Global Analysis of Cdc14 Dephosphorylation Sites Reveals Essential Regulatory Role in Mitosis and Cytokinesis*

Li Kao‡, Yi-Ting Wang§¶, Yu-Chen Chen‡, Shun-Fu Tseng‡, Jia-Cin Jhang‡, Yu-Ju Chen§¶** and Shu-Chun Teng‡**‡‡

Degradation of the M phase cyclins triggers the exit from M phase. Cdc14 is the major phosphatase required for the exit from the M phase. One of the functions of Cdc14 is to dephosphorylate and activate the Cdh1/APC/C complex, resulting in the degradation of the M phase cyclins. However, other crucial targets of Cdc14 for mitosis and cytokinesis remain to be elucidated. Here we systematically analyzed the positions of dephosphorylation sites for Cdc14 in the budding yeast *Saccharomyces cerevisiae*. Quantitative mass spectrometry identified a total of 835 dephosphorylation sites on 455 potential Cdc14 substrates in vivo. We validated two events, and through functional studies we discovered that Cdc14-mediated dephosphorylation of Smc4 and Bud3 is essential for proper mitosis and cytokinesis, respectively. These results provide insight into the Cdc14-mediated pathways for exiting the M phase. *Molecular & Cellular Proteomics* 13: 10.1074/mcp.M113.032680, 594–605, 2014.

All cells proliferate following a fixed, highly coordinated cycle. Mitosis especially requires elaborate coordination for proper chromosome segregation, mitotic spindle disassembly, and cytokinesis. Much of this activity is facilitated by numerous, diverse phosphorylation and dephosphorylation signals that orchestrate the precise progression of M phase.

Prior to mitosis, sister chromatids resulting from DNA replication during S phase are held together by the cohesion complex. Then, during prophase, chromosomes are condensed by the condensin (Smc2/4) complex (1) and microtubules are remodeled to form the mitotic spindle (2). Subsequently, in metaphase, the microtubules of the spindle apparatus attach to the chromosome kinetochores (3) and dissolution of the sister chromatids is triggered by the sepa-

rase-mediated cleavage of cohesin (4, 5). Finally, Cdc14, Cdh1, and APC/C work together in telophase to degrade the M phase cyclins (6), promote decondensation of chromosomes (7), and finish cytokinesis (8, 9).

Cdc14, a dual-specificity phosphatase that removes the phosphate group on both phosphotyrosine and phosphoserine/threonine residues (10), is required for mitosis (11, 12). Specifically, Cdc14 function is essential in late M phase: cells carrying a defective mutation arrest in telophase (13), whereas overexpression of Cdc14 results in G1 arrest (12). Cdc14 triggers mitotic cyclin-dependent kinase (CDK)1 inactivation, enabling cells to exit mitosis through dephosphorylation and activation of the inhibitors of CDKs. At interphase, Cdc14 is a subunit of the mitotic exit network (14–17), which usually localizes to the nucleolus. However, the Cdc14 early ana-

phase release network initiates the release of Cdc14 from its inhibitor, Net1/Cfi1 (18), and the mitotic exit network promotes further release of Cdc14 from its inhibitor, allowing it to spread into the nucleus and cytoplasm, where it dephosphory-

lates its major targets (8, 9), leading to exit from mitosis. In addition to this essential role in late M phase, Cdc14 substrates have also been identified in other stages of the cell cycle (19).

Cdc14 putatively regulates 27 proteins (19–22). Some studies have documented the substrates of Cdc14 via in vitro phosphatase assay, whereas others have provided in vivo evidence. However, dephosphorylation sites have been identified for only five of the target proteins (17, 22–25), suggesting that spurious relationships cannot be ruled out. Also, experi-

ments have not been carried out to demonstrate whether these modifications entail direct or indirect regulation. There-

fore, our understanding of Cdc14 function and regulation during mitosis in metazoans is incomplete. Conceivably,
Cdc14 may regulate many more substrates involved in aspects of chromosome condensation and cytokinesis. To examine this possibility we performed a systematic phosphoproteomic screen to identify new in vivo pathways regulated by Cdc14. Using this approach, we identified both known and potentially novel substrates of Cdc14, as well as their dephosphorylation sites. Many potentially novel substrates are physically associated with Cdc14 in public databases. We also provide biochemical evidence for direct dephosphorylation of the substrates, characterize the specificity of dephosphorylation in two substrates, Smc4 and Bud3, and further study their regulation and critical role in mitosis and cytokinesis.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**—All yeast operations were performed using standard methods. Strains used in this study are listed in [supplemental Table S1](#). Cultures were grown in rich medium (YPED: 1% yeast extract, 2% bacto-peptone, 2% dextrose) or, for plasmid transformation, in synthetic complete medium (0.67% yeast nitrogen base; 2% glucose, raffinose, or galactose). S288C and cdc28-4 (26) strains were used to detect phosphorylation and Y300 and cdc14-1 strains were used to detect dephosphorylation in vivo. CDC14, BUD3, and SMC4 were PCR-amplified and cloned into pRS306. Point mutations were introduced into plasmids using QuickChange site-directed mutagenesis (Stratagene, Santa Clara, CA). The cdc14-1 strain was constructed by insertion of pRS306-cdc14-D323G following 5-FOA-resistant selection. To generate chromosomal mutations of BUD3 and SMC4, pRS306-bud3 and pRS306-smc4 were linearized by EcoRI and Hpal, respectively, and transformed into the wild-type cells. The URA3 pop-out mutants were selected from the 5-FOA-resistant transformants. The mutations were checked by PCR and sequencing. pGH-bud3Cter (1534–1636) was constructed by ligating the BamHI/HindIII-treated fragment containing amino acids 1534–1636 of Bud3 into the BamHI/HindIII-digested pGH. Details of plasmid constructions are available upon request. Chromosomal tagging was constructed as previously described (27, 28).

**Gel-assisted Digestion**—Protein samples from yeast were subjected to gel-assisted digestion (29, 30). Samples were fixed into a gel directly in the Eppendorf vial with acrylamide/bisacrylamide solution (40%, v/v, 29:1), 10% (w/v) ammonium persulfate, 100% N,N,N’-tetramethyl-enediamine in a 14:5:0:7.0:0.3 ratio (v/v). The gel was cut into small pieces, washed several times with 25 mM triethylammonium bicarbonate containing 50% (v/v) ceric ammonium nitrate (CAN), further dehydrated with 100% CAN, and completely dried by vacuum centrifugation. Trypsin was then added into gels for proteolytic digestion (protein:trypsin = 50:1, g/g) in 25 mM triethylammonium bicarbonate with overnight incubation at 37 °C. Digestes were extracted three times with 5% (v/v) formic acid in 50% (v/v) acetonitrile for 30 min and completely dried by vacuum centrifugation at room temperature.

**Immobilized Metal Affinity Chromatography Procedure**—Phosphopeptide purification was performed using an immobilized metal affinity chromatography protocol (31). First, the column was capped at one end with a 0.5-μm frit disk encased in a stainless steel column-end fitting. The nickel-nitrilotriacetic acid resin was activated by 100 μl 0.2 mM FeCl3 and equilibrated with loading buffer for 15 min before sample loading. The loading-condition buffer was 6% (v/v) acetic acid (pH 3.0). The peptide samples from trypsin digestion were reconstituted in loading buffer and loaded into an activated immobilized metal affinity chromatography column pre-equilibrated with the same loading buffer for 12 min. The unbound peptides were then removed with 100 μl washing solution consisting of 75% (v/v) loading buffer and 25% (v/v) acetonitrile, followed by equilibration with loading buffer for 15 min. Finally, the bound peptides were eluted with 100 μl of 200 mM NH4H2PO4 (pH 4.4). Eluted peptide samples were dried and reconstituted in 0.1% (v/v) TFA (40 μl) for further desalting and concentration using ZipTips™ (Millipore, Bedford, CA).

**LC-MS/MS Analysis**—Purified phosphopeptides were reconstituted in buffer A (0.1% formic acid in H2O) and analyzed on an LTQ-Orbitrap (Thermo Electron, Bremen, Germany) in triplicate. For LTQ-Orbitrap analysis, LC-MS/MS was performed on an Agilent 1100 series HPLC (Agilent, Waldbronn, Germany) with a micro-T for flow splitting connected to an LTQ-Orbitrap XL hybrid mass spectrometer. Peptides were loaded onto a 25 cm × 75 μm fused-silica capillary column packed in-house with C18 Macherey-Nagel, GmbH & Co. KG, Düren, Germany) and separated using a segmented gradient over 100 min from 2% to 80% buffer B. Survey full-scan MS spectra were acquired in the Orbitrap (m/z 350–1600) with the resolution set to 60,000 at m/z 400 and the automatic gain control target at 106. The 10 most intense ions were sequentially isolated for collision-induced dissociation MS/MS fragmentation and detection in the linear ion trap (automatic gain control target at 7000) with previously selected ions dynamically excluded for 90 s. To improve the fragmentation spectra of the phosphopeptides, “multistage activation” at 97.97, 48.99, and 32.66 Thompson (Th) relative to the precursor ion was enabled in all MS/MS events. All the measurements in the Orbitrap were performed with the lock mass option for internal calibration.

**Database Search**—Raw MS/MS data from the LTQ-Orbitrap were searched against the Swiss-Prot database (Baker’s yeast) database (version 54.2, 6493 sequences) with the following exceptions: only tryptic peptides with up to two missed cleavage sites were allowed, and the fragment ion mass tolerance was set to 0.6 Da. Phosphorylation (STY) and oxidation (M) were specified as variable modifications. Peptides were considered identified if their Mascot ion scores were greater than 20 (p < 0.05). The false discovery rates for Orbitrap data were considered identified if their Mascot ion scores were greater than 20 (p < 0.05) from each LC-MS/MS run were loaded and merged to establish a global peptide information list (sequence, elution time, and mass-to-charge ratio). Alignment of elution time was then performed based on the peptide information list using linear regression in different LC-MS/MS runs followed by correction of aberrational chromatographic shifts across fragmental elution-time domains. To
increase correct assignment, the detected peptide peaks were validated by means of SCI validation using three criteria: (i) signal-to-noise ratio > 3, (ii) accurate charge state, and (iii) correct isotope pattern. To calculate the relative peptide abundance, the tool performs reconstruction of extracted ion chromatography and calculates the area of the extracted ion chromatograph. The fold-change of a given peptide was calculated based on the ratio of relative peptide abundance between different samples. Finally, the quantitation result for each phosphopeptide was manually checked. The analysis of identified peptides is presented in supplemental Tables S2 and S3.

Gene Ontology Analysis—Gene Ontology analysis was conducted using an online tool available at the Saccharomyces Genome Database website (version 8.03) (36). The default parameter settings were used and analyzed on October 3, 2013, using the “calculate false discovery rate” option for multiple hypothesis correction. The Bonferroni correction value was calculated using Generic GO Term Finder (with default options).

Generation of Phospho-specific Antibodies—Peptides were designed as indicated: RRLLELSLsPVKNSR for phosphorylation of Smc4 residue S128; epivpskpskidfl and qkdepivpskpskid for phosphorylation of Bud3 residue S1549; and rlktalkvpmtypl and lsrtkalk percept for phosphorylation of Bud3 residue T1566. A cysteine residue was added to the C terminus to facilitate conjugation with a carrier protein for greater immunogenicity. To generate antibodies, rabbits were boosted with carrier-conjugated phosphopeptides once per month. Pre-immune sera were collected before boost. Injection was conducted every 4 weeks, and blood samples were collected every 2 weeks. Blood samples were incubated at 37 °C for 30 min, and serum and blood cells were separated via high-speed centrifugation. Clarified serum was incubated at 56 °C for 30 min to remove complements. The specificity of antibodies was verified by means of peptide dot blot analysis.

Protein Analysis—Whole cell proteins were extracted via trichloroacetic acid precipitation and resolved via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Smc4 was detected with a Myc antibody (Roche) and the phospho-antibody, and Bud3 was detected with an HA antibody (Covance, Princeton, NJ, monoclonal antibody, HA.11) and the phospho-antibody. Cells were also stained with propidium iodide, and the cell cycle analysis was performed using a flow cytometer (FACS Calibur 200, BD Biosciences). Recombinant proteins were overexpressed in E. coli BL21 pLys strains and purified using glutathione Sepharose beads. Proteins were eluted by elution buffer (50 mM Tris-HCl, pH 8.0, 20 mM reduced glutathione, and 10% glycerol). The bound GST-fusion proteins were detected by means of Coomassie Blue staining.

In Vitro Kinase and Phosphatase Assays—Log phase cells were lysed and immunoprecipitated via incubation with an HA antibody (Covance, monoclonal antibody, HA.11) and protein G beads (Thermo). The immunoprecipitation–kinase assay was performed as described elsewhere (37). Cdc28-HA or the kinase-dead Cdc28<sup>K40L-HA</sup> were purified from a protease-deficient strain, BJ2168, carrying plasmid pKB174 (CDC28-HA) or pKB173 (cdc28<sup>K40L-HA</sup>), kindly provided by Dr. Katsunori Sugimoto. After immunoprecipitation–kinase assay, phosphorylated Bud3 and Smc4 were desalted and changed to phosphatase buffer by Microcon 10K (Millipore) for further phosphatase assay as described elsewhere (22).

Yeast Two-hybrid—β-galactosidase assays were performed as modified from Miller’s protocol (38, 39), pE2G02-LexA-CLB2, pJ4G–5-bud3Cter, and their derivatives were transformed into strain EGY191. The final A600 of the cell cultures was measured before cells were collected for β-galactosidase assays. Cells were suspended in 1 ml of Z buffer with 50 µl of chloroform and 20 µl of 0.1% SDS. 200 µl of o-nitrophenyl-β-D-galactoside (4 mg/ml in Z buffer) were added, and the solution was mixed and kept at 28 °C. Once the reaction mixture turned yellow, 400 µl of 1 M Na2CO3 was added to stop the reaction. The reaction mixture was microfiltered, and the A420 of the supernatant was measured. β-galactosidase units were calculated as follows:

\[ \text{β-galactosidase units} = (A420/A600) \times V \times t \]  
(Eq. 1)

where \( t \) is the reaction time in minutes and \( V \) is the volume of culture assayed in milliliters.

Quantitative Fluorescence Microscopy—Cells were cultured in YEPD medium for 20 h, washed by sterilized water, and stained with Calcofluor (Sigma-Aldrich) as described elsewhere (40). Calcofluor binds the chitin ring at the bud scar, which is retained on the mother cells after budding. Cells with three or more bud scars were scored for budding pattern under microscopy with UV excitation. At least 300 cells were scored for each strain. Induction and detection of GFP–Cib2 fusion protein were performed as described elsewhere (41).

Immunoprecipitation—Immunoprecipitation was performed as described (42) using EDB buffer (100 mM KCl, 50 mM HEPES-KOH (pH 7.5), 2.5 mM MgCl<sub>2</sub>, 50 mM NaF, 5 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 0.1 mM NaVO<sub>4</sub>, 0.25% Triton X-100) containing Complete protease inhibitors (Roche). Cell lysates were incubated for 2 h at 4 °C with anti-HA antibodies (Covance, monoclonal antibody, HA.11) and protein G beads (Thermo). The beads were washed three times with 1 ml of EBX. Proteins bound to antibodies were eluted in 50 µl of SDS-PAGE loading buffer.

Chromatin Fractionation—Cell lysates were fractionated into chromatin fractions according to Bilski’s protocol (43). Cells from 100 ml of logarithmically growing cultures were harvested via centrifugation, resuspended in buffer A (100 mM HEPES pH 8, 25 mM DTT), and incubated at 30 °C for 10 min. Cells were centrifuged and resuspended in buffer B (0.6 M sorbitol, 25 mM Tris pH 7.4, 4 mg/ml Zymolyase 20T, in yeast extract–peptone–dextrose medium plus ad- enine). Suspensions were incubated for a further 40 min at 30 °C. Spheroplasts were centrifuged at 2000 × g for 3 min and gently resuspended in buffer C (0.7 M sorbitol, 25 mM Tris pH 7.4, in yeast extract–peptone–dextrose medium plus adenine). Spheroplasts were allowed to recover at 30 °C for 15 min. Spheroplasts were centrifuged at 2000 × g for 3 min and washed in lysis buffer (0.4 M sorbitol, 150 mM KAc, 2 mM MgAc, 20 mM HEPES pH 6.8, protease inhibitors). Spheroplasts were chilled, washed in lysis buffer, and resuspended in 400 µl of lysis buffer. Triton X-100 was added to a final concentration of 1% to lyse the spheroplasts. The lysates were centrifuged at 4 °C and 20,000 × g for 15 min. Pellets (chromatin fraction) were washed three times in 1 ml of lysis buffer and resuspended in SDS-PAGE loading buffer.

RESULTS

Large-scale Identification of Potential Substrates of Cdc14 and Their Dephosphorylation Sites—To identify Cdc14-mediated dephosphorylation sites globally, we cultured G2/M arrested wild-type and cdc14-1 temperature-sensitive mutant yeast cells at a permissive temperature (23 °C) and then shifted them to a nonpermissive temperature (37 °C). We collected whole cell extracts that were proteolytically digested with trypsin and passed through a homemade immobilized metal affinity column (31) for phosphopeptide purification. The purified phosphopeptide samples were reconstituted in buffer and analyzed via liquid chromatography–tandem mass spectrometry (LC-MS/MS) for quantitative phosphoproteomics.
We collected 8484 MS/MS spectra, of which 5062 were successfully matched to phosphopeptide sequences ($p < 0.05$). In total, 3641 different phosphorylation sites were confidently assigned to 1243 proteins. The log$_2$ $\frac{cdc14-1}{CDC14}$ ratios (i.e. fold-change of expression in response to mutation) for phosphopeptides and phosphoproteins were broadly distributed (Fig. 1A). A rightward shift in the log$_2$ $\frac{cdc14-1}{CDC14}$ ratio of phosphopeptides indicated that the abundance of phosphopeptides increased when Cdc14 activity was inhibited, which is in agreement with the property of a phosphatase. It also demonstrated that Cdc14 is a major phosphatase in the cell cycle. To identify potential Cdc14 substrates, we filtered the log$_2$ $\frac{cdc14-1}{CDC14}$ ratio with a threshold of 2-fold determined from the technical replicate (34). A total of 835 dephosphorylation sites on 455 potential Cdc14 substrates showed a 2-fold increase in $\frac{cdc14-1}{CDC14}$ cells in vivo (Fig. 1B). A gene ontology analysis of the candidate substrates revealed a strong enrichment for cell-cycle-related functional categories (such as GO:0007049, Cell Cycle; hypergeometric $p$ value $< 10^{-14}$) (supplemental Table S4 and supplemental Fig. S1). At the stage of mitotic exit, Cdc14 tends to trigger mitotic exit by reversal of M phase kinase-mediated phosphorylation (12). We therefore compared the phosphorylation motifs of these phosphopeptides to find consensus sequences of Cdk1, Ipl1 (aurora), and Cdc5 (polo-like) (three major M phase kinases) consensus sequences (Fig. 1B), and many phosphopeptides indeed displayed the consensus of these kinases. Consistently, an enhancement of subunits in several pathways (especially the mitogen-activated protein kinase pathways) containing Cdc14 co-purified proteins (44) was observed (Fig. 2), suggesting that these phosphoproteins may be the direct in vivo substrates of Cdc14.

**Smc4 Is Dephosphorylated by Cdc14** —To validate our approach, two candidates important for mitosis and cytokinesis were chosen for further study. Chromosome condensation, which is facilitated by the condensin complex, Smc2/4, begins at the start of M phase, and chromosome decondensation is evident by telophase (45). Mass spectrometry analysis identified Smc4 as a potential Cdc14 substrate (supplemental Fig. S2 and supplemental Table S3). The condensin complex is required for the compaction of the long interphase DNA fibers into smaller units, which in turn facilitates segregation of the chromosomes during anaphase. In fission yeast, Cdk1-mediated Smc4 phosphorylation is required for nuclear localization of the condensin complex (46). We conducted an in vitro kinase assay and found that the budding yeast Smc4 was directly phosphorylated by Cdk1 (Fig. 3A). According to our mass data, serine 128 (over 3-fold increase in $\frac{cdc14-1}{CDC14}$) is a potential phosphorylation site (supplemental Fig. S2 and supplemental Table S3). Indeed, replacement of the serine 128 with alanine prevented phosphorylation in an in vitro kinase assay (Fig. 3A). We conducted an in vitro phosphatase assay (10) to demonstrate that phosphorylated Smc4 is dephosphorylated in a Cdc14-concentration-dependent manner (Fig. 3B). These results suggest that Smc4 is phosphorylated...
by Cdk1 and dephosphorylated by Cdc14 at S128. We next generated phosho-specific antibodies against S128 of Smc4 (Fig. 3C). The signal of phosphorylation of Smc4 was decreased in the cdk1 temperature-sensitive strain and elevated in the cdc14 temperature-sensitive strain (Figs. 3D and 3E), suggesting that Cdk1 and Cdc14 modify Smc4 in vivo. The expression of Smc4 began at S phase and decreased at late M phase, and the signal of phosphorylation of S128 of Smc4 approached the maximum at G2/M phase (Fig. 3F and supplemental Fig. S3). Together these results indicate that Smc4 is phosphorylated by Cdk1 during early mitosis and then dephosphorylated by Cdc14 at late M phase.

Phosphorylation of Smc4 at S128 Is Required for Chromosome Condensation and Chromosome Segregation—Gross defects in chromosome segregation and condensation were observed in S. cerevisiae condensin mutants (7, 47–50). In an attempt to understand the role of phosphorylation of Smc4 at S128, we asynchronously grew smc4 mutants including smc4S128A and smc4S128D and then microscopically monitored their DNA morphology. As anticipated, we observed an increase in unsegregated and missegregated nuclei in smc4S128A mutants (Fig. 4A). Next, we assessed condensin function in smc4S128A and smc4S128D cells using the LacO/LacR-GFP system, which has been developed to determine the functionality of the chromosome condensation pathway (51). Using two sets of LacO repeats spaced 450 kb apart on the right arm of chromosome IV, this system directly detects chromosome condensation at M phase. Condensin participates in the maintenance of cohesion, and inappropriate chromosome condensation and cohesion lead to multiple green dots (51). We synchronized wild-type, smc4S128A, and smc4S128D strains at G1 and released them into the cell cycle. Sixty minutes after release, when most cells enter the G2/M phase (supplemental Fig. S3A), 43.7% of wild-type cells contained a single dot indicating cells with condensed chromosomes, and 1.3% contained multiple dots. In contrast, smc4S128A cells showed a decreased proportion of large-budded cells containing one dot (16.7%) and a dramatic increase in cells containing multiple dots (22.3%) (Figs. 4B and 4C). These data revealed that phosphorylation of Smc4 at S128 is required for adequate chromosome condensation.

Given that S128 is located near the potential non-Smc subunit interaction domain of Smc4 (47), we asked whether the phosphorylation on S128 of Smc4 modulates the Smc4–Brn1 interaction. Co-immunoprecipitation assay did not detect any obvious difference between wild-type and mutant smc4 cells in the Smc4–Brn1 interaction. Co-immunoprecipitation assay did not detect any obvious difference between wild-type and mutant smc4 cells in the Smc4–Brn1 interaction (Fig. 4D). Instead, the loading of Smc4 and Brn1 to chromatin was reduced in the smc4S128A mutant (Fig. 4E). These results suggest that phosphorylation of Smc4 at S128 is required for the loading of condensin to chromatin.

**Fig. 2.** Cdc14-dephosphorylated candidates overlap with factors co-purified with Cdc14 in four MAPK pathways. Blue stars indicate previously reported proteins that co-purified with Cdc14 (44). Red and green colors indicate enriched (log2 (cdc14-1/CDC14)) and reduced (log2 (cdc14-1/CDC14)) phospho-proteins in this study, respectively.
Bud3 Is Dephosphorylated by Cdc14—In the budding yeast \textit{S. cerevisiae}, the site of cytokinesis is marked at the time of budding. Our mass spectrometry analysis identified Bud3 as another potential substrate of Cdc14 (supplemental Fig. S4 and supplemental Table S3). Cdc14 localizes to the bud neck at late mitosis (52). In agreement with this, Bud3 moves to the bud neck at the G2/M phase (53). Bud3 is required for cells to mark the site for axial budding in the next cell cycle (53) and is critical to target M phase cyclin Clb2 to the bud neck to modulate the timing of cytokinesis (41, 54). We therefore examined whether Bud3 is directly phosphorylated by Cdk1 using an in vitro kinase assay (Fig. 5A). Both serine 1549 (over 3-fold increase in cdc14-1) and threonine 1566 (over 16-fold increase in cdc14-1) were identified as phosphorylation sites of Bud3 in our mass data (supplemental Fig. S4 and supplemental Table S3). Accordingly, double mutations at S1549 and T1566A caused a reduction of phosphorylation in vitro (Fig. 5B). An in vitro phosphatase assay (10) demonstrated a Cdc14-dependent dephosphorylation in a concentration-dependent manner (Fig. 5C). Our data suggest that Cdk1 phosphorylates Bud3 at amino acids 1549 and 1566 and Cdc14 dephosphorylates these two sites. We further generated phospho-specific antibodies against S1549 and T1566 of Bud3 (Fig. 5D). Phospho-antibodies indeed detected a defect in Bud3 phosphorylation/dephosphorylation in \textit{cdk1} and \textit{cdc14} temperature-sensitive strains, respectively (Figs. 5E–F).

\textbf{Fig. 3. Smc4 is regulated by Cdk1 and Cdc14 \textit{in vitro} and \textit{in vivo}.} A, Cdk1-mediated phosphorylation of Smc4 \textit{in vitro}. Cdk1-HA and the kinase-dead \textit{cdk1}^{K40L}-HA were purified via immunoprecipitation with an HA antibody. Immunoprecipitated kinases were analyzed via SDS-PAGE and Western blot with an HA antibody. GST-Smc4 and GST alone were subjected to the immunoprecipitation-kinase assay as described (68). The gel was also stained by Coomassie Blue (CB). S128 is phosphorylated by Cdk1 \textit{in vitro}. GST-Smc4 and mutants were subjected to the immunoprecipitation-kinase assay. B, Cdc14-mediated dephosphorylation of Smc4 \textit{in vitro}. GST-Cdc14 and Cdc14 phosphatase dead were purified. Smc4 was phosphorylated by Cdk1 as described in A, followed by phosphatase treatment with or without the phosphatase inhibitor (22). λ Phosphatase was used as a control. The relative intensities after normalization are shown below. C, phospho-specific antibody against S128. Phospho- and nonphospho-peptides (left) and lysates (right) were subjected to dot blot and Western blot analyses, respectively, using phospho-specific antibodies from immunized rabbits. D, Cdk1-dependent phosphorylation of Smc4 \textit{in vivo}. Wild-type (+) and \textit{cdk1}^{(cdc28-4)} (−) strains were synchronized by nucodazole at 23 °C and shifted to 37 °C for 3 h. Lysates were subjected to Western blot analysis. E, Cdc14-dependent dephosphorylation of Smc4 \textit{in vivo}. Wild-type (+) and \textit{cdc14-1} (−) strains were synchronized by nucodazole at 23 °C and shifted to 37 °C for 3 h. Lysates were subjected to Western blot analysis. F, the phosphorylation of Smc4 is cell cycle dependent. Overnight culture was grown to early log phase in yeast peptone dextrose, arrested at G1 by \textit{α}-factor, and released into cell cycle. A second cell cycle was prevented by the addition of \textit{α}-factor at 75 min. Cells were collected at 15-min intervals for 135 min, and Smc4 phosphorylation was analyzed via Western blot analysis.
and 5F), suggesting that Cdk1 and Cdc14 modulate Bud3 function in vivo. The phosphorylation of S1549 and T1566 was observed at M phase (Fig. 5G and supplemental Fig. S5). Together these results indicate that direct phosphorylation/dephosphorylation of Bud3 by Cdk1/Cdc14 regulates the functions of Bud3.

Phosphorylation of Bud3 at S1549 and T1566 Is Required for Clb2 Recruitment and Swe1 Degradation—The haploid budding yeast exhibits bud scars clustered around one pole on the surface of mother cells (55). However, bud3 or bud4 mutations cause a bipolar budding phenotype (53, 56). Importantly, a single mutation at residue 1549 or 1566 to alanine caused a defect in bud scar clustering, and this phenotype was partially recovered by phospho-mimicking aspartic acid (Figs. 6A and 6B). Another mutation (S354A) at a predicted Cdk1-dependent consensus site was applied as a negative control and did not cause any defect (Fig. 6B). According to a previous report (41), the C terminus of Bud3 is critical for its interaction with Clb2 and the recruitment of Clb2 to the bud neck. A mutation at residue 1549 or 1566 to alanine caused a defect in the interaction with Clb2 in the yeast two-hybrid assay (Fig. 6C). Western blot analysis indicated that the reduction in the Bud3–Clb2 interaction was not due to different expression levels of Bud3 or Clb2 in the two-hybrid system (Fig. 6D). This reduced interaction further impeded the recruitment of GFP–Clb2 (Fig. 6E). Conversely, the Bud3–Clb2 interaction and bud neck localization of GFP–Clb2 were recovered with the phospho-mimicking aspartic acid mutation (Figs. 6C and 6E).

Swe1 (Saccharomyces wee1 homologue) inhibits mitotic Cdk1 activity by phosphorylating tyrosine 19 of Cdk1 (57). Thus, timely mitotic entry requires the inactivation of Swe1. Swe1 is localized to the bud neck (58). The Cdk1–Clb2 complex directly phosphorylates Swe1, and this modification serves as a priming step to promote subsequent Swe1 hyperphosphorylation and degradation (59). Because Bud3 controls the bud neck recruitment of Clb2, which may lead to Swe1 phosphorylation and degradation, we next examined whether Cdk1/Cdc14-mediated modification of Bud3 influences the Swe1 degradation. As shown in Fig. 6F, mutations at residues 1549 and 1566 to alanine caused a defect in the Swe1 degradation, suggesting that reduced phosphorylation on
Bud3 compromises Clb2 recruitment and further inhibits Swe1 degradation.

**DISCUSSION**

Within a cell cycle, multiple events need to be well coordinated. At the M phase, chromosome condensation, mitosis, and cytokinesis occur sequentially to guarantee precise entry and exit of the M phase. A few master regulators may control numerous signals, and Cdc14 seems to play such a role. Pinpointing the dephosphorylated residues for Cdc14 is essential for understanding its functions and detailed mechanisms. The identification of dephosphorylation sites provides the ultimate proof that the putative substrate is indeed dephosphorylated. In this study, we identified 455 potential Cdc14 substrates, and the majority of them are critical for M phase progression, confirming the master role of Cdc14 at M phase. Additionally, Cdc14 displays connections with many MAPK pathways. Cdc14 interacts with the pheromone and filamentous growth pathway MAPK Ste7 and the PKC pathway MAPKKK Bck1, and increases in the phosphorylation of Ste7 and Bck1 were observed in cdc14-1 cells (44). These results indicate that Ste7 and Bck1 may be direct targets of Cdc14 and that Cdc14 may serve as a hub for multiple environmental response signals.
To validate this broad screen, we demonstrated that two candidates, Smc4 and Bud3, are indeed Cdc14 direct substrates. Smc4 is the major component of condensin and is required for chromosome condensation. Our study demonstrated that the phosphorylation status of Smc4 S128 is critical for condensin function (Fig. 7). This phosphorylation does not control the interaction between Smc4 and the non-Smc subunit, but it regulates the loading ability of condensin to chromatin. Bud3 is important for the budding site selection at the early M phase. Not only is Bud3 required for budding site selection, but it also recruits Cib2 to the bud neck for Cdk1-mediated Swe1 degradation and cytokinesis. Elimination of Cdk1/Cdc14-controlled regulation of Bud3 impedes Cib2 recruitment and Swe1 degradation (Fig. 7). The C terminus of Bud3 was previously shown to be critical for cytokinesis (41). In agreement with this, exit from M phase was delayed in bud3 S1549T1566A cells (supplemental Figs. S5A and S5B). The regulation of both Smc4 and Bud3 is mediated by Cdk1 and Cdc14. Abolishing either modification deregulated normal M phase progression (Fig. 4A and supplemental Fig. S5). These results indicate that a single Cdc14-mediated regulation is critical for precise cell cycle movement.

Cdc14 is thought to dephosphorylate many substrates, and indeed many potential substrates were identified in our study (supplemental Table S5). Cdh1 is the best-known substrate of Cdc14 and is activated through Cdc14-dependent dephosphorylation (23, 60). In our screen, we did not identify Cdh1. The exact Cdc14 dephosphorylation site on Cdh1 has not been

Fig. 6. Bud3 phosphorylation regulates Cib2 localization and Swe1 degradation. A, cell walls of indicated yeast strains were stained with Calcofluor white and observed under a microscope. The percentage of abnormal bipolar cells is scored in B. bud3- and bud4-deleted strains were used as positive controls. C, two-hybrid assay was used to detect the Bud3–Cib2 interaction. D, Western blot analysis was conducted to examine the expression levels using antibodies against HA (Bud3) and LexA (Cib2). Pgk1 is a loading control. E, localization of the GFP-Cib2 fusion protein in indicated strains. Bud neck staining of Bud3 in large budded cells was observed under a microscope and counted. At least 200 cells were counted for each strain. F, BUD3 SWE1–3HA, bud3 S1549T1566A SWE1–3HA, and bud3 S1549T1566D SWE1–3HA cells were arrested in G1 by α-factor and released. Cells were taken at the indicated time points to determine Swe1 expression using anti-HA and anti-Pgk1 (loading control) antibodies.
mapped yet. Our approach was based on mass spectrometry analysis of trypsin-digested fragments. It is possible that the Cdc14 dephosphorylated site is located on a trypsin-digested fragment of Cdh1 greater than 35 amino acids, which is beyond the detection ability of the mass instrument (61, 62).

Our study globally identified Cdc14 substrates. Among all these potential substrates, many are well conserved in higher organisms. For example, minichromosome maintenance 2–7 proteins play a pivotal helicase role in DNA replication in eukaryotic organisms. Upon entry into S-phase, several subunits of the MCM hexameric complex are phosphorylated by CDK (63, 64). The MCM complex is highly conserved from yeast to human, and our study also identified Mcm2, Mcm3, and Mcm7 as potential Cdc14 substrates. Moreover, fine control of individual Cdc14-mediated regulation may be important in order for yeast to adapt to environmental challenges during the cell cycle in the wild and further help them to evolve. Future investigation will be required in order to understand this process.

Acknowledgments—We greatly appreciate Dr. Marie-Noëlle Simon for her suggestions and comments on the manuscript. We also thank Drs. Marie-Noëlle Simon, Duncan Clarke, Katsunori Sugimoto, and Steven Reed for the gifts of yeast strains and plasmids. The proteomics analysis was performed by the Academia Sinica Common Mass Spectrometry Facilities located at the Institute of Biological Chemistry.

Fig. 7. Cdk1/Cdc14-mediated stepwise mechanisms may facilitate multiple signal transmission at distinct locations in the cell. Cdc14 is thought to dephosphorylate many substrates. Gradual increase of the Cdc14 concentration at M phase instructs sequential dephosphorylation, and reversal of phosphorylation by Cdc14 regulates the temporal ordering of mitotic exit events. At S phase Cdk1 may phosphorylate Smc4 to promote the chromatin loading ability of condensin. During chromosome decondensation at telophase, dissociation between SMC complex and chromatin may be achieved by Cdc14. Cdk1 phosphorylates Bud3 to promote Cib2 localization to the bud neck. Localization of Cib2 to the bud neck induces proteolytical degradation of Swe1, a Cdk1 inhibitor, which further fully activates the Cdk1/Cib2 complex. Activated Cdk1 finally facilitates septin ring formation at the neck, and cytokinesis then should be the ultimate event that completes the exit from M phase (69, 70).

* This work was supported by grants from the National Science Council (101-2311-B-002-018-MY3), National Taiwan University (NTU CESRP-101R7602A1), and the National Health Research Institute (NHRI-EX10242BI) to S.C.T.

This article contains supplemental material.

‡‡ To whom correspondence should be addressed: Department of Microbiology, College of Medicine, National Taiwan University, No. 1, Sec. 1, Jen-Ai Road, Taipei, 10051, Taiwan, Tel.: 886-2-23123456 ext. 88289, Fax: 886-2-23915293, E-mail: shuchunteng@ntu.edu.tw.

REFERENCES

1. Hirano, T., and Mitchison, T. J. (1994) A heterodimeric coiled-coil protein required for mitotic chromosome condensation in vitro. Cell 79, 449–458
2. Haase, S. B., Winey, M., and Reed, S. I. (2001) Multi-step control of spindle pole body duplication by cyclin-dependent kinase. Nat. Cell Biol. 3, 38–42
3. Tanaka, T. U., Stark, M. J., and Tanaka, K. (2005) Kinetochore capture and bi-orientation on the mitotic spindle. Nat. Rev. Mol. Cell Biol. 6, 929–942
4. Uhmann, F., Lottspeich, F., and Nasmyth, K. (1999) Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. Nature 400, 37–42
5. Uhmann, F., Vernic, D., Poupart, M. A., Koonin, E. V., and Nasmyth, K. (2000) Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast. Cell 103, 375–386
6. Peters, J. M. (2002) The anaphase-promoting complex: proteolysis in mitosis and beyond. Mol. Cell 9, 931–943
7. Strunnikov, A. V., Hogan, E., and Koshland, D. (1995) SMC2, a Saccharomyces cerevisiae gene essential for chromosome segregation and condensation, defines a subgroup within the SMC family. Genes Dev. 9, 587–599
8. Shou, W., Seol, J. H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z. W., Jang, J., Charbonneau, H., and Deshaies, R. J. (1999) Exit from
mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. Cell 97, 233–244
26. Lorincz, A. T., and Reed, S. I. (1986) Sequence analysis of temperature-sensitive mutations of yeast SAD1. J. Bacterial. 168, 365–372
25. Khmelinskii, A., Lawrence, C., Roostalu, J., and Schiebel, E. (2007) Cdc14-dependent phosphorylation of Cut3 and Cdc14 from nucleolar RENT complex. Mol. Cell. Proteomics 6, 2217–2228
24. Dohmae, N., Takio, K., and Yanagida, M. (2002) Dual specificity protein phosphatase Cdc14: A dual specificity protein phosphatase Cdc14 from nucleolar RENT complex. Mol. Cell. Proteomics 1, 2217–2228
23. Tsai, C. F., Wang, Y. T., Chen, Y. R., Su, C. Y., Lin, Y. W., Pan, K. T., Chen, J. Y., Khoob, K. H., and Chen, Y. J. (2008) Immobilized metal affinity chromatography revisited: pH/acid control toward high selectivity in phosphopeptides. J. Proteome Res. 7, 4058–4069
22. Olsen, J. V., de Godoy, L. M., Li, G., Macek, B., Mortensen, P., Pesch, R., Makarov, A., Lange, O., Horning, S., and Mann, M. (2005) Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. Mol. Cell. Proteomics 4, 1010–2014
21. Vozziano, J. A., Cota, R. G., Corderas, A., Dianes, J. A., Fabregat, A., Foster, J. M., Griss, J., Alpi, E., Birim, M., Contell, J. O’Kely, G., Schonegger, A., Ovelleiro, D., Perez-Riverol, Y., Reisinger, F., Rios, D., Wang, R., and Hermjakob, H. (2013) The PRoteome IDEntifications (PRIDE) database and associated tools: status in 2013. Nucleic Acids Res. 41, D1063–D1069
20. Wang, Y. T., Tsai, C. F., Hong, T. C., Tsou, C. C., Lin, P. Y., Pan, S. H., Hsieh, J. M., Yang, P. H., Hsu, W. L., and Chen, Y. J. (2010) An informatics-assisted label-free quantitation strategy that depicts phosphopeptide profiles in lung cancer cell invasion. J. Proteome Res. 9, 5582–5597
19. Tsai, C. C., Tsai, C. F., Tsai, Y. H., Sudhir, P. R., Wang, Y. T., Chen, Y. J., Sung, T. Y., and Hsu, W. L. (2010) IDEAL-Q, an automated tool for label-free quantitation analysis using an efficient peptide alignment approach and spectral data validation. Mol. Cell. Proteomics 9, 131–144
18. Boyle, E. I., Weng, S., Gollub, J., Jin, H., Botstein, D., Cherry, J. M., and Sherlock, G. (2004) GO::TermFinder—open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. Bioinformatics 20, 3710–3715
17. Tala, C. S., Shen, Z. J., Tsai, H. J., Lin, Y. H., and Teng, S. C. (2009) Rapid Cdc13 turnover and telomere length homeostasis are controlled by Cdk1-mediated phosphorylation of Cdc13. Nucleic Acids Res. 37, 3602–3611
16. Liang, C., and Stillman, B. (1997) Persistent initiation of DNA replication and chromatin-bound MCM proteins during the S-phase of budding yeast. J. Cell Biol. 138, 517–530
15. Jaspersen, S. L., Charles, J. F., Tinker-Kulberg, R. L., and Morgan, D. O. (2000) A late mitotic regulatory network controlling cyclin destruction in Saccharomyces cerevisiae. J. Cell Biol. 150, 97–110
14. Bardin, A. J., Visintin, R., and Amon, A. (2000) A mechanism for coupling exit from mitosis to partitioning of the nucleus. Cell 102, 21–31
13. Fitzpatrick, P. J., Toyn, J. H., Millar, J. B., and Johnston, L. H. (1998) DNA damage checkpoint in Saccharomyces cerevisiae: epistasis to the cellular response to DNA damage. Science 280, 131–144
12. Guacci, V., Hogan, E., and Koshland, D. (1994) Chromosome condensation and the mechanism of paired-association in budding yeast. J. Cell Biol. 125, 517–530
11. Jaspersen, S. L., and Morgan, D. O. (2000) Cdc14 activates cdc15 to promote mitotic exit in budding yeast. Curr. Biol. 10, 615–618
10. Taylor, G. S., Liu, Y., Baskerville, C., and Charbonneau, H. (1997) The activity of Cdc14p, an oligomeric dual specificity protein phosphatase from Saccharomyces cerevisiae, is required for cell cycle progression. J. Biol. Chem. 272, 24054–24063
9. Visintin, R., Hwang, E. S., and Amon, A. (1999) Cft1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus. Nature 396, 818–823
8. Bloom, J., Cristea, I. M., Procko, A. L., Lubkov, V., Chait, B. T., Snyder, M., Akiyoshi, B., and Biggins, S. (2010) Cdc14-dependent dephosphorylation of a kinetochore protein prior to anaphase in Saccharomyces cerevisiae. Genetics 186, 1487–1491
7. Moccioara, A., and Schiebel, E. (2010) Cdc14: a highly conserved family of phosphatases with non-conserved functions? J. Cell Sci. 123, 2867–2876
6. Bloom, J., Cristea, I. M., Procko, A. L., Lubkov, V., Chait, B. T., Snyder, M., and Cross, F. R. (2011) Global analysis of Cdc14 phosphatase reveals diverse roles in mitotic processes. J. Biol. Chem. 286, 5434–5445
5. Zhai, Y., Yung, P. Y., Huo, L., and Liang, C. (2010) Cdc14p resets the competency of replication licensing by dephosphorylating multiple initiation proteins during mitotic exit in budding yeast. J. Cell Sci. 123, 3939–3943
4. Hall, M. C., Jeong, D. E., Henderson, J. T., Choi, E., Bremmer, S. C., Illek, A. B., and Charbonneau, H. (2008) Cdc28 and Cdc14 control stability of the anaphase-promoting complex inhibitor Acm1. J. Biol. Chem. 283, 10396–10407
3. Geymonat, M., Spanos, A., Wells, G. P., Smerdon, J. S., and Sedgwick, S. G. (2004) Cib2/Cib2/Cdc28 and Cdc14 regulate phosphorylation status and cellular localization of Swi6. Mol. Cell. Biol. 268, 22717–22825
2. Khmelinskii, A., Lawrence, C., Roostalu, J., and Schiebel, E. (2007) Cdc14-regulated midzone assembly controls anaphase B. J. Cell Biol. 177, 981–993
1. Lorincz, A. T., and Reed, S. I. (1986) Sequence analysis of temperature-sensitive mutations in the Saccharomyces cerevisiae gene CDC28. Mol. Cell. Biol. 6, 4099–4103

Global Identification of Cdc14-mediated Dephosphorylations