cln2d::TRP1; bar1:HisG                                                     |
| MK0267   | DOM0090 [Sic1wt-3XHA-pRS413]                                                |
| MK0268   | DOM0090 [Sic1wt-2rxl-3XHA-pRS413]                                           |
| MK0269   | DOM0090 [Sic1wt-3rxl-3XHA-pRS413]                                           |
| MK0270   | DOM0090 [Sic1wt-23rxl-3XHA-pRS413]                                          |
| MK0271   | DOM0090 [Sic1wt-14rxl-3XHA-pRS413]                                          |
| MK0272   | DOM0090 [Sic1wt-1234rxl-3XHA-pRS413]                                        |
| MK0273   | DOM0090 [Sic1wt-vllpp-3XHA-pRS413]                                          |
| MK0274   | MK0266 [Sic1wt-3XHA-pRS413]                                                 |
| MK0275   | MK0266 [Sic1wt-vllpp-3XHA-pRS413]                                           |
| MK0276   | MK0266 [Sic1wt-2rxl-3XHA-pRS413]                                            |
| MK0277   | MK0266 [Sic1wt-3rxl-3XHA-pRS413]                                            |
| MK0278   | MK0266 [Sic1wt-2rxl-vllpp-3XHA-pRS413]                                      |
| MK0279   | MK0266 [Sic1wt-3rxl-vllpp-3XHA-pRS413]                                      |
| MK0310   | DOM0090 [Sic1wt-1234rxl-vllpp-3XHA-pRS413]                                  |
Supplemental Discussion

The four major cyclin-Cdk1 complexes used in our experiments seem sufficient for describing the general dynamic changes of Cdk1 specificity during the cell cycle, as the rest of the cyclins, Clb1 and Clb4 (Grandin and Reed, 1993) as well as Cln3 and Clb6 (Cross et al., 2002; Tyers et al., 1993) are less abundant and therefore minor contributors to the total concentration of activated Cdk1, and most likely would not yield significant changes to the general model. In addition, we found that similarly to Clb3-Cdk1, Clb4-Cdk1, the other G2 complex, also exhibited an intermediate $K_M$ (210 $\mu$M) for the model substrate (data not shown). However, due to poor purification yields, this kinase was excluded from detailed analysis. Additionally, the other G1 complex Cln1-Cdk1, like the closely related Cln2-Cdk1 complex, showed low intrinsic activity towards the peptide substrate ($K_M > 1$ mM, data not shown).

Our findings have raised an intriguing question of how cyclins can change the specificity of the Cdk1 active site, so that its ability to bind and phosphorylate the consensus site motif is altered. These cyclin-specific effects could be due to specific conformational changes induced by cyclin in the Cdk1 molecule, resulting in altered accessibility and configuration of its active site. Interestingly, differential accessibility of the active site is also suggested by the differing ability of Swe1 to phosphorylate different cyclin-Cdk1 complexes at the active site-gating Y19 residue. The other gradually changing feature, the relative differences in RXL-dependent docking mechanisms among the B-type cyclin-Cdk1s, could also be due to different specific conformational changes induced by cyclins, resulting in changes in optimal distance requirements or structural hindrances between the RXL-hp docking site and the active site.
Supplemental Experimental Procedures

Plasmid constructs and yeast strains
For tandem affinity purification (TAP) of cyclin-Cdk1 complexes, the cyclin genes were cloned into the 2 micron vector pRSAB1234 containing a \textit{GAL1} promoter and the C-terminal TAP-tag. To purify different 6His-T33-Sic1ΔC forms for kinase assays, the C-terminally truncated fragment of Sic1 (with a stop codon inserted after amino acid 215) was cloned into the Nhel/BamHI site of the bacterial expression vector pET28a (Invitrogen). Mutagenesis of cyclins or substrates was performed using single-stranded pRSAB1234 or pET28a vector constructs as templates. The triple mutants in the hydrophobic patch (hpm) of Clb5 and Clb2 were described previously (Loog and Morgan, 2005), and the hpm of Clb3 (Phe-201, Leu-205 and Trp-208 mutated to alanines) was designed according to sequence homology with other B-type cyclins. In T33-Sic1ΔC constructs, the other Cdk sites bearing the consensus motif S/T-P were mutated to Ala-Ala in order to prevent any possible (pseudo)substrate competition. The other Cdk substrates were cloned as full-length forms into bacterial expression vectors pET28a or pGEX-4T-1. Cdh1 was purified using the baculovirus system and was a kind gift from Monica Rodrigo-Brenni (UCSF). For purification of 3HA-Cln2-Cdk1, a yeast strain DMY305 (a kind gift from Dr Doug Kellogg, UCSC) with the galactose promoter introduced along with the N-terminal 3HA-tag in the chromosomal locus of the \textit{CLN2} gene was used. The vector construct pCKS1 for bacterial expression of Cks1 was a kind gift from Dr. Adam Rudner. The C-terminally TAP-tagged tyrosine kinase Swe1 was cloned and purified using the 2 micron vector pRSAB1234 analogously to the cyclin-Cdk1 complexes. For C-terminal tagging of cyclin genes in their chromosomal loci, the Pringle method was applied (Longtine et al., 1998). Lists of plasmid vectors and yeast strains used in this study are presented in Tables S3 and S4, respectively.

Protein purification
The TAP method was applied for purification of cyclin-Cdk1 complexes and Swe1 as described previously for Clb5-TAP-Cdk1 and Clb2-TAP-Cdk1 (Puig et al., 2001; Ubersax et al., 2003). For purification of 3HA-Cln2-Cdk1, the yeast strain DMY305 was induced with 2% galactose for 1 hour, and the 3HA-Cln2-Cdk1 complex was purified according to published protocols (McCusker et al., 2007), exploiting immunoaffinity chromatography with a rabbit polyclonal antibody raised against the synthetic 2HA peptide (purchased from Labas, Estonia). We confirmed that the specificity of the Cln2-Cdk1 complex was independent of the nature of the affinity tag used, since Cln2-3HA showed...
similar substrate specificity as the Cln2-Cdk1 version purified using a TAP-tag. N-terminally 6His-tagged recombinant T33-Sic1ΔC constructs and substrates were cloned into the pET28a vector and purified by standard cobalt affinity chromatography and elution with 200 mM imidazole. 6His-Fin1 was purified as described previously (Woodbury and Morgan, 2007). Substrates cloned into the pGEX-4T-1 were purified on glutathione agarose columns using 5 mM reduced glutathione for elution. Cks1 was purified as described previously (Reynard et al., 2000). The optimal working concentration for purified Cks1 was taken as 500 nM based on optimization performed for cyclin-Cdk1 preparations and T33-Sic1ΔC as a substrate.

**Kinase assays**

For the quantitative phosphorylation assays of T33-Sic1ΔC constructs and recombinant substrates, substrate concentrations were kept in the range of 0.5-2 μM (in the linear [S] vs v0 range, several-fold below the estimated K_M value), and the initial velocity conditions were defined as an initial substrate turnover ranging up to 10% of the total turnover. The latter was estimated by a long-term incubation with excess amounts of cyclin-Cdk1 and ^{32}P-γ-ATP in the standard reaction mixture given below. About 1-10 nM of purified kinase complex was used, reaction aliquots were taken at two or more time points, and the reaction was stopped by SDS-PAGE sample buffer. The relative k_cat/K_M values for the substrates were calculated as the ratio of v0/[S], which was determined from at least two independent experiments. The basal composition of the assay mixture contained 50 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% NP-40, 20 mM imidazole, 0.1 mg/ml 2HA peptide, 2% glycerol, 2 mM EGTA, 0.2 mg/ml BSA, 80 μg/ml Cks1, and 500 μM ATP (with added ^{32}P-γ-ATP (Perkin Elmer)). About 1-10 nM of purified kinase complex was used, reaction aliquots were taken at two or more time points, and the reaction was stopped by SDS-PAGE sample buffer. For the steady-state peptide kinetics of the Histone peptide PKTPKKAKKL, a similar assay composition was used as for protein substrates and the standard phosphocellulose method was applied for the quantification of the phosphorylated substrate (Loog and Morgan, 2005). Synthetic peptides as competitor agents were used in 4 mM final concentrations.

Phosphorylation of Cdk1 by purified Swe1 was performed in the standard kinase assay mixture, except that 10 μM ATP was used to amplify the radioactive signal. The Swe1 concentration was optimized so that it was possible to take assay points below 10% of Cdk1 Y19 phosphorylation turnover, and the k_cat/K_M values for Swe1 as the enzyme and for cyclin-Cdk1 as the substrate were estimated as described above for the Cdk1 substrates. For the Cdk1 active site titration
experiments, similar conditions were used, except that more than a 10-fold higher concentration of purified Swe1 was included (Figure 1D).

**Western blotting**
The antibody used for the western blotting of 3HA-tagged proteins was HA.11 Clone 16B12 from Covance, USA. The antibody for the detection of Cdk1 was Cdc28 (yC-20) sc-6709 and the antibody to detect the zz-domain of the TAP-tag was cMyc (A-14) sc-789, both from Santa Cruz Biotechnology, USA. The antibody for the detection of inhibitory phosphorylation at Y19 in Cdk1 was Phospho-cdc2 (Y15) from Cell Signaling Technology and that for the detection of activating phosphorylation of Cdk1 at T169 was a kind gift from Dr Philip Kaldis (IMCB). In case of the time course experiments, the cells were lysed in lysis buffer containing 8M urea, 2M thiourea, 20 mM Tris pH7.4, 4% CHAPS, 1% DTT, 50 mM NaF, 89 mM β-glycerophosphate, 1 mM Na3VO4. The protein concentration of the samples was determined using the Bradford method.

**Peptide labeling with iTRAQ 4-plex reagents**
Purified cyclin-Cdk1 complexes were separated by 1D SDS-PAGE and the gel stained with Colloidal Coomassie blue. Protein bands containing Cdk1 were excised from the gel and the protein within them was submitted to reduction, alkylation and tryptic digestion in 100 mM triethyl ammonium bicarbonate (TEAB) (Applied Biosystems).

Dried tryptic peptides were dissolved in 100 mM triethyl ammonium bicarbonate (TEAB) containing the iTRAQ reagents (114, 115, 116 or 117) (Applied Biosystems) and final concentration of 25% (v/v) ethanol. The samples were incubated at room temperature for 1 h and the excess of reagent was quenched by adding MQ water. After 30 minutes incubation at room temperature samples were pooled together in a 1:1:1:1 ratio (v/v) and dried down in a vacuum centrifuge and purified on C18 StageTips (Rappsilber et al., 2007)

Peptides were separated by reverse-phase chromatography using an Agilent 1200 series nanoflow system (Agilent Technologies) connected to a LTQ Orbitrap classic mass-spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanoelectrospray ion source (Proxeon, Odense, Denmark), essentially as described in (Pulk et al., 2010). Data acquisition and analysis was done as described in (Kocher et al., 2009), with slight modifications. Peak lists were extracted from .raw files with Proteome Discoverer 1.1 and searched with Mascot 2.3 (<www.matrixscience.com>) against yeast database complemented with common contaminant sequences such as trypsin,
keratins etc. Peptides were identified based on CID MS/MS scans and reporter ions from HCD scans were extracted manually from corresponding .raw files.
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