Identification of Candidate Cyclin-dependent kinase 1 (Cdk1) Substrates in Mitosis by Quantitative Phosphoproteomics*§

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Cyclin-dependent kinase 1 (Cdk1) is an essential regulator of many mitotic processes including the reorganization of the cytoskeleton, chromosome segregation, and formation and separation of daughter cells. Deregulation of Cdk1 activity results in severe defects in these processes. Although the role of Cdk1 in mitosis is well established, only a limited number of Cdk1 substrates have been identified in mammalian cells. To increase our understanding of Cdk1-dependent phosphorylation pathways in mitosis, we conducted a quantitative phosphoproteomics analysis in mitotic HeLa cells using two small molecule inhibitors of Cdk1, Flavopiridol and RO-3306. In these analyses, we identified a total of 24,840 phosphopeptides on 4,273 proteins, of which 1,215 phosphopeptides on 551 proteins were significantly reduced by 2.5-fold or more upon Cdk1 inhibitor addition. Comparison of phosphopeptide quantification upon either inhibitor treatment revealed a high degree of correlation (R² value of 0.87) between the different datasets. Motif enrichment analysis of significantly regulated phosphopeptides revealed enrichment of canonical Cdk1 kinase motifs. Interestingly, the majority of proteins identified in this analysis contained two or more Cdk1 inhibitor-sensitive phosphorylation sites, were highly connected with other candidate Cdk1 substrates, were enriched at specific subcellular structures, or were part of protein complexes as identified by the CORUM database. Furthermore, candidate Cdk1 substrates were enriched in G2 and M phase-specific genes. Finally, we validated a subset of candidate Cdk1 substrates by in vitro kinase assays. Our findings provide a valuable resource for the cell signaling and mitosis research communities and greatly increase our knowledge of Cdk1 substrates and Cdk1-dependent signaling pathways. Molecular & Cellular Proteomics 15: 10.1074/mcp.M116.059394, 2448–2461, 2016.

An important objective of a cell is to accurately replicate its genetic material and evenly divide along with its subcellular components into two identical daughter cells. To do so, a cell reduces or halts growth, transcription, cap-dependent translation, and undergoes dramatic changes in cellular structure and organization. These changes include chromosome condensation, nuclear envelope breakdown, disassembly of the endoplasmic reticulum and Golgi apparatus, reorganization of the actin cortex, and the formation of the mitotic spindle. Deregulation and errors in these processes can produce non-identical daughter cells with aberrant chromosome numbers, a state known as aneuploidy and a hallmark of human cancer and the origin of many birth defects (1–5). Therefore, it is imperative that mitosis proceeds in a highly accurate and controlled manner. This is achieved by a sophisticated network of proteins that engage in a multitude of protein-protein interactions regulated by post-translational modifications, including dynamic protein phosphorylation by protein kinases and phosphatases (summarized in (6–8)).

One of the master regulators of mitosis that is conserved from yeast to human is the cyclin-dependent kinase 1 (Cdk1)1 (9). Cdk1 expression is constant across the cell cycle and the regulation of its activity relies on its association with cyclin A and B, as well as on post-translational modifications including phosphorylation. Specifically, mRNA and protein abundance of cyclin A and B oscillate during the cell cycle due to temporally regulated transcription, translation, and degradation cycles that restrict Cdk1 activity from S-phase to mitosis (10–14). In S-phase and G2, Cdk1 is nuclear and bound to cyclin A (9). In G2, cyclin B is synthesized in the cytoplasm.

1 The abbreviations used are: Cdk, Cyclin Dependent Kinase; PTM, Post-translational Modification; CAK, CDK Activating Kinase; DMEM, Dulbecco’s Modified Eagle’s Medium; FBS, Fetal Bovine Serum; SPE, Solid-phase Extraction; LC-MS, Liquid Chromatography Mass Spectrometry; HCD, Higher-energy Collisional Dissociation; NCE, Normalized Collision Energy; FDR, False Discovery Rate; H/L, Heavy to Light Ratio; MMFPh, Maximal Motif Finder for Phosphopeptides; MassChroQ, Mass Chromatogram Quantification; E. coli, Escherichia coli; LB, Luria-Bertani media; SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; SILAC, Stable Isotope Labeling by Amino Acids in Cell Culture; SCX, Strong Cation Exchange Chromatography; RNP, Ribonucleoprotein complex; GO, Gene Ontology; CORUM, Comprehensive Resource of Mammalian Protein Complexes.
and imported into the nucleus, where it binds and activates Cdk1 to initiate mitotic entry (15–17). In prophase, cyclin A is degraded, resulting in the majority of prometaphase and metaphase Cdk1 activity being mediated by Cdk1-cyclin B (9). Degradation of cyclin B in anaphase inactivates Cdk1, allowing for exit from mitosis (18, 19).

In addition, Cdk1-cyclin B activity is regulated by phosphorylation (20). In interphase, Cdk1 is inactivated through phosphorylation on threonine 14 (Thr14) and tyrosine 15 (Tyr15) by the dual specificity protein kinases Myt1 and Wee1, respectively (21–25) (Fig. 1A). At the G2/M transition, dephosphorylation of these sites by the dual-specificity protein phosphatase Cdc25c promotes Cdk1 activation (26–28) (Fig. 1A). Full activation of Cdk1-cyclin B is achieved through phosphorylation by a CDK-activating kinase (CAK) on threonine 161 (Thr161) (16, 29) (Fig. 1A). Once fully active, Cdk1-cyclin B activity triggers the G2/M transition and initiates the biological processes and changes in cellular structure and organization necessary for mitotic progression (30–34). Cdk1 reportedly phosphorylates substrates at a S/TPx(x)R/K consensus motif to initiate effector function or downstream signaling (31). Cdk1 likely phosphorylates hundreds of mitotic substrates to accurately coordinate mitotic progression in a timely and spatially controlled manner.

Because of the essential role of Cdk1 in the regulation of mitotic progression and the relatively high rate at which cancer cells cycle, small molecule inhibitors of Cdk1 activity have been developed as cancer chemotherapeutics (35–37). Flavopiridol, an ATP-competitive inhibitor that targets Cdk1, Cdk2, Cdk4, Cdk6, and Cdk9, was the first Cdk inhibitor to be tested in clinical trials (36, 38). It induces G1 and G2 cell cycle arrest due to inhibition of Cdk2, Cdk4, Cdk6, and Cdk1 resulting in cytostatic growth arrest instead of cell death (36). However, treatment with Flavopiridol after taxane-induced mitotic arrest is synergistic, leading to cytotoxicity by potentially promoting exit from abnormal mitosis and induction of cell death (36, 39). Clinical trials with Flavopiridol as a single agent (40) or in combination with taxanes (41, 42) have been conducted, but failed to show the same preclinical efficacy observed in both in vitro and in vivo models. Recently, an inhibitor with greater reported selectivity for Cdk1, RO-3306, was developed, which arrests cells at the G2/M transition and induces a rapid mitotic exit in cells in mitosis (43).

To increase our understanding of how mitotic progression is regulated by Cdk1-dependent phosphorylation pathways, we set out to identify Cdk1 substrates by combining quantitative phosphoproteomics and small molecule inhibitors of Cdk activity (40, 43–45) in mitotically-arrested HeLa cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% Fetal Bovine Serum (FBS; Hyclone, Logan, Utah) and penicillin-streptomycin (100 U/ml and 100 μg/ml; Gibco). Cells were incubated at 37 °C in a humidified chamber with 5% CO₂.

HeLa cells were grown in heavy or light DMEM (GIBCO) supplemented with 10% dialyzed FBS (Hyclone) and penicillin-streptomycin. “Heavy” media contained 100 mg/L $^{13}$C$_6$-$^{15}$N$_2$-lysine and 100 mg/L $^{13}$C$_6$-$^{15}$N$_2$-arginine (Sigma, St. Louis, MO). Cells were grown for a minimum of six doublings in the respective medium. To synchronize cells in mitosis, thymidine (1 mM, Sigma) was added for 22 h to both conditions, followed by a 3 h washout with PBS (Corning, Tewksbury, MA) and subsequent addition of taxol (Sigma) to both conditions for 16 h. Both heavy and light conditions were then treated with MG132 at a concentration of 10 μM for 30 min. After 30 min, either Flavopiridol (2 μM for 30 min) or RO-3306 (5 μM for 30 min) were added to the heavy condition. Heavy and light HeLa cells were counted, mixed 1:1, snap-frozen in liquid nitrogen, and stored at −80 °C until lysis. Experiments were performed in biological triplicates.

**Lysis and Digestion**—Frozen cell pellets were partially thawed on ice and immediately resuspended in lysis buffer containing 9 M urea (Sigma), 50 mM Tris pH 8.1 (Sigma), 2 mM sodium beta-glycerophosphate (Sigma), 2 mM sodium fluoride (Sigma), 2 mM sodium molybdate (Sigma), 1 mM sodium orthovanadate (Sigma), and protease inhibitors (1 tablet per 10 ml of lysis buffer) (Mini-Complete, Roche, Indianapolis, IN). Cells were sonicated on ice with a Branson microtip sonicator three times at 10 s bursts. Approximately 200 μl of lysis was removed to measure total protein using a BCA protein assay kit (Thermo Fisher Scientific, Grand Island, NY). Reduction of the lysis was performed by adding 5 mM dithiothreitol (DTT) (Sigma) to the remaining lysis with a 50 °C incubation period for 30 min with occasional swirling to distribute the heat. The sample was removed and cooled to room temperature, and 15 Mm iodoacetamide (Sigma) was added (final concentration) and the lysis was incubated in the dark for 60 mins. The alkylation reaction was quenched with 5 mM DTT for 15 min. The sample was diluted sevenfold in 25 mM Tris pH 8.1 and 1 μg of trypsin was added for every 200 μg of protein in lysate. Samples were incubated at 37 °C overnight. Trifluoroacetic acid was added to the digested peptide lysate to a final concentration of 0.25%, followed by centrifugation of the lysate at 3000 × g for 5 min. Peptides were desalted using a C$_18$ solid-phase extraction (SPE) cartridge (SepPak, Waters, Milford, MA) and placed in a vacuum centrifuge for 45 min to evaporate the organic solvent. Finally, the samples were snap frozen in liquid nitrogen and lyophilized overnight.

**Phosphopeptide Purification**—Phosphopeptide purification was performed using titanium dioxide microparticles essentially as described (46). Lyophilized peptides were dissolved in 2 M lactic acid (Sigma)/50% acetonitrile in water (Honeywell Burdick & Jackson, Morris Plains, NJ) and added to ~350 μl TiO$_2$ microspheres. The mixture was incubated for 1 h with agitation (46). The TiO$_2$ microspheres were removed by centrifugation and washed three times with 2 M lactic acid/50% acetonitrile and two times with 50% acetonitrile/0.1% TFA. Phosphopeptides were eluted twice with 50% acetonitrile/0.1% TFA. Peptides were measured using titanium dioxide microspheres essentially as described (46). Lyophilized peptides were dissolved in 2 M lactic acid (Sigma)/50% acetonitrile in water (Honeywell Burdick & Jackson, Morris Plains, NJ) and added to ~350 μl TiO$_2$ microspheres. The mixture was incubated for 1 h with agitation (46). The TiO$_2$ microspheres were removed by centrifugation and washed three times with 2 M lactic acid/50% acetonitrile and two times with 50% acetonitrile/0.1% TFA. Phosphopeptides were eluted twice with 50% acetonitrile/0.1% TFA. Peptides were measured using titanium dioxide microspheres essentially as described (46) for both conditions for 16 h. Both heavy and light conditions were then treated with MG132 at a concentration of 10 μM for 30 min. After 30 min, either Flavopiridol (2 μM for 30 min) or RO-3306 (5 μM for 30 min) were added to the heavy condition. Heavy and light HeLa cells were counted, mixed 1:1, snap-frozen in liquid nitrogen, and stored at −80 °C until lysis. Experiments were performed in biological triplicates.

**LC-MS/MS Analysis**—Phosphopeptides were analyzed on a Q-Exactive Plus hybrid quadrupole Orbitrap mass spectrometer (ThermoFisher Scientific) equipped with an Easy-nLC 1000 (ThermoFisher Scientific) and nanospray source (ThermoFisher Scientific). Peptides were desalted in 5% methanol/1% formic acid and loaded on to a trap column (1 cm length, 100 μm inner diameter, ReproSil, C$_18$ AQ 5 μm 120 Å pore (Dr. Maisch, Ammerbuch, Germany) for 5 min of trypsin digestion. The peptides were then treated with MG132 at a concentration of 10 μM for 30 min. After 30 min, either Flavopiridol (2 μM for 30 min) or RO-3306 (5 μM for 30 min) were added to the heavy condition. Heavy and light HeLa cells were counted, mixed 1:1, snap-frozen in liquid nitrogen, and stored at −80 °C until lysis. Experiments were performed in biological triplicates.

**Phosphoprotein Western Blotting**—Western blot analysis was performed as described (48). Blots were probed with anti-phospho-Cdk1 antibody (phospho-Thr161, Cell Signaling Technology, Danvers, MA). The molecular weight standards were visualized using a silver staining kit.

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column (35 cm length, 100 μm inner diameter, ReproSil, C18 AQ 3 μm 120 Å pore) pulled in-house (Sutter P-2000, Sutter Instruments, San Francisco, CA) with a 60 min gradient of 5–30% LC-MS buffer B (LC-MS buffer A: 0.0625% formic acid, 3% ACN; LC-MS buffer B: 0.0625% formic acid, 95% ACN). The Q-Exactive Plus was set to perform an Orbitrap MS1 scan (r = 70K; AGC target = 3e6) from 350–1500 Thomson, followed by HCD MS2 spectra on the 10 most abundant precursor ions detected by Orbitrap scanning (r = 17.5K; AGC target = 1e5; max ion time = 75ms) before repeating the cycle. Precursor ions were isolated for HCD by quadrupole isolation at width = 0.8 Thomson and HCD fragmentation at 26 normalized collision energy (NCE). Charge state 2, 3, and 4 ions were selected for MS2. Precursor ions were added to a dynamic exclusion list (AGC target 17.5K; AGC target = 1e5; max ion time = 75ms) for 20 s. Raw data were searched using COMET (release version 2014.01) in high resolution mode (48) against a target-decoy (reversed) version of the human proteome sequence database (UniProt; downloaded 2/2013, 40,482 entries of forward and reverse sequencing). The probability of phosphorylation site localization was assessed using PhosphoRS (50). Quantification of LC-MS/MS spectra was performed using MassChroQ (51). Phosphopeptide ratios were adjusted for mixing errors based on the median of the log2 H/L distribution.

Statistical Rationale and Data, Motif, and Homology Analysis—Phosphopeptides were filtered by their H/L log2 ratio averages <-1.4 and the corresponding p values <0.05, which were calculated using a two tailed Student’s t test assuming unequal variance. These peptides were then subjected to motif determination using an in-house modified version of the MMFPh algorithm (52).

Saccharomyces cerevisiae homologs of human candidate Cdk1 substrates were identified using Ensemble BioMart. UniProt accession numbers were mapped to Ensemble protein IDs using the UniProt conversion tool. In BioMart, Ensemble Genes 83 and hom sapiens genome (GRCh38.p5) were selected under databases. Ensemble protein IDs were input under filters, gene, and input into BioMart. Under “attributes,” homologs, yeast orthologs, yeast protein ID were selected. Yeast IDs were compared with previously identified Cdk1 phosphorylation sites in yeast (53), human and yeast homologs were aligned with Blast, and investigated for site conservation.

Protein-protein interactions of proteins belonging to phosphoprotein-tides with significant increase in phosphorylation occupancy were determined using the STRING database and analyzed in Cytoscape (54, 55). Edges represent protein-protein interactions based on the STRING database. GO analyses were performed in Cytoscape using BiNGO to test for ontology enrichment of biological processes and cellular components. To assess significance of enrichment of terms, a hypergeometric test and Benjamini & Hochberg false discovery rate (FDR) correction were used. For a processes or component to be considered as “enriched,” a corrected p value cutoff of 0.05 was applied.

Cloning, insect cell expression and purification of GST-Cdk1 and Cdk1—Cdk1 and Cyclin B were amplified via PCR using the following primers:

Cyclin B Forward 5’-CCGCTCGAGATGGCGCTCGAGTCACC-3’
Cyclin B Reverse 5’-CCAAAAGTTTTACACCTTTGTGGACAGCCTC-3’
Cdk1 Forward 5’-CGCGGAATCCATGGAAGATTATACCAAAA-3’
Cdk1 Reverse 5’-CCGGAATTCCATACATCCTTTAATCTGAT-3’—Both genes were cloned into the pFastBac1 (Life Technologies) vector using restriction digests. Cyclin B was cloned into a pFastBac1 vector containing a GST tag while Cdk1 was cloned into pFastBac1 with no tag. Constructs were sequenced and transformed into the DH10α E. coli strain to create bacmids. The resulting bacmid DNA was isolated, genotyped, and transfected into Sf9 cells to create recombinant baculovirus. For protein production, Sf9 cells were co-infected in a T75 dish with Cdk1 and GST-cyclin B viruses for 84 h. Prior to collection, cells were treated with okadaic acid for 2 h, collected, and washed in PBS. Cells were lysed in GST lysis buffer containing: PBS, 0.5% Triton X-100, 1 mM EDTA, 0.5 mM DTT, and a protease inhibitor tablet. Samples were sonicated three times for 15 s each. Cell lysate was clarified at 8000 g for 30 min. Glutathione-Sepharose previously washed in GST lysis buffer was added to the clarified lysate for 1 h with rotation at 4 °C. Kinase complex-bound glutathione-Sepharose was washed three times with PBS, followed by elution in 100 μl of GST elution buffer containing: 50 mM Tris-HCl pH 8.7, 150 mM NaCl, 50 mM reduced glutathione, 0.5 mM DTT, 0.1% CHAPS, pH 8 and dialyzed overnight at 4 °C against 10 mM HEPES-KOH pH 7.7, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol.

Purification of Substrates from E. coli—Substrates were amplified using PCR with the following primers

Vimentin Forward 5’-ATAAGAATTCGCGCGCAATGTTCCAGCA-3’, Vimentin Reverse 5’-CCGCTCGAGTATCATGTCATGTCGAGT-3’, HMGAI Forward 5’-CCGCTCGAGATGTCTGGGAGGCGAACG-3’, HMGAI Reverse 5’-TAACATCTGCGGTCGCTACGTCTC-3’, NUCKS Forward 5’-CCGCTCGAGATGTCCGCGTCTCAGAA-3’, NUCKS Reverse 5’-TAACATCTGCGGTCGCTACGTCTC-3’, LTOR1 Forward 5’-CCGCTCGAGATGTCTGCTGCTAAGC-3’, LTOR1 Reverse 5’-TAACATCTGCGGTCGCTACGTCTC-3’, PHILA2 Forward 5’-CCGCTCGAGATGAATCCTGCACCCCGACGAGG-3’, PHILA2 Reverse 5’-CCGCGATCTCCATGCGGCGGTTGTT-3’, TK1 Forward 5’-CCGCTCGAGATGATGCTGCTAATTACC-3’, TK1 Reverse 5’-TAACATCTGCGGTCGCTAATTACC-3’, Sec22B Reverse 5’-TAACATCTGCGGTCGCTAATTACC-3’, Sec22B Forward 5’-CCGCTCGAGATGTCCGCGTCTCAGAA-3’, Cdc55A Forward 5’-CCGCTCGAGATGTCCGCGTCTCAGAA-3’, Cdc55A Reverse 5’-TAACATCTGCGGTCGCTAATTACC-3’—Each substrate was cloned into the pET16b vector containing a 10×-His tag. The constructs were sequenced and transformed into BL21 (DE3) pLys E. coli. Colonies were grown overnight in LB liquid medium containing 0.1 mg/ml of ampicillin at 37 °C to saturation. Cultures were then diluted into LB liquid medium containing 0.1 mg/ml of ampicillin and grown at 37 °C until an OD600 reading of 0.6. Cultures were then induced with 1 mM IPTG and moved to 18 °C overnight. Soluble proteins were purified under native conditions. Pellets were resuspended in 6–7 ml of lysis buffer containing: 50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole pH 8. Cells were sonicated, preclarified, and incubated with nickel–NTA beads (Qiagen) for 3 h at 4 °C. Beads were collected and washed in wash buffer containing: 50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole pH 8. The beads were eluted with 100 μl of elution buffer containing: 50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole pH 8. Insoluble proteins were purified under denaturing conditions. The cell pellet was lysed in 7 ml urea, 0.1 M NaH2PO4, 0.01 M Tris-Cl pH 8, sonicated, preclarified, and incubated with nickel–NTA beads for 3 h at 4 °C. The beads were collected and washed in 8 ml urea, 0.1 M NaH2PO4, 0.01 M Tris-Cl pH 6.3. Beads were eluted in 8 ml urea, 0.1 M NaH2PO4, 0.01 M Tris-Cl pH 4.5. In both cases, purified protein was dialyzed overnight in dialysis buffer containing: 10 mM HEPES-KOH pH 7.7, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 10% glycerol.
In Vitro Kinase Assays—Kinase assays containing 75 ng of Cdk1-cyclin B, 1 μg of purified substrate, 20 mM HEPES-KOH pH 7.7, 10 mM MgCl₂, 0.1 mM EGTA, 0.1 mM DTT, 2.5 mM β-glycerophosphate, and 100 μM ATP were placed at 30 °C for 3 h, followed by addition of 5 mM DTT at 50 °C for 30 min. The reaction was allowed to cool to room temperature and then alkylated with 15 mM iodoacetamide in the dark for 1 h. The alklylation reaction was then quenched with 5 mM DTT for 15 min. Each reaction was analyzed by SDS-PAGE gel-LC-MS/MS.

RESULTS

Quantitative Analysis of the Phosphoproteome of Flavopiridol and RO-3306 Treated, Mitotically Arrested HeLa Cells—To determine the optimal concentration of Flavopiridol and RO-3306 necessary to inhibit Cdk1 activity in mitotically arrested HeLa cells, we performed a dilution series for each inhibitor. HeLa cells were synchronized in cell cycle progression by thymidine and arrested in mitosis using Taxol. Mitotically arrested HeLa cells were treated with the proteasome inhibitor MG-132 and increasing amounts of Flavopiridol or RO-3306 respectively (Fig. 1B) (56). HeLa cells were lysed and analyzed by Western blot using an anti-Cdc25c antibody. Cdc25c is a known Cdk1 substrate, which is hyperphosphorylated in mitosis by Cdk1 (57, 58). Inhibition of Cdk1 results in dephosphorylation of Cdc25c and collapse of the Cdc25c proteoforms into a faster migrating species by SDS-PAGE. Based on these results, we chose an inhibitor concentration of 2 μM for Flavopiridol and 5 μM for RO-3306 for the quantitative phosphoproteomics analysis.

To identify Cdk1 substrates, HeLa cells were metabolically-labeled with stable isotope-containing heavy and light amino acids were treated with Cdk inhibitor (heavy) or control DMSO (light), mixed, lysed, reduced, alkylated, and trypsin digested. Phosphopeptides were enriched using titanium dioxide microspheres. Phosphopeptides were separated by strong cation exchange (SCX) chromatography and analyzed by LC-MS/MS (n = 3 independent experiments).
nine and lysine in cell culture (SILAC) (59), synchronized in cell cycle progression, and arrested in mitosis with Taxol. Heavy-labeled HeLa cells were treated with MG132 and Flavopiridol (Fig. 1C) or RO-3306 (Fig. 1D), whereas light-labeled HeLa cells were treated with MG132 and DMSO. Post-treatment heavy- and light-labeled HeLa cells were mixed based on cell counts and digested into peptides with trypsin. Phosphopeptides were enriched using titanium dioxide microspheres, separated by strong cation exchange chromatography, and analyzed by LC-MS/MS.

In these analyses we identified 24,840 phosphopeptides on 4273 proteins, of which 13,139 phosphopeptides on 2996 proteins were quantified. Phosphopeptide quantification was carried out using conservative peak detection parameters to ensure accurate and reproducible peak area measurements. The majority of phosphopeptides that were identified but not quantified were present in only one of the six experiments and either too low in intensity or associated with a peak area in only the heavy or light condition. Some phosphopeptides were identified in two or three of the biological replicates for Flavopiridol or RO-3306, but were associated with a peak area in only the heavy or light condition, which could be due to complete dephosphorylation upon addition of Cdk inhibitor for light only phosphopeptides. Phosphopeptides present in only the heavy condition are potentially the result of dephosphorylation of multiply phosphorylated peptides with the same backbone sequence, but at additional phosphorylation site(s). We found that the ratio distribution of these phosphopeptides in both Flavopiridol (Fig. 2A) and RO-3306 (Fig. 2B) contained a large number of phosphopeptides with reduced abundance in the heavy condition, as seen by the tailing toward lower heavy-to-light ratios. To assess the statistical significance of the observed changes, we calculated the p value of the mean of independent quantifications in the biological replicates using Student’s t test. Of the 13,139 quantified phosphopeptides, 11,295 were quantified in at least two of the three biological replicates with either Flavopiridol or RO-3306. In the Flavopiridol experiment, 5057 phosphopeptides were quantified with a p value of <0.05, of which 527 had a heavy to light log₂ ratio > 1.4 (2.5-fold) and 1,152 had a heavy to light log₂ ratio < −1.4 (Fig. 2C). In the RO-3306 experiment, 4785 phosphopeptides were quantified with a p value of <0.05, of which 444 had a heavy to light log₂ ratio > 1.4 and 865 had a heavy to light log₂ ratio < −1.4 (Fig. 2D).
Comparison of ratio quantification upon treatment with Flavopiridol or RO-3306 on a per phosphopeptide basis revealed strong overall correlation of both datasets, with an R² value of 0.79 (Fig. 2E). When we limited this analysis to peptides that were quantified with a p value of 0.05 or less, the correlation further improved to an R² value of 0.87 (Fig. 2F). We identified a total of 1215 phosphopeptides on 551 proteins that were reduced by 2.5-fold or more (p value < 0.05) in the two experiments, of which 802 phosphopeptides on 420 proteins were identified and quantified upon treatment with Flavopiridol as well as RO-3306 (supplemental Fig. S1, supplemental Table S1). The high degree of correlation between both data sets and overlap of phosphopeptides that responded to inhibitor treatment supports the notion that in mitosis, effects on HeLa cells observed with either the more general Cdk inhibitor, Flavopiridol, or the more selective Cdk1 inhibitor, RO-3306, are predominately due to inhibition of Cdk1.

We compared candidate Cdk1 substrates identified in these analyses to a previously established data set of cyclin-specific interacting proteins (60) (supplemental Fig. S2). Of the 551 proteins we identified containing phosphorylation sites that were significantly decreased upon Cdk1 inhibition, only 53 were identified in the cyclin interactome analysis. Of these, 14 were specific to cyclin A, 20 to cyclin B, and 19 bound to both cyclin A and B (60) (supplemental Fig. S2). The limited overlap is likely due to the transient interaction of cyclin-dependent kinases with their substrates compared with more stable protein interactions that are essential for cyclin-dependent kinase function and localization.

We also sought to determine if any candidate Cdk1 substrates are conserved throughout evolution. Previously, Cdk1-dependent phosphorylation sites were reported in the budding yeast Saccharomyces cerevisiae (53). To compare both data sets, we first identified S. cerevisiae homologs of the 551 proteins containing candidate human Cdk1 phosphorylation sites and found that 158 proteins were conserved between these organisms (supplemental Table S2). Of these proteins, 98 were present in the yeast Cdk1 substrate analysis, but only 20 were sensitive to Cdk1 inhibition (53). Next, we investigated if the precise position of the phosphorylation site was conserved, and found that this was the case for three of the 20 proteins (NSF1C Ser²⁷² - YBL085W Ser³²², MINK1 Ser⁷⁷⁸ - YHR102W Ser⁷³⁵, and HFCF1 Ser⁹⁸⁸ - YHR158C Ser⁶¹³), while for the other 17 only the kinase-substrate relationship, but not the exact phosphorylatable position, was conserved. These results are consistent with observations made by us (61) and those authors (53) that evolutionary conservation of the exact phosphorylation site position is rare, and that functional kinase–substrate relationships do not require strict conservation of the position of the phosphorylation site.

Characterization of Candidate Substrates of Cdk1 Activity—To determine the chemical nature of Flavopiridol and RO-3306 sensitive phosphorylation sites, we performed motif analysis on phosphopeptides with reduced abundance upon inhibitor treatment (52, 62). In both Flavopiridol (Fig. 3A) and RO-3306 (Fig. 3B, we identified an enrichment of a proline-directed (phospho-S/T-P) motif, which is consistent with the canonical kinase substrate motif of cyclin-dependent kinases, including Cdk1 (63, 64). Of the 1215 phosphopeptides where the abundance was reduced upon inhibitor treatment, 68% contain a proline-directed motif. When we mapped these phosphopeptides onto their corresponding proteins (Fig. 3C), we found that 71% contained two or more regulated phosphopeptides and 46% contained three or more, suggesting that Cdk1 regulation of protein function occurs through cumulative multisite phosphorylation events. Multisite phosphorylation of Cdk substrates was previously reported in several yeast species and Xenopus laevis, and proposed to allow for differential regulation of substrate phosphorylation and function (57, 65–67).

To determine if cumulative Cdk1 phosphorylation occurs not only on specific proteins but also on multiple members of protein complexes or specific signaling pathways we used the STRING database (68, 69) and mapped the connectivity of Cdk1-regulated proteins (Figs. 3C) in Cytoscape (54, 55). Indeed, most proteins that contain Cdk1-regulated phosphorylation sites were highly connected with each other through known interactions, suggesting that Cdk1-dependent regulation in mitosis occurs through cumulative phosphorylation of individual as well as multiple proteins within defined signaling pathways.

To further investigate this, we mapped the localization of Cdk1 substrates to subcellular structures. Mitosis is characterized by the dramatic reorganization of major cellular architectures: DNA condenses into chromosomes; the nuclear envelope breaks down; the Golgi disassembles; the actin cytoskeleton rearranges to allow for cell rounding, cortical stiffening, and abscission; and the microtubule cytoskeleton forms the mitotic spindle. Indeed, in our analyses we identified enrichment (hypergeometric test, Benjamini and Hochberg false discovery rate (FDR) correction; p value < 0.05) of Cdk1 substrates at these subcellular structures (Fig. 4, supplemental Table S3), clearly demonstrating the important role of Cdk1 in the regulation of multiple aspects of mitosis. We identified many proteins that form the nuclear envelope and nuclear pore, which are disassembled in prophase. Furthermore, we identified proteins as part of the spliceosome and ribonucleoprotein (RNP) complex, the functions of which are ceased during mitosis although the proteins remain associated, pointing to a potential inhibitory function of Cdk1 phosphorylation (70). Conversely, a recent analysis of mitotic defects upon systematic deletion of all human genes found that deletion of 27 core spliceosome components resulted in mitotic defects (70, 71). In our analyses we identified phosphorylation sites on 17 of these proteins and found that two (SF3BP1 and MFAP1) are candidate Cdk1 substrates. Furthermore, there are many interactions between proteins local-
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ized to the same subcellular structure as well as between them, linking them together. For instance, the microtubule cytoskeleton that forms the mitotic spindle is tightly connected with kinetochores/centromeres, which are the anchor points of the spindle microtubules. Additional examples include condensed chromosomes, as well as centrosomes and spindle poles, which function as microtubule organizing centers and are essential for bipolar spindle formation.

Next, we performed gene ontology (GO) analysis of proteins that contained phosphorylation sites that were reduced upon Cdk1 inhibitor treatment. We found an enrichment (hypergeometric test, Benjamini and Hochberg FDR correction; \( p \) value \(< 0.05\)) of processes related to the different subcellular structures (supplemental Fig. S3, supplemental Table S4), including RNA splicing and processing, transcription, DNA replication, chromatin organization, chromosome condensation, sister chromatid cohesion and segregation, microtubule spindle organization, centrosome and microtubule organization, and nuclear pore assembly.

We also mapped candidate Cdk1 substrates onto the CORUM protein complex database to determine through which protein complexes and signaling pathways Cdk1 regulates these biological processes (72). The CORUM database is a collection of experimentally characterized protein complexes that are important for the function and regulation of cellular processes. Of the 551 proteins that contain Flavopiridol or RO-3306 sensitive phosphorylation sites, 215 were present in 433 CORUM protein complexes (supplemental Fig. S4). For the majority of CORUM protein complexes (317 of 433, 68.2%), we identified only one complex member as sensitive to Cdk1 inhibition. For the remaining 116 CORUM complexes, the number of Cdk1 inhibitor sensitive complex members ranged from 2 to 26, the latter of which was represented by the spliceosome. Of these 116 complexes, 68 were significantly enriched in our datasets (Fisher's exact test; \( p \) value \(< 0.05\)) (supplemental Table S5). Similar to the results of the GO analysis, the CORUM complexes enriched in our datasets indicate a function of Cdk1 in the regulation of the spliceosome (Fig. 5A) as well as chromosome segregation and condensation (Fig. 5B). Furthermore, we found an enrichment of candidate Cdk1 substrates in CORUM complexes involved in DNA replication (supplemental Fig. S5A), chromatin organization (supplemental Fig. S5B), transcription (supplemental Fig. S5C), translation and ribosome function (supplemental Fig. S5D), and nuclear pore complex assembly (supplemental Fig. S5E).

Cell Cycle-dependent Expression of Cdk1 Substrates—Cdk1 controls mitotic progression through substrate phosphorylation. Although Cdk1 is expressed throughout the cell

![Fig. 3. Characterization of candidate Cdk1 substrates. A and B, Motif analysis of phosphopeptides significantly (\( p \) value less than 0.05) decreased by 2.5-fold or more upon Flavopiridol (A) or RO-3306 (B) addition compared with control. C, STRING protein-protein interaction based network. Node size represents ratio fold-change, node color in green represents number of sequenced SP/TP phosphorylation (p-site) per protein, gray - no SP/TP site.](image-url)
cycle, its activity is restricted to G2 and mitosis by post-translational modifications and binding to cyclins, which are expressed in a cell cycle-dependent manner. In S-phase and in early G2, cyclin A is present, and binds to and activates Cdk1. Starting in G2, cyclin B is expressed and further binds to and activates Cdk1. In G2, the majority of Cdk1 activity depends on its regulation by cyclin A; however, in prophase cyclin A is degraded and the balance of Cdk1 activity shifts toward the Cdk1/cyclin B complex. To determine if cell cycle regulated Cdk1 activity is the main regulatory input that con-

Fig. 4. Subcellular localization of candidate Cdk1 substrates. Localization of candidate Cdk1 substrates to subcellular structures based on cellular component gene ontology (GO) analysis. Nodes indicate candidate Cdk1 substrates, edges indicate protein - protein interactions based on the STRING database.
Fig. 5. Mapping of candidate Cdk1 substrates to annotated CORUM protein complexes. A, Candidate Cdk1 substrates identified in CORUM complexes associated with RNA processing and splicing. B, Candidate Cdk1 substrates identified in CORUM complexes associated with the regulation of chromosome condensation, cohesion, and segregation. CORUM complexes are indicated as gray circular nodes, candidate Cdk1 substrates identified in this analysis are shown as turquoise squares.
Cdk1 Substrates in Mitosis

Fig. 6. Enrichment of candidate Cdk1 substrates in G2- and M-phase specific mRNA transcripts. Enrichment of candidate Cdk1 substrates identified by Flavopiridol or RO-3306 in cell cycle genes of different phases. Cell cycle genes in HeLa cells are sorted based on their peak expression during cell cycle progression. Each gene was assigned an angle range from 0° to 360° based on its peak time. Enrichment of substrates in cell cycle genes from each sliding window was calculated using Fischer’s exact test over a sliding window of 30° with a 10° overlap between neighboring windows. Blue to yellow gradient indicated enrichment of candidate Cdk1 substrates in different cell cycle phases.

d controls substrate function in mitosis, or if other inputs contribute to this process, we investigated substrate mRNA expression profiles (73) of candidate Cdk1 substrates across the cell cycle. It is conceivable that Cdk1 substrates are expressed throughout the cell cycle, and that Cdk1 phosphorylation activates or inhibits their function. RNA transcription and translation are strongly reduced in mitosis, thus it is possible that proteins with mitosis-specific functions that are expressed at early cell cycle stages are post-translationally modified by Cdk1 to fulfill their mitotic function. Alternatively, Cdk1 substrate expression could be cell cycle regulated, in which case Cdk1 phosphorylation would provide an additional layer of regulation to fine-tune substrate function. It was previously shown by Whitfield et al. (73) that of 13,912 genes profiled, 588 are cell cycle regulated (74). We mapped proteins on which we identified phosphorylation sites that responded to either Flavopiridol or RO-3306 to gene sequences present in that microarray analysis (73). Of the 527 and 444 proteins that were reduced in phosphorylation upon Flavopiridol or RO-3306 treatment (phosphorylation site reduced by at least 30-fold upon addition of ATP) were present in the data set of Whitfield et al. (73). Both datasets were strongly enriched for G2-(Flavopiridol: p value = 5.8e-22, RO-3306: p value = 2e-17) and M-(Flavopiridol: p value = 3.7e-26, RO-3306: p value = 6.42e-11) phase specific genes (Fig. 6), supporting the notion that mitotic processes are regulated on several levels, including cell cycle phase-specific expression as well as post translationally through Cdk1 phosphorylation to provide tighter control and increase the accuracy of mitotic progression.

Validation of Candidate Cdk1 Substrates by In Vitro Kinase Assay—We performed in vitro kinase assays using Cdk1-Cyclin B purified from SF9 insect cells and candidate substrates purified from bacteria to determine if reduction in phosphorylation abundance upon Flavopiridol or RO-3306 treatment is directly due to inhibition of Cdk1 activity. Purified Cdk1-Cyclin B and substrate were mixed with or without ATP and incubated at 30 °C for 3 h. Afterward, reactions were quenched by the addition of sample buffer and resolved by SDS-PAGE. After staining with Coomassie, bands corresponding to the substrates were excised from the gel, tryptic digested, and analyzed by LC-MS/MS to identify phosphorylation sites (Fig. 7A). Only two of the eight substrates were phosphorylated on candidate Cdk1 sites in the absence of ATP (supplemental Table S6), and in these two cases phosphopeptide abundance was increased by at least 30-fold upon addition of ATP. MS/MS spectra of phosphopeptides identified from HeLa cells that responded to Flavopiridol or RO-3306 inhibitor treatment were compared with MS/MS spectra of phosphopeptides obtained through in vitro kinase reactions (Fig. 7B, supplemental Fig. S6). We tested eight candidate Cdk1 substrates in this analysis and found that for all eight, the MS/MS spectra of phosphopeptides identified in cells mirrored those identified in vitro, supporting the notion that phosphorylation sites that respond to Flavopiridol or RO-3306 treatment are candidate Cdk1 substrates.

DISCUSSION

Accurate progression through mitosis is one of the most important objectives of a cell. The essential protein kinase Cdk1 plays a central role in the regulation of mitotic progression and is often dubbed the “master regulator of mitosis.” Cdk1 phosphorylation controls entry into mitosis, nuclear envelope breakdown, condensation of DNA into hallmark mitotic chromosomes, disassembly of the endoplasmic reticulum and Golgi apparatus, the formation of the microtubule-based mitotic spindle as well as checkpoints that ensure the fidelity of these processes. While the roles of Cdk1 in these processes are well established, known Cdk1 substrates are not sufficient to explain all the observed defects upon increased or reduced Cdk1 activity. To provide a more comprehensive view of the candidate mitotic substrate space, we conducted quantitative phosphoproteomics analyses using clinically evaluated small molecule inhibitors of Cdk1 activity in mitotically-arrested HeLa cells (35–37) (Fig. 1C and 1D).
Cdk1 is the founding member of a larger family of cyclin-dependent kinases, and early Cdk inhibitors lacked selectivity. However, in mitosis Cdk1 is thought to be the main acting Cdk, and we hypothesized that most mitotic Cdk substrates are indeed Cdk1 substrates. To test this hypothesis, we compared the phosphoproteome of mitotically-arrested HeLa cells treated with Flavopiridol, an ATP-competitive inhibitor that targets Cdk1, Cdk2, Cdk4, Cdk6, and Cdk9 (36, 38), and a more selective Cdk1 inhibitor, RO-3306 (43). Comparison of the changes in phosphorylation induced by both inhibitors revealed a high degree of correlation for all sequenced and quantified phosphopeptides ($R^2$ value of 0.79 (Fig. 2E)) and especially for phosphopeptides quantified with a $p$ value of 0.05 or less ($R^2$ value of 0.87 (Fig. 2F)), suggesting either that Cdk1 is the main acting Cdk or Cdk1 is responsible for the majority of Cdk substrate phosphorylation in mitosis. Motif analysis of significantly decreased phosphopeptides revealed a proline-directed motif, which is reminiscent of the Cdk1 consensus motif and further supported the selectivity of Flavopiridol and RO-3306 for Cdk-family kinases (63, 64).

Interestingly, we found that over 70% of proteins identified as candidate Cdk1 substrates contained two or more inhibitor-sensitive phosphorylation sites (Fig. 3C), suggesting that regulation of substrate activity, localization, or function by Cdk1-dependent multisite phosphorylation promotes switch-like transition during mitotic entry and progression when specific sites are phosphorylated. Multisite phosphorylation could provide an explanation for the differential phosphorylation of some Cdk1 substrates earlier or later in mitosis based on the number and order of sites that have to be phosphorylated to trigger downstream signaling. In addition, this mechanism allows for greater control by opposing phosphatases and fine tuning of the signal process based on the number of phospho-
ylated sites involved. Cdk1-dependent multisite phosphorylation has been observed in several yeast species and Xenopus laevis suggesting that this mechanism could be conserved in human cells (57, 65–67). Further investigation of Cdk1-dependent multisite phosphorylation will be important to determine if the underlying mechanisms are also conserved.

Furthermore, we found that multisite phosphorylation by Cdk1 is not restricted to individual substrates but extends to signaling pathways (Fig. 3C) and protein complexes (Fig. 5). Cdk1 is essential for many cellular processes that promote and control progression through mitosis, including structural changes to the actin and microtubule cytoskeleton, disassembly of the nuclear envelope, endoplasmic reticulum, and Golgi apparatus, as well as regulatory changes in chromatin organization, RNA processing, transcription, and translation. Temporal and spatial coordination of these processes is important to ensure mitotic fidelity. Cumulative multisite phosphorylation of specific proteins and signaling networks might contribute to a necessary robustness, as well as the dynamics observed in mitotic progression.

Our analyses greatly increase the known substrate space of Cdk1 and provide a resource for future mechanistic studies of the role of Cdk1-dependent substrate phosphorylation in mitotic progression and its dynamic regulation by counteracting phosphatases.

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Authors declare no competing interests.

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