Published in final edited form as:

Sci Signal. ; 4(198): rs12. doi:10.1126/scisignal.2001993.

Systematic Phosphorylation Analysis of Human Mitotic Protein Complexes

Björn Hegemann^{1,2,*}, James R.A. Hutchins^{1,3,*}, Otto Hudecz^{1,4}, Maria Novatchkova^{1,4}, Jonathan Rameseder⁵, Martina M. Sykora¹, Sihan Liu^{5,6}, Michael Mazanek^{1,4}, Péter Lénárt^{1,7}, Jean-Karim Hériché⁷, Ina Poser⁸, Norbert Kraut⁹, Anthony A. Hyman⁸, Michael B. Yaffe^{5,10}, Karl Mechtler^{1,4}, and Jan-Michael Peters^{1,†}

¹Research Institute of Molecular Pathology, Dr. Bohr-Gasse 7, 1030 Vienna, Austria

⁴Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Dr. Bohr-Gasse 3, 1030 Vienna, Austria

⁵Koch Institute for Integrative Cancer Research, Departments of Biology and Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

⁷European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany

⁸Max Planck Institute for Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, 01307 Dresden, Germany

⁹Boehringer Ingelheim RCV GmbH & Co KG, Dr. Boehringer-Gasse 5–11, 1121 Vienna, Austria

¹⁰Beth Israel Deaconess Medical Center, Dept. of Surgery, Harvard Medical School, 330 Brookline Ave., Boston, MA 02215

Abstract

Progression through mitosis depends on a large number of protein complexes that regulate the major structural and physiological changes necessary for faithful chromosome segregation. Most, if not all, of the mitotic processes are regulated by a set of mitotic protein kinases that control protein activity by phosphorylation. Although many mitotic phosphorylation events have been identified in proteome-scale mass spectrometry studies, information on how these phosphorylation

[†]To whom correspondence should be addressed: Jan-Michael Peters (Telephone +43-179730 3250; peters@imp.ac.at).

²Present address: Institute of Biochemistry, Eidgenössische Technische Hochschule Zürich, Schafmattstrasse 18, CH-8093 Zürich, Switzerland

³Present address: CNRS Institute of Human Genetics, 141 rue de la Cardonille, 34396 Montpellier, France

⁶Present address: Computation and Systems Biology, Singapore-MIT Alliance, 4 Engineering Drive 3, Singapore 117576, Republic of Singapore

*These authors contributed equally to this work.

Author contributions: B.H., J.R.A.H., M.M.S., P.L. and J.-M.P. designed and/or performed wet bench experiments, and interpreted results. O.H., M.N., M.M. and J.-K.H. performed data and bioinformatics analyses. J.R., S.L. and M.B.Y. designed and performed computational experiments. I.P., N.K. and A.A.H. generated essential reagents. A.A.H., M.B.Y., K.M. and J.-M.P. supervised the project. B.H., J.R.A.H. and J.-M.P. wrote the manuscript.

Competing interests: It is the policy of IMP, IMBA, CBG, and MIT to fulfil all reasonable requests for materials but in certain cases a material transfer agreement may be required.

Data availability: MS results and peptide spectra are available at the PRIDE database (www.ebi.ac.uk/pride): accession code 12224 ("interaction" dataset), and accession codes 11797–12223 ("four-condition" dataset). MS raw datafiles are available for download from an IMP-hosted server; see table S5 for full information.

sites are distributed within mitotic protein complexes and which kinases generate these phosphorylation sites is largely lacking. We used systematic protein-affinity purification combined with mass spectrometry to identify 1818 phosphorylation sites in more than 100 mitotic protein complexes. In many complexes the phosphorylation sites were concentrated on a few subunits, suggesting that these subunits serve as “switchboards” to relay the kinase-regulatory signals within the complexes. Consequent bioinformatic analyses identified potential kinase – substrate relationships for most of these sites. In a subsequent in-depth analysis of key mitotic regulatory complexes using the Aurora kinase B (AURKB) inhibitor Hesperadin and a new Pololike kinase (PLK1) inhibitor, BI 4834, we determined the kinase-dependency for 172 phosphorylation sites on 41 proteins. Combination of the results of the cellular studies with Scansite motif prediction enabled us to identify 14 sites on 6 proteins as direct candidate substrates of AURKB or PLK1.

Introduction

In eukaryotes, chromosome segregation depends on major structural and physiological changes in the dividing cell. These include centrosome maturation, chromosome condensation, assembly of a bipolar spindle and bi-orientation of all chromosomes on the spindle through the formation of microtubule-kinetochore attachments. Sister chromatids are only separated from each other once all of these events have been completed, and cytokinesis is only initiated once the sister chromatids are approaching the opposite spindle poles. Errors in any of these processes or their temporal order can lead to chromosome mis-segregation, an event that is thought to contribute to the evolution of malignant tumor cells and, when it occurs in human oocytes, is the cause of Down Syndrome (trisomy 21), a common human congenital disorder.

The molecular events that lead to chromosome segregation are mediated by numerous proteins. Most of these do not function in isolation but as multiprotein complexes, sometimes called “molecular machines” (1, 2). The properties of these “machines” are to a large part determined by the inherent properties of their subunits. For example, the ability of microtubules to assemble into bipolar spindles is primarily due to the physico-chemical properties of their α - and β -tubulin subunits (3). However, the precise control of the chromosome segregation “machinery” in space and time depends on numerous regulatory proteins. Most of these are enzymes that do not mediate chromosome segregation *per se*, but regulate these events indirectly by controlling the activities and locations of protein complexes with direct roles in chromosome segregation. Well known regulators of chromosome segregation are cyclin-dependent kinase 1 (CDK1) (4), the Aurora kinases A and B (AURKA, AURKB), Polo-like kinase 1 (PLK1) (5), and the ubiquitin ligase anaphase promoting complex/cyclosome (APC/C) (6). Activation of CDK1 leads to mitotic entry with concomitant activation of AURKA, AURKB, and PLK1 and, together, these kinases initiate the numerous events required for chromosome segregation. For example, CDK1 and AURKB are thought to confer rigidity to mitotic chromosomes by promoting the binding of condensin complexes to DNA, PLK1 and AURKB mediate the partial separation of sister chromatids by triggering the dissociation of cohesin complexes from chromosome arms, and all of these kinases are important for spindle assembly. The APC/C is essential for subsequent separation of sister chromatids and exit from mitosis. Precocious activation of

the APC/C is prevented by a surveillance mechanism known as the spindle assembly checkpoint (SAC) (7), which inhibits the APC/C until all chromosomes have been properly bi-oriented. Once bi-orientation has been achieved, the SAC is inactivated. This enables the APC/C to initiate sister chromatid separation by activating separase, a protease that destroys sister chromatid cohesion by cleaving residual cohesin complexes on chromosomes. Both the chromosome segregation “machinery,” as well as its regulatory enzymes, are therefore essential for proper chromosome segregation.

Although chromosome segregation has long been known to depend on the ability of mitotic kinases to phosphorylate other proteins, it is poorly understood what the substrates of these enzymes are. Numerous large-scale mass spectrometric studies have, therefore, searched for proteins that are specifically phosphorylated during mitosis (8–13). These experiments have identified thousands of mitotic phosphorylation sites, but in the majority of cases it has not been determined which of these sites are generated by which kinase. Furthermore, none of these large-scale studies has systematically addressed how protein kinases target protein complexes. Therefore, we have used information obtained by the MitoCheck project (14) to identify mitotic phosphorylation sites on more than 100 purified human mitotic protein complexes. With the help of an algorithm refined for the detection of major mitotic kinase motifs (15), we predicted which phosphorylation sites are targeted by which kinase. To further explore these kinase dependencies for a subset of mitotic complexes, we used small molecule inhibitors of AURKB [Hesperadin; (16)] and PLK1 (BI 4834; this study) in conjunction with tandem affinity protein purification and mass spectrometry with high sequence coverage (on average 77% for bait proteins and their main interactors). We generated a set of phosphorylation-specific antibodies that directly confirmed that the generation of phosphorylation sites on these complexes depends on these kinases. Our results show that, although most protein complexes required for chromosome segregation are phosphorylated on several complex subunits, in many cases one or two subunits are “hyperphosphorylated”, meaning that an unusually high number of phosphorylation sites exist on these proteins, and thus, these proteins might serve as “phosphate acceptors” for the phosphoregulation of the respective complex. Of the detected phosphorylation sites, we found that many were targeted by mitosis-specific protein kinases, but a considerable fraction depended on kinases that have so far not been associated with mitosis. Using the PLK1 and AURKB inhibitors, we identified sets of phosphorylation sites that depended on either or both of these kinases, with most proteins having only one or two sites dependent on these kinases. One striking exception is the cohesin subunit wings apart-like (WAPAL), on which we found that most of the phosphorylation sites are PLK1-dependent. Comparison of the kinase-dependent sites with the Scansite consensus motifs led to the identification of several high-confidence candidate substrates for PLK1 and AURKB in the following complexes: the cohesin complex, the RZZ [kinetochore-associated protein 1 (KNTC1, also known as rough deal, ROD), zeste white 10 (ZW10), ZWILCH] complex, the nucleoporin 107(NUP107)-nucleoporin 160 (NUP160) complex, and the mitotic checkpoint complex.

Results

Analysis of 107 mitotic protein complexes for the presence of phosphorylation sites

We previously characterized the intracellular location and subunit composition of 107 human protein complexes with various functions in mitosis (Fig. 1A) (14). Because numerous events in mitotic cells depend on mitotic protein kinases we reasoned that many of these complexes might be phosphorylated during mitosis. To test this hypothesis and to establish a database of mitotic phosphorylation sites that in the future can be used for functional studies, we searched for phosphorylation sites in subunits of all of these complexes (Fig. 1B). We first analyzed the mass spectrometry (MS) data obtained for 1175 proteins that are components of these complexes (239 “bait” and 936 “prey” proteins) for the presence of phosphorylated peptides. All of these complexes were purified from HeLa cells arrested in mitosis (14). By applying the phospho-search algorithm included in the Mascot software (17), we identified 1818 phosphorylation sites on 394 proteins (for a complete table of phosphorylated peptides and phosphorylation sites, see table S1). By using the Ascore algorithm (18), we could determine the precise position of the phosphorylated residue with a confidence above 95% (Ascore > 13) for 1451 of these sites. Of these phosphorylation sites, 656 have not been previously reported in the public phosphorylation database PhosphoSitePlus (19). The 1451 phosphorylation sites that we identified were found on different subunits of 89 out of the 107 different protein complexes (Fig. 2A), suggesting that the majority of protein complexes with mitotic functions may be regulated by phosphorylation. As an example, the distribution of phosphorylation sites among subunits of ten complexes is shown in Fig. 2B (see supplementary Cytoscape file S1 and fig. S1 for all complexes and tables S1 and S2 for a detailed listing of all phosphosites).

To test whether subunits of a given protein complex are phosphorylated to a similar extent, we calculated the phosphorylation-site density by dividing the number of sites per 100 amino acids actually detected by MS for each protein. Whereas the median of phosphorylation-site density was 1.07 sites / 100 amino acids across the whole dataset (1.07% of 63,568 amino acids covered), analysis of all 26 complexes with 4–20 members showed that in 17 of those (65%) a few subunits per complex had a phosphorylation-site density more than two times higher than the median of the entire complex (Fig. 2B, table S2, Supplementary Cytoscape file S1 and fig. S1). For example, most APC/C subunits had a phosphorylation-site density between 0.25% and 1.74% (APC/C median 1.02%), whereas CDC23, CDC26 and CDC27 had a phosphorylation-site density around 3% (3.04%, 2.99%, 3.29%, respectively). These findings suggest that phosphoregulation of many protein complexes might be mediated by few subunits that function as “phospho acceptors” and receive the majority of the kinase regulatory signal and possibly transmit this to other subunits in the complex.

To understand which kinases might phosphorylate these protein complexes, we analyzed the phosphorylation sites by applying the Scansite algorithm (15, 20, 21). Scansite uses position-specific scoring matrices (PSSM) to predict which phosphorylation sites can be recognized by which of 32 different protein kinases. The PSSMs describe the relative preference of the kinases for residues upstream and downstream of the phosphorylation site

as detected by incubating recombinant kinase with an oriented peptide library (22). Of the 1451 phosphorylation sites, 615 (42%) matched with high confidence (within the top 1.5% of Scansite hit scores) to 25 of the kinase motifs (Fig. 2C). More than a third of these phosphorylation sites (39%) are predicted to be targets of mitotic kinases: CDK1 [184 sites, subdivided into the CDK1 substrate subclasses identified by the Cdc2 (22), CDK1-1, and CDK1-2 motifs (15)], PLK1 (17 sites), AURKA (9 sites), AURKB (17 sites) and never in mitosis A (NIMA)-related protein kinase 2 (NEK2, 12 sites). To test if any of the kinase motifs occurred more frequently in our set of mitotic protein complex phosphorylation sites than on average in the human proteome, we compared the frequency of these motifs in our data set with their frequency in the entire phosphoproteome as represented by the phosphorylation sites present in PhosphoSitePlus (19). This comparison revealed that the frequency of phosphorylation sites that match the motifs of CDK1, CDK5, extracellular signal-regulated kinase 1 (ERK1), and protein kinase A (PKA) was higher in our data set than would be predicted by a random distribution in the human proteome ($p < 0.01$; fig. S2). Because the motifs of the proline-directed kinases CDK5 and ERK1 are similar to the motif recognized by CDK1 and because the PKA motif is similar to the motifs recognized by AURKA and AURKB, it is possible that the enrichment of the CDK5 and ERK1 motifs may be attributed to CDK1 activity and the enrichment of the PKA motif may be attributed to the activity of AURKA and AURKB. These results indicate that the majority of mitotic protein complexes are controlled by phosphorylation, in many cases through multiple phosphorylation events on a few subunits, and that many of these modifications are generated by mitosis-specific protein kinases.

Identification of phosphorylation sites sensitive to inhibitors of AURKB and PLK1 on 12 mitotic protein complexes

Although the analysis described above identified numerous phosphorylation sites, the analysis of the phosphorylation state of mitotic protein complexes remained incomplete because the average sequence coverage achieved in these experiments was only 38% per protein. Similar limitations apply to other large-scale phosphoproteomic studies in which similarly low sequence coverages have been obtained [see for example (11)]. Therefore, we selected a subset of 24 baits for high sequence-coverage MS analysis that are subunits of 12 protein complexes with well-established essential functions in mitosis. We chose these complexes because knowledge of their phosphorylation states might be particularly valuable for understanding the regulation of mitosis. This subset includes complexes with functions in the SAC; at the kinetochore, including mitotic checkpoint complex (MCC), APC/C, nuclear division cycle 80 (NDC80), minichromosome loss 12 (MIS 12), and associated proteins; and in chromosome segregation (cohesin and condensin), as well as regulators of the mitotic spindle [gamma-tubulin ring complex (γ -TuRC), centralspindlin] and the mitotic kinases CDK1, PLK1, and AURKB (see Table 1 for a full list).

We identified the phosphorylation sites on these complexes, determined which of these phosphorylation sites were mitosis specific, and which mitosis-specific sites were sensitive to treatment with an inhibitor of AURKB or of PLK1, thus which of these sites might be dependent on the activities of these kinases in mitotic cells. We synchronized HeLa cells in four different states: in interphase by harvesting them under conditions where they were

proliferating with a logarithmic proliferation curve (“LOG”), in mitosis by arresting them in prometaphase with nocodazole (“NOC”), or in mitosis where either AURKB activity was inhibited by treatment of the mitotic cells with the small-molecule inhibitor Hesperadin (“Hesp”) (16), or PLK1 activity was inhibited with a selective PLK1 inhibitor called BI 4834 (“BI”). In the Hesperadin experiments, cells were also treated with the proteasome inhibitor MG132, because Hesperadin treatment would otherwise lead to rapid exit from mitosis (16). To confirm that the treatment of cells with Nocodazole, BI 4834, or Hesperadin produced cells synchronized in prometaphase we analyzed protein extracts from these cell populations by immunoblotting (Fig. 3A) and measurement of histone H1 kinase activity (Fig. 3B), which depends largely on CDK1 (23). As predicted, these experiments revealed an electrophoretic mobility shift of the APC/C subunit CDC27 and elevated histone H1 kinase activity in the three cell populations treated with Nocodazole, and showed that BI 4834 did not inactivate AURKB activity, as measured by immunoblotting with antibodies specific to the activated phosphorylated form of AURKB (AURKB pThr²³²) and its substrate histone H3 (H3 pSer¹⁰), whereas these two phospho-epitopes were not detected in Hesperadin-treated cells (Fig. 3A).

For inhibition of PLK1, we used a novel small-molecule inhibitor, BI 4834, which is a dihydro-pteridinone derivative, similar in structure to BI 2536 (fig. S3A). This compound inhibits PLK1 (IC₅₀ 7.6 nM) with higher selectivity than it exhibits for the related enzyme PLK3 (IC₅₀ 198.4 nM) and this selectivity is greater than that of the previously characterized PLK1 inhibitor BI 2536 (24). To address if the cellular phenotypes caused by BI 4834 are consistent with selective PLK1 inhibition, we released HeLa cells from a thymidine-induced S-phase arrest into media containing increasing doses of BI 4834 and analyzed fixed cells by immunofluorescence microscopy. At BI 4834 concentrations between 250 and 500 nM, more than 85% of all cells arrested in prometaphase with monopolar spindles (“Polo phenotype”; fig. S3B and C) and with reduced amounts of tubulin gamma 1 (TUBG1) at centrosomes (fig. S3D and E). BI 4834 treatment also reduced the centrosomal signal of a PLK1-dependent phospho-epitope on the APC/C subunit cell division cycle 16 (CDC16 pSer⁵⁶⁰) (25) (fig. S3F) and reduced the dissociation of cohesin from chromosome arms (fig. S3G). Western blot analysis revealed that BI 4834 treatment abolished the electrophoretic mobility shifts of budding uninhibited by benzimidazoles 1B (BUB1B) and CDC25C, which depend on PLK1 (fig. S3H) (24, 26). All of these observations are consistent with inactivation of PLK1, indicating that the cellular phenotype caused by BI 4834 is primarily the result of PLK1 inhibition.

We isolated all 24 baits and their interactors from cells cultured under four conditions (LOG, NOC, BI, and Hesp). The baits were purified from HeLa cells stably expressing the bait protein from a bacterial artificial chromosome (BAC), by using a localization and affinity purification (LAP) tag (27) for tandem affinity purification (14, 28), or in a few cases by immunoprecipitation with bait-specific antibodies. In some cases, mouse proteins were used as baits because HeLa cells containing the corresponding mouse BACs had previously been used to test the functionality of the LAP-tagged bait proteins in RNAi “rescue” experiments (14, 28, 29). Of each of the resulting 96 protein isolates we analyzed a small fraction by SDS-PAGE and silver staining to assess their purity, subjected the remainder to digestion with three different proteases, and identified phospho-peptides in each of the resulting 288

samples (Fig. 3C, D). This procedure resulted in a high sequence coverage for the bait proteins and their main interactors [on average 77% (Table 1)] and in a total of 1183 phosphorylation sites. All phosphosites were validated by manual inspection of the corresponding MS spectra and were rated according to a quality score (see Materials and Methods and fig. S4). Spectra of poor quality (Score 0) were excluded from the analysis. For 615 sites of high quality, the position of the phosphorylated residue could be determined unambiguously, and 457 of these sites were covered by peptides in all four experimental conditions (either in their phosphorylated or their unphosphorylated form, depending on the experimental condition). Phosphorylation sites that were not covered in all four conditions were excluded from the subsequent comparison.

Of the 457 phosphorylation sites, only 21 sites were specifically phosphorylated in cells in interphase, the phosphorylated peptides covering these sites were only found in the “LOG” sample, whereas only unphosphorylated peptides covering these sites were found in the other three conditions. Conversely, 400 sites were found phosphorylated in mitotic cells (“NOC”, table S3). We analysed these mitotic phosphorylation sites with the Scansite algorithm containing motifs of 32 kinases. Of the 400 sites, 300 matched to the PSSM of one of 24 protein kinases (Fig. 3E). Similarly to what we found in the Scansite analysis of the large-scale interaction dataset (Fig. 2C), one third of all phosphorylation sites (34%) was predicted as targets of mitotic kinases, including PLK1 (16 sites; 5.3%) and AURKB (7 sites, 2.3%).

Because in this dataset the sequence coverage of the analysed protein complexes is higher than in the interaction dataset (see Fig 4A, B for a representative purification), we wanted to test the observation that most complexes are hyperphosphorylated on only one or two of their subunits. The median phosphorylation-site density was 1.4 sites per 100 amino acids covered (1.4% of 40,082 amino acids covered) across all 12 analysed complexes. In 8 of these complexes (67%), one or two subunits per complex had a phosphorylation-site density more than two times higher phosphorylated than the median of the entire complex (Fig. 4C, table S4). These data thus confirm the finding from the interaction dataset, and strengthen the hypothesis that a few subunits within a complex might act as target sites for various kinases to mediate phosphoregulation of a protein complex.

To determine experimentally which of the 400 mitotic phosphorylation sites were dependent on PLK1 or AURKB *in vivo*, we compared which phosphorylation sites were lost in samples from cells treated with BI 4834 or Hesperadin. In BI 4834-treated cells, 96 phosphorylation sites on 35 proteins could not be detected, and treatment of cells with Hesperadin resulted in the absence of 74 sites on 32 proteins, even though the corresponding non-phosphorylated peptides were recovered in all cases. Between these two treatments, 54 phosphorylation sites were overlapping, suggesting that these were indirectly dependent on PLK1 and AURKB activity. We, thus, identified 42 phosphorylation sites in 25 proteins (14% of all mitotic sites) that were only dependent on PLK1 and 20 phosphorylation sites in 18 proteins (5% of all mitotic sites) that were only dependent on AURKB (see Fig. 4C for a graphical representation and Table 2 for a summary of all identified sites and proteins). Because we had treated the cells with kinase inhibitors for 2 hours, it is likely that some of the phosphorylation sites that we identified as kinase-dependent are not direct substrates of

the inhibited kinases. To identify proteins that might be direct PLK1 or AURKB substrates we used the newly developed PLK1 and AURKB PSSMs in Scansite (15). Among the 42 experimentally determined BI 4834-sensitive sites were 11 sites located on 8 proteins and these 11 sites were predicted by Scansite to be PLK1 sites (Table 2, sites in bold). Five of these proteins [NUP107, radiation sensitive 21 (RAD21), stromal antigen 2 (STAG2), WAPAL, and ZWILCH] are previously unknown PLK1 substrates, whereas the other three [budding uninhibited by benzimidazoles 1 (BUB1), BUB1B, and mitotic arrest deficient-like 1 (MAD1L1)] are known PLK1 substrates (26, 30, 31). However, experimental support for the PLK1-dependent phosphorylation of these 11 sites had not been previously reported. Among the 20 Hesperadin-sensitive sites were three sites located on three proteins and these three sites were predicted by Scansite to be AURKB sites (Table 2, sites in bold). One protein, RAD21, is a previously unknown AURKB substrate, whereas the other two [chromobox protein homolog 5 (CBX5), inner centromere protein (INCENP)] are known AURKB substrates (32–34). Two of the three sites (on RAD21 and CBX5) were previously not known to depend on AURKB. The combination of the *in silico* Scansite predictions with our *in vivo* inhibitor results thus identifies one previously unknown, high confidence substrate candidate for AURKB, and five for PLK1.

Validation of selected phosphorylation sites by phosphospecific antibodies

Because our analysis of the dependence of phosphorylation sites on PLK1 and AURKB was, in part, based on negative evidence, the absence of phosphorylated peptides in certain experimental conditions, we generated phosphospecific antibodies to 13 of the identified phosphorylation sites. We used these antibodies to validate the mass spectrometry data in immunoblotting experiments, using either whole cell extracts, or in cases where the antibodies were not sensitive enough to detect their antigen in such extracts, using immunoprecipitated proteins. We raised four antibodies to BI 4834-sensitive sites that had been identified on the cohesin subunits STAG2 (Ser¹²⁶¹) and WAPAL (Ser⁴⁶⁵, Ser⁵²⁸, Ser¹¹⁵⁴). Immunoblot analysis of cell extracts or WAPAL immunoprecipitates (Fig. 5A) confirmed that the phosphorylation of all four sites was inhibited by BI 4834. When STAG2 or WAPAL immunoprecipitates obtained from interphase cells were incubated with purified PLK1 and ATP, the phosphorylated forms of these proteins that were recognized by the corresponding phosphorylation-specific antibodies could be generated *in vitro* (Fig. 5B), consistent with the possibility that these sites are direct substrates of PLK1. The Ser¹²⁶¹ site on STAG2 and the Ser⁴⁶⁵ site on WAPAL were also predicted by Scansite to be PLK1 targets; the *in vitro* kinase assays thus confirmed these *in silico* predictions. In the case of STAG2, immunofluorescence microscopy experiments further supported the notion that phosphorylation of Ser¹²⁶¹ is mediated by PLK1, because the mitotic staining obtained with pSer¹²⁶¹ antibodies was largely abolished by treatment of cells with BI 4834 (Fig. 5C).

We also generated antibodies to Hesperadin-sensitive sites on the AURKB interactor INCENP (residue Ser⁴⁴⁶), the nucleoporin NUP85 (Thr⁹⁰), BUB1B (Ser⁵⁵¹), and the condensin subunit non-SMC (structural maintenance of chromosomes) chromosome associated protein D2 (NCAPD2). For NCAPD2, the position of the phosphorylated residue could not be unambiguously identified and antibodies were, therefore, raised against both potential sites (Thr¹³⁸⁸ and Thr¹³⁸⁹). Immunoblot analysis of NUP85 purified by tandem

affinity purification of its interaction partner NUP107 and of INCENP in cell extracts (Fig. 5D) confirmed that the chosen phosphorylation sites on these proteins were sensitive to Hesperadin treatment, whereas no Hesperadin sensitivity could be observed when BUB1B immunoprecipitates were analyzed by immunoblotting with the corresponding phosphospecific antibodies. The two antibodies raised against sites on NCAPD2 could both recognize NCAPD2 in mitotic but not in interphase extracts (Fig. 5D), consistent with the possibility that NCAPD2 can be phosphorylated on either Thr¹³⁸⁸ or on Thr¹³⁸⁹, or both, but these reactions were not decreased in cells treated with Hesperadin (for a summary of all results obtained with the phospho-specific antibodies see Fig. 5E).

Finally, we raised one antibody to a site on the cohesin subunit precocious dissociation of sisters 5A (PDS5A, residue Ser¹²³³) that was mitosis specific but not inhibited by either BI 4834 or Hesperadin, and to four sites on the cohesin subunits RAD21 (Ser¹⁵³, Ser¹⁷⁵) and PDS5B (Ser¹³⁸⁴, Ser¹⁴¹⁸) that had been found in all experimental conditions. Immunoblot analysis of the corresponding immunoprecipitates confirmed that the PDS5A site was mitosis specific and that the two sites on PDS5B were present in interphase and mitosis (fig. S5A). However, the two sites on RAD21 were found to be mitosis specific and sensitive to BI 4834 treatment (fig. S5A and B).

In summary, the MS data for four out of the four tested BI 4834-sensitive sites, two out of four Hesperadin-sensitive sites, one out of one mitosis-specific site, and two out of four unregulated sites, in total 9 out of 13 sites, were confirmed by experiments with phosphospecific antibodies. In the remaining four cases, it is possible that the MS data were insufficient to determine the abundance change of these phosphorylation sites. These results indicate that the majority of the obtained MS data are reliable for a given phosphorylation site that is sensitive to BI 4834 or Hesperadin. Our MS experiments have, therefore, identified a large number of previously unknown candidate substrates of PLK1 and AURKB among human protein complexes essential for mitosis.

Data availability

These data complement the protein interaction study in Hutchins *et al.* (14). Phosphosites from this study have been incorporated into the PhosphoSitePlus (www.phosphosite.org) (19) and Phospho.ELM (phospho.elm.eu.org) (35) databases. Interpreted phosphopeptide spectrum data are accessible via the proteomics identifications database (PRIDE, www.ebi.ac.uk/pride/) (36), and raw MS datafiles are available for download; see table S5 for details.

Discussion

Oscillatory protein phosphorylation regulates the major phase transitions of the cell division cycle. The overall amount of phosphorylation is especially high during mitosis (37, 38), and several large-scale studies have identified sets of phosphorylation sites present during mitosis (8, 11–13, 39). These studies, although mostly performed on a “phosphoproteome” scale, are still far from complete due to the complexity and variance in protein abundance within the proteome (40). Because phosphoproteomic studies usually rely on phosphopeptide enrichment, the information about the unphosphorylated proteins is lost and

it thus remains difficult to estimate the protein coverage in these studies. In this study, we have taken a complementary approach to analyse mitotic phosphorylation within purified mitotic protein complexes. The much lower sample complexity allowed simultaneous analysis of phosphorylated and unphosphorylated peptides to obtain a measure of sequence coverage for each analysed protein and protein complex. Following the development of high-throughput tagging and protein purification for human cells within the MitoCheck project (14, 28), we have systematically analysed 107 mitotic protein complexes for protein phosphorylation. We identified a set of 1451 phosphorylation sites on 89 complexes, indicating that most mitotic protein complexes become phosphorylated in mitosis.

This large dataset on the phosphorylation of protein complexes allowed us to address how phosphorylation sites are distributed within protein complexes. We found that, in many cases, only one or two subunits carry most of the phosphorylation sites of the whole complex (Fig. 2B, Fig 4C, Supplementary Cytoscape file S1). This finding is consistent with phosphorylation site analyses of the APC/C and cohesin complexes (9, 25, 41, 42). In combination with available structural and functional data for some of these complexes, our data suggest that hyperphosphorylated subunits might serve as “phosphate acceptors” that relay phospho-regulatory signals within the protein complex. For example, the tetratricopeptide repeat (TPR) subunits of the APC/C (CDC16, CDC23, CDC27) were shown by electron microscopy studies to be located on the backbone of the complex, forming the so called “arc lamp” domain, and are suggested to control the positioning of the catalytic and regulatory subunits within the APC/C (43–46). We found that two of these TPR subunits, CDC23 and CDC27, had the highest number of phosphorylation events of any of the subunits of the complex (Fig. 4C, table S4), thus suggesting a mechanism by which TPR-subunit phosphorylation might regulate APC/C activity during mitosis. Within the cohesin complex the SMC subunits are thought to play an important structural role holding the sister chromatids together until the onset of anaphase, and the non-SMC subunits have been assigned several regulatory functions (47). The non-SMC subunit WAPAL, for example, is required to remove cohesin from chromosome arms in early mitosis, counteracting the small cohesin subunit cell division cycle associated 5 [CDCA5, also known as sororin (48–50)]. We found WAPAL was one of the highly phosphorylated cohesin subunits (Fig. 4C, table S4) and it is thus possible that hyperphosphorylation of WAPAL in mitosis plays a role in the regulatory mechanism required to remove chromosome arm-bound cohesin. Identification of PLK1 as the major WAPAL kinase in our study further strengthens this hypothesis, because PLK1 inhibition leads to the accumulation of cohesin on chromosome arms in mitosis (fig. S3G) (24, 51–53). Similar regulatory mechanisms could be at play in the NUP107-160 complex, in which NUP98 and NUP107 are phosphorylated to a much higher proportion than the remaining complex members (Fig. 4C, table S4). The NUP98 subunit is positioned within the complex so that it is exposed to cytosol or nucleoplasm, depending on its localization, and is thus well accessible to protein kinases (54). NUP98 phosphorylation by a number of NIMA-related kinases, but not by PLK1, leads to the disassembly of the nuclear pore complex at mitotic entry (55). We found that the NUP107 subunit (but not NUP98) is a direct PLK1 target (Table 2). It will be important to test how PLK1-dependent phosphorylation of NUP107 influences possible functions of this complex, for example at the kinetochore in prometaphase (56) where PLK1

is also located (5). Further identification of hyperphosphorylated complex components that possibly serve as kinase regulatory “switchboards” of these complexes opens a way for studying the function of these phospho-regulatory mechanisms during mitotic progression in more detail.

To predict which protein kinases are likely to target the identified phosphosites, we employed the Scansite algorithm, which includes PSSMs for 32 kinases, including a set of PSSMs for several mitotic kinases (CDKs, PLK1, AURKA, AURKB, and NEK2) (15). Comparison of the Scansite motifs matched to the sites within our dataset with all phosphorylation sites present in the PhosphoSitePlus database (19) revealed strong enrichment of the CDK1 motif, but also motif enrichment for other proline-directed kinases, such as CDK5 and ERK1. Although the PSSMs used identify minor differences between the CDK1, CDK5, and ERK1 motifs, they all share a strong requirement for proline at the –2 position and absolute requirement for proline at the +1 position (22, 57, 58). Thus, it is possible that the sites that match the motifs for these non-mitotic kinases are CDK1-dependent sites.

To assess the mitosis dependency of phosphorylation sites on mitotic complexes, and to identify potential *in vivo* targets of major mitotic kinases that may be important for the regulation of these complexes, we carried out a high sequence coverage analysis of a subset of 12 complexes that comprises major regulators of mitotic chromosome segregation. In contrast to previous proteome-wide studies, this approach allowed a much more complete sequence coverage of the analyzed proteins and yielded many previously unidentified phosphorylation sites on well-studied complexes [compare, for example, the 124 APC/C phosphorylation sites we detected in this study with 59 (11), 71 (9), and 45 sites (25, 42) that had been reported previously]. It will thus be important to expand such systematic high-coverage analyses to more protein complexes, because these data are likely to yield more informative results on specific subsets of protein complexes and their phosphorylation than previous “proteome-scale” studies.

Small-molecule inhibitors have been used successfully for establishing the involvement of particular protein kinases in biological systems in cell biological experiments (59). With the availability of potent and specific inhibitors of PLK1 and AURKB, we identified several sites that are dependent on PLK1 and AURKB [42/400 (11%), 20/400 (5%), respectively]. The percentage of PLK1-dependent sites is lower than that reported in another analysis of the mitotic spindle phosphoproteome, where 18% of all sites were PLK1-dependent [698 of 3984, (13)]. One possibility for this discrepancy is that our non-quantitative MS approach might have missed some phosphorylation sites that were reduced but not completely dephosphorylated after inhibitor treatment. To assess the approximate number of false negative identifications, we generated a panel of phosphospecific antibodies against sites classified as PLK1-dependent, AURKB-dependent, or independent of either or both of these kinases (Fig. 5 and fig. S5). Two phosphorylation sites on RAD21 (Ser¹⁵³ and Ser¹⁷⁵), which we had classified by MS as PLK1-independent, were PLK1-dependent in this Western blot analysis, suggesting that our approach might have missed several phosphorylation sites that are either only partially dependent on PLK1 or AURKB or that are not fully dephosphorylated after kinase inhibition. A second possibility why in our

approach we identified relatively few PLK1- and AURKB-dependent sites is that we acutely inhibited these two kinases in cells that had already entered mitosis. This is in contrast to the study by Santamaria *et al.* where cells entered mitosis in the absence of active PLK1. The sites we identified as lost on complexes purified from inhibitor-treated cells must thus have been actively dephosphorylated by phosphatases. As our understanding of the substrate targeting and activity regulation of phosphatases at present lags behind that of kinases, a new generation of tools will be required to identify the phosphatases that act on PLK1- and AURKB-dependent phosphorylation sites.

Our experiments also revealed that of the eight kinase-dependent sites we tested using phosphospecific antibodies, two sites originally classified as AURKB-dependent (Ser⁵⁵¹ on BUB1B and Thr^{1388/89} on NCAPD2) were not AURKB-dependent in Western blot analysis. These sites might have been mis-classified because of the low abundance of the corresponding phosphopeptides. In the shotgun MS approach we applied, peptides are selected stochastically for identification and thus low abundance peptides have a higher chance of going undetected in complex mixtures of peptides. The targeted MS technique single reaction monitoring (SRM) would improve accuracy in the measurement of abundance changes. This is, however, at the cost of the need to select the peptides to be measured prior to the experiment, thus preventing unbiased sample analysis. Nevertheless, our approach likely identified a large number of true positive PLK1 and AURKB targets, because 75% (6/8) of the phosphospecific antibodies confirmed that the phosphorylation sites identified as kinase sensitive by mass spectrometry were dependent on PLK1 or AURKB.

Pharmacological kinase inhibition likely results in phosphorylation changes not only on direct targets but also on targets further downstream in the kinase-dependent signaling cascade. Because obtaining evidence for direct kinase-substrate relationships *in vivo* remains virtually impossible, we combined our *in vivo* analysis with Scansite predictions to identify direct targets of PLK1 and AURKB with high confidence. This led to the identification of new candidate direct substrates of PLK1 (RAD21, STAG2, WAPAL, ZWILCH, NUP107) and AURKB (RAD21), two of which (pSer¹²⁶¹ on STAG2 and pSer⁴⁶⁵ on WAPAL) were phosphorylated on the experimentally identified and computationally predicted sites in *in vitro* kinase assays using recombinant PLK1.

Our study complements the phosphoproteome-wide approaches by an in-depth analysis of many key regulatory complexes and their phosphorylation pattern in mitosis. Because the present study was not designed to generate quantitative data on the stoichiometry of phosphorylation, nor to determine the consequences of phosphorylation (or absence of phosphorylation) at each site, detailed follow-up studies are required to determine the extents and regulatory effects of these phosphorylations on the protein complexes in question. Combination of the MitoCheck localization, interaction, and phosphorylation data [(14, 28), this study], publicly accessible through the MitoCheck (www.mitocheck.org), PhosphoSitePlus (www.phosphosite.org), Phospho.ELM (phospho.elm.eu.org) and PRIDE (www.ebi.ac.uk/pride) databases will serve as a resource for further research into the regulation of mitosis. This study shows that the MitoCheck-developed LAP-tagged cell lines allow identification of not only protein localization and protein interactors but also

posttranslational modification in a high-throughput fashion. Because many more LAP-tagged proteins are becoming available (60), these cell lines will greatly facilitate systematic analyses of protein complexes regulating many important cellular processes, and our study lays the groundwork for further systematic studies on the phosphorylation of protein complexes.

Materials and methods

Cell culture

HeLa cells were cultured on ten 24-cm square cell culture trays, in DMEM supplemented with 10% Fetal Bovine Serum (Gibco/Invitrogen), plus 0.2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Sigma-Aldrich). For transfected cell lines the medium was supplemented with 500 µg/ml G418 (Gibco). For Tet-On cell lines the medium was supplemented with 100 µg/ml G418 and 200 or 400 µg/ml hygromycin for maintenance or selection, respectively.

Cell synchronization and harvesting

To obtain an interphase cell population, the cells were harvested whilst undergoing exponential growth and with a confluence of <90. For those baits (AURKB, CDCA5), whose protein concentrations are low in early interphase but higher in G2 phase, the cells were harvested following a double-thymidine arrest and release procedure. In one case (PDS5B), the cells were arrested in S-phase by inclusion of hydroxyurea in the growth medium for 18 hr. To obtain a cell population arrested in mitosis (prometaphase), the culture media of exponentially-growing cells was supplemented with nocodazole at 100 µg/ml, and the cells were incubated for a further 18 hr before harvesting; these cells typically had a mitotic index (as judged by cell morphology) of ~90%. To obtain cells arrested in mitosis but in which the activity of endogenous PLK1 was inhibited, nocodazole-treated cells were incubated for a further 2 hr with 250 nM of BI 4834 (a gift from Boehringer Ingelheim, Vienna, Austria). To obtain cells arrested in mitosis but in which the activity of the endogenous AURKB was inhibited, nocodazole-arrested cells were incubated for a further 2 hr with 100 nM Hesperadin (a gift from Boehringer Ingelheim), plus 10 µM N-(benzyloxycarbonyl)leucinylleucinylleucinal (MG132, Sigma-Aldrich). Cells were harvested by scraping into their medium, washed with ice-cold phosphate-buffered saline (PBS), snap-frozen in liquid nitrogen, and stored at -80°C until required.

Protein extract preparation

All steps were performed on ice or at 4°C. Frozen cell pellets were thawed then resuspended in one pellet-volume of extract buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 20 mM β-glycerophosphate, 10 mM NaF, 10% glycerol, 0.1% NP-40, 1 µM okadaic acid [Alexis/Enzo Life Sciences], 0.2 mM Na₃VO₄, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and protease inhibitor mix [10 µg/ml each of leupeptin, pepstatin and chymostatin]). Cells were lysed using a Dounce homogenizer, and the crude extract clarified by centrifugation at 14,000 rpm in a microfuge for 15 min. The protein concentration of the clarified extracts was estimated by the method of Bradford (61), using reagents from Bio-Rad (Hercules, CA, USA). For a given bait, for

each of the different conditions equal amounts of extract (by mass of protein) were used for the initial affinity purification step.

Affinity purification of protein complexes

Endogenous proteins were immunopurified using antibodies described in the antibody section. After immunoprecipitation with antibodies crosslinked with dimethyl pimelimidate (62) to Affi-Prep Protein A beads (Bio-Rad), purified complexes were washed extensively in 10x bead volume of IP buffer [extract buffer without phosphatase inhibitors (no β -glycerophosphate, NaF, okadaic acid or Na_3VO_4)] for a total of 1 hr at 4°C. For mass spectrometric analysis, the last three washing steps were performed in IP-buffer without detergent.

Purification of protein complexes with LAP-tagged baits was performed as described (28). LAP lysis buffers additionally contained 1 μM okadaic acid and either BI 2536, BI 4834, or Hesperadin combined with MG132 when proteins were purified from inhibitor-treated cells. Purified proteins were eluted from beads using 0.2 M glycine pH 2.0 and the eluate was neutralized by addition of 1/10th volume 1.5 M Tris-HCl, pH 9.2.

Affinity purification of condensin-I and condensin-II complexes was performed from HeLa cell lines expressing either NCAPH-GFP-FLAG (63) or NCAPH2-GFP-FLAG (64) fusion proteins, respectively. Agarose beads coupled to the M2 antibody recognizing FLAG (65) (Sigma-Aldrich) were washed three times with 150 mM KCl; then three times with 0.2 M glycine, pH 2.0; then three times with IP buffer. Extracts were prepared in LAP lysis buffer containing 1 μM okadaic acid and the relevant kinase inhibitors, as described above. Clarified extract was then mixed with 100 μl FLAG M2 beads for 1 hr. The beads were pelleted, and washed five times with LAP wash buffer (LAP lysis buffer with NP-40 at 0.05% instead of Triton X-100 at 1%) supplemented with 1 μM okadaic acid, then three times with 150 mM KCl. Purified proteins were eluted from the beads using one bed-volume of 0.2 M glycine, pH 2.0, then the eluate was neutralized by addition of 1/10th volume 1.5 M Tris-HCl, pH 9.2.

In-solution digestion of eluted phosphoprotein complexes

Eluted and neutralized protein samples were reduced, acetylated, and subjected to in-solution digestion using trypsin, chymotrypsin, subtilisin, or Glu-C, as described previously (25). Approximately equal amounts of digested peptide mixtures were analyzed by MS.

MS analysis

All nano HPLC separations were performed using an UltiMate 3000 Nano-LC system (Dionex Benelux, Amsterdam, The Netherlands) equipped with a trap column (PepMap C18, 300 μm ID $\times$ 5 mm length, 3 μm particle size, 100 $\text{\AA}$ pore size, Dionex) for sample desalting and concentration, and an analytical column (PepMap C18, 75 μm ID $\times$ 150 mm length, 3 μm particle size, 100 $\text{\AA}$ pore size, Dionex) for the chromatographic separation. The loading buffer used contained 0.1% trifluoroacetic acid (Pierce/Thermo Scientific). For chromatographic separation, mobile phase A contained 5% acetonitrile (Merck, Darmstadt, Germany) and 0.1% formic acid (Merck), and mobile phase B contained 80% acetonitrile

and 0.08% formic acid. Mass spectrometric analyses were conducted on a hybrid linear ion trap/Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (LTQ-FT Ultra, Thermo Fisher Scientific) with a 7-Tesla superconducting magnet, except for samples derived from NCAPH and NCAPH2 baits, which were analyzed on a 4000 QTRAP mass spectrometer (Applied Biosystems). Mass spectrometers were equipped with a nano-electrospray ionization (ESI) source (Proxeon Biosystems, Odense, Denmark). Metal-coated nano ESI needles were used (New Objective, Woburn, MA, USA).

Samples were loaded onto the trap column at a flow rate of 20 μ l/min loading buffer and were washed for 10 min. Afterwards the sample was eluted from the trap column and separated on the separation column with a gradient from 20% to 50% mobile phase B in 180 min at a flow rate of 300 nl/min.

Peptides eluting from the HPLC were ionized by ESI, with a spray voltage set to 1.5 kV. Full-scan measurements (m/z range 400–1800) were conducted in the ICR cell, yielding a survey scan with resolution of 100,000 and a typical mass accuracy of <2 ppm. Collision-induced dissociation (CAD) fragmentation and spectrum acquisition were carried out in the linear ion trap using a multi-stage activation (MSA) method (66). The target values of the automatic gain control were set to 10,000 for CAD in the ion trap, and to 500,000 for FT-ICR full-scan spectra. Fragmentation was performed on the five most intense signals of the survey scan using MSA of the neutral loss of phosphoric acid. Singly-charged ions were excluded for precursor selection and precursors of MS^2 spectra acquired in previous scans were excluded for further fragmentation for a period of 3 min, whereas the exclusion mass tolerance was set to 5 ppm.

Phosphopeptide identification and manual evaluation

MS raw data files were submitted for database searching using the Mascot program (17) (Matrix Science, London, UK), running on a local computing cluster. For peptide identification, we used a custom sequence database of mouse baits and proteome-wide human proteins, as previously described (14). The following parameters were used for the database search: Carboxymethylation (+58.0055 u) of cysteine was set as fixed and oxidation (+15.9949 u) of methionine and phosphorylation (+79.966331 u) as variable modification; enzymatic cleavage was specified as trypsin, chymotrypsin, or no specificity (for subtilisin digests). Mass tolerances of the parent ion and the fragments were set to 10 ppm and 0.80 Da, respectively. Unphosphorylated peptides with a Mascot score greater than 20 were kept and used to calculate the sequence coverage of detected proteins after combining all parallel digests (usually trypsin, chymotrypsin, and subtilisin). The result of the Mascot database search for each MS run was exported from the Mascot server as a spreadsheet file in xml format.

Following the analysis of phosphorylation sites in the mitotic interaction dataset, the relevant Mascot MS data were processed with the Ascore algorithm (18) to provide an additional quality control of the quality of the spectrum-phosphopeptide match.

Following Mascot database search of the four-condition dataset, for each peptide that Mascot predicted to contain one or more phosphorylated residues, the corresponding MSA

spectrum was manually inspected and rated, by a single skilled MS data analyst, on a score from 0 to 3, using the criteria described in fig. S4. For each manually-rated phosphopeptide, the position(s) of phosphorylation were indicated within the peptide sequence using the following notation (example given for serine residues): S# represents a phosphorylated residue of certain position; S% represents one possible position at which a residue may be phosphorylated (the spectrum provides insufficient information to be sure).

Phosphorylation dataset processing

For the four-condition dataset, following Mascot database search and manual rating the total complement of peptide and phosphopeptide data from all experimental conditions was compiled into a custom database. This allowed the following filters to be applied, for each experimental condition: Firstly, where multiple phosphopeptides with identical sequence (and annotation of phosphorylated residues) were present, only the highest-rated ones were kept. Secondly, where peptides (modified or unmodified) with identical sequence (and annotation of phosphorylated residues, where applicable) were present, only the one with the highest Mascot ions score was kept.

For each bait, the set of sequences of identified peptides (modified or unmodified) was mapped to protein entries in the Ensembl (67) database, and used to identify the corresponding Ensembl gene entries, as previously described (14). Peptides mapping to the set of genes defined by our previous analysis (14) as “contaminant” or “background” proteins were identified and removed.

Phosphosite interpretation

Phosphorylation sites that could not be unambiguously assigned to a single residue were not used further. Unambiguous phosphorylation sites from all four cell cycle conditions were compared: interphase (LOG), mitosis (NOC), mitosis + PLK1 inhibition (BI), and mitosis + AURKB inhibition (Hesp). For each cell cycle state, the phosphorylation site could either be present (P), absent with an unmodified peptide covering the site (N), or absent with no peptide covering the site (nd). We considered only sites that were covered by N, P, or both in all four conditions.

Antibodies

Phosphospecific antibodies (listed in Fig. 5E) were raised against 8-mer to 13-mer synthetic peptides (generated in-house using fluorenylmethyloxycarbonyl chemistry) in rabbits, and affinity purified as described (25). The lab internal accession numbers are as follows (the first number corresponds to those shown in Fig. 5E, codes in brackets are accession codes relevant for antibody requests): 2067 [A768-G], 1706 [A759-M], 1707 [A760-M], 1710 [A761-M], 2063 [A764-M], 1746 [A762-M], 2062 [A763-M], 2263 [A772-G], 2262 [A771-G], 2275 [A775-M], 2068 [A769-G], 2065 [A766-M], 2264 [A773-M], 2265 [A774-M]. Further antibodies used in immunopurification, Western blotting, and immunofluorescence were as follows: rabbit antibodies recognizing CDC27 (68), PDS5A [antibody number 521, (69)], PDS5B [antibody number 1531: QLKGLEDTKSPQFNRYFC and antibody number 2230: VSTVNVRRRSAKRERR (70)], STAG1 [antibody number 444 (69)], STAG2 [antibody number 446 (69)], WAPAL [antibody number 986 and 987 (49)], NUP85 (71),

NCAPH (72), CDC25C (Cell Signaling Technology), BUB1B (a gift from Gregor Kohlmaier), INCENP (41), TUBG1 (T3559, Sigma-Aldrich), phosphorylated CDC16 (25), RAD21 (05–908, Millipore), or PDS5B (BL2352, Bethyl Laboratories); mouse antibodies recognizing phosphorylated Ser¹⁰ Histone 3 (H3) (05–499, Millipore), CCNB1 (GNS1, Santa Cruz), MYC (4A6, 05–724, Millipore), SMC2 (72), TUBA1B (clone B-5-1-2, Sigma-Aldrich); goat antibody recognizing H3 (sc-8654, Santa Cruz Biotechnology); and a human calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia (CREST) antiserum (Cortex Biochem).

Secondary antibodies used for Western blotting were antibodies recognizing mouse, rabbit, or goat coupled to horseradish peroxidase (HRP); for immunofluorescence Alexa 488, Alexa 568, and Alexa 633-labeled secondary antibodies were used (Molecular Probes/Invitrogen).

In vitro kinase assays

WAPAL and STAG2 were immunopurified as described above. In vitro phosphorylation was performed as described (25) using 10 mM ATP and recombinant 6×His-tagged PLK1. Recombinant human PLK1 was expressed as a 6×His-tagged fusion protein using a baculoviral expression system and purified by affinity chromatography using Ni-nitrilotriacetic acid (NTA)-coupled agarose beads (QIAGEN). Where indicated, reactions were supplemented with 1 μM BI 2536.

Histone H1 kinase assays

The activity of a cell extract to catalyze the addition of ³²P-phosphate from [γ -³²P] ATP into histone H1 was assayed as described (73). Duplicate measurements were performed twice, then mean and standard deviation values calculated. The Bradford method (61) was used to determine the concentration of protein in HeLa cell extracts, and this was used to calculate specific kinase activities (pmol phosphate incorporated per min per μg of extract).

Immunofluorescence microscopy

Cells were grown on coverslips and fixed with 4% formaldehyde in PBS at room temperature, or with methanol at –20°C for 15 min. RAD21-9×MYC cells were centrifuged onto slides (Cytospin, Thermo Scientific), extracted with 0.5% Triton X-100 in PBS for 2 min, and fixed with 4% formaldehyde in PBS (41). After fixation samples were permeabilized with 0.5% Triton X-100 in PBS for 15 min and thereafter blocked with 10% FCS in PBS containing 0.01% Triton X-100. Coverslips were incubated for 1 hr each at room temperature with primary and secondary antibodies (at 2 μg/ml in 3% bovine serum albumin (BSA) in PBS containing 0.01% Triton X-100), DNA was counterstained with Hoechst 33342, and samples were mounted with either ProLong Gold (Molecular Probes / Invitrogen) or Vectashield Mounting Medium (H1000, Vector Laboratories) onto slides. Image acquisition was performed as described (74) or (for BI 4834 characterization) images were taken on a Zeiss Axioplan 2 microscope using a 100× Plan-Apochromat objective lens (Carl Zeiss, Jena, Germany) and equipped with a CoolSnap HQ chargecoupled device camera (Photometrics). For signal intensity quantification, images were acquired as raw 12-bit files, captured at identical exposure times within each experiment. Images were

processed using *ImageJ* software (<http://rsb.info.nih.gov/ij/>) (75). Images shown in the same panel have been scaled identically.

For quantification of TUBG1 intensities a circular region with fixed diameter was measured on the centrosome and a same-sized region was also measured in the cytoplasm. After subtracting the background outside the cells ratio of these values was calculated and average and standard deviation of these measurements were plotted. For each measurement at least 40 centrosomes in 20 cells were quantified. Mitotic phenotypes were classified based on the staining pattern produced by 4',6-diamidino-2-phenylindole (DAPI) and TUBA1B antibodies; for each measurement 100 cells were counted.

Scansite and statistical analyses

Scansite predicts potential target sites for a protein kinase by scanning through a set of protein sequences, evaluating for each Ser, Thr, or Tyr residue the match between its surrounding sequence and the preferred amino acids in each position relative to the target site, as represented by a PSSM (20, 21).

We established an automated computing workflow enabling the Scansite algorithm, running on a local server, to scan a large set of input protein sequences, employing multiple PSSMs in succession. This approach was used to predict which kinases may target particular Ser, Thr, or Tyr residues among the set of protein sequences in which phosphosites were identified by MS. Each match between a PSSM and a Ser, Thr, or Tyr residue and its surrounding sequence is given a score (the “Scansite score”) and is assigned a percentile. The percentile is based on the position of the Scansite score in the empiric score distribution derived from a reference dataset. As the phosphosites in our dataset are recorded as positions relative to protein entries from the Ensembl database (www.ensembl.org)(67), a complete set of Ensembl human and mouse protein sequences was used as the reference dataset. We considered Scansite hits within the 5%, 1.5% and 0.2% as “low,” “medium,” and “high” stringency, respectively. We only included hits of “high” or “medium” stringency in the analytical outputs.

Statistical analyses were performed to determine whether the number of Scansite hits matching MS-derived phosphosites was significantly greater than that which we would expect based on the known distribution of phosphosites in the human proteome. The number of matches of Scansite hits to phosphosites recorded in the PhosphoSite Plus database (www.phosphosite.org)(19) was determined. For each kinase motif, a Bonferroni-corrected Fisher’s exact test (76) was used to calculate the probability (p-value) that our set of MS-identified phosphosites would have been found at the same frequency by chance alone. To make the reporting of p-values (which vary across an exponential range) more manageable, they were expressed as a probability score, equal to $-\log_{10}(p)$. Kinase motifs with a probability score of 2 or greater (where the corrected probability of a random match was 1 in 100, or lower) were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank E. Kreidl, G.G. Leparc, M. Madalinski, G. Mitulovi, M. Schutzbier, I. Steinmacher, Y. Sun, S. Zettl (IMP-IMBA, Vienna); M. Augsburg, F. Buchholz, D. Richter, A. Szykora (MPI-CBG, Dresden) for valuable advice and assistance; V. Doye (Institut Curie, Paris) for the kind gift of the anti-NUP85 antibody; S. Beausoleil (Harvard University) for Ascore analysis of Mascot results, M. Peter (ETH, Zürich) for providing lab space and reagents during revision experiments, and R. Durbin, J. Ellenberg and K. Nasmyth for helpful discussions.

Funding: This work was funded by the European Commission via the Sixth Framework Programme Integrated Project "MitoCheck" (LSHG-CT-2004-503464). Work in the laboratories of J.-M.P. and K.M. received support from Boehringer Ingelheim, the Austrian Science Fund Special Research Programme F34 "Chromosome Dynamics", and the Austrian Research Promotion Agency FFG. Work in the laboratory of M.B.Y. was funded, in part, by NIH grants GM-60594, GM-68762, ES-015339, and CA-112967.

N.K. is an employee of Boehringer Ingelheim RCV GmbH & Co KG, which supplied the compounds Hesperadin and BI 4834 used in this study, holds two patents on some of the phosphorylation sites published here (EP1884522 for WAPAL, EP1884523 for STAG2), and in part funded this research.

References

1. Alberts B. The cell as a collection of protein machines: preparing the next generation of molecular biologists. *Cell*. 1998; 92:291–294. [PubMed: 9476889]
2. Chari A, Fischer U. Cellular strategies for the assembly of molecular machines. *Trends Biochem Sci*. 2010; 35:676–683. [PubMed: 20727772]
3. Mitchison TJ. Microtubule dynamics and kinetochore function in mitosis. *Annu Rev Cell Biol*. 1988; 4:527–549. [PubMed: 3058165]
4. HUGO, In this paper, proteins are referred to by their official gene symbols: for human genes according to the Human Genome Organization (HUGO) nomenclature (<http://www.genenames.org>); for mouse genes, according the Mouse Genome Informatics (MGI) nomenclature (<http://www.informatics.jax.org>)
5. Nigg EA. Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol*. 2001; 2:21–32. [PubMed: 11413462]
6. Peters JM. The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat Rev Mol Cell Biol*. 2006; 7:644–656. [PubMed: 16896351]
7. Musacchio A, Salmon ED. The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol*. 2007; 8:379–393. [PubMed: 17426725]
8. Nousiainen M, Sillje HH, Sauer G, Nigg EA, Korner R. Phosphoproteome analysis of the human mitotic spindle. *Proc Natl Acad Sci U S A*. 2006; 103:5391–5396. [PubMed: 16565220]
9. Steen JA, Steen H, Georgi A, Parker K, Springer M, Kirchner M, Hamprecht F, Kirschner MW. Different phosphorylation states of the anaphase promoting complex in response to antimetabolic drugs: a quantitative proteomic analysis. *Proc Natl Acad Sci U S A*. 2008; 105:6069–6074. [PubMed: 18420821]
10. Malik R, Lenobel R, Santamaria A, Ries A, Nigg EA, Korner R. Quantitative analysis of the human spindle phosphoproteome at distinct mitotic stages. *J Proteome Res*. 2009; 8:4553–4563. [PubMed: 19691289]
11. Olsen JV, Vermeulen M, Santamaria A, Kumar C, Miller ML, Jensen LJ, Gnäd F, Cox J, Jensen TS, Nigg EA, Brunak S, Mann M. Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Sci Signal*. 2010; 3:ra3. [PubMed: 20068231]
12. Dephoure N, Zhou C, Villen J, Beausoleil SA, Bakalarski CE, Elledge SJ, Gygi SP. A quantitative atlas of mitotic phosphorylation. *Proc Natl Acad Sci U S A*. 2008; 105:10762–10767. [PubMed: 18669648]
13. Santamaria A, Wang B, Elowe S, Malik R, Zhang F, Bauer M, Schmidt A, Sillje HH, Korner R, Nigg EA. The Plk1-dependent Phosphoproteome of the Early Mitotic Spindle. *Mol Cell Proteomics*. 2011; 10:M110 004457. [PubMed: 20860994]
14. Hutchins JRA, Toyoda Y, Hegemann B, Poser I, Hériché JK, Sykora MM, Augsburg M, Hudecz O, Buschhorn BA, Bulkescher J, Conrad C, Comartin D, Schleiffer A, Sarov M, Pozniakovskiy A,

- Slabicki MM, Schloissnig S, Steinmacher I, Leuschner M, Ssykor A, Lawo S, Pelletier L, Stark H, Nasmyth K, Ellenberg J, Durbin R, Buchholz F, Mechtler K, Hyman AA, Peters JM. Systematic analysis of human protein complexes identifies chromosome segregation proteins. *Science*. 2010; 328:593–599. [PubMed: 20360068]
15. Alexander J, Lim D, Joughin BA, Hegemann B, Hutchins JR, Ehrenberger T, Ivins F, Sessa F, Hudecz O, Nigg EA, Fry AM, Musacchio A, Stukenberg PT, Mechtler K, Peters JM, Smerdon SJ, Yaffe MB. Spatial exclusivity combined with positive and negative selection of phosphorylation motifs is the basis for context-dependent mitotic signaling. *Sci Signal*. 2011; 4:ra42. [PubMed: 21712545]
 16. Hauf S, Cole RW, LaTerra S, Zimmer C, Schnapp G, Walter R, Heckel A, van Meel J, Rieder CL, Peters JM. The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J Cell Biol*. 2003; 161:281–294. [PubMed: 12707311]
 17. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*. 1999; 20:3551–3567. [PubMed: 10612281]
 18. Beausoleil SA, Villen J, Gerber SA, Rush J, Gygi SP. A probability-based approach for high-throughput protein phosphorylation analysis and site localization. *Nat Biotechnol*. 2006; 24:1285–1292. [PubMed: 16964243]
 19. Hornbeck PV, Chabra I, Kornhauser JM, Skrzypek E, Zhang B. PhosphoSite: A bioinformatics resource dedicated to physiological protein phosphorylation. *Proteomics*. 2004; 4:1551–1561. [PubMed: 15174125]
 20. Obenauer JC, Cantley LC, Yaffe MB. Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res*. 2003; 31:3635–3641. [PubMed: 12824383]
 21. Yaffe MB, Leparo GG, Lai J, Obata T, Volinia S, Cantley LC. A motif-based profile scanning approach for genome-wide prediction of signaling pathways. *Nat Biotechnol*. 2001; 19:348–353. [PubMed: 11283593]
 22. Panchamoorthy G, Fukazawa T, Stolz L, Payne G, Reedquist K, Shoelson S, Songyang Z, Cantley L, Walsh C, Band H. Physical and functional interactions between SH2 and SH3 domains of the Src family protein tyrosine kinase p59fyn. *Mol Cell Biol*. 1994; 14:6372–6385. [PubMed: 7520528]
 23. Gautier J, Norbury C, Lohka M, Nurse P, Maller J. Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene *cdc2+*. *Cell*. 1988; 54:433–439. [PubMed: 3293803]
 24. Lénárt P, Petronczki M, Steegmaier M, Di Fiore B, Lipp JJ, Hoffmann M, Rettig WJ, Kraut N, Peters JM. The small-molecule inhibitor BI 2536 reveals novel insights into mitotic roles of polo-like kinase 1. *Curr Biol*. 2007; 17:304–315. [PubMed: 17291761]
 25. Kraft C, Herzog F, Gieffers C, Mechtler K, Hagting A, Pines J, Peters JM. Mitotic regulation of the human anaphase-promoting complex by phosphorylation. *Embo J*. 2003; 22:6598–6609. [PubMed: 14657031]
 26. Elowe S, Hummer S, Uldschmid A, Li X, Nigg EA. Tension-sensitive Plk1 phosphorylation on BubR1 regulates the stability of kinetochore microtubule interactions. *Genes Dev*. 2007; 21:2205–2219. [PubMed: 17785528]
 27. Cheeseman IM, Desai A. A combined approach for the localization and tandem affinity purification of protein complexes from metazoans. *Sci STKE*. 2005; 2005:pl1. [PubMed: 15644491]
 28. Poser I, Sarov M, Hutchins JRA, Hériché JK, Toyoda Y, Pozniakovsky A, Weigl D, Nitzsche A, Hegemann B, Bird AW, Pelletier L, Kittler R, Hua S, Naumann R, Augsburg M, Sykora MM, Hofemeister H, Zhang Y, Nasmyth K, White KP, Dietzel S, Mechtler K, Durbin R, Stewart AF, Peters JM, Buchholz F, Hyman AA. BAC TransgeneOmics: a high-throughput method for exploration of protein function in mammals. *Nat Methods*. 2008; 5:409–415. [PubMed: 18391959]
 29. Neumann B, Walter T, Hériché JK, Bulkescher J, Erfle H, Conrad C, Rogers P, Poser I, Held M, Liebel U, Cetin C, Sieckmann F, Pau G, Kabbe R, Wunsche A, Satagopam V, Schmitz MH, Chapis C, Gerlich DW, Schneider R, Eils R, Huber W, Peters JM, Hyman AA, Durbin R,

- Pepperkok R, Ellenberg J. Phenotypic profiling of the human genome by time-lapse microscopy reveals cell division genes. *Nature*. 2010; 464:721–727. [PubMed: 20360735]
30. Qi W, Tang Z, Yu H. Phosphorylation- and polo-box-dependent binding of Plk1 to Bub1 is required for the kinetochore localization of Plk1. *Mol Biol Cell*. 2006; 17:3705–3716. [PubMed: 16760428]
 31. Chi YH, Haller K, Ward MD, Semmes OJ, Li Y, Jeang KT. Requirements for protein phosphorylation and the kinase activity of polo-like kinase 1 (Plk1) for the kinetochore function of mitotic arrest deficiency protein 1 (Mad1). *J Biol Chem*. 2008; 283:35834–35844. [PubMed: 18922800]
 32. Hirota T, Lipp JJ, Toh BH, Peters JM. Histone H3 serine 10 phosphorylation by Aurora B causes HP1 dissociation from heterochromatin. *Nature*. 2005; 438:1176–1180. [PubMed: 16222244]
 33. Fischle W, Tseng BS, Dormann HL, Ueberheide BM, Garcia BA, Shabanowitz J, Hunt DF, Funabiki H, Allis CD. Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. *Nature*. 2005; 438:1116–1122. [PubMed: 16222246]
 34. Honda R, Körner R, Nigg EA. Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis. *Mol Biol Cell*. 2003; 14:3325–3341. [PubMed: 12925766]
 35. Dinkel H, Chica C, Via A, Gould CM, Jensen LJ, Gibson TJ, Diella F. Phospho.ELM: a database of phosphorylation sites—update 2011. *Nucleic Acids Res*. 2011; 39:D261–267. [PubMed: 21062810]
 36. Vizcaíno JA, Cote R, Reisinger F, Foster JM, Mueller M, Rameseder J, Hermjakob H, Martens L. A guide to the Proteomics Identifications Database proteomics data repository. *Proteomics*. 2009; 9:4276–4283. [PubMed: 19662629]
 37. Maller J, Wu M, Gerhart JC. Changes in protein phosphorylation accompanying maturation of *Xenopus laevis* oocytes. *Dev Biol*. 1977; 58:295–312. [PubMed: 885290]
 38. Karsenti E, Bravo R, Kirschner M. Phosphorylation changes associated with the early cell cycle in *Xenopus* eggs. *Dev Biol*. 1987; 119:442–453. [PubMed: 3803713]
 39. Malik R, Nigg EA, Korner R. Comparative conservation analysis of the human mitotic phosphoproteome. *Bioinformatics*. 2008; 24:1426–1432. [PubMed: 18426804]
 40. Bodenmiller B, Wanka S, Kraft C, Urban J, Campbell D, Pedrioli PG, Gerrits B, Picotti P, Lam H, Vitek O, Brusniak MY, Roschitzki B, Zhang C, Shokat KM, Schlaphach R, Colman-Lerner A, Nolan GP, Nesvizhskii AI, Peter M, Loewith R, von Mering C, Aebersold R. Phosphoproteomic analysis reveals interconnected system-wide responses to perturbations of kinases and phosphatases in yeast. *Sci Signal*. 2010; 3:rs4. [PubMed: 21177495]
 41. Hauf S, Roitinger E, Koch B, Dittrich CM, Mechtler K, Peters JM. Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. *PLoS Biol*. 2005; 3:e69. [PubMed: 15737063]
 42. Herzog F, Mechtler K, Peters JM. Identification of cell cycle-dependent phosphorylation sites on the anaphase-promoting complex/cyclosome by mass spectrometry. *Methods Enzymol*. 2005; 398:231–245. [PubMed: 16275332]
 43. Dube P, Herzog F, Gieffers C, Sander B, Riedel D, Muller SA, Engel A, Peters JM, Stark H. Localization of the coactivator Cdh1 and the cullin subunit Apc2 in a cryoelectron microscopy model of vertebrate APC/C. *Mol Cell*. 2005; 20:867–879. [PubMed: 16364912]
 44. Herzog F, Primorac I, Dube P, Lénárt P, Sander B, Mechtler K, Stark H, Peters JM. Structure of the anaphase-promoting complex/cyclosome interacting with a mitotic checkpoint complex. *Science*. 2009; 323:1477–1481. [PubMed: 19286556]
 45. Buschhorn BA, Petzold G, Galova M, Dube P, Kraft C, Herzog F, Stark H, Peters JM. Substrate binding on the APC/C occurs between the coactivator Cdh1 and the processivity factor Doc1. *Nat Struct Mol Biol*. 2011; 18:6–13. [PubMed: 21186364]
 46. da Fonseca PC, Kong EH, Zhang Z, Schreiber A, Williams MA, Morris EP, Barford D. Structures of APC/C(Cdh1) with substrates identify Cdh1 and Apc10 as the D-box co-receptor. *Nature*. 2011; 470:274–278. [PubMed: 21107322]
 47. Peters JM, Tedeschi A, Schmitz J. The cohesin complex and its roles in chromosome biology. *Genes Dev*. 2008; 22:3089–3114. [PubMed: 19056890]

48. Gandhi R, Gillespie PJ, Hirano T. Human Wapl is a cohesin-binding protein that promotes sister-chromatid resolution in mitotic prophase. *Curr Biol.* 2006; 16:2406–2417. [PubMed: 17112726]
49. Kueng S, Hegemann B, Peters BH, Lipp JJ, Schleiffer A, Mechtler K, Peters JM. Wapl controls the dynamic association of cohesin with chromatin. *Cell.* 2006; 127:955–967. [PubMed: 17113138]
50. Nishiyama T, Ladurner R, Schmitz J, Kreidl E, Schleiffer A, Bhaskara V, Bando M, Shirahige K, Hyman AA, Mechtler K, Peters JM. Sororin mediates sister chromatid cohesion by antagonizing Wapl. *Cell.* 2010; 143:737–749. [PubMed: 21111234]
51. Losada A, Hirano M, Hirano T. Cohesin release is required for sister chromatid resolution, but not for condensin-mediated compaction, at the onset of mitosis. *Genes Dev.* 2002; 16:3004–3016. [PubMed: 12464631]
52. Sumara I, Vorlaufer E, Stukenberg PT, Kelm O, Redemann N, Nigg EA, Peters JM. The dissociation of cohesin from chromosomes in prophase is regulated by Pololike kinase. *Mol Cell.* 2002; 9:515–525. [PubMed: 11931760]
53. Gimenez-Abian JF, Sumara I, Hirota T, Hauf S, Gerlich D, de la Torre C, Ellenberg J, Peters JM. Regulation of sister chromatid cohesion between chromosome arms. *Curr Biol.* 2004; 14:1187–1193. [PubMed: 15242616]
54. Griffiths ER, Xu S, Powers MA. Nup98 localizes to both nuclear and cytoplasmic sides of the nuclear pore and binds to two distinct nucleoporin subcomplexes. *Mol Biol Cell.* 2003; 14:600–610. [PubMed: 12589057]
55. Laurell E, Beck K, Krupina K, Theerthagiri G, Bodenmiller B, Horvath P, Aebersold R, Antonin W, Kutay U. Phosphorylation of Nup98 by Multiple Kinases Is Crucial for NPC Disassembly during Mitotic Entry. *Cell.* 2011; 144:539–550. [PubMed: 21335236]
56. Güttinger S, Laurell E, Kutay U. Orchestrating nuclear envelope disassembly and reassembly during mitosis. *Nat Rev Mol Cell Biol.* 2009; 10:178–191. [PubMed: 19234477]
57. Songyang Z, Lu KP, Kwon YT, Tsai LH, Filhol O, Cochet C, Brickey DA, Soderling TR, Bartleson C, Graves DJ, DeMaggio AJ, Hoekstra MF, Blenis J, Hunter T, Cantley LC. A structural basis for substrate specificities of protein Ser/Thr kinases: primary sequence preference of casein kinases I and II, NIMA, phosphorylase kinase, calmodulin-dependent kinase II, CDK5, and Erk1. *Mol Cell Biol.* 1996; 16:6486–6493. [PubMed: 8887677]
58. Manke IA, Nguyen A, Lim D, Stewart MQ, Elia AE, Yaffe MB. MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation. *Mol Cell.* 2005; 17:37–48. [PubMed: 15629715]
59. Cohen P. Targeting protein kinases for the development of anti-inflammatory drugs. *Curr Opin Cell Biol.* 2009; 21:317–324. [PubMed: 19217767]
60. Hubner NC, Bird AW, Cox J, Splettstoesser B, Bandilla P, Poser I, Hyman A, Mann M. Quantitative proteomics combined with BAC TransgeneOmics reveals in vivo protein interactions. *J Cell Biol.* 2010; 189:739–754. [PubMed: 20479470]
61. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248–254. [PubMed: 942051]
62. Harlow, E.; Lane, D. Using antibodies: a laboratory manual. 1. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1999.
63. Hirota T, Gerlich D, Koch B, Ellenberg J, Peters JM. Distinct functions of condensin I and II in mitotic chromosome assembly. *J Cell Sci.* 2004; 117:6435–6445. [PubMed: 15572404]
64. Gerlich D, Hirota T, Koch B, Peters JM, Ellenberg J. Condensin I stabilizes chromosomes mechanically through a dynamic interaction in live cells. *Curr Biol.* 2006; 16:333–344. [PubMed: 16488867]
65. Brizzard BL, Chubet RG, Vizard DL. Immunoaffinity purification of FLAG epitope-tagged bacterial alkaline phosphatase using a novel monoclonal antibody and peptide elution. *Biotechniques.* 1994; 16:730–735. [PubMed: 8024796]
66. Schroeder MJ, Shabanowitz J, Schwartz JC, Hunt DF, Coon JJ. A neutral loss activation method for improved phosphopeptide sequence analysis by quadrupole ion trap mass spectrometry. *Anal Chem.* 2004; 76:3590–3598. [PubMed: 15228329]

67. Flicek P, Amode MR, Barrell D, Beal K, Brent S, Chen Y, Clapham P, Coates G, Fairley S, Fitzgerald S, Gordon L, Hendrix M, Hourlier T, Johnson N, Kahari A, Keefe D, Keenan S, Kinsella R, Kokocinski F, Kulesha E, Larsson P, Longden I, McLaren W, Overduin B, Pritchard B, Riat HS, Rios D, Ritchie GR, Ruffier M, Schuster M, Sobral D, Spudich G, Tang YA, Trevanion S, Vandrovcova J, Vilella AJ, White S, Wilder SP, Zadissa A, Zamora J, Aken BL, Birney E, Cunningham F, Dunham I, Durbin R, Fernandez-Suarez XM, Herrero J, Hubbard TJ, Parker A, Proctor G, Vogel J, Searle SM. Ensembl 2011. *Nucleic Acids Res.* 2011; 39:D800–806. [PubMed: 21045057]
68. Gieffers C, Peters BH, Kramer ER, Dotti CG, Peters JM. Expression of the CDH1-associated form of the anaphase-promoting complex in postmitotic neurons. *Proc Natl Acad Sci U S A.* 1999; 96:11317–11322. [PubMed: 10500174]
69. Sumara I, Vorlaufer E, Gieffers C, Peters BH, Peters JM. Characterization of vertebrate cohesin complexes and their regulation in prophase. *J Cell Biol.* 2000; 151:749–762. [PubMed: 11076961]
70. Losada A, Yokochi T, Hirano T. Functional contribution of Pds5 to cohesin-mediated cohesion in human cells and *Xenopus* egg extracts. *J Cell Sci.* 2005; 118:2133–2141. [PubMed: 15855230]
71. Loïdice I, Alves A, Rabut G, Van Overbeek M, Ellenberg J, Sibarita JB, Doye V. The entire Nup107–160 complex, including three new members, is targeted as one entity to kinetochores in mitosis. *Mol Biol Cell.* 2004; 15:3333–3344. [PubMed: 15146057]
72. Yeong FM, Hombauer H, Wendt KS, Hirota T, Mudrak I, Mechtler K, Loregger T, Marchler-Bauer A, Tanaka K, Peters JM, Ogris E. Identification of a subunit of a novel Kleisin-beta/SMC complex as a potential substrate of protein phosphatase 2A. *Curr Biol.* 2003; 13:2058–2064. [PubMed: 14653995]
73. Clarke, PR. *Cell Cycle: Materials and Methods.* Pagano, M., editor. Springer; Heidelberg: 1995. p. 103–116.
74. Waizenegger IC, Hauf S, Meinke A, Peters JM. Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. *Cell.* 2000; 103:399–410. [PubMed: 11081627]
75. Collins TJ. ImageJ for microscopy. *Biotechniques.* 2007; 43:25–30. [PubMed: 17936939]
76. Fisher RA. *Statistical Methods for Research Workers.* 1925
77. Student, The Probable Error of a Mean. *Biometrika.* 1908; 6:1–25.

Fig. 1. Overview of the phosphorylation analysis of the mitotic interaction network

(A) Schematic outline of the workflow for mitotic gene selection, BAC tagging, phosphoprotein complex purification, and analysis.

(B) Summary table of data obtained from the phosphorylation analysis of the mitotic interaction dataset.

Fig. 2. Identification of phosphosites in proteins from the mitotic interaction network

(A) Frequency distribution graph of the number of phosphosites identified per interaction cluster in the mitotic interaction dataset.

(B) Interaction map for a selection of 9 out of 107 small clusters as determined in (14), showing the phosphorylation-site density in percent for each protein. Complexes containing reciprocal interactions are enclosed by solid gray lines; those without reciprocal interactions are denoted by dashed gray lines. Interactions between complexes are indicated by dashed blue lines with the number of interactions represented by line thickness. SFCM is the name of the clustering method.

(C) Percent kinase motifs found within 615 high confidence hits found in 1451 phosphorylation sites. Note that 39% of all hits correspond to the mitotic kinases CDK1 (represented by three motif variants named Cdc2, CDK1-1, and CDK1-2), PLK1, AURKA, AURKB, and NEK2.

nocodazole arrest with additional treatment of 250 nM BI 4834 for last two hours, Hesp = 18 hours nocodazole arrest with additional treatment of 100 nM Hesperadin and 10 μ M MG132 for last two hours.

(D) Summary table of data obtained from phosphorylation analysis of 24 baits from samples as described in (C).

(E) Percent kinase motifs found within 455 high confidence hits found in 618 phosphorylation sites. Note that 33% of all hits correspond to the mitotic kinases CDK1 (represented by three motif variants named Cdc2, CDK1-1, and CDK1-2), PLK1, AURKA, AURKB, and NEK2.

Fig. 4. Identification of phosphorylation sites in protein complexes from the four-condition dataset

(A) Silver-stained SDS-PAGE gel showing proteins purified from the four cell-treatment conditions (LOG, NOC, BI, Hesp) following a LAP-TAP purification using mouse Bub1b as bait. This gel illustrates the level of purity achieved in purifications carried out with 24 different baits. Annotated protein bands are based on their expected migration positions, the intensity of the marker bands corresponds to the annotated protein amounts in ng.

(B) Phosphoproteins and phosphosites identified by MS analysis of the three protein complexes MCC, APC/C, and MIS12/NDC80 and the copurifying protein UBR5, the first column (c) indicates complex membership, affinity purified using the mouse Bub1b-LAP bait, from the four conditions, as shown in (A). The percentages results from the sequence coverages obtained after combining peptides generated by all proteases.

(C) Interaction map for a selection of 8 out of 12 complexes analysed in the four treatment conditions, projected onto the interaction clusters determined in Hutchins et al. (14), showing the phosphorylation site density (in percent) for each protein and indicating proteins with sites dependent on PLK1 and AURKB.

Fig. 5. Phosphospecific antibodies validate MS results

(A) Phosphospecific antibodies (listed in panel E) were tested on cell extract (XT) or immunopurified protein (IP). XT were from interphase (LOG), noc-arrest (NOC), and noc-arrest in combination with 2 hours of BI 4834 (BI) or a combination of Hesperadin and MG132 (Hesp). The STAG2 pSer¹²⁶¹ antibody was tested on XT from synchronised cells harvested either in G2, in mitosis by shake-off (SO), in mitosis with 3 hours nocodazole treatment (sN), or in mitosis with 3 hours nocodazole and last 2 hours either in BI 4834

(sBI) or in Hesperadin plus MG132 (sHesp). Cell cycle stage was verified using the indicated antibodies. N(experiments)=2.

(B) STAG2 and WAPAL were immunopurified from LOG and incubated with PLK1, ATP, and 1 μ M of BI 2536. Western blots were performed using indicated antibodies.

N(experiments)=2.

(C) Immunofluorescence images of cells at different cell-cycle stages treated or not with 100 nM of BI 2536 for 30 min prior to fixation were stained with indicated antibodies and DAPI. N=3.

(D) Phosphospecific antibodies were tested on XT or IP using the indicated antibodies or LAP purifications from indicated BAC-cells. N(experiments)=2.

(E) Phosphospecific antibodies and antigenic peptides (#= ID number). The phosphosite category as determined by MS and Western blotting (WB) is: i/m = detected in LOG and NOC, m = only in NOC, unphosphorylated in LOG, bi = unphosphorylated in BI, hes = unphosphorylated in Hesp).

Table 1
Summary of complexes, baits (with their sequence coverage) and interactors, identified phosphoproteins and phosphorylation sites in the four-condition dataset

Sp. bait, the organism of the bait protein; Pf., purification method, Int., interactors; LOG, interphase; NOC, noc-arrest; BI, noc-arrest in combination with 2 hours of BI 4834; Hesp, nocarrest in combination with a combination of Hesperadin and MG132; ph-proteins, the number of phosphorylated proteins; ph-sites, the number of phosphorylated sites; CPC: chromosomal passenger complex [AURKB, INCENP, baculoviral IAP repeat-containing protein 5 (BIRC5), CDCA8]

Complex	Sp. bait	Bait	Pf.	Int.	% seq. coverage bait				ph-proteins	ph-sites
					LOG	NOC	BI	Hesp		
Dynactin	Mm	Actr1a	LAP	5	93	94	93	96	1	6
γ-TuRC	Mm	Tubg1	LAP	96	96	93	94	92	4	14
CPC	Mm	Aurkb	LAP	13	77	79	78	81	4	43
CDK1	Mm	Cdk1	LAP	9	78	84	82	88	5	7
PLK1	Mm	PLK1	LAP	9	72	34	36	74	3	4
MIS12/NDC80	Mm	Bub1	LAP	25	84	89	89	88	8	41
MIS12/NDC80	Mm	Mis12	LAP	30	84	74	81	83	7	26
MIS12/NDC80	Hs	NSL1	Ab	65	78	67	na	na	4	19
RZZ	Mm	Zw10	LAP	23	85	85	84	85	4	13
MCC	Mm	Bub1b	LAP	55	83	86	86	89	13	49
MCC	Mm	Mad2l1	LAP	105	98	95	93	95	6	30
APC	Hs	CDC27	Ab	64	87	78	81	94	17	124
NUP107-160	Mm	Nup107	LAP	39	82	87	86	12	6	53
Centralspindlin	Mm	Kif23	LAP	7	31	36	37	47	5	23
Centralspindlin	Mm	Racgap1	LAP	9	58	57	61	54	7	64
cohesin	Mm	Cdca5	LAP	23	78	75	65	66	1	8
cohesin	Hs	PDS5A	Ab	238	76	91	64	80	4	27

Complex	Sp. bait	Bait	Pf.	Int.	% seq. coverage bait				ph-proteins	ph-sites
					LOG	NOC	BI	Hesp		
cohesin	Hs	PDS5B	Ab	42	81	75	58	73	3	26
	Hs	STAG1	Ab	298	65	69	na	na	6	18
	Hs	STAG2	Ab	243	60	78	74	72	11	53
	Hs	WAPAL	Ab	27	19	68	77	31	5	32
condensin-I	Hs	NCAPH	FLAG	6	20	18	0	22	4	19
condensin-II	Hs	NCAPH2	FLAG	7	0	10	0	0	2	6
SMC5/6	Mm	Smc6	LAP	40	42	43	43	42	5	19
			average:		78	76	77	77		

Table 2
Summary of proteins identified containing **PLK1- or AURKB-dependent phosphorylation sites, with sites matching Scansite sequence motifs in bold**

Sp. bait, the organism of the bait protein; mit. sites, mitotic sites; BI-sens, all sites sensitive to BI 4834 treatment; BI-only, all BI-sensitive sites that are not also sensitive to Hesperadin treatment; Hesp-sens, all sites sensitive to Hesperadin treatment; Hesp-only, all Hesp-sensitive sites that are not also sensitive to BI 4834 treatment.

Complex	Sp	Name	mit. sites	BI-sens. (BI-only)	Hesp-sens (Hesp-only)	BI-sens. sites	Hesp-sens. sites
γ-TURC	Mm	Tubg1	3	2 (1)	2 (1)	Ser ¹³¹ , Ser ³²	Ser ²⁸⁴ , Ser ³²
γ-TURC	Hs	TUBGCP6	5	1 (1)	0	Ser ²⁴²	–
CPC	Mm	Aurkb	4	2 (0)	3 (1)	Ser ⁷¹ , Ser ²⁵	Ser ⁷¹ , Thr ²³⁷ , Ser ²⁵
CPC	Hs	CDCA8	3	2 (0)	2 (0)	Thr ¹⁸⁵ , Thr ¹⁰⁶	Thr ¹⁸⁵ , Thr ¹⁰⁶
CPC	Hs	INCENP	29	9 (5)	5 (1)	Thr ⁸³² , Ser ⁸⁹⁴ , Ser ⁸⁹³ , Ser ⁹¹⁴ , Thr ²³⁹ , Thr ⁵⁰⁷ , Thr ¹⁴⁵ , Ser ⁸²⁸ , Ser ⁸³¹	Ser ⁸⁹⁴ , Ser ⁸⁹³ , Thr ⁵⁰⁷ , Ser ⁹¹⁴ , Ser ⁴⁴⁶
CDK1	Mm	Cdk1	3	0	1 (1)	–	Tyr ¹⁵
PLK1	Mm	Plk1	2	1 (1)	0	Ser ³³¹	–
MIS12/NDC80	Mm	Bub1	12	4 (2)	2 (0)	Ser ⁶⁸⁹ , Ser ³⁸⁷ , Ser ³²¹ , Ser ⁴⁰⁰	Ser ⁶⁸⁹ , Ser ³⁸⁷
MIS12/NDC80	Hs	CASC5	8	2 (0)	5 (3)	Ser ⁶³³ , Ser ¹⁶⁷⁵	Thr ⁵¹³ , Ser ⁶³³ , Ser ¹⁶⁷⁵ , Thr ⁵¹⁷ , Ser ³²
MIS12/NDC80	Hs	DSN1	8	0	1 (1)	–	Ser ⁴¹
RZZ	Mm	Zw10	3	0	1 (1)	–	Ser ⁷⁵⁵
RZZ	Hs	ZWILCH	7	3 (2)	1 (0)	Ser ⁵⁶⁵ , Ser ⁷⁵ , Thr ⁷⁴	Ser ⁵⁶⁵
MCC	Mm	Bub1b	10	2 (2)	1 (1)	Ser ⁵²⁹ , Ser ⁵²⁷	Thr ⁶⁰¹
MCC	Hs	BUB1B	7	2 (1)	2 (1)	Ser ⁷⁴⁷ , Ser ³⁸¹	Ser ⁷⁴⁷ , Ser ⁵⁵¹
MCC	Hs	BUB3	2	1 (1)	0	Ser ²¹¹	–

Complex	Sp	Name	mit. sites	BI-sens. (BI-only)	Hesp-sens (Hesp-only)	BI-sens. sites	Hesp-sens. sites
APC	Hs	ANAPC1	16	3 (1)	2 (0)	Thr ⁵³⁰ , Ser ³³³ , Ser ³⁶²	Ser ³³³ , Ser ³⁶²
APC	Hs	ANAPC5	5	4 (2)	2 (0)	Ser ¹⁷⁹ , Thr ¹⁷⁸ , Ser ¹⁹⁵ , Ser ²²¹	Ser ¹⁷⁹ , Ser ¹⁹⁵
APC	Hs	ANAPC7	2	1 (0)	1 (0)	Ser ¹²⁵	Ser ¹²⁵
APC	Hs	CDC16	3	1 (1)	0	Ser ¹¹²	–
APC	Hs	CDC23	8	2 (2)	0	Ser ⁵⁷⁶ , Ser ⁵⁹³	–
APC	Hs	CDC27	12	1 (1)	0	Ser ²²⁰	–
NUP107–160	Mm	Nup107	12	5 (0)	5 (0)	Ser ⁶⁸ , Ser ⁶⁵³ , Thr ⁷⁸⁴ , Ser ⁴ , Ser ¹³⁸	Ser ⁶⁵³ , Ser ⁶⁸ , Thr ⁷⁸⁴ , Ser ⁴ , Ser ¹³⁸
NUP107–160	Hs	NUP133	11	1 (1)	1 (1)	Thr ⁵¹²	Ser ⁷²
NUP107–160	Hs	NUP85	2	1 (1)	1 (1)	Ser ²²³	Thr ⁹⁰
NUP107–160	Hs	NUP98	15	4 (3)	2 (1)	Ser ⁹⁴⁹ , Ser ⁹³¹ , Ser ¹⁰⁰⁶ , Thr ⁹⁸³	Ser ¹⁴⁴⁸ , Ser ⁹³¹
cohesin	Hs	PDS5A	13	6 (1)	5 (0)	Ser ⁸⁷⁰ , Ser ⁸⁶⁵ , Ser ¹¹⁷⁷ , Ser ¹⁰⁹⁷ , Thr ¹¹⁷⁸ , Ser ¹¹⁷²	Ser ⁸⁷⁰ , Ser ¹¹⁷⁷ , Ser ⁸⁶⁵ , Thr ¹¹⁷⁸ , Ser ¹¹⁷²
cohesin	Hs	PDS5B	16	4 (1)	3 (0)	Ser ¹¹⁶⁵ , Ser ¹³¹⁹ , Ser ¹¹⁶² , Thr ¹³⁰¹	Ser ¹¹⁶⁵ , Ser ¹¹⁶² , Ser ¹³¹⁹
cohesin	Hs	RAD21	8	4 (2)	3 (1)	Ser ¹³⁴ , Thr ⁶²³ , Ser ⁵⁴⁵ , Ser ¹³⁸	Thr ³⁶⁶ , Ser ¹³⁴ , Ser ⁵⁴⁵
cohesin	Hs	SMC1A	2	1 (0)	1 (0)	Ser ⁷³⁵	Ser ⁷³⁵
cohesin	Hs	SMC3	2	1 (0)	1 (0)	Ser ⁸⁵	Ser ⁸⁵
cohesin	Hs	STAG2	12	9 (1)	9 (1)	Ser ¹⁰⁹¹ , Ser ⁷⁵² , Thr ¹¹²⁴ , Ser ¹²⁶¹ , Thr ¹¹⁵¹ , Thr ¹¹⁴⁶ , Ser ¹¹³⁷ , Ser ¹¹⁴⁰ , Ser ⁷⁵¹	Ser ⁸⁴³ , Ser ¹⁰⁹¹ , Ser ⁷⁵² , Thr ¹¹²⁴ , Ser ¹²⁶¹ , Thr ¹¹⁵¹ , Thr ¹¹⁴⁶ , Ser ¹¹³⁷ , Ser ¹¹⁴⁰ , Ser ⁷⁵¹
cohesin	Hs	WAPAL	20	10 (7)	3 (0)	Ser ⁵⁰⁵ , Ser ¹¹⁵⁴ , Ser ⁹⁸⁹ , Thr ²⁷⁶ , Ser ¹¹⁶¹ , Ser ⁴⁶⁵ , Ser ⁶²⁹ , Ser ⁵²⁸ , Ser ³⁷¹ , Thr ¹¹⁶⁰	Ser ⁵⁰⁵ , Thr ²⁷⁶ , Thr ¹¹⁶⁰
condensin	Hs	NCAPD2	2	0	1 (1)	–	Thr ^{1388/1389}
SMC5/6	Hs	NSMCE4A	4	1 (1)	0	Thr ⁵⁸	–

Complex	Sp	Name	mit. sites	BI-sens. (BI-only)	Hesp-sens (Hesp-only)	BI-sens. sites	Hesp-sens. sites
SMC5/6	Hs	SMC5	2	0	1 (1)	–	Ser ¹²
–	Hs	CBX5	1	1 (0)	1 (0)	Ser ⁹²	Ser ⁹²
–	Hs	KIF2C	4	2 (0)	2 (0)	Ser ⁶³³ , Ser ¹⁶⁶	Ser ⁶³³ , Ser ¹⁶⁶
–	Hs	MAD1L1	8	1 (1)	1 (1)	Ser ⁴⁹⁰	Ser ⁵⁵¹
–	Hs	MAD2L1BP	1	1 (0)	1 (0)	Ser ⁴²	Ser ⁴²
–	Hs	MAD2L1BP	2	1 (1)	0	Ser ⁷⁸	–
–	Hs	UBR5	4	1 (0)	2 (1)	Ser ³²⁷	Ser ¹¹⁰ , Ser ³²⁷
TOTAL				96 (42)	74 (20)	35 (25)	32 (18)