Quantitative Phospho-proteomics to Investigate the Polo-like Kinase 1-Dependent Phospho-proteome*§

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Polo-like kinase 1 (PLK1) is a key regulator of mitotic progression and cell division, and small molecule inhibitors of PLK1 are undergoing clinical trials to evaluate their utility in cancer therapy. Despite this importance, current knowledge about the identity of PLK1 substrates is limited. Here we present the results of a proteome-wide analysis of PLK1-regulated phosphorylation sites in mitotic human cells. We compared phosphorylation sites in HeLa cells that were or were not treated with the PLK1-inhibitor BI 4834, by labeling peptides via methyl esterification, fractionation of peptides by strong cation exchange chromatography, and phosphopeptide enrichment via immobilized metal affinity chromatography. Analysis by quantitative mass spectrometry identified 4070 unique mitotic phosphorylation sites on 2069 proteins. Of these, 401 proteins contained one or multiple phosphorylation sites whose abundance was decreased by PLK1 inhibition. These include proteins implicated in PLK1-regulated processes such as DNA damage, mitotic spindle formation, spindle assembly checkpoint signaling, and chromosome segregation, but also numerous proteins that were not suspected to be regulated by PLK1. Analysis of amino acid sequence motifs among phosphorylation sites down-regulated under PLK1 inhibition in this data set identified two potential novel variants of the PLK1 consensus motif. Molecular & Cellular Proteomics 10: 10.1074/mcp.M111.008540, 1–11, 2011.

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Progression through the cell cycle is controlled by cyclin-dependent kinases (CDKs)1. In mitosis, several other kinases, including Aurora A and B (AURKA/B) and PLK1, are activated to orchestrate the different events that are required for chromosome segregation and subsequent cell division. PLK1 has several essential roles during mitotic entry, early mitosis, and late mitosis (1, 2). Before mitotic entry, PLK1 is required for the release from a DNA-damage-induced G2-phase arrest (3). During mitotic entry, PLK1 amplifies cyclin-dependent kinase 1 (CDK1) activation, enabling efficient onset of mitosis (4) and mediates centrosome maturation, the accumulation of γ-tubulin complexes on centrosomes (5, 6). In prometaphase, PLK1 is required for the generation of stable kinetochore-microtubule attachments (7–10). PLK1 also promotes dissociation of cohesin from chromosome arms in prophase and prometaphase by phosphorylating cohesin’s STAG2 subunit (11–14), as well as multiple aspects of cytokinesis by phosphorylating activators and effectors of RhoA (1, 15).

For each of these processes, only few PLK1 substrates have been identified so far, and in most cases potential substrates have often only been identified by testing candidate proteins in in vitro kinase assays, lacking the context of cellular regulatory systems (16, 17). The function of PLK1 in these processes is therefore incompletely understood. Furthermore, it remains to be determined if PLK1 also phosphorylates proteins that have functions in cellular processes other than the ones mentioned above. Because PLK1 is essential for cell division and because its inhibition leads to a mitotic arrest followed by apoptotic cell death (8) several small molecule inhibitors of PLK1 are presently undergoing clinical trials to test their potential utility in cancer therapy (reviewed in 1 The abbreviations used are: CDK, cyclin-dependent kinase; PLK1, Polo-like kinase 1; SCX, strong cation exchange chromatography; AURKA/B, Aurora A and B; FT, flow through; CENPF, centromere protein F; WAPAL, wings apart-like; EIF4B, eukaryotic translation initiation factor 4B; noc, nocodazole; ph, phospho; I, BI 4834 induced; S, BI 4834 sensitive; N-R, BI 4834 unregulated; MSA, multistage activation collisional induced dissociation; IVK, in vitro kinase assay.
A more comprehensive knowledge about the identity of PLK1 substrates will therefore not only be important to understand the role of PLK1 in basic cellular functions, but also to understand the cellular effects of PLK1 inhibitors in cancer patients. We therefore developed a systematic, proteome-wide approach for the unbiased identification of potential PLK1 substrates by combining treatment of human mitotic cultured cells with a highly selective PLK1 inhibitor with quantitative mass spectrometric analysis of phosphopeptides. This approach led to the identification of 519 PLK1 inhibitor sensitive phosphorylation sites on 401 proteins but also revealed that the abundance of 134 phosphorylation sites on 122 proteins was increased upon inhibition of PLK1. These results provide important new insight into the functions of PLK1.

EXPERIMENTAL PROCEDURES

Cell Synchronization—The medium composition was used as described (20). For cell cycle synchronization HeLa cells were first arrested at 50% confluency and a second time after release into fresh medium by using 2 mM thymidine (Sigma-Aldrich) followed by a nocodazole arrest. Prometaphase cells were harvested by a mitotic shake-off, washed twice with PBS (containing noc or noc and BI 4834, respectively, in the same concentrations as in the cell culture buffer), frozen in liquid nitrogen and stored at −80 °C.

Immunofluorescence Microscopy—After harvesting and washing with PBS, cells were cytospun (Thermo Fisher Scientific, Shandon Brand) and fixed onto microscopy slides (12). The nuclear envelopes of the cells were stained with a Lamin A antibody and DNA was counterstained with 4′,6-Diamidino-2-phenylindole (DAPI, Molecular Probes, Invitrogen, UK). For further details see Supplementary experimental procedures.

Sample Preparation for MS Analysis—Cell lysis was performed as described in Poser et al. (21). Proteins were precipitated in ice-cold acetone for further purification before proteolytic cleavage (see Supplementary experimental procedures).

Sample preparation included enzymatic digestion with Lys-C and trypsin and was followed by isotopic labeling. The two samples were differentially labeled with either heavy or light methanol and subsequent desalting was performed as previously described (22). Dried peptide pellets (each containing 2 mg of trypsin-digested cell lysate) were dissolved in 1.5 ml of strong cation exchange (SCX) buffer A (buffer composition and the SCX separation gradient are shown in the Supplementary experimental procedures). The peptides were separated on a PolySULFOETHYL A column (Poly LC, USA) of the dimension: 4.6 mm ID × 15 cm length (operated at 1 ml/min) using a 100 min separation gradient. Elution of peptides was facilitated by increasing pH and salt concentration in parallel. Twenty-five separate fractions were collected, desalted and phosphopeptides were enriched by IMAC as described in Morandell et al. (22).

Nano-HPLC Separation—Chromatographic separation of enriched phosphopeptides and unphosphorylated peptides from the IMAC flow-through was performed on an Ultimate Plus Nano-LC chromatography system (consisting of Famos, Switchos, Ultimate Pumping system and UV detector, Dionex Benelux). First, samples were applied to a reversed-phase trap column (PepMap C18, 5 mm × 0.3 mm × 5 μm, 100 Å) and washed with 0.1% trifluoroacetic acid (Pierce, Thermo Fisher Scientific) at a flow rate of 20 μl/min for 40 min. Separation of peptides was achieved on a C18 column (PepMap C18, 150 mm × 0.075 mm × 3 μm, 100 Å) at a flow rate of 275 nl/min. For all liquid chromatography (LC)-MS experiments, a linear gradient from 100% solvent A (5% acetonitrile (Sigma-Aldrich), 0.1% formic acid (Merck, Germany)) to 70% solvent B (30% acetonitrile, 0.08% formic acid) in 205 min and further to 100% solvent B in 15 min was applied for peptide separation. This separation was followed by a high organic wash with 90% solvent C (80% acetonitrile, 10% trifluoroethanol (Sigma-Aldrich), 0.08% formic acid) for the next 10 min.

MS detection—The nano-HPLC system was directly coupled to a hybrid linear ion trap/Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT Ultra, Thermo Fisher Scientific) with a 7-Tesla superconducting magnet (LTQ-FT Ultra was used for the definition of the cutoff) or an LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific) via a nano-electrospray ionization source (Proxeon Biosystems, Denmark). Metal-coated nano ESI emitters (New Objective) generated the spray at a needle voltage of 2 kV. Mass spectra were acquired in positive ionization mode with the following settings: Fullscan: 400–1800 Th., resolution 60,000, AGC target = 5e6 for LTQ Orbitrap XL measurements, resolution 100,000, AGC target = 5e5 for LTQ-FT measurements. For all full scan measurements in the orbitrap detector a lock-mass from siloxane (m/z 429.088735) was used for internal calibration as described earlier (23). In the applied MS method, fragmentation was performed on the five most intense signals of the survey scan using collisional induced dissociation with multistage activation of the neutral loss of phosphoric acid at 49 and 32.7 Da (24). Singly charged ions were excluded from precursor selection and precursors of MS² spectra acquired in previous scans were temporarily restricted for further fragmentation for a period of 3 min with an exclusion mass window from ~0.005 Da to ~2 Da around the precursor m/z.

Data Quality

Labeling Efficiency by Flow Through Measurement—The unphosphorylated peptides descending from the IMAC flow-through (flow-through data set contains pools 1–25 merged into one file for the determination of the labeling efficiency) was searched twice. For peptide identification of the flow through data, all MS/MS spectra were searched using Mascot 2.2.0 (Matrix Science, London, UK). The generation of dta-files for Mascot was performed using the Extract MSn program (version 4.0, Thermo Fisher Scientific). First the light esterification was applied as fixed modification and second, the heavy modification on the C-term and DE in addition to the following modifications: peptide mass tolerance ± 0.5 ppm; fragment mass tolerance ± 0.5 Da; database nr-human 2010–06-28 comprising 232,368 sequences containing 82,489,472 residues, trypsin; fixed modification carboxymethyl (C); variable modifications oxidation (M), phosphorylation (STY); max. number of missed cleavages 3. One peptide was required for the protein identification. The two exported peptide lists were merged so that only the highest scoring peptide interpretation for each spectrum was retained, and filtered to obtain a list of rank 1 peptides with an expect value < 0.05 and a minimum Mascot score of 20. This resulted in 5940 peptide-spectrum matches carrying one or more light methyl modifications, 6136 peptide-spectrum matches carrying one or more heavy methyl modifications, and 74 peptide-spectrum matches containing 76 DE residues that were not methyl labeled at all (0.6%). Subsequently, the fraction of labeled glutamates, aspartates, and C termini was determined and the labeling efficiency was calculated separately for the heavy and methyl esterified samples, taking into account that completely unlabeled peptides could be derived from either the heavy or the light methyl esterification reactions.

Sequest Search Parameters for Phosphopeptide Identification, Cutoff Determination and False Discovery Rate Calculation—Acquired
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RESULTS AND DISCUSSION

Identification of PLK1-dependent Phosphorylation Sites—Because PLK1 is active in mitosis we searched for phosphopeptides whose abundance is affected by inhibition of PLK1 in cells arrested in prometaphase, using the strategy outlined in Fig. 1A. We used the compound BI 4834 to inhibit PLK1 in this study because this compound inhibits PLK1 with similar efficacy (IC50 7.6 nm) but with higher selectivity over the related enzyme PLK3 (IC50 198.4 nm) as the previously characterized compound BI 2536 (8). Treatment of cells with increasing amounts of BI 4834 showed that at 250 nm BI 4834 caused a cellular phenotype indistinguishable from BI 2536 treatment, e.g. with prometaphase arrested cells displaying monopolar spindles, reduced amounts of tubulin gamma 1 (TUBG1) at centrosomes, and an abolished electrophoretic mobility shift of budding uninhibited by benzimidazoles 1B (BUB1B) and CDC25C (8). To obtain a homogenous population of cells arrested in prometaphase we synchronized HeLa cells using a double thymidine arrest and release protocol. Seven hours after the second release cells were arrested in prometaphase with nocodazole for 3 h. During this treatment, the PLK1 inhibitor BI 4834 was added for various periods of time to determine the shortest inhibitor treatment necessary for full PLK1 inhibition and to keep secondary effects to a minimum. Mitotic cells were subsequently harvested by shake off (Fig. 1B). Immunofluorescence microscopy experiments revealed that our synchronization protocol yielded a mitotic index of 94% in the absence or presence of BI 4834 (supplementary Fig. S1). Synchronization of cells in G2/M-phase was also confirmed by FACS analysis (supplementary Fig. S1). Using 250 nm of BI 4834 we tested five different treatment durations of 15, 30, 45, 60, and 120 min. To measure PLK1 inhibition and subsequent dephosphorylation of PLK1 substrates we analyzed cell lysates by immunoblotting, using phospho-specific antibodies that recognize a PLK1-dependent phosphorylation site (S1224ph) on the cohesin subunit STAG2 (12, 31). This phosphopeptide became undetectable after 15 min of BI 4834 treatment (Fig. 1C). To address if BI 4834 also affected the activities of other mitotic kinases we also analyzed phosphorylation of serine 10 on histone H3, a substrate of Aurora B (32),

CDK1, AURKA, AURKB, NEK2, and PLK1 were provided by the Yaffe laboratory (29). Two-sided Fisher’s exact test was performed to compare the incidence of kinase motifs in the BI 4834 sensitive, BI 4834 induced and BI 4834 not-regulated sets.

A greedy search for statistically significantly enriched motifs in the up-regulated and down-regulated foregrounds (either BI 4834-sensitive or -inhibited), relative to the background of all sites identified in this study, was carried out as described (30). Significances were not calculated for, nor was search-recurs on, any motif that was not contained in at least 2% of the sites in the foreground being searched (see also supplementary Experimental Procedures).

Bioinformatic Analysis—For every detected phosphopeptide, quantitative information obtained from the 25 SCX pools was combined to give a median ratio in case of multiple evidences; in case of even number of observations the geometric mean of the median was used. Normalization factor was determined by measuring the nonphosphopeptides (FT) of all 25 pools and calculating the median log2 ratio of all nonphosphopeptides identified was ~0.03, therefore we did not consider it further. Phosphopeptides with precise phosphorylation site localization by SEQUEST were considered separately, because of the higher confidence of the phospho-site to be dependent on PLK1 (supplementary Table S1). In case of doubly or triply phosphorylated peptides it was impossible to determine the PLK1-dependent phosphorylation site (all phosphopeptides seesupplementary Table S2). In case of doubly or triply phosphorylated peptides it was impossible to determine the PLK1-dependent phosphorylation site (all phosphopeptides seesupplementary Table S2). For the later motif and Scansite analysis, phosphopeptides containing only one phosphorylation site, were generated as a 15-mer peptide consisting of the site of phosphorylation and seven residues on both the N- and C-terminal side.

Scansite analysis of all obtained phosphoproteins was performed as described in Obenauer et al. (28). For this analysis the whole protein sequences were searched for Scansite predictions. Later the phosphorylation sites obtained by the Scansite analysis with high and medium thresholds were compared with the MS data. PSSMs for

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and the phosphorylation dependent electrophoretic mobility shift of the APC/C subunit CDC27, which depends on CDK1 activity (33). As an additional marker of CDK1 activity we also analyzed threonine 14/tyrosine 15 phosphorylation on CDK1, modifications that are known to inhibit this enzyme. BI 4834 treatment for 15 min did not cause a detectable reduction in H3 phosphorylation or of the CDC27 mobility shift or an increase of inhibitory CDK1 phosphorylation, indicating that the phosphorylation states of Aurora B and CDK1 substrates were not or not to the same degree affected as the phosphorylation of PLK1 substrates. These data are consistent with the previous observation that inhibition of PLK1 causes activation of the spindle assembly checkpoint and an arrest in prometaphase, which depends on Aurora B and CDK1 activity (8, 34).

Using this synchronization protocol we isolated HeLa cells in prometaphase that were or were not treated for 15 min with BI 4834, digested proteins by trypsinization, and labeled peptides via methyl esterification with heavy and light methanolic HCl (35). The labeling efficiency was similar for both cell populations (97.5% light label, 97.7% heavy label) as determined by comparing all unphosphorylated peptides that were identified (supplementary Table S3). After the labeling step the two peptide samples were combined, separated by SCX into 25 fractions, phosphopeptides were enriched from each fraction by Fe³⁺-IMAC and analyzed by mass spectrometry.

We identified a total of 9313 phosphopeptides corresponding to 5742 unique phospho-peptides of which 1158 were phosphorylated on multiple sites and 4584 on a single site (singly phosphorylated peptides are listed in supplementary Table S1, singly and multiply phosphorylated peptides are listed in supplementary Table S2). To unambiguously identify PLK1-dependent phosphorylation sites we focused our further analysis on the 4070 unique phosphorylation sites identified in the set of 4584 singly phosphorylated peptides. The 4070 phosphorylation sites analyzed were present on 2069 proteins (supplementary Table S1) and 1262 of these sites (31%) had not been reported before (www.phosphosite.org).
We considered phosphopeptides as being regulated by BI 4834 treatment if their abundance varied at least twofold because an analysis of identical mitotic samples that were differentially labeled with light and heavy methanol and analyzed as above, showed that 91% of all phosphopeptides had less than twofold variation in abundance between the two samples (Fig. 2A, supplementary Fig. S2). Using this threshold we found 519 phosphorylation sites (12.8%) down-regulated whereas the abundance of 134 phosphopeptides (3.3%) was increased (Table I, supplementary Table S1). At a regulatory cutoff above 1:45, no inhibitor-induced phosphorylation sites were detected whereas 51 inhibitor-sensitive sites were identified with a regulatory ratio ranging from 45:1 up to 86:1, the maximum median ratio observed (supplementary Table S1). The 519 down-regulated phosphorylation sites were present on 401 proteins. These proteins may represent substrates of PLK1, or substrates of other protein kinases whose activity depends on PLK1, or in some cases substrates of unidentified kinases that are also inhibited by BI 4834. Table II shows 32 proteins that contain three to nine BI 4834-sensitive phosphorylation sites.

In a biological replicate analysis we identified 6261 unique phosphopeptides, confirming 42.5% of the 5742 unique phosphopeptides initially identified. Of this set of common peptides, 60% confirmed the original regulatory category (peptides which were detected in both replicates within the same regulatory categories are listed in supplementary Tables S1 and S2).

The abundance of phosphopeptides might not only change due to dephosphorylation, but also because of a change in protein stability or because the monitored phosphopeptide becomes phosphorylated at a site close to the one monitored. To test if protein stability is changing after 15 min BI 4834 treatment we determined the abundance of 12523 nonphosphorylated peptides, corresponding to 2761 proteins in the samples obtained from cells treated with or without BI 4834. The median ratio of these peptides was 1:1.02, suggesting that changes in protein levels had little if any influence on the abundance of phosphopeptides (Fig. 2A). To test if any of the identified BI 4834-sensitive phosphorylation sites derive from singly phosphorylated phosphopeptides, which were phosphorylated on a second site or if any of the BI 4834-induced phosphorylation sites derive from doubly phosphorylated peptides on which one site was dephosphorylated, we identified those singly phosphorylated peptides that overlapped with multiply phosphorylated peptides. Because none of the BI 4834-sensitive phosphorylation sites and 4 (supplementary Table S4) of the identified BI 4834-induced phosphorylation sites overlap with these 282 phosphopeptides, we can thus exclude that any of the changes in phosphopeptide abundance of BI 4834-sensitive phosphopeptides we detected are attributable to additional phosphorylation of these peptides and that only in very few cases abundance of BI 4834-induced phosphopeptides are attributable to dephosphorylation of a residue on the same peptide.

To confirm some of the mass spectrometry data we raised antibodies against BI 4834-sensitive phosphopeptides derived from centromere protein F (CENPF), wings apart-like protein (WAPAL) and the eukaryotic translation initiation factor 4B (EIF4B). In immunoblot experiments, all three antibodies recognized bands at the expected size in extracts from mitotic HeLa cells, but not in extracts from cells synchronized in G2-phase, consistent with the possibility that these antibodies recognize only the mitotically phosphorylated forms of their antigens. Importantly, in all three cases the immunoblot signals were reduced after 15 min of BI 4834 treatment, and the extent of reduction correlated with the regulatory ratios determined by quantitative mass spectrometry (Fig. 2B). The results indicate that the changes in phosphopeptide abundance detected by quantitative mass spectrometry are in
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Before we carried out our analyses, 42 PLK1 proteins had been reported to be PLK1 substrates, but for most of them the exact phosphorylation site had not been determined (1, 37). Our experiments identified phosphorylation sites in 29 of 42 previously reported PLK1 substrates, but only seven of these contained BI 4834-sensitive phosphorylation sites, and only for CDC27 and KIF23 the same sites were found regulated by us and the previous studies (Table III). This small degree of overlap between our and previous results could be because of experimental differences because most previous studies determined phosphorylation sites by using in vitro kinase reactions. However, we cannot exclude that our approach failed to detect some PLK1-regulated phosphorylation sites, either because the corresponding phosphopeptides were not recovered, or because the 15 min BI 4834 treatment was not sufficient to allow dephosphorylation of phosphorylation sites that had been generated by PLK1. Very recently, another phospho-proteomic study identified 866 PLK1-dependent phosphorylation sites on 304 mitotic spindle proteins (38). We identified only 25 of these 866 phosphorylation sites. Also in this case, the low degree of overlap might result from differences in experimental conditions, for example because Santamaria et al. used a different PLK1 inhibitor and shRNA-mediated PLK1 depletion and cells already entered mitosis in the absence of active PLK. However, it is also possible that both studies only identified subsets of the total number of PLK1-dependent phosphorylation sites in the human proteome.

**Bioinformatic Analysis of BI 4834-regulated Phosphorylation Sites**—Using the PLK1 inhibitor we observed the reduction of many phosphorylation sites but also the induction of some phosphorylation sites. Although we optimized for a short inhibitor treatment, this observation suggests that we are also detecting secondary effects of PLK1 inhibition. To assess which BI 4834-sensitive phosphorylation sites might be generated by PLK1 we made use of the Scansite algorithm. This algorithm identifies amino acid sequences matching kinase consensus motifs based on in vitro kinase assays on oriented peptide libraries (28, 39). In addition to the initial set of 28 kinase motifs (28) currently available in Scansite, the mitotic kinases PLK1, Aurora kinase A and B, as well as NEK2 were also recently characterized and included in Scansite (29). We analyzed our data set of 4070 phosphorylation sites for the existence of all Scansite motifs and could relate 44% of the identified sites to 27 Scansite kinase motifs with medium and high stringency. Interestingly, the 27 kinase motifs that showed significant hits in the total data set of phosphorylation sites are mostly associated with kinases implicated in mitosis (49% match to motifs for the major mitotic kinases AURKA/B, PLK1, and CDK1 (Scansite uses three distinct motifs for CDK1: Cdc2, CDK1.1, and CDK1.2) and NEK2 (Fig. 3A and supplementary Fig. S3). Importantly, the PLK1 motif is 4-fold enriched among BI 4834-sensitive phosphorylation sites, while none of the other kinase motifs show significant enrichment in the set of BI 4834-sensitive phosphorylation sites. This indicates that our approach identified 12 potential direct PLK1 substrates: NFKB2, TUBA1C, PDCD11, CAMSAP1L1, ZNF295, GOLGA2, NCK1, OPTN, NUMA1, INF2, RAD18, and COPS8. The analysis

| Unique          | Number of phosphopeptides with one or more phosphorylation sites | %    | Number of phosphopeptides with one phosphorylation sites | %    |
|-----------------|------------------------------------------------------------------|------|---------------------------------------------------------|------|
| Total number of phosphopeptides                        | 5742 (5643)                                                      | 100  | 4584 (4070)                                             | 100  |
| BI 4834-sensitive phosphopeptides (cutoff 1:2)         | 713 (761)                                                       | 12   | 559 (519)                                               | 12   |
| BI 4834-induced phosphopeptides (cutoff 2:1)           | 157 (175)                                                       | 3    | 135 (134)                                               | 3    |
| BI 4834-sensitive phosphopeptides (cutoff 1:5)         | 379 (412)                                                       | 7    | 293 (274)                                               | 6    |
| BI 4834-induced phosphopeptides (cutoff 5:1)           | 17 (21)                                                         | <1   | 14 (14)                                                 | <1   |
| BI 4834-sensitive phosphopeptides (cutoff 1:10)        | 267 (292)                                                       | 5    | 211 (200)                                               | 5    |
| BI 4834-induced phosphopeptides (cutoff 10:1)          | 6 (8)                                                           | <1   | 5 (5)                                                   | <1   |
of all phosphopeptides with 2 or more phosphorylation sites revealed similar results (supplementary Fig. S4). Besides the significant distribution of the PLK1 motif in this analysis also the CK2 motif hits are significantly distributed. The increase in matches to the CK2 motif in the BI 4834 induced pool might suggest a repressing function of PLK1 activity toward CK2 substrate phosphorylation.

About 60% of the inhibitor-sensitive sites cannot be matched to the PLK1 motif or any of the other Scansite motifs. This suggests that either PLK1 or other kinases can phosphorylate sites that differ from their characterized consensus motif or that other, yet unidentified kinases generated these sites. Similarly, many of the inhibitor-induced phosphorylation sites could not be matched to any of the 32 kinase motifs.

To identify amino sequence motifs that are enriched among the inhibitor-sensitive or inhibitor-induced phosphorylation sites, we used a previously described algorithm (30). All 134 BI 4834-induced and 519 BI 4834-sensitive phosphorylation sites were transformed into 15-mer peptides consisting of the site of phosphorylation and seven residues on both the N- and C-terminal sides (shown in supplementary Table S1). 15-mers from the induced and sensitive data subsets were analyzed for motifs enriched in comparison to the entire set of phosphorylation sites. Two motifs predicted to be highly surface accessible were significantly enriched in the induced phosphopeptide group by eight- and 19-fold, respectively (Fig. 3 B, logos a and b (40), supplementary Table S4). The motifs generally show a basophilic group on the positions upstream of the phospho-

### Table II

| Entrez-GenID | Entrez name | Protein-name | BI 4834-sensitive phospho-peptides | Implicated in |
|--------------|-------------|--------------|-----------------------------------|--------------|
| 3875         | KRT18       | keratin 18   | 9                                 | Member of the intermediate filament gene family with KRT8 |
| 3856         | KRT8        | keratin 8    | 9                                 | Cellular structural integrity |
| 10579        | TACC2       | transforming, acidic coiled-coil containing protein 2 | 6          | Centrosome- and microtubule-interacting protein |
| 9414         | TJP2        | tight junction protein 2 | 5                   | Proper assembly of tight junctions |
| 4008         | LMO7        | LIM domain 7 | 5                                 | Protein-protein interactions |
| 23451        | SF3B1       | splicing factor 3b, subunit 1, 155kDa | 4     | Member of the U2 small nuclear ribonucleoprotein complex |
| 1063         | CENPF       | centromere protein F, 350/400ka (mitosin) | 4     | Chromosome segregation during mitosis |
| 7916         | BAT2        | HLA-B associated transcript 2 | 4     | Inflammatory process of pancreatic beta-cell destruction during the development of insulin-dependent diabetes mellitus |
| 7165         | TPDS2L2     | tumor protein D52-like 2 | 3     | Uncharacterized |
| 254427       | C10orf47    | chromosome 10orf 47 | 3     | Uncharacterized |
| 7175         | TPR         | translocated promoter region (to activated MET oncogene) | 3     | Directly interacts with several components of the nuclear pore complexes |
| 22837        | COBL1       | COBL-like 1 | 3     | Uncharacterized |
| 6294         | SAFB        | scaffold attachment factor B | 3     | Attaching the base of chromatin loops to the nuclear matrix |
| 8661         | Eif3A       | eukaryotic translation initiation factor 3, subunit A | 3     | Development and differentiation |
| 26135        | SERBP1      | SERPIN1 mRNA binding protein 1 | 3     | Tumor invasion and metastasis |
| 85379        | CTA-221G9.4 | KIAA1671 protein | 3     | Uncharacterized |
| 4791         | NFkB2       | nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100) | 3     | Apoptosis, inappropriate immune cell development, delayed cell growth |
| 85456        | TNKS1BP1    | tankyrase 1 binding protein 1 | 3     | Assembly of bipolar spindles |
| 23271        | CAMSAP1L1   | calmodulin regulated spectrin-associated protein 1-like 1 | 3     | Uncharacterized |
| 9100         | USP10       | ubiquitin specific peptidase 10 | 3     | Cleavage of ubiquitin from ubiquitin-conjugated substrates |
| 55604        | LRRC16A     | leucine rich repeat containing 16A | 3     | Uncharacterized |
| 283489       | ZNF828      | zinc finger protein 828 | 3     | Uncharacterized |
| 10397        | NDRG1       | N-myc downstream regulated 1 | 3     | Stress responses, hormone responses, cell growth, differentiation and apoptosis |
Our bioinformatic analysis thus identified two potential variations of the published PLK1 consensus motif (43). Both revealed an asparagine at position –2 and in motif (f) also the leucine at –3 is prominent. In a recent summary of PLK1 substrates (2), nine out of 40 substrates contained a D/E at –2 whereas six out of 40 substrates contained an asparagine in position –2.

Our results suggest that these motif variations are quite common in PLK1 substrates. These variations should thus be taken into account when searching for PLK1 substrates by sequence analysis. 22 other motifs were found which contain a tryptophan in close proximity to the phosphorylation site. One reason for this environmental tryptophan enrichment could be the preference of the PLK1 inhibitor toward tryptophan-containing phosphorylation sites, but further experiments have to be done to test this assumption (supplementary Table S6).

To classify the identified BI 4834-regulated phosphoproteins, we allocated a subset of them into five biological processes associated with mitosis (Fig. 3C) derived from manual literature curation in combination with GO term analysis (44). This places the identified substrates into the PLK1-regulated processes DNA damage, mitotic progression regulation, mitotic spindle formation, chromosome segregation and spindle assembly checkpoint signaling.

Until recently, PLK1’s only role in DNA damage was thought to be the promotion of degradation of the mitotic entry inhibitors WEE1 and Claspin to release cells from the G2 damage checkpoint. A recent publication (3), however, identified TP53BP1, a mediator protein important early in the DNA damage response, and Chk2 as PLK1 targets required for DNA damage checkpoint release. In our set of PLK1 targets we found a large number of proteins involved in DNA damage detection and repair (Fig. 3C). These findings thus support the idea that efficient inactivation of the DNA damage checkpoint requires phosphorylation and possibly inactivation of a large set of DNA damage response components. The cohesin-complex holds sister chromatids together from S-phase to mitosis (45). Its dissociation from chromosomes arms in prophase depends on WAPAL and on phosphorylation of STAG2 by PLK1 (12, 31). Here, we also identified the cohesin-associated proteins PDS5A/B, and WAPAL as potential in vivo PLK1 targets. This finding will enable further analysis of the role of PLK1 in cohesin release from chromosomes. Furthermore, PLK1 is required for proper kinetochore function to allow faithful chromosome segregation and is also enriched at kinetochores during mitosis (2). Nevertheless, only few PLK1-substrates at the kinetochore are known to date. We identified the published substrate BUB1 for which the PLK1

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**TABLE III**

Comparison of PLK1 substrates reported in the literature with proteins containing BI 4834 sensitive phosphorylation sites. (* Phosphorylation sites that were reported in the literature and found to be BI 4834-sensitive (IVK in vitro kinase assay, MS: mass spectrometry))

| Protein | BI 4834-sensitive phosphosite(s) | ID method | reported phosphosite(s) | Ref. |
|---------|----------------------------------|-----------|-------------------------|------|
| BUB1    | T306                             | IVK       | ND                      | Qi W et al., 2006 |
| CDC27   | S427                             | IVK, MS   | T209, S427*, S434, S435 | Kraft et al., 2003 |
| KIF23   | S911*, S912*, T913               | IVK       | S911*, S912*            | Liu et al., 2004 |
| ASPM    | S348, S367, S370                  | IVK       | ND                      | Maria do Carmo Avides et al., 2001 |
| TB53BP1 | S1618, T951, S598, S692           | IVK       | ND                      | van Vugt et al., 2010 |
| CDC25B  | S563                             | IVK       | ND                      | Valerie Lobjois et al., 2009 |
| PIN1    | S41                              | IVK       | S65                     | Eckerd et al., 2005 |

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3 Hegemann et al., in preparation.
phosphorylation site, however, has not been described before (46, 47). In addition we found the kinetochore regulatory protein ZWINT (48) and the mitotic checkpoint activator MAD1L1 (49) to contain BI 4834-sensitive phosphorylation sites. Interestingly, also several components of the NUP107–160 nuclear pore subcomplex were detected as potential PLK1 substrates; consistent with the possibility that nuclear pore disassembly in mitosis is regulated by PLK1.

**CONCLUSIONS**

By using quantitative phosphoproteomics in combination with a small molecule kinase inhibitor we have identified a...
large number of potential PLK1 substrates. This method represents a generic method applicable for the identification of other kinase substrates in vivo for which inhibitors are available. Of 4070 identified mitotic phosphorylation sites, motif analysis assigned 46% of the sites to 27 kinases and half of all sites were matched to mitotic kinases. Within the 519 BI 4834-sensitive phosphorylation sites, the PLK1 motif was significantly enriched, indicating that some if not many of these sites are direct targets of PLK1. Further, unbiased motif analysis identified a minimal PLK1 motif containing only D/E in position –2 and a variation of the classical PLK1-motif containing N at position –2. Only few of the known PLK1 substrates contain the novel motif N-x-S/T or D/E-x-S/T respectively. We have now identified many PLK1 substrates with these motifs, suggesting D/E-x-S/T and N-x-S/T represent a new subclass of PLK1 motifs. Our data provide an important resource for further biological and biochemical experiments investigating the function of PLK1. Further, as PLK1 is over-expressed in many cancers and PLK1 inhibitors are in clinical trials as anti-cancer drugs, the identified PLK1 substrates may also serve as powerful diagnostic or clinical biomarkers.

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