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

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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.

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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 separase-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 anaphase 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 dephosphorylates 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, experiments have not been carried out to demonstrate whether these modifications entail direct or indirect regulation. Therefore, our understanding of Cdc14 function and regulation during mitosis in metazoans is incomplete. Conceivably,

1 The abbreviations used are: CDK, cyclin-dependent kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
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. Strains were constructed and analyzed via standard genetic methods. Cells were grown in rich medium (YPD: 1% yeast extract, 2% bacto-peptone, 2% dextrose) or, for plasmid selection, 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. pHG-bud3Cter (1534–1636) was constructed by ligating the BamHI/Xhol-treated fragment containing amino acids 1534–1636 of Bud3 into the BamHI/Xhol-digested pHG. 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’,N’-tetramethylenediamine in a 14:5:0.7:0.3 ratio (v/v). The gel was cut into small pieces, washed several times with 25 mM triethylammonium bicarbonate with the addition of 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. Digested peptides 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.

Immobile 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 glass disk enclosed in a stainless steel column-end fitting. The nickel-nitrilotriacetic acid resin was activated by 100 μL 0.2 M FeCl₃ and equilibrated with loading buffer for 15 min before sample loading. The loading/concentration 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, 5 μm, Nucleosil 120–5 C18, Macherey-Nagel, GmbH & Co. KG, Düren, Germany) and separated using a linear 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 transformed to mrm files using the software RAW2M+MSM (version 1.1) (32). The mrm files were searched using Mascot (version 2.2.1) against the Swiss-Prot Saccharomyces cerevisiae (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, the fragment ion mass tolerance was set at 10 ppm, and the parent ion tolerance was set at 0.6 Da. Phosphorylation (STY) and oxidation (M) were specified as variable modifications. Peptides were considered identified if their Mascot individual ion score was greater than 20 (p < 0.05). The false discovery rates for Orbitrap data were determined with a Mascot score greater than 20 (p < 0.05) in this study. All of the raw datasets, peak lists, spectra of identified phosphopeptides, and identification results have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (33) with the dataset identifier PXD000375 and DOI 10.6019/PXD000375.

Quantitative Analysis by IDEAL-O—The quantitative analysis of phosphopeptides was performed with the SEMI label-free algorithm (34) and IDEAL-Q software (35). The raw data files acquired from the LTQ-Orbitrap were converted into files of mzXML format by the program ReAdW (XCalibur, Thermo Finnigan), and the search results in MASCOT were exported in Extensible Markup Language data (.xml) format. After data conversion, the confident peptide identification results (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 fragment elution-time domains. To

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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 Benferroni correction value was calculated using Generic GO Term Finder (with default options).

Generation of Phospho-specific Antibodies—Peptides were designed as indicated: RRLELQLS-PVKNSR for phosphorylation of Smc4 residue S128; epipwpskphd and qkdepipwpskphd for phosphorylation of Bud3 residue S1549; and rktakEatmptvl and lsnrkAtEatmptvl 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 Cdc28K40L-HA were purified from a protease-deficient strain, BJ2168, carrying plasmid pKB174 (CDC28-HA) or pKB173 (cdc28K40L-HA), 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), pEG202-LexA-CLB2, pJG4–5-bud3Ctcr, 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 microfuged, 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–CIBZ2 fusion protein were performed as described elsewhere (41).

Immunoprecipitation—Immunoprecipitation was performed as described (42) using EBX buffer (100 mM KCl, 50 mM HEPES-KOH (pH 7.5), 2.5 mM MgCl2, 50 mM NaF, 5 mM Na4P2O7, 0.1 mM NaVO3, 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 Bilisland’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 mM sorbitol, 25 mM Tris pH 7.4, 4 mg/ml Zymolyase 20T, in yeast extract–peptone–dextrose medium plus adenine). 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 mM 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 mM 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 we 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 ($p \leq 0.05$). In total, 3641 different phosphorylation sites were confidently assigned to 1243 proteins. The log$_2$ $cdc14$-$1$/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 $cdc14$-$1$/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 $cdc14$-$1$/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 $cdc14$-$1$ 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.

**Fig. 1. Large-scale identification of Cdc14 substrates in vivo.** A, distributions of log$_2$ $cdc14$-$1$/CDC14 ratios for phosphopeptides (blue) and phosphoproteins (red). B, Venn diagrams represent the number of proteins and unique phosphorylation sites identified in this experiment. The black square indicates the total number of phosphorylation sites and proteins. The red rectangle indicates phosphopeptides and proteins that increased in abundance by over 50% (log$_2$ > 1), and the blue rectangle indicates phosphopeptides and proteins that decreased in abundance by over 50% (log$_2$ < −1). The green oval indicates phosphopeptides and proteins containing a consensus motif of Cdk1 (65), the orange oval indicates phosphopeptides and proteins containing a consensus motif of Ipl1 (66), and the gray oval indicates phosphopeptides and proteins containing a consensus motif of Cdc5 (67).

**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 $cdc14$-$1$) 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 phospho-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 smc4 S128A and smc4 S128D and then microscopically monitored their DNA morphology. As anticipated, we observed an increase in unsegregated and missegregated nuclei in smc4 S128A mutants (Fig. 4A). Next, we assessed condensin function in smc4 S128A and smc4 S128D 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, smc4 S128A, and smc4 S128D 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, smc4 S128A 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 (Fig. 4D). Instead, the loading of Smc4 and Brn1 to chromatin was reduced in the smc4 S128A mutant (Fig. 4E). These results suggest that phosphorylation of Smc4 at S128 is required for the loading of condensin to chromatin.
Bud3 Is Dephosphorylated by Cdc14—
In the budding yeast *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 *cdk1* and *cdc14* temperature-sensitive strains, respectively (Figs. 5E and 5F).

**Fig. 3.** Smc4 is regulated by Cdk1 and Cdc14 *in vitro* and *in vivo*. A, Cdk1-mediated phosphorylation of Smc4 *in vitro*. Cdk1-HA and the kinase-dead *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 *in vitro*. GST-Smc4 and mutants were subjected to the immunoprecipitation-kinase assay. B, Cdc14-mediated dephosphorylation of Smc4 *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 *in vivo*. Wild-type (+) and *cdk1* *K40L* (−) strains were synchronized by nocodazole at 23 °C and shifted to 37 °C for 3 h. Lysates were subjected to Western blot analysis. E, Cdc14-dependent dephosphorylation of Smc4 *in vivo*. Wild-type (+) and *cdc14* (−) strains were synchronized by nocodazole 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 α-factor, and released into cell cycle. A second cell cycle was prevented by the addition of 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 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

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**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.

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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. Uhlmann, 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. Uhlmann, F., Wernic, 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 Kosher, 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...
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50. Ouspenski, I. I., Cabello, O. A., and Brinkley, B. R. (2000) Chromosome condensation factor Brn1p is required for chromatid separation in mitosis. Mol. Biol. Cell 11, 1305–1313
51. Vas, A. C., Andrews, C. A., Kirkland Matesky, K., and Clarke, D. J. (2007) In vivo analysis of chromosome condensation in Saccharomyces cerevisiae. Mol. Biol. Cell 18, 557–568
52. Bembenek, J., Kang, J., Kurischko, C., Li, B., Raab, J. R., Belanger, K. D., Luca, F. C., and Yu, H. (2005) Crm1-mediated nuclear export of Cdc14 is required for the completion of cytokinesis in budding yeast. Cell Cycle 4, 961–971
53. Chant, J., Mischke, M., Mitchell, E., Herskowitz, I., and Pringle, J. R. (1995) Role of Bud3p in producing the axial budding pattern of yeast. J. Cell Biol. 129, 767–778
54. Eluere, R., Offner, N., Varlet, L., Motteux, O., Signon, L., Picard, A., Baillie, E., and Simon, M. N. (2007) Compartmentalization of the functions and regulation of the mitotic cyclin Clb2 in S. cerevisiae. J. Cell Sci. 120, 702–711
55. Chant, J., and Herskowitz, I. (1991) Genetic control of bud site selection in yeast by a set of gene products that constitute a morphogenetic pathway. Cell 65, 1203–1212
56. Sanders, S. L., and Herskowitz, I. (1996) The BUD4 protein of yeast, required for axial budding, is localized to the mother/BUD neck in a cell cycle-dependent manner. J. Cell Biol. 134, 413–427
57. Booher, R. N., Deshaies, R. J., and Kirschner, M. W. (1993) Properties of Saccharomyces cerevisiae wee1 and its differential regulation of e34cdc28 in response to G1 and G2 cyclins. EMBO J. 12, 3417–3426
58. Longtine, M. S., Theesfeld, C. L., McMillan, J. N., Weaver, E., Pringle, J. R., and Lew, D. J. (2000) Septin-dependent assembly of a cell cycle-regulatory module in Saccharomyces cerevisiae. Mol. Cell. Biol. 20, 4049–4061
59. Asano, S., Park, J. E., Sakchaiari, K., Yu, L. R., Song, S., Supavilai, P., Veenstra, T. D., and Lee, K. S. (2005) Concerted mechanism of Swe1/Wee1 regulation by multiple kinases in budding yeast. EMBO J. 24, 2194–2204
60. Jaspersen, S. L., Charles, J. F., and Morgan, D. O. (1999) Inhibitory phosphorylation of the APC regulator Hct1 is controlled by the kinase Cdc28 and the phosphatase Cdc14. Curr. Biol. 9, 227–236
61. Xu, H., and Freitas, M. A. (2009) MassMatrix: a database search program for rapid characterization of proteins and peptides from tandem mass spectrometry data. Proteomics 9, 1548–1555
62. Xu, H., Hsu, P. H., Zhang, L., Tsai, M. D., and Freitas, M. A. (2010) Database search algorithm for identification of intact cross-links in proteins and peptides using tandem mass spectrometry. J. Proteome Res. 9, 3384–3393
63. Montagnoli, A., Valsasina, B., Brotherton, D., Troiani, S., Rainoldi, S., Tenca, P., Molinari, A., and Santocanale, C. (2006) Identification of Mcm2 phosphorylation sites by S-phase-regulating kinases. J. Biol. Chem. 281, 10281–10290
64. Mortani, M., and Ishimi, Y. (2013) Inhibition of DNA binding of MCM2–7 complex by phosphorylation with cyclin-dependent kinases. J. Biochem. 154, 363–372
65. Ubersax, J. A., Woodbury, E. L., Quang, P. N., Paraz, M., Blethrow, J. D., Shah, K., Shokat, K. M., and Morgan, D. O. (2003) Targets of the cyclin-dependent kinase Cdk1. Nature 425, 859–864
66. Cheeseman, I. M., Anderson, S., Jwa, M., Green, E. M., Kang, J., Yates, J. R., 3rd, Chan, C. S., Drubin, D. G., and Barnes, G. (2002) Phospho-regulation of kinetochore-microtubule attachments by the Aurora kinase lpl1p. Cell 111, 163–172
67. Yoshida, S., Kono, K., Lowery, D. M., Bartolini, S., Yaffe, M. B., Ohya, Y., and Pellman, D. (2006) Polo-like kinase Cdc5 controls the local activation of Rho1 to promote cytokinesis. Science 313, 108–111
68. Tseng, S. F., Lin, J. J., and Teng, S. C. (2006) The telomerase-recruitment domain of the telomere binding protein Cdc13 is regulated by Mec1p/Tel1p-dependent phosphorylation. Nucleic Acids Res. 34, 6327–6336
69. Altman, R., and Kellogg, D. (1997) Control of mitotic events by Nap1 and the Gin4 kinase. J. Cell Biol. 138, 119–130
70. Li, C. R., Yong, J. Y., Wang, Y. M., and Wang, Y. (2012) CDK regulates septin organization through cell-cycle-dependent phosphorylation of the Nim1-related kinase Gin4. J. Cell Sci. 125, 2533–2543