

Kinase-Selective Enrichment Enables Quantitative Phosphoproteomics of the Kinome across the Cell Cycle

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SUMMARY

Protein kinases are pivotal regulators of cell signaling that modulate each other's functions and activities through site-specific phosphorylation events. These key regulatory modifications have not been studied comprehensively, because low cellular abundance of kinases has resulted in their underrepresentation in previous phosphoproteome studies. Here, we combine kinase-selective affinity purification with quantitative mass spectrometry to analyze the cell-cycle regulation of protein kinases. This proteomics approach enabled us to quantify 219 protein kinases from S and M phase-arrested human cancer cells. We identified more than 1000 phosphorylation sites on protein kinases. Intriguingly, half of all kinase phosphopeptides were upregulated in mitosis. Our data reveal numerous unknown M phase-induced phosphorylation sites on kinases with established mitotic functions. We also find potential phosphorylation networks involving many protein kinases not previously implicated in mitotic progression. These results provide a vastly extended knowledge base for functional studies on kinases and their regulation through site-specific phosphorylation.

INTRODUCTION

Reversible phosphorylation of proteins represents a fundamental and evolutionarily conserved type of posttranslational modification that regulates protein function on the molecular and signal transmission on the cellular level (Blume-Jensen and Hunter, 2001; Ubersax and Ferrell, 2007). Cellular protein phosphorylation events are mediated by protein kinases, which comprise

one of the largest enzyme superfamilies in higher eukaryotes (Manning et al., 2002). Due to their crucial functions as molecular switches in intracellular signaling pathways, protein kinases have to be tightly controlled with both temporal and spatial resolution. Considerable evidence supports the notion that members of the protein kinase complement (also termed the kinome) not only form an interconnected and dynamic network by which they govern cellular signaling pathways in a cooperative fashion, but also directly modulate each other's functions through site-specific phosphorylation as one means of cellular activity control (Shi et al., 2006). In addition, many protein kinases undergo intermolecular autophosphorylation in the course of their activation through conformational changes upon, for example, association with extracellular ligands, interacting protein factors, or second messenger molecules (Blume-Jensen and Hunter, 2001; Colbran, 2004; Pirruccello et al., 2006). Moreover, phosphorylation in the activation loop segment, which represents a conserved structural element within the protein kinase domain, has been implicated in the stabilization of the catalytically active state of many eukaryotic protein kinases (Nolen et al., 2004). While autophosphorylation events and activation loop phosphorylation can provide a biochemical read-out for cellular protein kinase activity, site-specific phosphorylations also generate recognition sites for the recruitment of substrate proteins and thereby couple protein kinases to downstream signaling pathways (Blume-Jensen and Hunter, 2001; Seet et al., 2006).

However, despite its key importance in cellular signaling, the current picture of protein kinase regulation through site-specific phosphorylation is far from being complete. Due to the rather low cellular abundance of many protein kinases compared to their substrate proteins, kinase-derived phosphopeptides have been underrepresented even in large-scale phosphorylation analyses of total cell extracts by mass spectrometry (MS) (Beausoleil et al., 2004; Bodenmiller et al., 2007; Olsen et al., 2006; Villen et al., 2007). Therefore, to increase the analytical sensitivity for protein kinases, they have to be enriched from total cell extracts prior to MS analysis. Immunoprecipitation techniques

can be employed for the focused analysis of individual protein kinases. Moreover, antibody-mediated purification of tyrosine-phosphorylated proteins can be used to enrich for protein kinases bearing this posttranslational modification (Kratchmarova et al., 2005). However, none of these approaches permits the prefractionation of major parts of the expressed kinome from total cellular extracts. Concurrent enrichment of considerable numbers of protein kinases has been demonstrated with affinity purification techniques employing immobilized low-molecular-weight inhibitors as selective capture reagents for protein kinases (Brehmer et al., 2005; Godl et al., 2003, 2005). Moreover, immobilized kinase inhibitors with distinct kinase-binding properties can be combined for increased coverage of the expressed kinome in highly enriched cellular subfractions (Bantscheff et al., 2007; Wissing et al., 2007).

Here, we report the combination of efficient kinase enrichment with quantitative mass spectrometry using stable isotope labeling by amino acids in cell culture (SILAC). We apply this experimental strategy to analyze protein kinase regulation in cell-cycle progression. To our knowledge, cell-cycle-dependent regulation has been analyzed neither quantitatively nor with a kinase-selective approach in previous large-scale phosphoproteomics studies (Beausoleil et al., 2006; Nousiainen et al., 2006). Due to its analytical sensitivity, our proteomics approach uncovers regulatory phosphorylations in protein kinases to an extent, which goes beyond previous assessments of cellular kinase biochemistry.

RESULTS

Protein Kinase Enrichment

To analyze cell-cycle-dependent regulatory events on protein kinases with high analytical sensitivity, we developed a quantitative phosphoproteomics strategy that combines SILAC with selective kinase enrichment by affinity chromatography (Mann, 2006; Ong et al., 2002). Two populations of HeLa S3 cells were quantitatively labeled by growing them in medium containing either normal arginine and lysine (Arg⁰ and Lys⁰) or the heavy isotopic variants [¹³C₆, ¹⁴N₄]-arginine and [¹³C₆]-lysine (Arg¹⁰ and Lys⁶) for six cell doublings. Subsequently, cells were synchronized in early S phase by a double thymidine block in suspension culture. One of the cell populations was harvested at this point, whereas cells of the second synchronized population were released into a nocodazole-induced mitotic arrest (Figure 1A).

To enrich as many protein kinases as possible from the vast majority of other cellular protein, the pooled lysates from M and S phase cells were loaded onto a series of three affinity columns displaying a total of five different immobilized kinase inhibitors with distinct, overlapping kinase binding profiles (Wissing et al., 2007) (Figure 1B). Sample preparation and loading were performed at 4°C in the presence of chelating agents to disrupt ATP-Mg²⁺ complexes, which together with high salt concentrations eliminated the risk of phosphorylation reactions occurring after cell lysis (Wissing et al., 2007). In addition to the previously described kinase inhibitor resins with the immobilized compounds bisindolylmaleimide X, AX14596, SU6668, and purvalanol B (Godl et al., 2005; Wissing et al., 2007), we further included a capture molecule termed VI16832 with a high degree of nonselectivity with respect to kinase binding.

After extensive washing and equilibration to elution conditions, the three columns were disconnected and eluted separately. All column eluates were pooled prior to further sample processing to avoid redundant analysis of proteins fractionating into column eluates from different affinity columns. This can arise from overlapping inhibitor selectivities in conjunction with non-quantitative kinase binding. Our kinase-selective enrichment strategy represents an advance over previous chromatography procedures in terms of protein kinase coverage and experimental straightforwardness (Wissing et al., 2007). As is evident from a comparison of the total lysate and the column flow-through with a dilution series of the combined elution fractions, multicolumn chromatography led to the affinity purification of a set of proteins, which accounted for only about 0.3% of the initially loaded protein mass (Figure 1C). Parallel immunoblotting revealed partial to nearly complete sequestration of protein kinases by the resin combination, with purification yields ranging from 30% to 70%. Thus distinct members of the protein kinase superfamily were enriched about 100- to 200-fold, thereby providing an experimental basis for an in-depth characterization of expressed protein kinases with a substantially higher analytical sensitivity than achievable from much more complex, unfractionated total cell extracts.

Quantification of Kinome-wide Expression and Phosphorylation Changes

To obtain high coverage and analytical sensitivity with respect to phosphopeptide identification, we devised an experimental strategy that involved splitting of the kinase-enriched cellular subfraction into two parts. One of them was resolved by gel electrophoresis followed by tryptic digestion of proteins separated into different molecular weight regions. The remaining part was directly digested prior to fractionation of the resulting peptides by strong cation exchange (SCX) chromatography (Beausoleil et al., 2004; Olsen et al., 2006; Villen et al., 2007). Resulting peptide fractions were subjected to phosphopeptide enrichment on TiO₂ beads prior to analysis by nanoscale liquid chromatography-tandem MS (LC-MS/MS) on a high-resolution linear ion trap/orbitrap (LTQ-Orbitrap) hybrid mass spectrometer (Larsen et al., 2005; Olsen et al., 2006) (Figure 2A). Thus, in contrast to earlier phosphoproteomics studies, we here combined both gel-based and gel-free separation strategies with phosphopeptide enrichment (Li et al., 2007; Olsen et al., 2006; Villen et al., 2007). This strategy considerably increased the overall number of detected phosphopeptides. For example, multiply phosphorylated peptides were preferentially found in the flow-through fraction of the SCX peptide chromatography, whereas phosphopeptides from gel slices could be identified within a larger dynamic range of ion intensities. Additionally, total peptide fractions were also analyzed on the LTQ-Orbitrap without phosphopeptide enrichment to obtain data sets of unphosphorylated peptides for the quantification of protein ratios (Figure 2A).

Due to the SILAC strategy employing either Arg⁰/Lys⁰ or Arg¹⁰/Lys⁶ for metabolic labeling, arginine- and/or lysine-containing peptides coeluted in LC-MS as doublets with a defined mass difference and the ion signals allowed the accurate measurement of relative peptide abundance. As shown in Figure 2B for an M

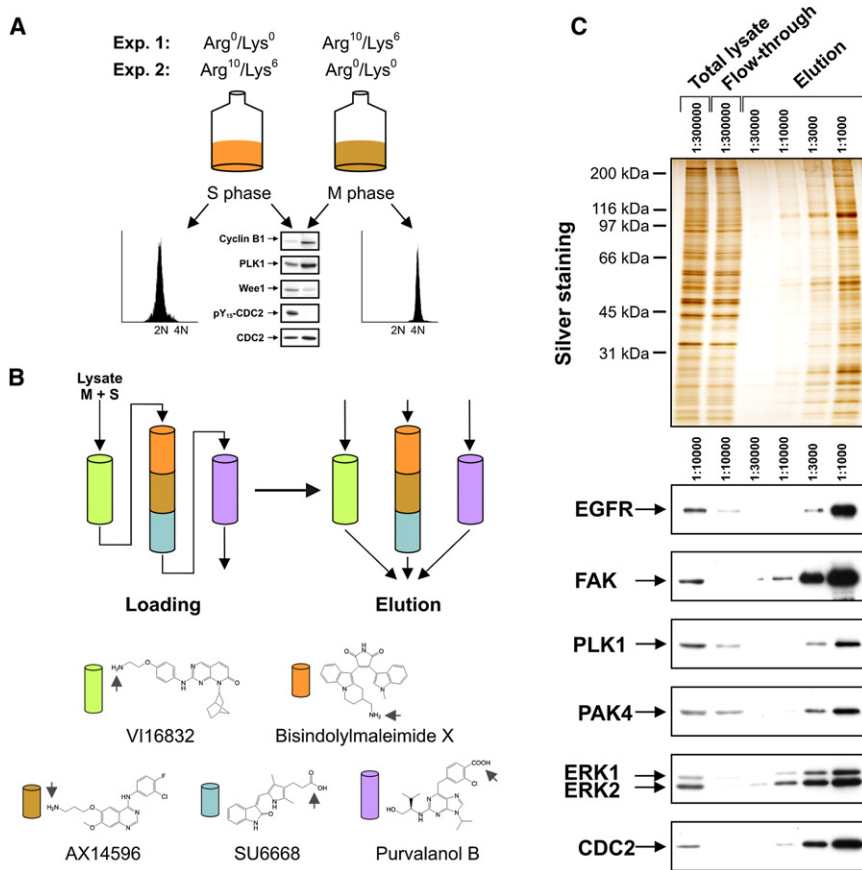


Figure 1. Protein Kinase Enrichment for Cell-Cycle Analysis

(A) HeLa S3 cells grown in the presence of normal or stable isotope-labeled arginine and lysine were synchronized prior to the harvest of S and M phase-arrested cell populations. Synchronization of cells was verified by flow cytometry analysis, and immunoblotting of total cell extracts further indicated the expected upregulation of cyclin B1 and PLK1 protein levels in M phase cells, whereas Wee1 expression and CDC2 phosphorylation at Tyr15 were reduced upon nocodazole treatment. Two independent experiments were done with inverse SILAC encoding of the M and S phase proteomes.

(B) Protein kinase enrichment by multicolumn affinity chromatography. The combined M and S phase extracts were loaded onto three consecutive columns containing five distinct kinase inhibitor resins for selective affinity capture. Each column was eluted separately prior to pooling of the enriched proteins. Immobilization of affinity ligands was established through the amino and carboxy groups, which are marked by arrows in the chemical structures of the kinase inhibitors.

(C) To determine the efficiency of protein kinase enrichment, aliquots representing the 300,000th part of the loaded total lysate and multicolumn flow-through were compared with a dilution series ranging from the 30,000th to the 1000th part of the combined elution fractions (upper panel). In parallel, the same aliquots of pooled eluate and the 10,000th portion of the initial extract and flow-through fractions were analyzed by immunoblotting with kinase-specific antibodies.

phase-induced phosphopeptide from the protein kinase NEK2, the ratio of the heavy versus the light peptide version (M versus S phase) was inverted in a second SILAC experiment using inverse labeling conditions (crossover experiment). The peptide fragmentation spectra in the two experiments resulted in the assignment of the same phosphorylation site within the peptide by computational posttranslational modification (PTM) scoring (Olsen et al., 2006) (Figure 2B).

After quantification as described above, we retained all phosphopeptides identified with greater than 99% certainty (Beausoleil et al., 2006). For 40% of kinase-derived phosphopeptides we had also sequenced and quantified the nonphosphorylated form of the peptide (see Table S1 available online; Figures S1 and S2A). Since phosphorylation of a site can lead to reduction of the nonphosphorylated peptide signal in addition to an increase in the relative phosphopeptide level, these peptide pairs contain additional information about phosphorylation site occupancy. Next, we used the SILAC ratios of the nonphosphorylated peptides of each kinase to quantify its expression level in S compared to M phase. Phosphopeptide ratios were then normalized for changes in protein abundance between M and S phase-arrested cells, which was possible for more than 90% of all protein kinase-derived phosphopeptides (Tables S1–S3; Figures S1 and S2A).

The principles of our quantification strategy are exemplified for a set of identified peptides from the protein kinase TTK (Fig-

ure 2C). As seen from the relative peak intensities in the respective MS spectra, the M phase-derived, heavier versions of the TTK phosphopeptides VPVLLNpSPDCDVK (amino acids 274–287) and NSVPLpSDALLNK (amino acids 217–228) are more abundant than their SILAC partners from S phase-arrested cells (Figure 2C). In contrast, only the nonphosphorylated counterpart of VPVLLNpSPDCDVK is downregulated in M phase-arrested cells, whereas the other partner peptide as well as other unphosphorylated TTK peptides exhibited increased M phase abundance. The measured M/S ratios were corrected for proline conversion and the initial SILAC pooling ratio. Based on these corrected peptide ratios, an about two-fold increase in M versus S phase protein abundance was determined for the kinase TTK (Figure 2C, right panels). Subsequently, all TTK phosphopeptide and phosphopeptide counterpart ratios were divided by this factor to get the final normalized M versus S phase ratios. Interestingly, although both phosphopeptides were induced in M versus S phase-enriched cells, only the unphosphorylated peptide VPVLLNpSPDCDVK showed a reciprocal behavior indicative of a stoichiometric phosphorylation event at Ser281 of TTK (Figure 2C). In contrast, as the counterpart of NSVPLpSDALLNK did not change significantly, suggesting that Ser222 was only phosphorylated in a smaller subfraction of M phase-derived TTK. To our knowledge, our current study is the first that integrates information about phosphoprotein abundance and phosphorylation site stoichiometry into a large-scale, quantitative MS-based

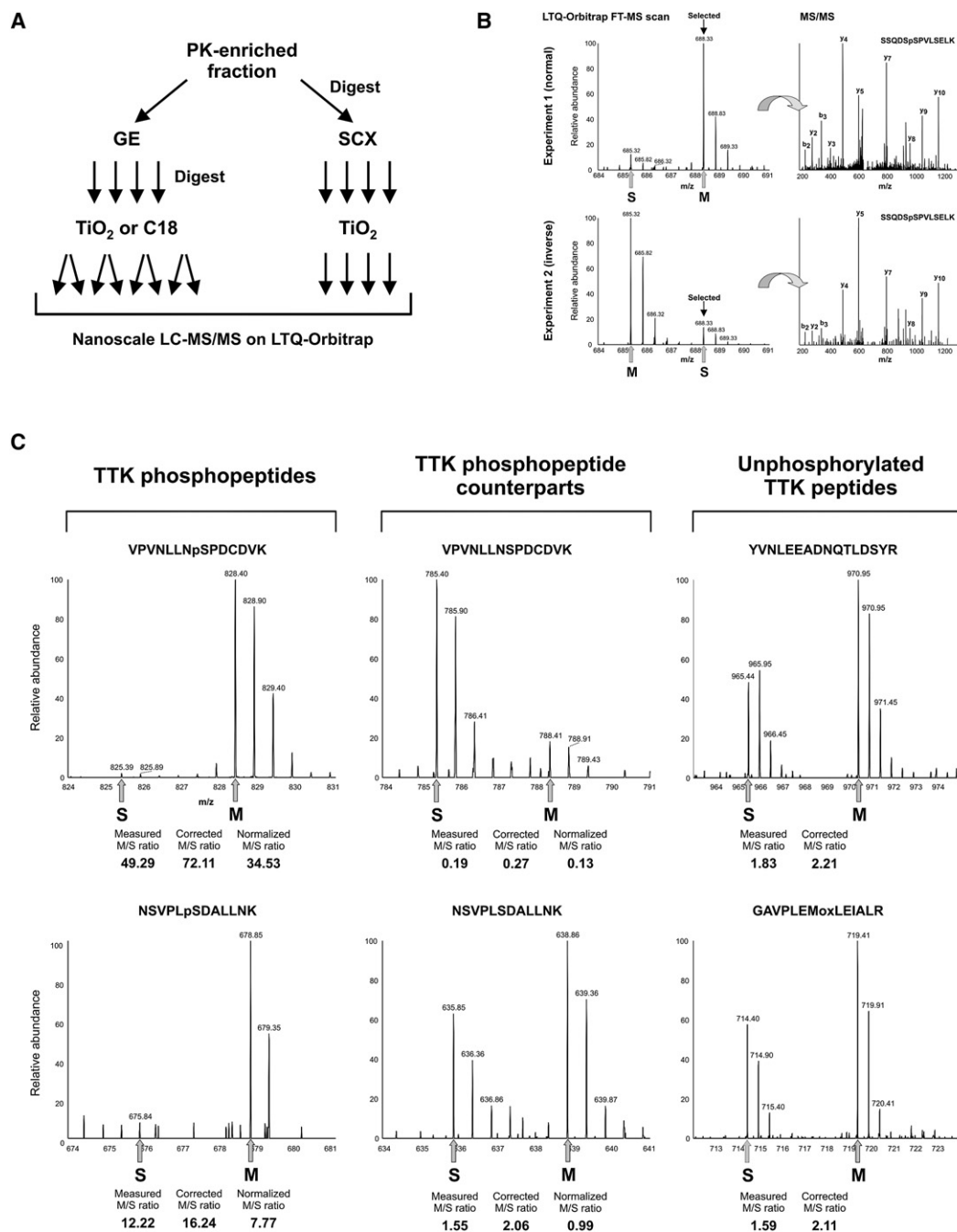


Figure 2. Quantitative Phosphoproteomics

(A) MS sample preparation strategy. Parts of the protein kinase-enriched fraction were resolved either by gel electrophoresis (GE) prior to or by SCX chromatography after trypsin digestion, followed by phosphopeptide enrichment (TiO₂) or peptide capture (C18) and subsequent MS analysis.

(B) Reciprocal SILAC labeling in the experiments 1 and 2 resulted in inverse peptide ratios in the MS spectra, whereas the tandem mass spectra were similar as shown for a NEK2-derived phosphopeptide.

(C) Determination of phosphopeptide, phosphopeptide counterpart, and protein ratios for selected peptides of the protein kinase TTK. Representative MS spectra with the measured, corrected, and normalized peptide ratios are shown.

phosphoproteomic analysis. Importantly, we can discern post-translationally regulated modifications from phosphopeptide changes merely due to altered protein expression. This advance enables the quantitative comparison of site-specific phosphory-

lations from rather different cellular states or even different biological systems and thereby considerably expands the utility of phosphoproteomics in the fields of signal transduction research and cell biology.

Cell-Cycle Regulation of the Protein Kinase Complement

In our analyses of protein kinase-enriched samples from HeLa S3 cells, we uniquely identified and quantified peptides from 219 different members of the human protein kinase superfamily. Based on a comparison of the kinases enriched by inhibitor affinity chromatography with the about 100 protein kinases detected in a previous large-scale phosphoproteomic analysis of total HeLa cell lysate, the kinase coverage of our prefractionation strategy was about 70% (Olsen et al., 2006). This estimated representation of the expressed kinome is also in good agreement with data from earlier transcriptome analyses (Su et al., 2002).

About 14% of all analyzed proteins were protein kinases, demonstrating considerable enrichment by our affinity approach compared to previous large-scale analyses of unfractionated cell extracts. Moreover, this percentage still represents an underestimation of the actual enrichment efficiency, as 35% (1528 of 4338) of all distinct, quantified phosphopeptides and almost 70% of the total phosphopeptide intensity were derived from protein kinases (Figures 3A and S2B). The identified kinase phosphopeptides harbored at least 1182 phospho-sites, of which 1007 could be assigned to specific serine, threonine, or tyrosine residues with high confidence (Figures 3A and S2C). Thus, by our estimate, we identified and quantified more than four times as many distinct phosphorylation sites and phosphopeptides derived from protein kinases compared to the reported analysis of kinase-enriched fractions (Bantscheff et al., 2007). The vast majority of these identified phospho-sites in protein kinases have not been reported earlier. Therefore, our current study not only creates an extended inventory for cellular kinase biochemistry, but also shows that most phosphorylation events had not yet been revealed despite the long-standing research interest in protein kinases and their cellular regulation through site-specific phosphorylation.

Although the focus of our study was on assessing protein kinase regulation, quantitative phosphorylation data were also obtained for a large number of additional proteins, including other enzymes such as phosphatidylinositol kinases, as well as for a considerable number of regulatory kinase subunits such as different members of the cyclin family (Table S1). As expected, we observed that cyclin B1 and B2 were highly upregulated at the protein level in M phase, whereas the cyclins A2, E1, and E2 were induced in S phase. All other identified cyclins (C, H, K, T1, and T2) were not significantly regulated at the level of protein abundance. Interestingly, for all mitotic cyclins phosphorylation sites were found that were highly induced in M phase.

For each of the two independent large-scale analyses performed here, about two-thirds of all kinase phosphopeptides and more than 85% of all protein kinases were identified in the crossover SILAC experiment (Figure S3). As verified for a selection of protein kinases, similar phosphopeptide ratios were measured after either inhibitor-based enrichment or direct kinase immunoprecipitations (Table S4). These control experiments further exemplify that the chemical proteomics approach delivers reliable quantitative data about phosphorylation events on protein kinases. Moreover, as investigated for those set of peptides, in which the phosphorylation site could be precisely determined in both experiments, more than 95% were found in

similar ratios (Figure S3). Thus, due to the good technical and biological reproducibility, we consider the overall data set a high-quality resource for further data evaluation and cell-cycle-related follow-up studies (Table S1). Table S1 provides further information where all respective MS/MS spectra can be found in the original raw data files, which are publicly available through the Tranche file-sharing system (<http://tranche.proteomecommons.org/>; see the Supplemental Data for further information). All quantitative phosphorylation data and annotated MS/MS spectra can also be retrieved from the Phosida database (Figure S4; <http://www.phosida.com/>). Moreover, extensive information about the evolutionary conservation of the identified phosphorylation sites has been deposited in Phosida. This provides a valuable resource for the identification of regulated phosphorylation events with potentially conserved functions across a variety of species including several eukaryotic organisms from yeast to man.

Intriguingly, more than 50% of all kinase phosphopeptides were upregulated at least two-fold in mitotically arrested HeLa cells, whereas only about 10% showed increased S phase abundance (Figure 3A). At the protein abundance level, regulation by a factor two or more was observed for less than 10% of all protein kinases. For example, we measured elevated protein amounts of polo-like kinase 1 (PLK1) and Aurora kinases as well as reduced NEK2, Wee1, and Myt1 kinase expression in M phase-arrested cells. This is in accordance with previously published data (Hayes et al., 2006; Honda et al., 2000; Nigg, 2001; Strebhardt and Ullrich, 2006) (Table S3). Formally, it cannot be ruled out that some of the observed changes in kinase abundance result from selective retention of S or M phase-derived protein instead of altered cellular expression levels. However, high-salt conditions during enrichment should have reduced activation state-specific kinase binding to a minimum (Brehmer et al., 2004). The substantial overall induction of mitotic kinase phosphorylation was also clearly evident from the comparison of peptide and phosphopeptide distributions according to their M to S phase abundances (Figure 3B). This shows that the kinome is primarily cell-cycle-regulated at the level of post-translational modification rather than protein expression levels. We then asked what fraction of protein kinases were associated with cell-cycle-regulated phosphopeptides (greater than two-fold upregulated in S phase or M phase) and found that this was the case for 75% of all detected protein kinases (Figure 3C and Table S3). Thus, cell-cycle progression is accompanied by massive kinome-wide phosphorylation changes and a global and dynamic reorganization of cellular signaling networks.

Regulated Site-Specific Phosphorylation of Kinases with Known and Potential Mitotic Functions

Major kinase regulators of cell-cycle progression such as CDC2/CDK1 and PLK1 are phosphorylated in *trans* at their activation segments by upstream acting kinases, whereas others such as the Aurora kinases most likely undergo autophosphorylation in the same region when they become activated during mitotic cell division (Nolen et al., 2004). Our quantitative MS analysis recapitulated these as well as other known cell-cycle events such as the mitotic dephosphorylation at the inhibitory sites Thr14 and

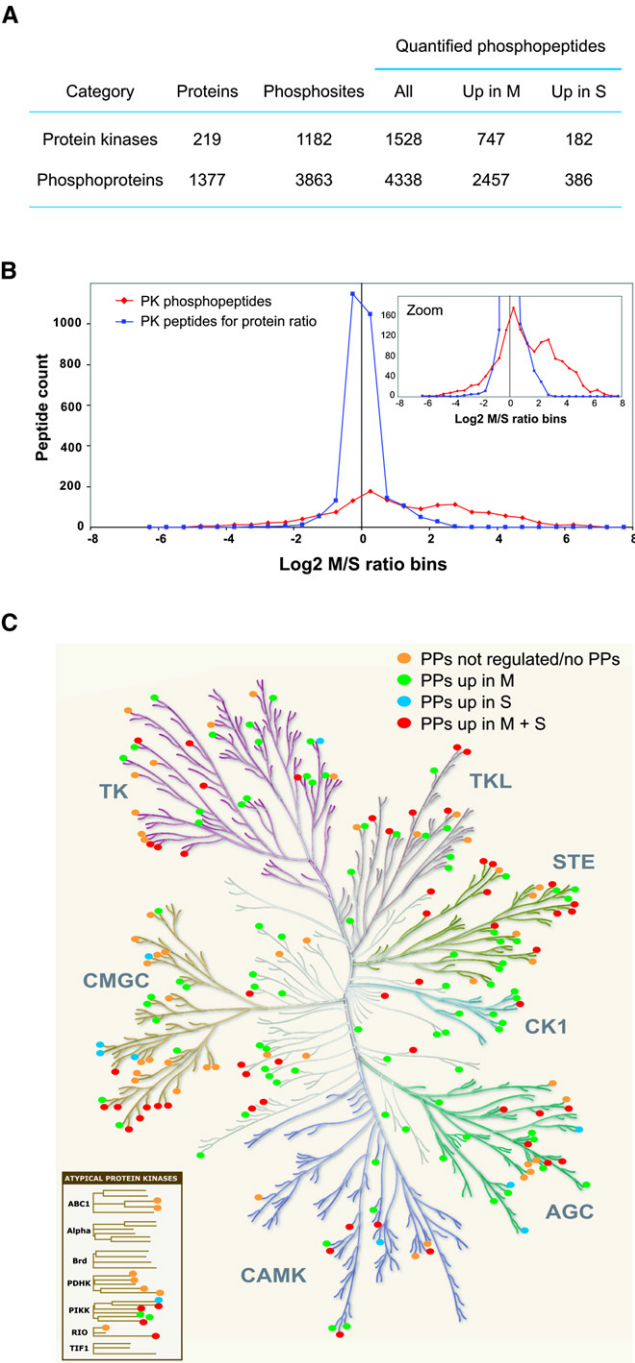


Figure 3. Kinome-wide Phosphopeptide Regulation in M versus S Phase-Arrested HeLa S3 Cells

(A) Numbers of phosphorylation sites and quantified phosphopeptides for protein kinases and all phosphoproteins identified in this study. Phosphopeptides with at least 2-fold changes in abundance were considered as regulated. (B) Distribution of all protein kinase phosphopeptides, which could be normalized for changes in protein abundance (red), and all unphosphorylated protein kinase peptides, which were used for calculating protein ratios (blue). Peptides were binned according to the $\log_2$ values of their respective M versus S phase ratios. (C) Protein kinases identified in this study are marked in a dendrogram of the human kinome. The identification of at least 2-fold upregulated phosphopep-

Tyr15 of CDC2 (Ferrari, 2006; Meraldi et al., 2004; Strebhardt and Ullrich, 2006) (Figures 4A and 5). By extension, we consider our experimental results a reliable data set of regulatory phosphorylation events. Strikingly, even for intensely studied cell-cycle kinases such as CDC2, AurB, and PLK1, a large number of new phosphorylation sites were found, demonstrating again the high analytical sensitivity of our experimental approach (Figures 4A and 6). Most of these phosphorylation sites were strongly upregulated in nocodazole-arrested cells and might be of particular relevance for further functional studies of mitosis.

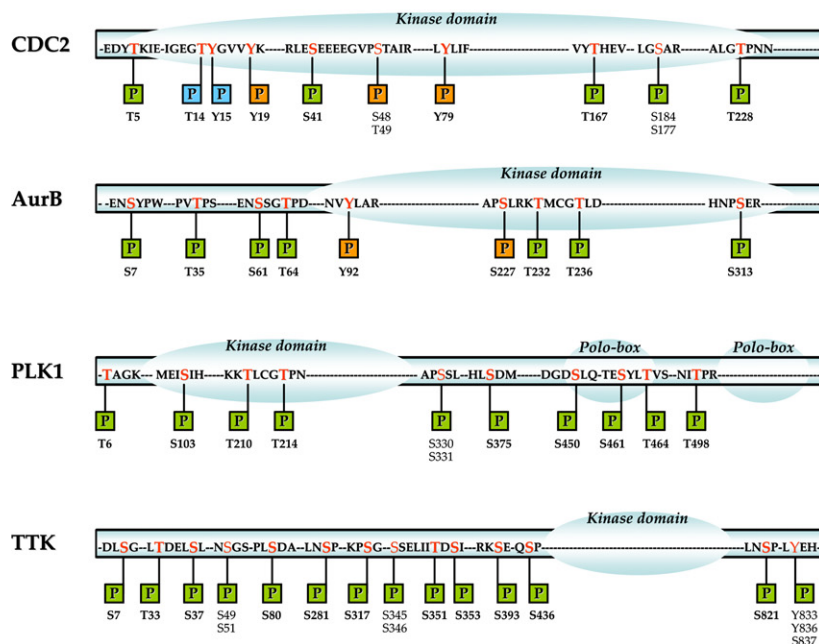
In addition to CDC2, PLK1, and Aurora kinases, we found M phase-induced phosphorylation in the activation segment for several other members of the protein kinase superfamily. This was evident, for example, from phosphopeptides of the AMP-activated protein kinase (AMPK) and microtubule affinity-regulating kinase (MARK) Ser/Thr kinases (Figure 5). Interestingly, induced phosphorylation within the AMPK activation segment indicates a mechanism by which high ATP levels are maintained through increased AMPK activity in mitosis, in line with a recently reported mitotic arrest upon of AMPK α 2 knockdown in HT29 cells (Moffat et al., 2006). Mitotic phosphorylation within the activation segment region can likely indicate induced kinase activity, which might be functionally associated with progression through G₂ and/or M phase as suggested for ULK3 and MAP2K1 in siRNA-mediated knockdown experiments (Figure S5). In addition, we also observed mitotic activation loop phosphorylations for both cytoplasmic and receptor tyrosine kinases, while S phase-increased activation loop phosphorylation was found for a different set of kinases. Interestingly, members of the mitogen-activated protein kinase family such as Erk1, Erk2, JNK1, and p38 α showed higher activity in S phase-arrested cells, as evident from induced phosphorylation of the TXY motifs within their activation loop sequences (Figure 5 and Table S5).

To explore potential contributions of the M phase-activated CDC2, PLK1, and the Aurora kinases to the observed kinome-wide phosphorylation changes, we screened our kinase data for highly upregulated mitotic phosphorylation on CDK, PLK, and Aurora kinase substrate recognition sites (Figure 4B and Table S5). This analysis revealed potential kinase-substrate interactions between these pivotal mitotic regulators and a number of protein kinases such as ZAK, PCTAIRE3, LIMK2, GAK, and IRAK1, which have only recently been linked to mitotic functions in large-scale RNA interference studies (Moffat et al., 2006; Mukherji et al., 2006). In the context of the limited overlap of previous RNAi screens, possibly caused by cell type-specific differences or incomplete knockdown efficiencies, our identification of more than 30 additional putative CDC2, PLK1, and Aurora substrate kinases suggests potential mitotic functions of these kinases (Figure 4B).

Moreover, as our study revealed a large number of additional regulated phosphorylation sites on human kinases previously implicated in mitotic progression, these sites are now accessible

tides (PPs) in M and/or S phase-derived protein kinases is indicated by different colors. The kinase dendrogram was adapted with permission from Cell Signaling Technology, Inc. (<http://www.cellsignal.com/>).

A



B

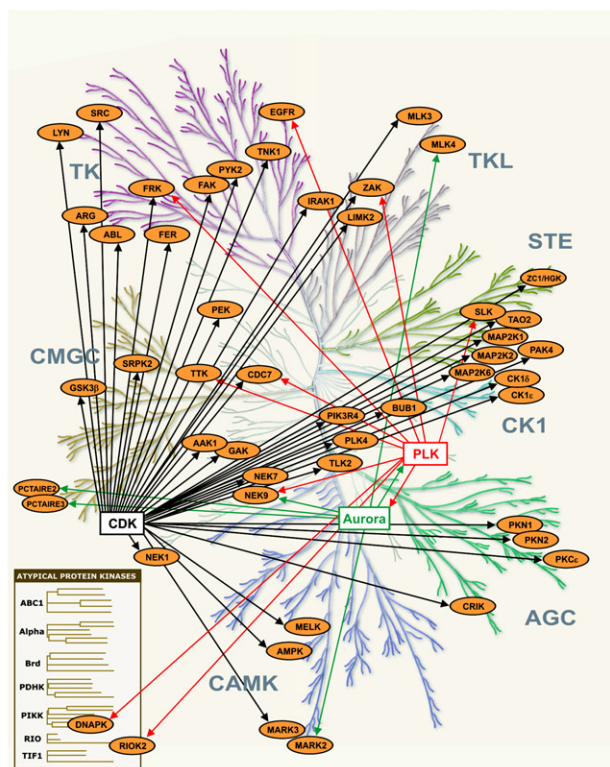


Figure 4. Regulation and Potential Substrates of Selected Mitotic Kinases

(A) Phosphorylation sites and their regulation in selected mitotic protein kinases. Precisely assigned phosphorylation sites are printed in bold. Potential alternative positions are shown in the absence of phosphorylation site-determining information. Phospho-groups marked in green indicate at least 2-fold upregulation in M phase, whereas those highlighted in blue were at least 2-fold induced in S phase. Phosphorylation sites without regulation are displayed in orange. Numbering of phosphorylated amino acids in CDC2 according to the matching entry in the IPI database, which differs for Ser41 onward from the frequently used numbering according to the UniProt/Swiss-Prot entry CDC2_HUMAN.

(B) Protein kinase networks in mitosis. Protein kinases with identified phosphopeptides, which were more than 4-fold upregulated in M phase and contain consensus phosphorylation sites for CDK, PLK, or Aurora kinases, are indicated in the dendrogram of the human kinome adapted with permission from Cell Signaling Technology, Inc. (<http://www.cellsignal.com/>).

been identified as being required for mitotic progression within a cluster of several other components of tumor necrosis factor signaling (Mukherji et al., 2006). Moreover, with Ser131 of IRAK1 and Ser1291 of MAP3K1, two of the up-regulated phospho-sites match the consensus motif for CDK-mediated phosphorylation, suggesting that different components of an interconnected kinase module can be controlled by mitotically activated CDC2 in a coordinated fashion.

DISCUSSION

We have developed a chemical phosphoproteomics strategy that combines efficient kinase enrichment with SILAC-based, quantitative mass spectrometry and allowed us to monitor cell-cycle-dependent protein kinase regulation with improved analytical sensitivity, as highlighted by the more than 1000 phosphorylation sites identified on protein kinases. Moreover, we found more than 50% of all kinase-derived phosphopeptides significantly upregulated in mitotic cells, demonstrating that a major part of the expressed kinome is regulated through

for targeted approaches addressing mechanistic aspects of cell-cycle-dependent kinase regulation (Figure 6; Tables S3 and S6). For example, we found strongly induced mitotic phosphorylation in the kinases IRAK1, IKK β , and MAP3K1, which have recently

site-specific phosphorylations in mitotic cells. These results strongly support the notion that progression through mitosis requires global rearrangements within cellular signaling networks, which, on the basis of our data set, can now be accessed in

M Phase-induced

Activation segment

M/S ratio

ABL	-----LVKVA DFGL SL MTGD ----- TYTAHAGAKF -----PIKWT AE SLAYNK--	2.47
ARG	-----LVKVA DFGL SL MTGD ----- TYTAHAGAKF -----PIKWT AE SLAYNT--	2.00
AMPKα1	-----MNAK ADFG LSNM--SDGE-----FLR SCGSPNYAAPEVISGR -----	7.41
AMPKα2	-----MNAK ADFG LSNM--SDGE-----FLR SCGSPNYAAPEVISGR -----	8.52*
AMPKα1	-----MNAK ADFG LSNM--SDGE-----FLR SCGSPNYAAPEVISGR -----	> 11.43
AMPKα2	-----MNAK ADFG LSNM--SDGE-----FLR SCGSPNYAAPEVISGR -----	> 10.12
AurA	-----LKI ADFG WSVHAPSS-----RRT LCGTLDL YPPEMIEGR--	24.38
AurB	-----LKI ADFG WSVHAPSL-----RRK TCGTLDL YPPEMIEGR--	5.31
AurB	-----LKI ADFG WSVHAPSL-----RRK TCGTLDL YPPEMIEGR--	3.50
CDC2	-----DKGT IKLADFG LAR--AFGVP--LR----- YV HEVVT LMYR SPVLLGS--	5.35
CDK3	-----ELGA IKLADFG LAR--AFGVP--LR----- Y HEVVT LMYR SPVLLGS--	24.83*
FRK	-----IYK ADFG LARVFPVDN-----ED IESR HEIKL-----PVKWT AE PAIRSNK--	2.40
IGF1R	-----TVKIG DFG MT DI YETD----- YY --RKGGKGLL-----PVRWMS PE SLKDGVS--	14.74
INSR	-----TVKIG DFG MT DI YETD----- YY --RKGGKGLL-----PVRWMS PE SLKDGVS--	15.01
IRAK1	-----TPKIG DFGL ARS RFAG ----- SSPSQSSMVAR QTVR-----GTAY IL SEETIKTGR--	> 10.42*
MAP2K1	-----DFVSGQLID-----MANS--FVGTR GYMS PERILQG--	26.74
MARK1	-----MNIK ADFG SNF--TVGN-----K LD TCG SP PYAA PELFQCK -----	> 5.59
MARK2	-----MNIK ADFG SNF--TVGN-----K LD TCG SP PYAA PELFQCK -----	> 3.39
MARK3	-----MNIK ADFG SNF--TVGG-----K LD TCG SP PYAA PELFQCK -----	> 4.02
NEK2	-----LGD GLGR FFS-----HDT SAFT TVGT PPYMS PEQMNR--	> 21.90
NEK7	-----LGD GLGR FFS-----SK TTAAHS LVGT PPYMS PERIEK--	5.97
PLK1	-----VKIG DFGL ATKVEYDGE-----R KK TCGT PPYMS PEVLSK--	38.05
ULK3	-----EKPH LK AD FG FAQHMSPW-----DEKH VT CG SP LYMA PEMVQR -----	10.45
Wee1	-----DL GHVT -----R I SP--QVEGDS RF ANS--VLQENY--	15.50

S Phase-induced

Erk1	-----TTC DLK IC DFGL AR-- I --AD PEHD HTG-----FL E VAT RYR PEIMLNS--	0.38
Erk2	-----TTC DLK IC DFGL AR-- V --AD PEHD HTG-----FL E VAT RYR PEIMLNS--	0.26
JNK1	-----SDCT LK IL DFGL AR-----TAGT SF -----MM PYVTR Y RYR PEVILG--	0.35
JNK3	-----SDCT LK IL DFGL AR-----TAGT SF -----MM PYVTR Y RYR PEVILG--	0.40
MST1	-----L ADFG VAGQL DT-----MAK RNT VI GT PF WMA PEVIO--	0.03
MST2	-----L ADFG VAGQL DT-----MAK RNT VI GT PF WMA PEVIO--	0.03
NEK7	-----LGD GLGR FFS-----SK TTAAHS LVGT PPYMS PERIEH--	< 0.16
p38a	-----EDCEL KIL DFGLAR-----HT DD -----EMT GV VAT RYR PEIMLNW--	0.23
p38a	-----EDCEL KIL DFGLAR-----HT DD -----EMT GV VAT RYR PEIMLNW--	0.23
PKD1	-----VK IC DF GF ARI IG EKS-----FR R SV VTG PAY LA PEVL RNK -----	0.50
RSK2	-----LRIC DF GF AKQ LR AE -----NGL MT PCY TAN FV AE VL RQ -----	0.36
RSK3	-----LRIC DF GF AKQ LR AE -----NGL MT PCY TAN FV AE VL RQ -----	0.33

Figure 5. Cell-Cycle-Regulated Activation Segment Phosphorylation in Protein Kinases

Identified phosphopeptides are highlighted in yellow. Identified class I and class III phosphorylation sites are shown in bold and light red, respectively (Olsen et al., 2006). M/S ratios which could not be normalized for protein expression are marked by an asterisk. For shared phosphopeptides, a normalized M/S ratio is displayed for each protein kinase.

erable potential to pinpoint kinase-substrate relationships on a system-wide level. We think that such experimental data in combination with advanced bioinformatics might significantly extend current knowledge of cellular signaling pathways and their underlying phosphorylation networks (Linding et al., 2007).

The fact that the cell cycle, which is de-regulated in tumors, is driven by phos-

phorylations makes kinases prime targets for anticancer drugs (Malumbres and Barbacid, 2007).

As our approach is based on immobilized drugs and drug-like small molecules, it can be easily implemented for comparative drug target analysis. Such experimental strategies enable the study of dynamic drug target behavior in one particular cell line, or the comparison of target expression and posttranslational modifications in different cell lines characterized by, for example, differential biological sensitivities to a small-molecule drug of interest. Our approach might prove particularly relevant for the assessment of multitargeted drugs and drug candidates and their potential to reduce the risk of molecular resistance formation (Daub et al., 2004). This could be combined with other proteomic techniques aiming at the quantification of cellular drug-target interactions (Bantscheff et al., 2007). Therefore, we not only expect contributions to a far more comprehensive understanding of protein kinases and their central regulatory functions in cellular signaling, but also envision potential applications in drug and drug target research aiming at better strategies to treat life-threatening diseases such as human cancer.

Further mechanistic and functional studies addressing kinase-controlled, mitotic progression.

Remarkably, even for the most intensely studied mitotic kinases, our analysis revealed numerous additional M phase-induced phosphorylation events of potential functional relevance. For example, Thr228 in CDC2 maps to the binding site of for the small CDC2 subunit CKS, suggesting a control mechanism for a protein-protein interaction of importance for mitotic CDC2 function (Bourne et al., 1996; Patra et al., 1999). The carboxy-terminal polo box domain of PLK1, which targets this kinase to its primed mitotic substrates (Elia et al., 2003; Strebhardt and Ullrich, 2006), was found to harbor four additional phosphorylation sites that showed strong induction in mitosis, suggesting a role in the regulation of PLK1 substrate recognition. Furthermore, we quantified mitosis-specific phosphorylations on AurB kinase and its targeting factor inner centromere protein (INCENP). Departure of these chromosomal passengers from kinetochores to the spindle midzone in anaphase is inhibited by constitutively active CDC2/CDK1 (Murata-Hori et al., 2002). Furthermore, in budding yeast, this process requires Cdc14 phosphatase-dependent dephosphorylation of the INCENP homolog Sli15 (Pereira and Schiebel, 2003). Therefore, it is tempting to speculate that M phase-induced phosphorylation events on AurB and INCENP found in this study might regulate the relocalization of this crucial mitotic kinase complex (Table S1).

Importantly, our approach is entirely generic and can be used to study kinase regulation in any field of signal transduction and cell biology. Further studies could also address protein kinase regulation by using three distinct isotope forms of arginine and lysine. Combinations of such SILAC experiments, when done in parallel, would allow to monitor phosphorylation changes on protein kinases with temporal resolution (Olsen et al., 2006). When linked to the dynamics of cellular substrate protein phosphorylation measured in total cell extracts, the parallel and in-depth analysis of kinase regulation profiles should have consid-

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections

For stable isotope labeling by amino acids in cell culture (SILAC), HeLa S3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% dialyzed fetal bovine serum (Invitrogen) and either unlabeled L-arginine (Arg⁰) at 42 mg/l and L-lysine (Lys⁰) at 71 mg/l or equimolar amounts of the isotopic variants L-arginine-U-¹³C₆-¹⁴N₄ (Arg¹⁰) and L-lysine-U-¹³C₆ (Lys⁶) (from Cambridge Isotope Laboratories). After five cell doublings on culture dishes, cell cultures were expanded in spinner flasks prior to their synchronization at early S phase by a double thymidine block as described (Krek and DeCaprio, 1995). One population of the early S phase-arrested cells was harvested by centrifugation, whereas the population with different SILAC encoding was washed, released in fresh medium for 4 hr and then arrested in the presence of 0.15 μg/l nocodazole for 12 hr before harvesting. Frozen pellets of collected M and S phase-arrested cells were stored at -80°C until cell lysis.

Protein kinase	Phosphorylation site(s)
AMPK α 2	S176, S377
ARG	Y116, S203, S275, S602, S620, S671, T864, S915
AurA	S41, S51
AurB	T35, S45, S61, T64, T236, S313
AXL	S868, S877
BUB1	T461, S593 , S596, S655
BUBR1	S670
CDC2	T5, S41, T228
CDKL5	S646, S761
CHK1	S286, S331
CK1 ϵ	S323, T362, S363 , S389
CRK7	S24, S25, T57, S400, T514
DYRK1A	S748, S758
EphA2	S570, Y575, S579, T647
GAK	S456 , S770, T780, S826, S829, S834, S1096
GSK3 β	T275 , T277, T392, T402
IKK β	S672
IRAK1	S131 , S371
LIMK1	S196, T239, T260, S337
LIMK2	S191, T210 , S287, S293, S298
MAP2K1	S230
MAP3K1	S169, S267, S288, S366, S376, S641, S645, S1152, S1177, S1291
MET	S966, T977, S988, S990
MST4	S4
NEK2	S184, S296, S300
NEK7	S204
NEK9	S13, S16, S20, S76 , S331, S750, S793, S827, S829, S832, S868, T883, T886, S978
PAK4	S41, S181, T187, S258, T267, S291
PCTAIRE3	S89 , S98 , S117
PKC ϵ	T309 , S316, S329, S339, S388
PLK1	T6, S103 , T214, S375, S450, S461, T464, T498
PLK4	S499 , S592, S671, S817
RAF1	S244
SLK	S330 , S546, S655, S777, S779, T1097
TTK	S7, T33, S37 , S80, S317, S393, S821
TYK2	S499, S884
ULK3	S219
YES	S21, S50, S121
ZAK	S3, S302 , T328, S339, T628, S733 , S727, S781

Figure 6. Additional M Phase-Induced Phosphorylation Sites on Known Mitotic Protein Kinases

Class I sites in at least two-fold induced phosphopeptides of kinases required for mitotic progression are depicted (Olsen et al., 2006). Phosphorylation sites in CDK, PLK, and Aurora kinase motifs are typed in bold black, red, and green, respectively.

Affinity Chromatography with Immobilized Kinase Inhibitors

Bisindolylmaleimide X and purvalanol B were purchased from Alexis Biochemicals and Tocris, respectively. The kinase inhibitors AX14596 and SU6668 were synthesized as previously described (Brehmer et al., 2005; Godl et al., 2005). Immobilization was done as described previously (Godl et al., 2005; Wissing et al., 2007). The preparation and immobilization of V16832 are described in the Supplemental Data. Cell lysis was performed as reported previously (Wissing et al., 2007), and equal protein amounts of M and S phase cell extracts were pooled prior to multicolumn affinity chromatography on an ÄKTA explorer system (GE Healthcare). Total protein amounts of the combined extracts were 375 mg in experiment 1 (normal) and 315 mg in experiment 2 (inverse). For sample loading, the lysate was passed through three consecutive columns containing five different inhibitor resins at a flow rate of 70 μ l/min. The first and third column was filled with 0.5 ml of the V16832 and purvalanol B matrices, respectively. In the second column, 0.33 ml of the resins containing immobilized SU6668, AX14596, and bisindolylmaleimide X were packed on top of each other. After sample loading and a high- and low-salt washing steps, the columns were disconnected and the purvalanol B column was eluted with a combination of free inhibitor and ATP analogs as described (Wissing et al., 2007). All three columns were then reconnected in the initial order and washed with detergent- and salt-free buffer containing 20 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10 mM NaF, and 0.1 mM sodium orthovanadate. Subsequently, a separate elution step was performed for every column with 0.5% SDS solution at room temperature to release the resin-bound material. The protein-containing fractions were identified by silver staining, lyophilized, and then resuspended with water in one-fifth of the initial volume followed by a final protein precipitation step (Godl et al., 2003).

Mass Spectrometric Analysis

Upon in-solution or in-gel protein digestion, TiO₂ enrichment of phosphopeptides and peptide extraction with StageTips, MS analyses were performed by nanoflow LC connected to a LTQ-Orbitrap (Thermo Fisher Scientific, Bremen, Germany) essentially as described (Olsen et al., 2006). Briefly, the effluent from the capillary column was directly electrosprayed into the mass spectrometer that was operated in data-dependent mode to automatically switch between full-scan MS and MS/MS acquisition (see the Supplemental Experimental Procedures for further details).

Peptide Identification and Quantitation

MS/MS peak lists were extracted from the raw MS files and searched by MASCOT against a concatenated forward and reversed version of the IPI human database v. 3.13. The initial mass tolerance in MS mode was set to 25 ppm, and MS/MS mass tolerance was 0.5 Da. Cysteine carbamidomethylation was searched as a fixed modification, whereas N-acetyl protein, N-pyroglytamine, oxidized methionine, phosphorylation of serine, threonine, and tyrosine and SILAC labels on arginine and lysine were searched as variable modifications. The resulting MASCOT html-output file was linked to the raw MS files and loaded into the MSQuant software (<http://msquant.sourceforge.net/>; versions 1.4.0 and 1.4.3 were used for peptide quantification and PTM scoring, respectively). MSQuant calculated the ratio average over the peptide elution time, and the assignments used for quantitation were visually displayed and validated. In cases where just one of the two SILAC partners of a phosphopeptide was detectable, the minimal difference in ion intensity to its undetectable counterpart was estimated against the ion intensities close to the background level in the respective MS scans. To minimize the false-discovery rate (FDR), all phosphopeptide identifications were filtered by thresholds on peptide length, mass error, SILAC state, and MASCOT score. We accepted peptides based on the criteria that the number of forward hits in the database were at least 200-fold higher than the number of reversed database hits (incorrect peptide sequences), which gives an estimated FDR of less than 1% ($p < 0.01$) (Beausoleil et al., 2006). The final filtering criteria at which p was less than 0.01 were peptide length ≥ 6 , mass error < 5 ppm, MASCOT score ≥ 14 (experiment 1), or MASCOT score ≥ 15 (experiment 2), and the peptide should be identified in the correct SILAC form (heavy or light) and contain the correct number of lysine and arginine residues specified by the mass difference observed in the full scan between the SILAC partners. Finally, to pinpoint the actual phosphorylated amino acid residue within the identified phosphopeptide sequences in

an unbiased manner, we calculated the localization probabilities of all serine, threonine, and tyrosine phosphorylation sites using the PTM score algorithm in MSQuant as described (Olsen et al., 2006). Class I sites had a localization probability of the phosphorylation site of at least 0.75, whereas class III sites had a localization probability between 0.25 and 0.75. The localization p values of all phosphorylation sites are provided at <http://www.phosida.com/>. Quantification of unphosphorylated peptides was done by following essentially the same strategy, except that PTM scoring was not performed and the FDR was determined before peptide quantification with MSQuant (see Figure S1 for further details). All measured M/S peptide ratios were first corrected to account for the metabolic conversion of arginine to proline, which occurred for 9.2% and 2.9% of cellular proline in experiments 1 (normal) and 2 (inverse), respectively. To obtain the final corrected peptide ratios, a second correction step was performed based on the initial SILAC pooling ratio, which was calculated by determining the median of all protein ratios. Further information about the overall quantitation strategy and subsequent data analysis is provided in the Supplemental Data.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, six figures, and seven tables and can be found with this article online at <http://www.molecule.org/cgi/content/full/31/3/438/DC1/>.

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REFERENCES

- Bantscheff, M., Eberhard, D., Abraham, Y., Bastuck, S., Boesche, M., Hobson, S., Mathieson, T., Perrin, J., Rida, M., Rau, C., et al. (2007). Quantitative chemical proteomics reveals mechanisms of action of clinical ABL kinase inhibitors. *Nat. Biotechnol.* 25, 1035–1044.
- Beausoleil, S.A., Jedrychowski, M., Schwartz, D., Elias, J.E., Villen, J., Li, J., Cohn, M.A., Cantley, L.C., and Gygi, S.P. (2004). Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proc. Natl. Acad. Sci. USA* 101, 12130–12135.
- Beausoleil, S.A., Villen, J., Gerber, S.A., Rush, J., and Gygi, S.P. (2006). A probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nat. Biotechnol.* 24, 1285–1292.
- Blume-Jensen, P., and Hunter, T. (2001). Oncogenic kinase signalling. *Nature* 411, 355–365.
- Bodenmiller, B., Mueller, L.N., Mueller, M., Domon, B., and Aebersold, R. (2007). Reproducible isolation of distinct, overlapping segments of the phosphoproteome. *Nat. Methods* 4, 231–237.
- Bourne, Y., Watson, M.H., Hickey, M.J., Holmes, W., Rocque, W., Reed, S.I., and Tainer, J.A. (1996). Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1. *Cell* 84, 863–874.
- Brehmer, D., Godl, K., Zech, B., Wissing, J., and Daub, H. (2004). Proteome-wide identification of cellular targets affected by bisindolylmaleimide-type protein kinase C inhibitors. *Mol. Cell. Proteomics* 3, 490–500.
- Brehmer, D., Greff, Z., Godl, K., Blencke, S., Kurtenbach, A., Weber, M., Muller, S., Klebl, B., Cotten, M., Keri, G., et al. (2005). Cellular targets of gefitinib. *Cancer Res.* 65, 379–382.
- Colbran, R.J. (2004). Targeting of calcium/calmodulin-dependent protein kinase II. *Biochem. J.* 378, 1–16.
- Daub, H., Specht, K., and Ullrich, A. (2004). Strategies to overcome resistance to targeted protein kinase inhibitors. *Nat. Rev. Drug Discov.* 3, 1001–1010.
- 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.
- Ferrari, S. (2006). Protein kinases controlling the onset of mitosis. *Cell. Mol. Life Sci.* 63, 781–795.
- Godl, K., Wissing, J., Kurtenbach, A., Habenberger, P., Blencke, S., Gutbrod, H., Salassidis, K., Stein-Gerlach, M., Missio, A., Cotten, M., and Daub, H. (2003). An efficient proteomics method to identify the cellular targets of protein kinase inhibitors. *Proc. Natl. Acad. Sci. USA* 100, 15434–15439.
- Godl, K., Gruss, O.J., Eickhoff, J., Wissing, J., Blencke, S., Weber, M., Degen, H., Brehmer, D., Orfi, L., Horvath, Z., et al. (2005). Proteomic characterization of the angiogenesis inhibitor SU6668 reveals multiple impacts on cellular kinase signaling. *Cancer Res.* 65, 6919–6926.
- Hayes, M.J., Kimata, Y., Wattam, S.L., Lindon, C., Mao, G., Yamano, H., and Fry, A.M. (2006). Early mitotic degradation of Nek2A depends on Cdc20-independent interaction with the APC/C. *Nat. Cell Biol.* 8, 607–614.
- Honda, K., Mihara, H., Kato, Y., Yamaguchi, A., Tanaka, H., Yasuda, H., Furukawa, K., and Urano, T. (2000). Degradation of human Aurora2 protein kinase by the anaphase-promoting complex-ubiquitin-proteasome pathway. *Oncogene* 19, 2812–2819.
- Kratzmarova, I., Blagoev, B., Haack-Sorensen, M., Kassem, M., and Mann, M. (2005). Mechanism of divergent growth factor effects in mesenchymal stem cell differentiation. *Science* 308, 1472–1477.
- Krek, W., and DeCaprio, J.A. (1995). Cell synchronization. *Methods Enzymol.* 254, 114–124.
- Larsen, M.R., Thingholm, T.E., Jensen, O.N., Roepstorff, P., and Jorgensen, T.J. (2005). Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol. Cell. Proteomics* 4, 873–886.
- Li, X., Gerber, S.A., Rudner, A.D., Beausoleil, S.A., Haas, W., Villen, J., Elias, J.E., and Gygi, S.P. (2007). Large-scale phosphorylation analysis of alpha-factor-arrested *Saccharomyces cerevisiae*. *J. Proteome Res.* 6, 1190–1197.
- Linding, R., Jensen, L.J., Ostheimer, G.J., van Vugt, M.A., Jorgensen, C., Miron, I.M., Diella, F., Colwill, K., Taylor, L., Elder, K., et al. (2007). Systematic discovery of in vivo phosphorylation networks. *Cell* 129, 1415–1426.
- Malumbres, M., and Barbacid, M. (2007). Cell cycle kinases in cancer. *Curr. Opin. Genet. Dev.* 17, 60–65.
- Mann, M. (2006). Functional and quantitative proteomics using SILAC. *Nat. Rev. Mol. Cell Biol.* 7, 952–958.
- Manning, G., Whyte, D.B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002). The protein kinase complement of the human genome. *Science* 298, 1912–1934.
- Meraldi, P., Honda, R., and Nigg, E.A. (2004). Aurora kinases link chromosome segregation and cell division to cancer susceptibility. *Curr. Opin. Genet. Dev.* 14, 29–36.
- Moffat, J., Grueneberg, D.A., Yang, X., Kim, S.Y., Kloepper, A.M., Hinkle, G., Piqani, B., Eisenhaure, T.M., Luo, B., Grenier, J.K., et al. (2006). A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* 124, 1283–1298.
- Mukherji, M., Bell, R., Supkova, L., Wang, Y., Orth, A.P., Batalov, S., Miraglia, L., Huesken, D., Lange, J., Martin, C., et al. (2006). Genome-wide functional

- analysis of human cell-cycle regulators. *Proc. Natl. Acad. Sci. USA* **103**, 14819–14824.
- Murata-Hori, M., Tatsuka, M., and Wang, Y.L. (2002). Probing the dynamics and functions of aurora B kinase in living cells during mitosis and cytokinesis. *Mol. Biol. Cell* **13**, 1099–1108.
- Nigg, E.A. (2001). Mitotic kinases as regulators of cell division and its checkpoints. *Nat. Rev. Mol. Cell Biol.* **2**, 21–32.
- Nolen, B., Taylor, S., and Ghosh, G. (2004). Regulation of protein kinases; controlling activity through activation segment conformation. *Mol. Cell* **15**, 661–675.
- Nousiainen, M., Sillje, H.H., Sauer, G., Nigg, E.A., and Korner, R. (2006). Phosphoproteome analysis of the human mitotic spindle. *Proc. Natl. Acad. Sci. USA* **103**, 5391–5396.
- Olsen, J.V., Blagoev, B., Gnäd, 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.
- Ong, S.E., Blagoev, B., Kratchmarova, I., Kristensen, D.B., Steen, H., Pandey, A., and Mann, M. (2002). Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* **1**, 376–386.
- Patra, D., Wang, S.X., Kumagai, A., and Dunphy, W.G. (1999). The xenopus *Suc1/Cks* protein promotes the phosphorylation of *G(2)/M* regulators. *J. Biol. Chem.* **274**, 36839–36842.
- Pereira, G., and Schiebel, E. (2003). Separase regulates INCENP-Aurora B anaphase spindle function through *Cdc14*. *Science* **302**, 2120–2124.
- Pirruccello, M., Sondermann, H., Pelton, J.G., Pellicena, P., Hoelz, A., Chernoff, J., Wemmer, D.E., and Kuriyan, J. (2006). A dimeric kinase assembly underlying autophosphorylation in the p21 activated kinases. *J. Mol. Biol.* **361**, 312–326.
- Seet, B.T., Dikic, I., Zhou, M.M., and Pawson, T. (2006). Reading protein modifications with interaction domains. *Nat. Rev. Mol. Cell Biol.* **7**, 473–483.
- Shi, Z., Resing, K.A., and Ahn, N.G. (2006). Networks for the allosteric control of protein kinases. *Curr. Opin. Struct. Biol.* **16**, 686–692.
- Strebhardt, K., and Ullrich, A. (2006). Targeting polo-like kinase 1 for cancer therapy. *Nat. Rev. Cancer* **6**, 321–330.
- Su, A.I., Cooke, M.P., Ching, K.A., Hakak, Y., Walker, J.R., Wiltshire, T., Orth, A.P., Vega, R.G., Sapinoso, L.M., Moqrich, A., et al. (2002). Large-scale analysis of the human and mouse transcriptomes. *Proc. Natl. Acad. Sci. USA* **99**, 4465–4470.
- Ubersax, J.A., and Ferrell, J.E., Jr. (2007). Mechanisms of specificity in protein phosphorylation. *Nat. Rev. Mol. Cell Biol.* **8**, 530–541.
- Villen, J., Beausoleil, S.A., Gerber, S.A., and Gygi, S.P. (2007). Large-scale phosphorylation analysis of mouse liver. *Proc. Natl. Acad. Sci. USA* **104**, 1488–1493.
- Wissing, J., Jansch, L., Nimtz, M., Dieterich, G., Hornberger, R., Keri, G., Wehland, J., and Daub, H. (2007). Proteomics analysis of protein kinases by target class-selective prefractionation and tandem mass spectrometry. *Mol. Cell. Proteomics* **6**, 537–547.