Combination of Chemical Genetics and Phosphoproteomics for Kinase Signaling Analysis Enables Confident Identification of Cellular Downstream Targets*

Felix S. Oppermann‡§¶, Kathrin Grundner-Culemann‡§¶, Chanchal Kumar||**, Oliver J. Gruss‡¶, Prasad V. Jallepalli§¶¶, and Henrik Daub‡¶

Delineation of phosphorylation-based signaling networks requires reliable data about the underlying cellular kinase-substrate interactions. We report a chemical genetics and quantitative phosphoproteomics approach that encompasses cellular kinase activation in combination with comparative replicate mass spectrometry analyses of cells expressing either inhibitor-sensitive or resistant kinase variant. We applied this workflow to Plk1 (Polo-like kinase 1) in mitotic cells and induced cellular Plk1 activity by wash-out of the bulky kinase inhibitor 3-MB-PP1, which targets a mutant kinase version with an enlarged catalytic pocket while not interfering with wild-type Plk1. We quantified more than 20,000 distinct phosphorylation sites by SILAC, approximately half of which were measured in at least two independent experiments in cells expressing mutant and wild-type Plk1. Based on replicate phosphorylation site quantifications in both mutant and wild-type Plk1 cells, our chemical genetic proteomics concept enabled stringent comparative statistics by significance analysis of microarrays, which unveiled more than 350 cellular downstream targets of Plk1 validated by full concordance of both statistical and experimental data. Our data point to hitherto poorly characterized aspects in Plk1-controlled mitotic progression and provide a largely extended resource for functional studies. We anticipate the described strategies to be of general utility for systematic approaches are required for the comprehensive and confident identification of cellular protein kinase substrates. Molecular & Cellular Proteomics 11: 10.1074/mcp.O111.012351, 1–12, 2012.

Reversible protein phosphorylation by protein kinases represents a key control mechanism in signal transmission and

controls nearly all aspects of cellular physiology. Quantitative proteomics approaches that incorporate techniques such as stable isotope labeling by amino acids in cell culture (SILAC),1 phosphopeptide fractionation and enrichment by strong cation exchange (SCX), and ion metal affinity chromatography (IMAC) together with sensitive high resolution MS analysis and automated peptide identification and quantification have made it possible to monitor phosphorylation-based signaling on a global scale (1–4). Because signaling networks are defined by the underlying kinase-substrate relationships, systematic approaches are required for the comprehensive and confident assignment of cellular kinase substrates (5). To identify cellular substrates, the catalytic activity of a kinase of interest needs to be rapidly regulated to capture a high fraction of direct phosphorylation events. This implies that protein kinase ablation by genetic knockout or RNA interference can be of limited utility, because of secondary changes that can accumulate during the time required for cellular kinase depletion (3, 5). In contrast, pharmacological interference by small molecules allows for rapid modulation of kinase activity and should therefore enable unbiased monitoring of signaling perturbations when combined with advanced MS-based proteomics. Such approaches are ideally based on mono-selective kinase inhibition. Although generally difficult to achieve for naturally occurring kinases, this is considered feasible by chemical genetics using drug-sensitized kinase mutants possessing an enlarged catalytic pocket to accommodate bulky kinase inhibitors (6). Recently, this inhibition strategy has been combined with large scale quantitative phosphoproteomics in efforts to identify cyclin-dependent kinase 1 substrates upon the addition of the purine analog NM-PP1 in yeast cells (7). However, even supposedly selective kinase inhibitors such as the purine analog NM-PP1, which was designed for specific

The abbreviations used are: SILAC, stable isotope labeling by amino acids in cell culture; SCX, strong cation exchange; IMAC, ion metal affinity chromatography; IPI, International Protein Index; FDR, false discovery rate; GO, Gene Ontology; RPE, retinal pigment epithelial; SAM, significance analysis of microarrays; mTOR, mammalian target of rapamycin; EGFR, epidermal growth factor receptor.

Received June 30, 2011, and in revised form, November 18, 2011

Published, MCP Papers in Press, December 22, 2011, DOI 10.1074/mcp.O111.012351

Want to cite this article? Please look on the last page for the proper citation format.
inhibition of mutationally sensitized kinase variants, exhibit off-target activity in vitro (6–8). Therefore, cellular selectivity control is warranted to confirm assignment of kinase-substrate relationships.

Here, our interest was to advance strategies for unbiased and confident identification of cellular downstream targets of protein kinases by using Plk1 (Polo-like kinase 1) signaling in human cells as a model system. Plk1 is a central regulator of cell division with key roles in mitotic entry, bipolar spindle assembly, and chromosome segregation, as well as cytokinesis (9). Consistent with these important functions throughout the M phase, human Plk1 localizes to centrosomes at mitotic entry, then associates with kinetochores, and later accumulates at the central spindle and the midbody in the late M phase (9). Plk1 possesses a carboxyl-terminal Polo box domain involved in the phosphorylation-dependent recruitment of substrate proteins through specific interactions with a Ser-(Ser(P)/Thr(P))-(Pro/Xaa) motif (10). Plk1 has been analyzed in previous studies to gain further insights into the mechanisms that execute Plk1 functions in mitosis, including a targeted analysis of selected candidates (11) and a proteomic screening of Plk1-interacting proteins using recombinant Polo box domain as bait (12). Moreover, a recent phosphoproteomics screening reported Plk1 substrate candidates in the mitotic spindle. In this study, phosphorylation changes were measured upon prolonged Plk1 inhibition by either small interference RNA expression or small molecule inhibitor treatment (13). However, none of the previous studies has systematically interrogated the effect of short term Plk1 modulation on a proteome-wide scale in a specificity-controlled manner. Here, we report an advanced concept that implements these criteria for the identification of kinase-regulated phosphoproteins in intact cells. Our goal was to acquire comprehensive and confident data about phosphorylation of Plk1 downstream targets as proof-of-concept for a tightly controlled approach of general utility for kinase signaling research.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—Telomerase-expressing human retinal pigment epithelial (hTERT-RPE) cells in which both genomic PLK1 loci were disrupted and that expressed either wild-type Plk1 (Plk1wt) or an analog-sensitive Plk1 mutant (C67V/L130G, referred to as Plk1as) as EGFP fusions were used throughout this study (14). For differential SILAC encoding, the cells were grown in a 1:1 mixture of arginine- and lysine-deficient Dulbecco’s Modified Eagle’s medium (Invitrogen) and F12 Ham’s medium (Invitrogen) supplemented with L-glutamine (2 mm; PAA Laboratories), sodium pyruvate (1 mm; Invitrogen), 1% penicillin/streptomycin (PAA Laboratories), 10% dialyzed fetal bovine serum (Invitrogen), and either 175 μM unlabeled l-arginine (Arg9) and 250 μM unlabeled l-lysine (Lys9) or the same concentrations of l-[U-13C6,15N2]arginine (Arg9) and l-[U-13C6,15N2]lysine (Lys9) (Sigma). After six cell doublings to ensure complete proteome labeling, 1.6 × 10^8 cells were seeded per 15-cm dish (in total 12–20 dishes/experiment). 18 h later, 1 μg/ml aphidicoline (Sigma) was added for a further 12 h to synchronize cells in the early S phase. The cells were then washed with PBS and cultured for another 13 h in fresh SILAC medium containing 50 ng/ml nocodazole (Sigma) and 5 μg/ml 3-MB-PP1 (Piraxon AG, Munich, Germany) to arrest Plk1as or Plk1wt cells in M phase in addition to inhibiting mutant kinase activity in Plk1as cells. The cells were then washed once prior to adding either fresh SILAC medium containing 50 ng/ml nocodazole and 5 μM 3-MB-PP1 to Arg9/Lys9-labeled cells or the same medium without 3-MB-PP1 to Arg9/Lys9-labeled cells for a further 30 min until cell lysis. This labeling scheme was used in two biological replicate experiments with Plk1wt (referred to as as1 and as3) and Plk1as cells (wt1 and wt3), whereas in two further replicate experiments with either cell line reciprocal labeling conditions were used (as2, as4, wt2, and wt4).

Cell Lysis and Immunoblotting—The cells were lysed in 500 μl of 8 mM urea, 50 mM Tris-HCl, pH 8.2, 75 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mM NaF, 2.5 mM Na3VO4, 50 ng/ml calyculin A (Alexis Biochemicals), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% phosphatase inhibitor mixture 1 and 2 (v/v) (Sigma) per two 15-cm dishes for 5 min on ice. The cell extracts were sonicated three times for 1 min on ice. Cell debris was then removed by centrifugation, and equal protein amounts from differentially SILAC-encoded cells were pooled for subsequent MS sample preparation. For immunoblot analysis, Plk1as cells were seeded at 180,000 cells/well in 3-well cell culture dishes. The cells were synchronized and treated as in the SILAC experiments with the only modification that the 13-h nuclease incubation was done both in the presence and the absence of 3-MB-PP1. Lysis was performed with buffer containing 50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 0.05% Triton X-100, and all phosphatase and protease inhibitors used for urea lysis of SILAC-encoded cells prior to gel electrophoresis and immunoblotting with monoclonal mouse anti-BubR1 antibody (Cell Signaling Technology, Inc.).

Mass Spectrometry Sample Preparation—Pooled lysates were adjusted to 6 M urea, 1.5 M thiourea and reduced, alkylated, and digested in solution with endoproteinase Lys-C and trypsin as detailed before (15). Tryptic peptides were then filtered through a 0.22-μm polyvinylidene fluoride membrane (Millipore) and desalted using reversed phase C18 SepPak cartridges (500-mg sorbent weight; Waters) as described previously (16). Desalted peptide samples were snap-frozen in liquid nitrogen, lyophilized, and stored at −20 °C. Lypophilized peptides were dissolved in 600 μl of 7 mM KH2PO4, pH 2.65, 30% ACN and loaded onto a 250 × 5.4 mm polySULFOETHYL A column (200 Å pore size and 5-μm particle size; PolyLC) operated on an linear ion trap (LTQ)-Orbitrap (Thermo Fisher Scientific) as described (2, 7). Desalted peptide samples were concentrated to 4 μl and mixed with an equal volume of 0.2% TFA, 4% ACN prior to MS analysis of technical replicates of each sample.

Mass Spectrometry Analysis—All of the LC-MS/MS analyses were performed on an linear ion trap (LTQ)-Orbitrap (Thermo Fisher Scientific) connected to a nanoflow HPLC system (Agilent 1100) via a nanoelectrospray ion source (Proxeon Biosystems) as described (2, 15). Briefly, phosphopeptide-enriched samples were resolved by a 15-cm analytical column (75-μm inner diameter) packed with 3-μm
C18 beads (Reprosil-AQ Pur; Dr. Maisch, GmbH, Germany) in 140-min runs by a gradient from 5 to 40% acetonitrile in 0.5% acetic acid and at a flow rate of 250 nl/min. Eluting peptides were directly electrosprayed into the mass spectrometer. The LTQ-Orbitrap was operated with Xcalibur 2.0 in the data-dependent mode to automatically switch between full scan MS acquisition in the orbitrap analyzer (resolution r = 60,000 at m/z = 400) and the acquisition of tandem mass spectra of 10 multiply charged ions by in the LTQ part of the instrument (18). Multi-stage activation was enabled in the linear ion trap to activate neutral loss species of phosphopeptides at 97.97, 48.99, or 32.66 m/z below the precursor ion for 30 ms during fragmentation (19). For all full scans in the orbitrap detector, a lock-mass ion from ambient air (m/z 429.08875) was used for internal calibration as described (2). Typical mass spectrometric conditions were: spray voltage, 2.4 kV; no sheath and auxiliary gas flow; heated capillary temperature, 175 °C; normalized collision energy 35% for multi-stage activation in LTQ. The ion selection threshold was 500 counts for MS/MS. An activation of q = 0.25 was used.

Data Processing and Statistical Analysis—All 192 raw files acquired in this study were collectively processed with the MaxQuant software suite (version 1.0.13.12), which performs peak list generation, SILAC-based quantification, estimation of false discovery rates, peptide to protein group assembly, and phosphorylation site localization as described previously (4, 20). Peak lists were searched against a concatenated forward and reversed version of the human International Protein Index (IPI) database version 3.37 (containing 69,141 protein entries and 175 frequently detected contaminants such as porcine trypsin, human keratins, and Lys-C) using the Mascot search engine (Matrix Science; version 2.2.04). Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine, amino-terminal acetylation, losses of ammonia from amino-terminal glutamine and cysteine, and phosphorylation on serine, threonine, and tyrosine were set as variable modifications. SILAC spectra detected by presearch MaxQuant analysis were searched with the additional fixed modifications Arg\(^{15}\) and/or Lys\(^{6}\), whereas spectra for which a SILAC state was not assignable prior to database searching were searched with Arg\(^{15}\) and Lys\(^{6}\) as variable modifications. Accepted mass tolerances were 7 ppm for the MS and to 0.5 Da for MS/MS peaks. The minimum required peptide length was six amino acids, and up to three missed cleavages and three labeled amino acids were allowed. The accepted estimated FDR determined on the basis peptide-spectral matches in the reversed database version was set to 1% for both peptide and protein identifications. Phosphorylation sites were assigned by the post-translational modification scoring algorithm implemented in MaxQuant as described (18, 20).

For phosphorylation site analysis, only those with a localization probability of at least 0.75 (class I sites) were considered. Phosphosite ratios were averaged for each biological replicate experiment in case of SILAC-based quantification in both technical replicate analyses. The resulting quantified phosphosite ratios from biological Plk1\(^{17}\) and Plk1\(^{18}\) replicate experiments were considered for all further comparative and statistical analyses. The distribution of phosphosite ratios was visualized by box plots with whiskers indicating five times the interquartile range. Box plots were computed in the R statistical environment (21) and were generated for all individual biological replicate experiments in Plk1\(^{17}\) and Plk1\(^{18}\) cells.

For phosphopeptide analysis, all peptide evidences were filtered for a Mascot score of at least 7 and a mass error of less than 5 ppm. Phosphopeptides were specified by their amino acid sequence and number of phosphorylation sites. In each technical replicate experiment, the median SILAC ratio was calculated for repeatedly quantified phosphopeptides. Phosphopeptide SILAC ratios were also averaged for each biological replicate experiment in case such ratios were available for both technical replicates.

To identify differential 3-MB-PP1 phosphoregulation in Plk1\(^{17}\) versus Plk1\(^{18}\) cells, all phosphosite or phosphopeptide ratios measured in at least two biological replicate analyses of both Plk1\(^{17}\) and Plk1\(^{18}\) cells were \(\log_2\)-transformed and subjected to two-class, unpaired significance analysis of microarrays (SAM) analysis (version 3.0, http://www-stat.stanford.edu/~tibs/SAM/sam.pdf) (22). SAM computes a statistics \(d_i\) for each ratio to identify significantly different ±3-MB-PP1 ratios in Plk1\(^{17}\) versus Plk1\(^{18}\) cells and uses permutations of the repeated measurements to calculate false discovery rates (q values) for different values of a threshold parameter \(\Delta\). Missing ratio values were imputed by SAM via a K-Nearest Neighbor algorithm normalization (number of neighbors set to 15).

For all regulated phosphosites reported by SAM (up to an FDR of 60%), we further analyzed whether either the Plk1\(^{17}\) or the Plk1\(^{18}\) ratio deviated more strongly from one for different \(\Delta\) parameters. For further bioinformatics analysis, phosphosites and phosphopeptides of the SAM output with a FDR (or q value) of 0% obtained for \(\Delta \geq 2.136\) and \(\Delta \geq 2.363\), respectively, were considered as regulated.

CK1\(^{16}\) Kinase Assay—In vitro CK1\(^{16}\) activity in the presence of different concentrations of 3-MB-PP1 was measured on the substrate CK1tide (200 \(\mu\)M; Upstate Biotechnology Inc.) in buffer containing 8 mM MOPS-NaOH, pH 7.0, 0.2 mM EDTA, 10 mM magnesium acetate, 0.1 \(\mu\)M ATP, 0.5 \(\mu\)M of [\(\gamma\)-\(^32\)P]ATP, and different 3-MB-PP1 concentrations. After a 30-min preincubation with inhibitor on ice, kinase reactions were started by ATP addition and performed for 10 min at 30 °C in a final volume of 25 \(\mu\)L. Phosphate incorporation and IC\(_{50}\) values were determined as described (23).

Bioinformatics Analysis—Sequence stretches of ≤6 residues for all up-regulated serine phosphorylation sites were analyzed by Motif-X to extract significantly overrepresented motifs compared with the background of all SAM-analyzed class I serine sites (24). In Motif-X analysis, the minimum number of occurrences and the required motif significance were set to 10 and 10\(^{-4}\), respectively. Furthermore, sequence windows of all phosphosites were analyzed for the presence of the Plk1 consensus motif (25), a (D/E/N)p(S/T) motif, and the Polo box domain recognition motif (10). For each of these three motifs, we then determined the fraction of Plk1-regulated phosphoproteins and the fraction of all proteins (harboring SAM-analyzed phosphosites) in which they were identified. Respective \(p\) values were calculated with Fisher’s exact test, one-tailed.

Enrichment analysis in the Gene Ontology (GO) cellular component or molecular function categories was performed for Plk1-up-regulated phosphoproteins (according to SAM analysis at an FDR of 0%, 372 IPI entries) compared with either all phosphoproteins with quantified phosphorylation changes from Plk1\(^{18}\) cells (4463 IPI entries) or the entire IPI database by nonconditional hypergeometric testing using the GO stats package (26) in the R statistical environment (21).

For network visualizaion, all of the identified phosphoproteins with quantified phosphorylation changes that were annotated with the GO cellular component terms centrosome, kinetochore, microtubule, and nuclear pore were submitted to the web-based Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) version 8 (27). Prior to retrieving protein-protein interactions according to the prediction methods “experiments” and “databases,” all of the IPI identifiers were converted to Ensembl gene identifiers using the BioMart web server (28). In cases where no Ensembl gene identifier could be assigned, the protein names were retained for STRING. Only interactions of high confidence (score > 0.7) were considered, and the resulting network was visualized using the Cytoscape software (29).

Accession Information—All of the MS raw data files from this study are accessible in the public repository Tranche (https://tranche.ucsc.edu/). The MS raw data files can be accessed through the following links: Plk1\(^{17}\) cell experiments; N7b51Xc861fjzudud.
RESULTS AND DISCUSSION

Pharmacological Induction of Cellular Plk1 Activity—To examine Plk1 inhibition in human retinal pigment epithelial (RPE) cells expressing analog-sensitive mutant (Plk1<sup>as</sup>) cells, we added the bulky purine analog 3-MB-PP1 to noclodazole-arrested mitotic cells similar to the reported strategy used for Cdk1 substrate identification in yeast (7, 14, 25). Plk1<sup>as</sup> harboring a mutationally enlarged hydrophobic pocket at the ATP-binding site was previously shown to be inhibited by 3-MB-PP1 in <i>vitro</i> and <i>in vivo</i>, whereas wild-type Plk1 was not affected by the inhibitor (14). However, even 30 min of 3-MB-PP1 treatment did not suppress phosphorylation of the known Plk1 substrate BubR1 (Fig. 1A)(30). This was likely due to inefficient dephosphorylation by cellular phosphatases and indicated a conceptual weakness in the experimental design. We therefore conceived a strategy to rapidly induce suppressed Plk1 activity in mitotically synchronized cells and first arrested cells by prolonged nocodazole treatment with the Plk1<sup>as</sup> inhibitor 3-MB-PP1 added during synchronization. Plk1-medi-
ated BubR1 phosphorylation was suppressed upon this treatment and, importantly, could then be induced when we washed out the inhibitor as demonstrated by the appearance of the phosphorylated, slower migrating band of BubR1 in Plk1<sup>as</sup>-expressing cells (Fig. 1A). In contrast, 3-MB-PP1 had no effect on BubR1 in control cells expressing wild-type Plk1 (data not shown).

**Combination of Chemical Genetics and Quantitative Phosphoproteomics**—Next, we performed SILAC of RPE cells expressing either Plk1<sup>as</sup> or 3-MB-PP1-insensitive wild-type enzyme (Plk1<sup>wt</sup>) followed by 30 min of inhibitor wash-out from nocodazole-arrested cells to reactivate 3-MB-PP1-inhibited kinases in mitotic cells prior to lysis (1, 18). For quantitative phosphoproteome assessment, we separated proteolytically derived peptides by SCX into 12 fractions prior to phosphopeptide enrichment by IMAC. We performed technical replicate analyses of all IMAC samples by LC-MS on a LTQ-Orbitrap (1, 18). Raw data files were processed with the MaxQuant software for peptide and protein identification and the quantification of phosphorylation changes (4). Finally, phosphorylation site ratios measured in both technical replicate experiments were averaged prior to further data analysis.

By applying this workflow, we identified and quantified ~9,000 distinct phosphorylation sites in either cell line that could be assigned to a specific serine, threonine, or tyrosine residue with a localization probability of at least 0.75 (class I sites) (18). Only class I phosphorylation sites were considered for further analysis throughout this study. Notably, although SILAC quantification reported for 482 sites at least 2-fold changes upon 3-MB-PP1 removal in Plk1<sup>as</sup> cells, we also recorded as many as 75 such apparent regulations in cells expressing 3-MB-PP1-insensitive Plk1<sup>wt</sup> (Fig. 1B and supplemental Table 1). Because the two cell lines were identical apart from either Plk1<sup>as</sup> or Plk1<sup>wt</sup> expression, any phosphorylation changes exclusively recorded in analog-sensitive cells should account for Plk1-mediated phosphorylation events, whereas those occurring in both cell lines would reflect Plk1-independent off-target effects of 3-MB-PP1. The latter could not be excluded because related purine analogs such as NM-PP1 and NA-PP1 were found to inhibit nonmutated protein kinases in vitro despite being designed as specific antagonist of mutationally sensitized kinase variants (8). However, the vast majority of sites quantified with greater than 2-fold changes in the Plk1<sup>wt</sup> cells did not exhibit similar regulation in Plk1<sup>as</sup> cells upon 3-MB-PP1 wash-out (supplemental Table 1). Of the 46 sites that we quantified with greater than 2-fold changes in Plk1<sup>wt</sup> cells and that we also quantified in the parallel Plk1<sup>as</sup> cell analysis, only four exhibited more than 2-fold change in the same direction upon 3-MB-PP1 wash-out from Plk1<sup>as</sup> cells (supplemental Table 1). Thus, the vast majority of apparent changes in Plk1<sup>as</sup> cells most likely reflected the inherent biological and technical variability of the overall experimental workflow. A similar number of such “false positives” in the Plk1<sup>as</sup> cell analysis would imply that ~15% of measured changes are not related to cellular Plk1 activity control by 3-MB-PP1, resulting in an estimated FDR we considered as inappropriate.

**Evaluation of Biological Replicate Strategy and 3-MB-PP1 Off-Target Identification**—Therefore, we extended our analysis to four replicate SILAC analyses per cell line with reversed labeling schemes in consecutive experiments (Fig. 1C). In total, we obtained quantitative phosphorylation data for 4803 phosphoproteins and quantified the effect of 3-MB-PP1 wash-out for 20,185 class I sites phosphorylation sites (median localization probability, 0.996) (Fig. 1D and supplemental Tables S1 and S2). Our experimental design enabled multiple pair-wise comparisons of independently measured phosphosite ratios across biological replicate analyses (Fig. 2A). Almost 3% (on average ~200) of all phosphosites were consistently regulated by more than 2-fold upon 3-MB-PP1 wash-out in the overlap of Plk1<sup>as</sup> experiments. In contrast, very few phosphosites exhibited reproducible changes in Plk1<sup>wt</sup> cell comparisons (Fig. 2A), and only two of them were present on the same doubly phosphorylated peptide were found in consistent ratios across all experiments (supplemental Fig. 1A). These sites mapped to casein kinase 1δ (CK1δ) and were also regulated in Plk1<sup>as</sup> cells (supplemental Fig. 1A). Notably, regulation was most likely due to direct cellular CK1δ inhibition, because low micromolar concentrations of 3-MB-PP1 abrogated CK1δ kinase activity in vitro (supplemental Fig. 1B). However, as these were the sole off-target effects in our large scale analyses, our results indicate high cellular 3-MB-PP1 selectivity against mitotically active protein kinases.

Consequently, our results verified that almost all ratio changes measured in individual Plk1<sup>wt</sup> cell experiments were due to experimental fluctuations and that two biological replicates dramatically reduced such false positive identifications. Moreover, the number of at least duplicate measurements increased considerably by ~60% when all four instead of just two replicate analyses were considered for Plk1<sup>as</sup> or Plk1<sup>wt</sup> cells (Fig. 2B).

**Statistical Approach to Identify Plk1-regulated Phosphorylations with High Confidence**—Our experimental design with parallel biological replicate analyses of Plk1<sup>as</sup> or Plk1<sup>wt</sup> cells resulted in a data structure rather similar to gene expression data from microarray measurements, except that SILAC ratios instead of mRNA intensities were measured and missing values were more prevalent in our proteomics data. Building on the similarities, we subjected our data to SAM, which is widely used in transcriptomics and provides FDR estimates based on adjustable thresholds and permutations of replicate data (22).

To identify specific Plk1-mediated phosphorylation events that are differentially induced in Plk1<sup>as</sup> over Plk1<sup>wt</sup> control cells upon 3-MB-PP1 wash-out, we restricted our further analysis to the 9204 phosphosite ratios available from at least two biological replicate experiments in both Plk1<sup>as</sup> and Plk1<sup>wt</sup>
Cells (Fig. 2C and supplemental Table 1). Values for missing phosphosite ratios were imputed in the resulting data matrix via a K-nearest neighbor algorithm normalization. SAM retrieved significantly regulated phosphosite subsets along with the corresponding FDRs for different delta (Δ) thresholds (Fig. 3A, Table I, and supplemental Table 1). At Δ = 2.136, the 396 most highly ranked phosphosites did not contain any remaining false positive regulation according to SAM (estimated FDR = 0%). Importantly, our experimental data were fully concordant with the statistical analysis, because not a single regulated site according to SAM exhibited stronger 3-MB-PP1-related change in Plk1wt compared with Plk1as cells (Table I and supplemental Table 1). Such apparent behavior would have indicated a false positive identification of a regulated phosphosite, which in this case would have an equal chance to appear more strongly regulated in either cell line, irrespective of Plk1 status. This verified SAM as an adequate tool for stringent statistical analysis that was not compromised by the more frequent missing values in our phosphoproteomics compared with transcriptomics data. Moreover, our results implied apparently error-free identification of phosphorylation and thus indicated a very high level of confidence. As seen in Fig. 3B, as many as 386 of the 396 highly significant changes were due to Plk1as cell-selective up-regulation upon 3-MB-PP1 wash-out and ranged from 1.22-fold to almost 50-fold induction (supplemental Table 1). By contrast, only 10 phosphosites were significantly suppressed upon 3-MB-PP1 wash-out from Plk1as cells, which might
 specifier sites in Plk1as cells. Average ratios from Plk1as cell SILAC analyses were compared with Plk1wt ratios in Plk1wt experiments. The scatter plot displays the observed relative difference $d(i)$ versus the expected score for differential phosphorylation changes by SAM. The solid line indicates identity of observed and expected scores, whereas the dashed lines represent thresholds of $d = 2.136$ beyond which phosphorylation sites were identified according to an FDR of 0%. B, regulated phosphosites with significantly different $\pm 3$-MB-PP1 ratios in Plk1as compared with Plk1wt cells. Average ratios from Plk1wt cell SILAC analyses were plotted against the respective ratios from Plk1as experiments. Selectively up- and down-regulated sites in Plk1as versus Plk1wt cells are shown as red and blue circles, respectively.

Cellular Sites of Plk1 Action—Next, we performed GO analysis to validate our data in the context of known Plk1 functions (31). We also included proteins harboring Plk1-regulated phosphopeptides in which phosphoacceptor sites could not be confidently localized in at least two Plk1as and Plk1wt experiments (supplemental Table 3). Using the same criteria as for phosphosites analysis, SAM identified 534 distinct phosphopeptides exhibiting significant regulation Plk1as cells upon 3-MB-PP1 wash-out (supplemental Table 3). In total, our analysis identified 382 distinct proteins as cellular Plk1 downstream targets. We subjected these Plk1-regulated proteins to GO term enrichment analysis using the cellular component annotations of all quantified phosphoproteins from Plk1as cells as reference data (supplemental Table 4). Generally, we found Plk1-regulated phosphoproteins significantly enriched in cellular components such as the centrosome, kinetochore, or spindle apparatus in accordance with known mitotic sites of Plk1 action (Fig. 5A) (9). Downstream targets of Plk1 were also prominent among constituents of the nuclear pore, which has recently been appreciated as a major cellular site of Plk1-mediated phosphorylation (13). Notably, we found Plk1 regulation for 7–29% and identified phosphopeptides for 42–74% of all proteins annotated to the above mentioned and other cellular components enriched for Plk1-regulated phosphoproteins (supplemental Fig. 3A). In line with such extensive regulation and high proteome coverage, network analysis revealed Plk1-mediated regulation at multiple nodes within interconnected phosphoprotein networks, thus indicating coordinated control of mitotic processes instead of targeted perturbation of individual components (supplemental Fig. 3B and supplemental Table 5).

Plk1-regulated Phosphoproteins—To refine our data analysis to the level of site-specific modifications, we then screened our results for reported in vivo and in vitro phosphorylation sites of Plk1. Overall, 45 of the 386 up-regulated phosphosites upon cellular Plk1 activation had been described previously, indicating that our experimental approach faithfully recapitulates known mechanisms of cellular Plk1 signaling (supplemental Table 1). Among these 45 sites, 36...
were reported as down-regulated upon either small molecule 
or small hairpin RNA inhibition of Plk1 in a recent proteomics
analysis of human spindle proteins (13) (supplemen-
tal Table 1). In addition to characterized 
in vivo 
Plk1 sites on 
mitotic proteins such as Ser436 of CEP55, Ser4 of nucleophos-
min, or Ser194 of FADD, our data confirmed cellular regulation 
of reported 
in vitro 
Plk1 phosphorylation sites, such as Ser404 
of NEDD1 and Ser164 of HsCYK-4/MgcRacGAP, as well as of 
various residues of the anaphase-promoting-complex 
components APC1, Cdc16, and Cdc27 (Table II). Moreover, be-
cause almost 90% of the Plk1-regulated phosphosites found 
in our current study have not been reported previously, our 
data provide a vastly extended resource for further functional 
studies on Plk1-controlled mitotic progression. These studies 
might include the investigation of new site-specific phosphor-
ylation events on both known and unreported Plk1 substrates 
localizing to major mitotic compartments, including proteins 
as diverse as DNA topoisomerase 2α, BubR1, nuclear mitotic 
apparatus protein 1, RanBP2, and stathmin (Table II). We also

found prominent induction of Cdc25C phosphorylation on 
Ser191. Phosphorylation at this site has been characterized as 
a modification by the related kinase Plk3, promoting nuclear 
localization of the Cdc25C phosphatase (32). By extension,
our finding suggests an additional mechanism by which Plk1 
triggers G2/M transition through Cdc25C regulation. Plk1-
mediated phosphoregulation of protein phosphatases might 
explain the suppression we observed for a few phosphosites 
in our study upon 3-MB-PP1 wash-out from Plk1as cells.

Notably, our results point to possible Plk1 effects on protein 
ubiquitination beyond the aforementioned regulation of APC 
components. Cellular Plk1 activation induced site-specific 
phosphorylations on the E3 ligase HUWE1 and on two com-
ponents of the SCF (Skp1-Cul1-F-box) ubiquitin ligase com-
plex, SUGT1 and FBXO30. We also identified the ubiquitin 
carboxyl-terminal hydrolases USP5 and USP47 as cellular 
Plk1 targets, suggesting coordinated regulation of both ubiq-
uitylation and deubiquitylation during mitotic progression (Ta-
ble II). To further characterize Plk1-regulated proteins, we

| Δ       | No. called significant | No. falsely significant | FDR (SAM) | No. regulated in Plk1as cells | No. regulated in Plk1wt cells |
|---------|------------------------|------------------------|-----------|-------------------------------|-------------------------------|
| 2.136   | 396                    | 0.0                    | 0.0%      | 396                           | 0                            |
| 2.011   | 406                    | 0.8                    | 0.2%      | 406                           | 0                            |
| 1.810   | 442                    | 0.8                    | 0.2%      | 442                           | 0                            |
| 1.609   | 473                    | 2.3                    | 0.5%      | 472                           | 1                            |
| 1.407   | 509                    | 3.9                    | 0.8%      | 508                           | 1                            |
| 1.206   | 576                    | 8.5                    | 1.5%      | 575                           | 1                            |
| 1.005   | 635                    | 17.8                   | 2.8%      | 631                           | 4                            |
| 0.804   | 784                    | 44.1                   | 5.6%      | 767                           | 13                           |
| 0.603   | 1214                   | 159.5                  | 13.1%     | 1152                          | 54                           |
| 0.402   | 2765                   | 943.0                  | 34.1%     | 2432                          | 288                          |

*a For different Δ parameters, all phosphosites for which SAM reported significantly different ratios upon 3-MB-PP1 wash-out in Plk1as or in Plk1wt cells were analyzed whether their ratios deviated either in Plk1as or in Plk1wt cells more strongly from one.

Fig. 4. Enriched sequence motifs in Plk1-induced phosphorylation sites. A, phosphorylation motifs were extracted with the Motif-X algorithm (24) using all class I serine phosphorylation sites significantly up-regulated upon cellular Plk1 activation. Additional information is provided in supplemental Fig. 2.

B, frequencies of the Plk1 consensus motif (D/E/N)(pS/pT)-φ, where X represents any amino acid, and φ denotes a hydrophobic residue (25), its reduced version (D/E/N)(pS/pT) without a hydrophobic residue in +1 position, and the Polo box domain recognition motif S(pS/pT)(P/X) motif in all proteins with induced phosphosites upon Plk1 activation compared with all phosphoproteins in our SAM analysis.
performed GO enrichment analyses on the level of molecular function. Remarkably, enzymes with helicase activity emerged as a prominent class of Plk1 targets (supplemental Table 6). We identified several Plk1-dependent modification sites on PICH, a Plk1-interacting SNF2 family ATPase implicated in spindle checkpoint signaling (33). Our identification of various other regulated phosphorylations on proteins with ATP-dependent DNA helicase activity points to a rather prominent theme in Plk1 signaling that has been largely unappreciated in previous studies (Table II).

We further analyzed our data for Plk1-regulated phospho-proteins with known functions in signal transduction processes. This revealed several Plk1-dependent modification sites on PICH, a Plk1-interacting SNF2 family ATPase implicated in spindle checkpoint signaling (33). Our identification of various other regulated phosphorylations on proteins with ATP-dependent DNA helicase activity points to a rather prominent theme in Plk1 signaling that has been largely unappreciated in previous studies (Table II).

Collectively, our data point to Plk1 as a cellular regulator of numerous proteins, including many that have not yet been assigned to key mitotic processes. We identified more than 300 Plk1-regulated phosphoproteins in addition to those listed in Table II, and all information about significantly and reproducibly Plk1-regulated phosphosites and phosphopeptides can be accessed in the supplemental data (supplemental Tables 1 and 3). Thus, our data may serve as a valuable resource of numerous new starting points for research in kinase-controlled mitotic progression.

More generally, our integrated chemical genetics and phosphoproteomics approach introduces the concepts of cellular

Fig. 5. Plk1 regulation in cellular compartments and mTOR signaling. A, cellular localization of Plk1-regulated phosphoproteins. Selected GO cellular component terms are shown that were significantly over-represented for Plk1-regulated phosphoproteins compared with all proteins quantified in Plk1α cells (p < 0.05). A full list of all GO terms, as well as their enrichment compared with all entries in the human IPI database, is provided in supplemental Table 4. The ratios represent the numbers of proteins with the indicated GO annotation divided by the number of all GO annotated proteins in the respective categories. B, Plk1 regulation of the mTOR pathway. Components of the mTOR reference pathway (from the Kyoto Encyclopedia of Genes and Genomes) are shown with Plk1-regulated proteins highlighted in red, all other identified phosphoproteins depicted in orange, and additional pathway members in blue. PRAS40 has been further added based on literature evidence (40).
## Table II

### Selected Plk1-regulated phosphorylation sites

| Protein | Short name<sup>a</sup> | Site | Sequence<sup>b</sup> | Average<sup>c</sup> | No. ratios<sup>d</sup> | Average<sup>c</sup> | No. ratios<sup>d</sup> |
|---------|-----------------|------|-------------------|-----------------|----------------|-----------------|----------------|
| Centrosomal protein of 55 kDa | CEP55 | Ser<sup>366</sup> | TALNSELVCEPK | 7.72 | 4 | 0.94 | 4 |
| Nucleophosmin | NPM1 | Ser<sup>147</sup> | MEDSMDMMDMS | 2.41 | 4 | 0.98 | 4 |
| FAS-associated death domain protein | FADD | Ser<sup>149</sup> | NRSGAMSPMSWNS | 12.88 | 2 | 0.90 | 2 |
| Rac GTPase-activating protein 1 | MgcRacGAP | Ser<sup>157</sup> | ESGSIILSDFDK | 10.39 | 3 | 0.94 | 2 |
| Rac GTPase-activating protein 1 | MgcRacGAP | Ser<sup>170</sup> | SILVDSSLVKTFK | 3.89 | 3 | 0.74 | 3 |
| Protein NEDD1 | NEDD1 | Ser<sup>404</sup> | KQNDKSSFDGTK | 1.79 | 3 | 0.99 | 4 |
| Anaphase-promoting complex subunit 1 | APC1 | Thr<sup>377</sup> | ISSHNSQPKRHIS | 5.75 | 4 | 0.99 | 4 |
| Cell division cycle protein 16 homolog | Cdc16 | Ser<sup>112</sup> | KYLKDESFGKDFS | 20.19 | 2 | 1.03 | 4 |
| Cell division cycle protein 27 homolog | Cdc27 | Ser<sup>426</sup> | ONPINDSLEITKL | 18.06 | 4 | 0.98 | 4 |
| Cell division cycle protein 27 homolog | Cdc27 | Ser<sup>435</sup> | ITKLSSEJSGEG | 9.37 | 4 | 0.99 | 4 |
| Cell division cycle protein 27 homolog | Cdc27 | Ser<sup>467</sup> | LFTSDDSSTKENS | 2.12 | 4 | 1.01 | 3 |
| DNA topoisomerase 2α | TOP2A | Thr<sup>1280</sup> | KIKNENETSPOE | 5.13 | 2 | 1.11 | 4 |
| Mitotic checkpoint serine/threonine-protein kinase BUB1β | BubR1 | Thr<sup>710</sup> | PEKLENTNETSEN | 13.62 | 2 | 0.83 | 1 |

**Protein Short name a** Short protein name according to PhosphoSitePlus database (http://www.phosphosite.org).

**Site** Residues in bold type are phosphorylated by Plk1.

**Sequence** Phosphorylation sites are highlighted in bold type, and residues in –2 and/or +1 positions are underlined if consistent with Plk1 substrate consensus motif (D/E/N(X)pS/pT)(F/L/I/Y/W/V/M) (25).

**Average** Average ± 3-MB-PP1 ratio measured in Plk1<sup>αs</sup> and Plk1<sup>αt</sup> cell experiments.

**No. ratios** Number of biological replicate experiments in which the phosphosite ratio was quantified.

---

These data are used to highlight the importance of Plk1 in regulating these proteins and their roles in cellular processes.

---

<sup>a</sup> Short protein name according to PhosphoSitePlus database (http://www.phosphosite.org).

<sup>b</sup> Phosphorylation sites are highlighted in bold type, and residues in –2 and/or +1 positions are underlined if consistent with Plk1 substrate consensus motif (D/E/N(X)pS/pT)(F/L/I/Y/W/V/M) (25).

<sup>c</sup> Average ± 3-MB-PP1 ratio measured in Plk1<sup>αs</sup> and Plk1<sup>αt</sup> cell experiments.

<sup>d</sup> Number of biological replicate experiments in which the phosphosite ratio was quantified.
kinase activation, off-target surveillance, and high confidence identification of downstream targets according to stringent FDR filtering. In principle, our experimental set-up can be applied to any protein kinase that can be sensitized or desensitized to small molecule inhibition. We believe these advances will be highly significant for future efforts to delineate kinase-substrate networks in the physiological cellular context. More generally, experimental strategies similar to those described here might be adopted for a wide range of comparative MS studies and enable the identification of even subtle modifications or protein changes with very high confidence.

Conclusions—We have advanced the combination of chemical genetics and large scale, MS-based phosphoproteomics for the systematic identification of cellular downstream targets regulated by a protein kinase. Our refined approach employs inhibitor-sensitive and -resistant kinase variants and introduces three concepts that enabled confident identification of cellular phosphoregulation through the mitotic regulator Plk1. First, Plk1 activity was induced by cellular inhibitor wash-out to directly trace kinase substrate phosphorylation. Second, in addition to analyzing cells expressing Plk1 mutant with engineered sensitivity to a small molecule inhibitor, we performed parallel control experiments with wild-type Plk1 cells for systematic surveillance of inhibitor off-target effects. Third, we undertook independent replicate analyses of cells harboring wild-type and inhibitor-sensitive Plk1. This enabled highly confident identification of regulated phosphorylation sites on Plk1 downstream targets, which we could verify by full concordance of statistical and experimental data. Our analysis unveiled Plk1-regulated phosphorylations on more than 300 distinct proteins and points to a number of uncharacterized aspects of Plk1-controlled mitotic progression. Most of these have not been reported previously. Therefore our data provide a vastly extended resource for further studies of cellular Plk1 function. Because our experimental strategies can be adopted for any protein kinase amenable to small molecule sensitization or desensitization, the concepts introduced in this study should be of general utility for the systematic and confident identification of cellular kinase-substrate relationships.

Acknowledgments—We thank Axel Ullrich for generous support of our work. We further thank Matthias Mann for continued support and Jürgen Cox for early access to the MaxQuant software. We further thank Renate Hornberger for excellent technical assistance and Mark Burkard for advice on RPE cell culture. F.S.O., K.G.C., and H.D. are employees of Evotec AG, Martinsried, Germany. H.D. is also a shareholder of Evotec AG.

* This work was supported by a grant from the German Bundesministerium für Bildung und Forschung awarded to Kinaxo Biotechnologies GmbH, Martinsried (now Evotec AG). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

† Present address: Evotec AG, Am Klopferspitz 19a, 82152 Martinsried, Germany.

** Present address: MSD, Translational Medicine Research Centre, 8 Biomedical Grove, 04-01/05, Neuros Bldg., Singapore 138665.

¶ Supported by National Institutes of Health Grant R01 GM094972.

|| To whom correspondence should be addressed. E-mail: henrik.daub@evotec.com.

REFERENCES

1. Dephoure, N., Zhou, C., Villén, J., Beausoleil, S. A., Bakalets, C. E., Elledge, S. J., and Gygi, S. P. (2008) A quantitative atlas of mitotic phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10762–10767

2. 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, 2010–2021

3. Schreiber, T. B., Määsbacher, N., Breitkopf, S. B., Grundner-Culemann, K., and Daub, H. (2008) Quantitative phosphoproteomics: An emerging key technology in signal-transduction research. *Proteomics* 8, 4416–4432

4. Cox, J., and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 26, 1367–1372

5. Tan, C. S., and Linding, R. (2009) Experimental and computational tools useful for (re)construction of dynamic kinase-substrate networks. *Proteomics* 9, 5233–5242

6. Bishop, A. C., Ubersax, J. A., Petsch, D. T., Matheos, D. P., Gray, N. S., Bleirow, J., Shimizu, E., Tsen, J. Z., Schultz, P. G., Rose, M. D., Wood, J. L., Morgan, D. O., and Shokat, K. M. (2000) A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature* 407, 395–401

7. Holt, L. J., Tuch, B. B., Villén, J., Johnson, A. D., Gygi, S. P., and Morgan, D. O. (2009) Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. *Science* 325, 1682–1686

8. Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C. J., McLauchlan, H., Klevernic, I., Arthur, J. S., Alesi, D. R., and Cohen, P. (2007) The selectivity of protein kinase inhibitors: A further update. *Biochem. J.* 408, 297–315

9. Archambault, V., and Glover, D. M. (2009) Polo-like kinases: Conservation and divergence in their functions and regulation. *Nat. Rev. Mol. Cell Biol.* 10, 265–275

10. Elia, A. E., Cantley, L. C., and Yaffe, M. B. (2003) Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science* 299, 1228–1231

11. Sneed, J. L., Sullivan, M., Lowery, D. M., Cohen, M. S., Zhang, C., Randle, D. H., Taunton, J., Yaffe, M. B., Morgan, D. O., and Shokat, K. M. (2007) A coupled chemical-genetic and bioinformatic approach to Polo-like kinase pathway exploration. *Chem. Biol.* 14, 1261–1272

12. Lowery, D. M., Clauser, K. R., Hjerdlid, M., Lim, D., Alexander, J., Kishi, K., Ong, S. E., Gammeltoft, S., Carr, S. A., and Yaffe, M. B. (2007) Proteomic screen defines the Polo-box domain interactome and identifies Rock2 as a Plk1 substrate. *EMBO J.* 26, 2282–2273

13. Santamaria, A., Wang, B., Elovse, S., Malik, R., Zhang, F., Bauer, M., Schmidt, A., Silljé, H. H., Körner, R., and Nigg, E. A. (2010) The Plk1-dependent phosphoproteome of the early mitotic spindle. *Mol. Cell. Proteomics* 10, 10.1074/M110.004457

14. Burkard, M. E., Randall, C. L., Larochelle, S., Zhang, C., Shokat, K. M., Fisher, R. P., and Jallepalli, P. V. (2007) Chemical genetics reveals the requirement for Polo-like kinase 1 activity in positioning RhoA and triggering cytokinesis in human cells. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4383–4388

15. Daub, H., Olsen, J. V., Bariein, M., Gnadt, F., Oppermann, F. S., Körner, R., Greff, Z., Kéri, G., Stemmann, O., and Mann, M. (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. *Mol. Cell* 31, 435–445

16. Villel, J., and Gygi, S. P. (2008) The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry. *Nat. Protoc.* 3, 1630–1638

17. Rappaport, J., Mann, M., and Ishihama, Y. (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat. Protoc.* 2, 1896–1906
Cellular Kinase Substrate Proteomics

1. Olsen, J. V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635–648

2. Schroeder, M. J., Shabanowitz, J., Schwartz, J. C., Hunt, D. F., and Coon, J. J. (2004) A neutral loss activation method for improved phosphopeptide sequence analysis by quadrupole ion trap mass spectrometry. Anal. Chem. 76, 3590–3598

3. Olsen, J. V., Vermeulen, M., Santamaria, A., Kumar, C., Miller, M. L., Jensen, L. J., Gnad, F., Cox, J., T. S., Nigg, E. A., Brunak, S., and Mann, M. (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. Sci. Signal. 3, r3a

4. R Development Core Team (2008) R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria

5. Burkard, M. E., Maciejowski, J., Rodriguez-Bravo, V., Repka, M., Lowery, D. M., Clauser, K. R., Zhang, C., Shokat, K. M., Carr, S. A., Yaffe, M. B., and Jallepalli, P. V. (2009) Plk1 self-organization and priming phosphorylation of HsCYK-4 at the spindle midzone regulate the onset of division in human cells. PLoS Biol. 7, e1000111

6. Falcon, S., and Gentleman, R. (2007) Using GOstats to test gene lists for GO term association. Bioinformatics 23, 257–258

7. Jensen, L. J., Kuhn, M., Stark, M., Chaffron, S., Creevey, C., Muller, J., Doerks, T., Julien, P., Roth, A., Simonovic, M., Bork, P., and von Mering, C. (2009) STRING 8: A global view on proteins and their functional interactions in 630 organisms. Nucleic Acids Res. 37, D412–D416

8. Haider, S., Ballester, B., Smedley, D., Zhang, J., Rice, P., and Kasprzyk, A. (2009) BioMart Central Portal: Unified access to biological data. Nucleic Acids Res. 37, W23–W27

9. Cline, M. S., Smoot, M., Cerami, E., Kuchinsky, A., Landys, N., Workman, D., Christmas, R., Avila-Campilo, I., Creech, M., Gross, B., Hanspers, K., Isserlin, R., Kelley, R., Kicillof, S., Lotia, S., Maere, S., Morris, J., Ono, K., Pavlović, V., Pico, A. R., Vailaya, A., Wang, P. L., Adler, A., Conklin, B. R., Hood, L., Hu, J., Ideker, T., and Bader, G. D. (2007) Integration of biological networks and gene expression data using Cytoscape. Nat. Protoc. 2, 2366–2382

10. Elowe, S., Hümmer, S., Uldschmid, A., Li, X., and Nigg, E. A. (2007) Tension-sensitive Plk1 phosphorylation on BubR1 regulates the stability of kinetochore microtubule interactions. Genes Dev. 21, 2205–2219

11. Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matase, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M., and Sherlock, G. (2000) Gene Ontology: Tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. 25, 25–29

12. Bahassi, M., Hennigian, R. F., Myer, D. L., and Stambrook, P. J. (2004) Cdc25C phosphorylation on serine 191 by Plk3 promotes its nuclear translocation. Oncogene 23, 2658–2663

13. Baumann, C., Körner, R., Hofmann, K., and Nigg, E. A. (2007) PICH, a centromere-associated SNF2 family ATPase, is regulated by Plk1 and required for the spindle checkpoint. Cell 128, 101–114

14. Wang, X., and Poud, C. G. (2009) Nutrient control of TORC1, a cell-cycle regulator. Trends Cell Biol. 19, 260–267

15. Astrinidis, A., Senapedis, W., Coleman, T. R., and Henske, E. P. (2003) Cell cycle-regulated phosphorylation of hamartin, the product of the tuberous sclerosis complex 1 gene, by cyclin-dependent kinase 1/cyclin B. J. Biol. Chem. 278, 51372–51379

16. Kiyokawa, N., Lee, E. K., Karunagaran, D., Lin, S. Y., and Hung, M. C. (1997) Mitosis-specific negative regulation of epidermal growth factor receptor, triggered by a decrease in ligand binding and dimerization, can be overcome by overexpression of receptor. J. Biol. Chem. 272, 18656–18665

17. Naslavsky, N., and Caplan, S. (2011) EHD proteins: Key conductors of endocytic transport. Trends Cell Biol. 21, 122–131

18. Astle, M. V., Horan, K. A., Ooms, L. M., and Mitchell, C. A. (2007) The inositol polyphosphate 5-phosphatases: traffic controllers, waistline watchers and tumour suppressors? Biochem. Soc. Symp. 161–181

19. Ross, J. (1997) A hypothesis to explain why translation inhibitors stabilize mRNAs in mammalian cells: mRNA stability and mitosis. Bioessays 19, 527–529

20. Sancak, Y., Thoreen, C. C., Peterson, T. R., Lindquist, R. A., Kan, S. A., Spooner, E., Carr, S. A., and Sabatini, D. M. (2007) PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol. Cell 25, 903–915

21. Fabbro, M., Zhou, B. B., Takahashi, M., Sarcevic, B., Lai, P., Graham, M. E., Gabrielli, B. G., Robinson, P. J., Nigg, E. A., Ono, Y., and Khanna, K. K. (2005) Cdk1/Erk2 and Plk1-dependent phosphorylation of a centrosome protein, Cep55, is required for its recruitment to midbody and cytokinesis. Dev. Cell 9, 477–488

22. Zhang, H., Shi, X., Paddoon, H., Hampong, M., Dai, W., and Pechle, S. (2004) B23/nucleophosmin serine 4 phosphorylation mediates mitotic functions of polo-like kinase 1. J. Biol. Chem. 279, 35726–35734

23. Alappat, E. C., Feig, C., Boyerinas, B., Voikland, J., Samuels, M., Murmann, A. E., Thorburn, A., Kidd, V. J., Slaughter, C. A., Osborn, S. L., Winoto, A., Tang, W. J., and Peter, M. E. (2005) Phosphorylation of FADD at serine 194 by PKCθ regulates its nonapoptotic activities. Mol. Cell 19, 321–332

24. Zhang, X., Chen, Q., Feng, J., Hou, J., Yang, F., Liu, J., Jiang, Q., and Zhang, C. (2009) Sequential phosphorylation of Nedd1 by Cdk1 and Plk1 is required for targeting of the γTuRC to the centrosome. J. Cell Sci. 122, 2240–2251

25. Kraft, C., Herzog, F., Gieffers, C., Mechtler, K., Hagting, A., Pines, J., and Peters, J. M. (2003) Mitotic regulation of the human anaphase-promoting complex by phosphorylation. EMBO J. 22, 6598–6609

In order to cite this article properly, please include all of the following information: Oppermann, F. S., Grundner-Culemann, K., Kumar, C., Gruss, O. J., Jallepalli, P. V., and Daub, H. (2012) Combination of Chemical Genetics and Phosphoproteomics for Kinase Signaling Analysis Enables Confident Identification of Cellular Downstream Targets. Mol. Cell. Proteomics 11(4):O111.012351. DOI: 10.1074/mcp.O111.012351.