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Spatial Exclusivity Combined with Positive and Negative Selection of Phosphorylation Motifs Is the Basis for Context-Dependent Mitotic Signaling

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Abstract

The timing and localization of events during mitosis is controlled by the regulated phosphorylation of proteins by the mitotic kinases, which include Aurora A, Aurora B, Nek2, Plk1, and the cyclin-dependent kinase complex Cdk1/cyclin B. Although mitotic kinases can have overlapping subcellular localizations, each kinase appears to phosphorylate its substrates on distinct sites. To gain insight into the relative importance of local sequence context in kinase selectivity, identify previously unknown substrates of these five mitotic kinases, and explore potential mechanisms for substrate discrimination, we determined the optimal substrate motifs of these major mitotic kinases by Positional Scanning Oriented Peptide Library Screening (PS-OPLS). We verified individual motifs with in vitro peptide kinetic studies and used structural modeling to rationalize the kinase-specific selection of key motif-determining residues at the molecular level. Cross comparisons among the phosphorylation site selectivity motifs of these kinases revealed an evolutionarily conserved mutual exclusion mechanism in which the positively and negatively selected portions of the phosphorylation motifs of mitotic kinases, together with their subcellular localizations, result in proper substrate targeting in a coordinated manner during mitosis.

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Introduction

Reversible protein phosphorylation is a major mechanism for regulating signal transduction events in mammalian cells (1, 2). For many kinases, substrate selection depends not only on the amino acid sequence motif surrounding the phosphorylation site, but also on additional contextual information provided by kinase-substrate subcellular colocalization or formation of multicomponent complexes through binding proteins, adaptor molecules, or additional protein-domain-mediated interactions, or combinations thereof. Contextual information is critical for correctly identifying substrates of specific DNA damage-activated protein kinases, protein kinase C family members, and growth factor receptor tyrosine kinases (3). During mitosis, however, when the genomic content of the nucleus is being equally partitioned into two daughter cells through cell division, much of this contextual information may be lost because many subcellular boundaries are dissolved or disrupted.

The mitotic process includes centrosome separation and maturation, chromatin condensation, nuclear envelope breakdown, Golgi disassembly, spindle formation, attachment of chromosomes to the mitotic spindle, and chromosome segregation (4-7). The timing and mechanics of each of these events are carefully regulated, because uncorrected errors committed during these processes often result in aneuploidy and genetic instability, and can lead to cancer (8, 9). Regulation of these dynamic processes involves the precise phosphorylation of mitotic effector proteins at specific sites through the actions of mitotic kinases, as well as other posttranslational events, such as ubiquitin-mediated proteolysis (6, 10). Major mitotic kinases include the complex of cyclin-dependent kinase 1 and cyclin B (Cdk1/cyclin B), the kinases Aurora A and Aurora B, Nek2 (never in mitosis kinase 2), and Plk1 (Polo-like kinase 1), all of which play key roles during various mitotic substages (6). Cdk1/cyclin B is a master regulator of mitosis with roles in chromatin condensation, nuclear envelope breakdown, and spindle formation (11-16), and its activation marks entry into mitosis. Aurora A is involved in centrosome separation and maturation; Aurora B is involved in chromosome condensation and assuring chromosome attachment to the spindle (amphitelic chromosome attachment) prior to the metaphase to anaphase transition (17-23). Nek2 is required for centrosome separation and plays a role in centrosome maturation (24, 25). Plk1 facilitates Cdk1/cyclin B activation, centrosome maturation, spindle formation, amphitelic chromosome attachment, and cytokinesis, and thus is involved in each of the different substages of mitosis (26-34).

Because these kinases cooperate to regulate processes within mitosis, they have variously overlapping localizations (Fig. 1). At and just prior to the G2/M transition, Aurora A and Plk1 are localized to the pericentriolar material, while Nek2 localizes to the proximal centriole at the core of the centrosome and Aurora B localizes to the centromeres of the decondensed, but duplicated, chromosomes (24, 35-37). Cdk1/Cyclin B activity is low at this time, but increases rapidly as cells progress into prophase and prometaphase (28, 38). By metaphase, Cdk1/cyclin B is maximally active and diffusely localized throughout the cell. Intense activity of Cdk1/cyclin B, which first appeared on centrosomes during prophase, remains at the centrosomes, and also extends to spindle microtubules and kinetochores (11, 28, 38-40). Aurora A and Plk1 colocalize with Cdk1/Cyclin B to the pericentriolar material and the spindle microtubules early in mitosis (37, 41), whereas Plk1 later colocalizes with Aurora B and Cdk1/Cyclin B at the kinetochores during and after prometaphase (11, 35, 37).

Various substrates for these kinases are known, but more remain to be identified because the known substrates are not sufficient to explain all of the phenotypic events regulated by these kinases. In addition, despite overlapping subcellular localizations, and therefore access to overlapping sets of potential phosphorylation sites on substrates, each of these mitotic

kinases appears to have distinct and apparently mutually exclusive substrate phosphoryation sites in vivo. How the activities of these kinases, along with their substrate targeting, are coordinated so that each kinase maintains a distinct set of phosphorylation sites is unclear. With Positional Scanning Oriented Peptide Library Screening (PS-OPLS) (42), we identified optimal motifs for Cdk1/cyclin B, Aurora A and B, Nek2, and Plk1 in order to shed light on the issue of substrate selection, as well as to identify previously unknown substrates of these kinases. Our analysis revealed both positively selected motifs and "anti-motifs", which represented specific residues that were strictly selected against. Integration of the motif data with localization data suggested that these mitotic kinases exist in two functionally orthogonal spaces, a localization space and a motif space, such that the major mitotic kinases with overlapping localizations do not have overlapping motifs and major mitotic kinases with overlapping motifs do not have overlapping localizations.

Results

Comparison of the optimal consensus phosphorylation motifs of Cdk1/cyclin B and Plk1 identified with PS-OPLS

To investigate the role of sequence specificity in substrate selection by mitotic kinases, and to facilitate further substrate identification, we determined and compared the optimal consensus phosphorylation motifs of Cdk1/cyclin B and Plk1 (Fig. 2) using PS-OPLS (42), in which the kinase of interest is incubated individually in solution with each of 180 different peptide libraries. Each library contains a C-terminal biotin tag, a central Ser or Thr that acts as the phosphoacceptor, and a second fixed amino acid located at any of the residues from five before the phosphoacceptor site (Ser/Thr-5) to the residue four after the phosphoacceptor site (Ser/Thr+4) (fig. S1). At all other positions within this 10 amino acid window, a degenerate mixture containing all 20 naturally occurring amino acids except Cys, Ser, or Thr is present. The position and identity of the second fixed residue acts as the primary determinant for substrate phosphorylation.

We performed experiments with Cdk1/cyclin B as a control, because the optimal phosphorylation motif for this kinase is generally accepted as Ser/Thr-Pro-X-Arg/Lys, where X is any amino acid, and the slashes represent "or" (43-47), and a large number of in vivo substrates are known (12, 48-54). Peptide library phosphorylation by Cdk1/cyclin B showed a near absolute requirement for Pro in the +1 position, as well as strong selection for Arg or Lys in the +3 position, as expected (Fig. 2A). We also found a strong positive selection for Lys in the +4 position and modest selection for Pro or Cys in the −2 position, along with weaker selection for other amino acids in this and other positions. On the basis of these results, we expanded the optimal Cdk1/cyclin B peptide phosphorylation motif to [P/C/X]-X-[S/T]-**P**-X-[**R/K**]-**K**, where bold indicates the most strongly selected residues.

To examine whether the amino acid preferences revealed by this peptide library screen are physiologically relevant, we compared our results with the reported collection of Cdk1/cyclin B substrates and phosphorylation sites determined by with an analog-sensitive human Cdk1 combined with thiophosphate tagging and covalent capture (49). Approximately 25% of Cdk1 substrates contained a Lys in the Ser/Thr+4 position, either alone (pS/pT-P-X-X-K motif) or together with an Arg/Lys in the Ser/Thr+3 position (pS/pT-P-X-R/K-K motif) (Fig. 2B). Many of the substrates also contained a Pro in the Ser/Thr-2 position, consistent our peptide library results. Thus, the PS-OPLS approach can reveal new motif information even for well-studied kinases like Cdk1.

The results of PS-OPLS for Plk1 (using the active phosphomimicking T210D form of the kinase) revealed a strong positive selection for Asp, Asn, or Glu in the Ser/Thr-2 position along with modest selection for aromatic residues, particularly Tyr (Fig. 2C). There was less

amino acid selectivity in the Ser/Thr-1 position, although peptides containing either Gly or Pro in this position were poorly phosphorylated. In addition, Plk1 showed strong positive selection for hydrophobic amino acids in the Ser/Thr+1 position, particularly Phe, Tyr, Ile, and Met, as well as some additional hydrophobic selection in the Ser/Thr+2 position. The presence of a Pro residue in the Ser/Thr+1 position was very strongly discriminated against, as revealed by an almost complete absence of phosphorylation of this sub-library of peptides (Fig. 2C, arrowhead). These data suggest an optimal substrate phosphorylation motif for Plk1 of [D/N/E/Y]-X-[S/T]-[F/Φ; no P]-[Φ/X], where Φ is any hydrophobic amino acid.

To validate the PS-OPLS results, we measured kinetic parameters for Plk1-dependent phosphorylation of an optimal peptide substrate based on the PS-OPLS consensus motif (GHDTSFYWAAYKKKK) and for several peptide variants containing single amino acid substitutions (Fig. 3A, B). In general, the V_{max}/K_m ratios of the optimal and variant peptides followed the same trends as those displayed in the PS-OPLS data. The optimal peptide had neither the lowest K_m nor the highest V_{max}, but had the highest V_{max}/K_m ratio, which is consistent with the peptide library screening approach identifying optimal kinase motif sequences on the basis of the maximal substrate turnover rate rather than on K_m or V_{max} alone (55). All variations from the optimal peptide displayed decreased V_{max}/K_m ratios indicating that the peptide chosen as the optimal substrate by individually selecting the best amino acid in each position was correct, and verifying that optimal phosphorylation motifs can be determined from PS-OPLS data by choosing the most highly selected amino acid in each position independently. Changing the Asp to an Ala in the Ser/Thr −2 position resulted in a greater than 20-fold drop in the V_{max}/K_m ratio (due to a 6.3-fold rise in K_m, and a 3.3-fold drop in V_{max} relative to those of the optimal peptide), and the ability of Plk1 to phosphorylate the peptide with a Pro in the Ser/Thr+1 was so minimal that it was not possible to fit the data to a Michaelis-Menten curve, recapitulating the lack of activity observed in the PS-OPLS screen for the sublibrary of peptides that all contained Pro in the Ser/Thr+1 position (Fig. 2C, arrowhead).

The motif that we determined is consistent with previously mapped Plk1 phosphorylation sites on known substrates (Fig. 3C) (34, 56-65). Most of these Plk1 phosphorylation sites contain an Asp or Glu in the Ser/Thr-2 position and a hydrophobic amino acid in the Ser/Thr +1 position, in agreement with the consensus motif revealed by the PS-OPLS method. Hydrophobic amino acids in the Ser/Thr+2 position were also occasionally observed, but did not appear to be a strong discriminator for known in vivo Plk1 sites. The PS-OPLS-derived motif is similar to a previously published motif for Plk1, D/E-X-S/T-Φ-X-D/E, which was determined by mutagenic analysis of a single peptide sequence surrounding Ser^{198} in Cdc25C, which is phosphorylated by Plk1 (59). The PS-OPLS-derived motif differs from the other motif by having a strong selection for Asn in the Ser/Thr-2 position and no general selectivity for Asp or Glu in the Ser/Thr+3 position.

This Plk1 phosphorylation motif revealed by PS-OPLS screening suggested the possible existence of previously unrecognized Plk1 substrates with phosphorylation sites containing Asn in the Ser/Thr-2 position. Although no such Plk1 substrates matching this motif have been identified in higher eukaryotes, an in vivo study of yeast mitotic cohesins revealed eleven Cdc5 (the yeast Plk1 ortholog) sites on Rec8, of which 5 contained Asn in the Ser/Thr-2 position (66). To directly explore whether a subset of mitotic Plk1 substrates in mammalian cells contained a similar N-X-S/T motif, we performed a mass-spectrometry based screen in nocodazole-arrested HeLa cells for phosphorylated mitotic proteins containing Asn in the pSer/pThr-2 position that were not present in the nocodazole-treated cells if they were pre-incubated with the Plk1 inhibitor BI 4834 (fig. S2).

We identified three Plk1-dependent phosphorylation sites containing an Asn in the Ser/Thr-2 position (Fig. 3D, E). Only the nonphosphorylated forms of these same peptides, but not the phosphorylated forms, were observed following Plk1 inhibition. Two of the proteins containing these Plk1 sites, Bub1 and $p31^{comet}$, are involved in spindle assembly checkpoint and the third, Scc1 (also known as Rad21), is involved in sister chromatid cohesion in mitosis (23, 67, 68), processes that are controlled by Plk1 (69, 70).

Determination of the optimal consensus motif of Aurora A and Aurora B

We used the PS-OPLS method to determine the optimal sequence motifs phosphorylated by Aurora A and Aurora B, and compared the results with those obtained for Cdk1/cyclin B and Plk1. Both Aurora A and Aurora B showed similar motifs, with extremely strong selection for Arg in the Ser/Thr-2 position and strong discrimination against all other amino acids at this position (Fig. 4A). Even Lys in the Ser/Thr-2 position was a poor substitute for Arg. There is a smaller selectivity for Arg in the Ser/Thr-3 position. In the Ser/Thr+1 position, although both Aurora A and B selected hydrophobic amino acids, the specific set of preferred amino acids differed slightly between them. Aurora B showed some preference for Ile and Met in this position, whereas residues preferred by Aurora A also included Phe and Leu. Like Plk1, both Aurora A and Aurora B showed very strong discrimination against libraries containing Pro in the Ser/Thr+1 position (Fig. 4A, arrowheads). Moderate selection for certain aromatic or hydrophobic amino acids, along with Gly and Pro, was noted in the Ser/Thr+2 position for both kinases, with little selectivity for particular amino acids beyond the Ser/Thr-2 to Ser/Thr+2 positions for either kinase.

The position of maximal substrate selectivity for both of the Aurora kinases revealed by PS-OPLS screening -- a strongly basic amino acid in the Ser/Thr-2 position -- is the opposite of the requirement for an acidic (or Asn) residue in the Ser/Thr-2 position that we observed for Plk1. We verified the PS-OPLS-predicted kinase selectivity by measuring the kinetics of Aurora B-dependent phosphorylation of an optimal PS-OPLS-determined peptide (ARRHSMGWAYKKK), along with two peptide variants in which the Arg in the Ser/Thr-2 position was changed to an Asp (rendering it a more Plk1-like substrate), or the Met in the Ser/Thr+1 position was changed to a Pro (rendering it a more Cdk1-like substrate) (Fig. 4B). Substitution of Asp for Arg in the Ser/Thr-2 position both increased the K_m and decreased the V_{max}, resulting in a 13-fold drop in the V_{max}/K_m ratio relative to the Aurora B optimal peptide. As with Plk1, the ability of Aurora B to phosphorylate an otherwise consensus peptide that contained Pro in the Ser/Thr+1 position was so low that it was not possible to fit the data to a Michaelis-Menten equation. From these data, the minimal optimal consensus phosphorylation sequence shared by both Aurora A and Aurora B is R-X-S/T-Φ, where X is any amino acid and Φ is any hydrophobic amino acid except Pro. These motifs are consistent with mapped Aurora A and Aurora B phosphorylation sites (Fig. 4C), nearly all of which contain an Arg in the Ser/Thr −2 position. Many of these previously mapped sites also contain a hydrophobic amino acid in the Ser/Thr+1 position, particularly those that are subtrates of Aurora A (18, 19, 71-87).

Determination of the optimal consensus motif of Nek2

In contrast to Plk1 and Aurora A and B, which discriminated against both Cdk1 sites by selection against Pro in the Ser/Thr+1 position, and against each other by selection for or against acidic or basic residues in the Ser/Thr-2 position, the Nek2 motif [determined for the active stabilized T175A form (88)] exhibited strong amino acid selectivity in the Ser/Thr-3 and Ser/Thr+2 positions (Fig. 5A). In the Ser/Thr-3 position, Nek2 selected for specific hydrophobic amino acids, particularly Phe, Met, and Leu, and strongly discriminated against hydrophilic amino acids. Both basic (Arg or Lys) and acidic (Asp or Glu) residues were disfavored in the Ser/Thr-3 position. Unlike Plk1 and Aurora A and B kinases, Nek2 did not

show a strong and narrow preference for specific amino acids in the Ser/Thr-2 position, although Arg, Lys, Phe, Tyr, and Trp were somewhat favored. Pro was strongly disfavored in both the Ser/Thr-2 and Ser/Thr-1 positions. In the Ser/Thr+1 position, Nek2 also showed moderately strong selection for hydrophobic amino acids, along with strong discrimination against Pro, similar to the strong Pro deselection observed for Plk1 and Aurora A and B. Nek2 also showed strong discrimination against Asp and Glu in the Ser/Thr+1 position, as well as against Glu in the Ser/Thr+2 position, where instead we found pronounced selection for Arg, along with modest selection for His.

To verify the PS-OPLS data, we measured the kinetic parameters for the Nek2-dependent phosphorylation of its optimal PS-OPLS-determined peptide (WFRMSIRGGYKKK) along with those of three peptide variants containing single amino acid substitutions (Fig. 5B). Replacement of the strongly selected Phe in the Ser/Thr-3 position with Val, a similarly hydrophobic residue but one that was somewhat disfavored in the PS-OPLS screen, resulted in an approximately 8-fold decrease in the V_{max}/K_m ratio due primarily to an increase in K_m relative to that of the optimal peptide. In contrast, complete charge reversal in the Ser/Thr-2 position of the Nek2 optimal peptide by substitution of Asp for Arg resulted in only a 5-fold decrease in the V_{max}/K_m ratio due to compensating effects on K_m and V_{max}. As with Plk1 and Aurora B, the kinase activity of Nek2 against an otherwise optimal peptide containing a Pro substitution in the Ser/Thr+1 position was minimal, precluding any determination of kinetic parameters.

These data reveal an optimal consensus phosphorylation motif for Nek2 as [F/L/M]-X′-X″-S/T-Φ-[R/H/X], where both X′ and X″ denote any amino acid except Pro, and X′ also includes some positive selection for basic and hydrophobic residues, Φ denotes any hydrophobic amino with Pro, Asp, and Glu excluded from this position, and X denotes any amino acid including Pro. The motif that we determined for Nek2 agrees reasonably well with the known phosphorylation sites that are present in the few well-verified Nek2 substrates, including Nek2 itself and the centrosomal and centromeric protein Hec1 (88, 89) (Fig. 5C).

An evolutionarily conserved structural basis for mitotic kinase motif exclusivity

The mitotic kinase phosphorylation motifs identified with PS-OPLS suggested a mechanism for dictating mitotic protein kinase specificity. If the identity of the Ser/Thr+1 residue of the substrate site is a Pro residue, then the substrate is recognized by Cdk1/cyclin B, and not by Plk1, Aurora A, Aurora B, or Nek2. The presence of amino acids other than Pro, particularly hydrophobic ones, in the Ser/Thr +1 position determines whether phosphorylation is performed by Plk1, Aurora A or B, or Nek2. This critical Ser/Thr+1 specificity determinant prevents Cdk1 from phosphorylating putative Plk1, Aurora A, Aurora B, or Nek2 sites and likewise prevents Plk1, Aurora A, Aurora B and Nek2 from phosphorylating Cdk1 sites. However, additional criteria, must dictate whether the non-Cdk1 sites are preferentially phosphorylated by Plk1, one of the Aurora kinases, or Nek2. A comparison of the PS-OPLS blots and motif logos for Plk1, Aurora A, Aurora B, and Nek2 suggested that additional substrate-kinase specificity for mitotic kinases appears to arise from mutual exclusivity of the different mitotic kinase motifs themselves (Fig. 6A, B). This mutual exclusivity means that the strongest specificity-determining residues for one mitotic kinase were strongly deselected by the others, particularly in the case of Plk1 and Aurora A or Aurora B.

We compared the ability of Aurora B, Nek2, and Plk1 to phosphorylate a common set of peptides, each of which had been optimized for a particular family member (Fig. 7A). Each kinase phosphorylated its optimal peptide the best. Neither Aurora B nor Nek2 displayed much activity against the optimal Plk1 peptide, producing <1% and ~8%, respectively, of the phosphorylation displayed by Plk1 in this assay. Similarly, neither Plk1 nor Nek2 had

much activity against the optimal Aurora B peptide, resulting in ~1% or less of the phosphorylation displayed by Aurora B. In contrast, although Plk1 had essentially no activity for the optimal Nek2 peptide (<1%), Aurora B did phosphorylate the Nek2-optimized peptide (~23% of the amount observed for Nek2 kinase itself).

To examine the molecular basis for this motif exclusivity, we performed molecular modeling studies of kinase-substrate complexes (Fig. 7B) based on published X-ray crystal structures (see Materials and Methods for details). Despite the near absolute requirement for a Ser/Thr-Pro motif for substrate phosphorylation by Cdk1, we did not find any structural evidence in the Cdk1/cyclin B model for a direct interaction between the cyclin or Cdk subunits and the Ser/Thr+1 Pro residue in the substrate peptide (Fig. 7B), which is consistent with the reported Cdk2/cyclin A:peptide crystal structure (90). Instead, the strong selection for Pro observed in this position likely results from a combination of relatively weak effects. The backbone conformation of Val^{164} in the activation loop prevents hydrogen bonding between its carbonyl oxygen and the NH group of any amino acid (but would allow Pro to interact because it lacks an NH) in the Ser/Thr+1 position as described for the Cdk2/cyclin A structure (90). This conformation of Val^{164}, combined with the need to correctly position the phosphoacceptor Ser/Thr residue near the end of the peptide-binding cleft, while simultaneously avoiding a steric clash of the remaining C-terminal residues of the peptide substrate with the cyclin subunit, means that the kink in the substrate main chain backbone resulting from the Ser/Thr+1 Pro would facilitate favorable interactions of basic side chains in the Ser/Thr+3 and Ser/Thr+4 positions with the cyclin and Cdk subunits. The peptide Ser/Thr+3 Lys in the Cdk2/cyclin A:peptide structure made a salt bridge to the regulatory phosphothreonine ($pThr^{160}$) in the activation loop of the kinase domain, as well as a hydrogen bond to the main chain carbonyl of Ile^{270} on cyclin A; we expect that equivalent residues in the Cdk1/cyclin model ($pThr^{161}$ in Cdk1 and the main chain carbonyl of Met^{261} in cyclin B1) are likely responsible for the observed Ser/Thr +3 Arg or Lys selectivity. The selection that we observed for Lys in the Ser/Thr+4 position of the substrate peptides appears to arise from interactions with acidic side chains of residues Glu^{265} and Asp^{268} in cyclin B, rather than from any direct contact with residues in the substrate-binding cleft of the kinase itself.

For Plk1, selection for Asp, Asn, and Glu in the Ser/Thr-2 position of the optimal substrate motif likely results from electrostatic or hydrogen-bonding interactions with the side chains of Lys^{178} and Asn^{216}, or a combination of these kinds of interactions (Fig. 7B). This interaction model is supported by the observation that mutation of the Ser/Thr-2 residue to one incapable of forming either type of interaction (for example, Ala) increased the K_m by 6-fold, whereas replacement with a positively charged residue (for example, Arg) increased the K_m nearly 10-fold relative to the optimal Plk1 peptide. Hydrogen-bonding interactions may dominate over electrostatic interactions for favorable substrate selection, because substitution of Asn for Asp at position Ser/Thr-2 lowered the peptide K_m by half, but decreased the V_{max} by greater than a factor of 5 relative to the optimal Plk1 peptide, suggesting that the high degree of negative charge on the Asp-containing peptide following phosphorylation contributes to its dissociation from the catalytic cleft. Both Lys^{178} and Asn^{216} are conserved in the Plk1 homologs of vertebrates, worms, flies, and yeast, implying evolutionary conservation of motif selection for Asp, Asn, and Glu, and strong deselection for Arg or Lys, in the Ser/Thr-2 position of potential substrates (fig. S3). Phe, along with other aromatic or bulky hydrophobic amino acids that were selected in the Ser/Thr+1 position in the PS-OPLS screening can be accommodated in a hydrophobic pocket formed by Leu^{211}, Pro^{215}, Ile^{218}, and Val^{222} in the activation loop. Substitution of small or negatively charged amino acids in this position increased the K_m by a factor of 20 or greater relative to the optimal Plk1 peptide. The residues that comprise this hydrophobic pocket are similarly conserved in the Plk1 homologs of other species, although the *Saccharomyces*

cerevesiae and *Saccharomyces pombe* homologs have an Ile replacement for Leu^{211}, and in fission yeast and *Drosophila melanogaster* there is an Ile in place of Val^{222}. Furthermore, strong deselection for Pro in the Ser/Thr+1 position of the motif would direct the substrate peptide chain outside of the kinase's substrate-binding cleft and preclude favorable interactions with any additional C-terminal residues, because unlike kinases of the Cdk family, which function with a cyclin partner, Plk1 does not function in complex with a binding partner that could restore these contacts.

In the case of Aurora B, the weak selection for Arg that is observed in the Ser/Thr-3 position probably arises from an electrostatic interaction with Glu^{177}, whereas very strong selection for Arg in the Ser/Thr-2 is likely due to an electrostatic interaction with Glu^{220} and Glu^{281}. All three of these Glu residues form an extended acidic patch in the kinase active site, and all are conserved in Aurora kinase sequences from yeast to man (fig. S4), implying that selection for basic residues and exclusion of acidic residue in the Ser/Thr-3 and Ser/Thr-2 positions of the substrate motif is an evolutionarily important feature. Consistent with this model, replacement of the Ser/Thr-2 Arg by an Asp resulted in a 5-fold increase in K_m and over a two-fold drop in V_{max} relative to the optimal Aurora B peptide. Met and other hydrophobic amino acids selected in the Ser/Thr+1 position appear to fit into a hydrophobic pocket formed by Trp^{237} and Met^{249} on the activation loop and Leu^{256} of the active site cleft. These residues are conserved in vertebrate, worm, fly, and yeast Aurora kinases, although Met^{249} is replaced by Leu in *D. melanogaster* and *S. pombe* and by Val in *S. cerevesiae*. Thus, despite similar selection for hydrophobic residues in the Ser/Thr+1 position, evolutionary conservation of the acidic and basic Ser/Thr-2 selection pockets may ensure substrate exclusivity between Plk1 and the Aurora kinases.

For Nek2, the selection for Phe and other hydrophobic amino acids in the Ser/Thr-3 position likely arises from the presence of a spatially flat hydrophobic surface formed by two alanines, Ala^{95} and Ala^{145}. Small residues at this location are found in all Nek2 homologs, but are rarely found in this part of the substrate-binding cleft in the other mitotic kinases (see Plk1 and Aurora B in Fig. 7B) or in PKA, where these positions are occupied by Phe and Glu. Selection for Arg in the Ser/Thr-2 position of the motif likely arises from an electrostatic interaction with Glu^{208}, and the selected Ile and other hydrophobic amino acids in the Ser/Thr+1 position of the peptide likely fit into a hydrophobic pocket formed by Phe^{176} in the activation loop and Pro^{180} and Met^{183} of the active site. The Arg and His selection that we observed in the Ser/Thr+2 position of the substrate motif may arise from electrostatic interactions with Glu^{48}. The identity of these specificity-determining residues is also conserved among mammals, zebrafish, frog, and yeast Nek2 family members (fig. S5).

Substrate enrichment, colocalization, and motif exclusion--A unified model for mitotic kinase selectivity

The modeling results suggested that the structural elements responsible for selection or exclusion of specific amino acids within mitotic kinase phosphorylation motifs reflect evolutionarily conserved features that may be critically important in substrate selection during cell division, when multiprotein mitotic complexes undergo phosphorylation at exclusive sites by the cooperative action of multiple distinct kinases. Figure 8A, for example, shows three representative mitotic protein complexes that were experimentally identified in a systematic mass-spectrometry screen (91), with proteins in each complex annotated for previously mapped mitotic phosphorylation sites (17, 92-100). These complexes comprise the γ-tubulin ring complex (γ-TuRC) important for spindle assembly, the anaphase-promoting complex (APC/C) important for chromosome segregation, and the outer kinetochore complex MIS12/NDC80 important for binding kinetochores to spindle

microtubules (101). In each case, multiple proteins within each mitotic complex are targeted by one or more different mitotic kinases.

During cell division, the localization of Cdk1, Plk1 and the Aurora A and B kinases overlap at specific mitotic substructures, particularly along the outer portions of the centrosomes, at the kinetochores, and along the mitotic spindle. This contrasts with Nek2, which localizes to the proximal centriole within the core of the centrosome (24) (Fig. 1). To examine whether substrates for these kinases might be enriched at the subcellular structures where these kinases reside, we performed a bioinformatic analysis for enrichment of potential phosphorylation sites for Cdk1, Plk1, Nek2, and Aurora kinases on the subproteome found at the centrosome (102) and the spindle apparatus (103), relative to the entire human proteome (International Protein Index-version 3.23) (see Materials and Methods for details). All three variations of the Cdk1/cyclin B motif revealed by PS-OPLS were statistically over-represented in proteins found on the mitotic spindle and none of the Cdk1 motifs were over-represented on the centrosome subproteome (Fig. 8B). The Plk1 motif defined by PS-OPLS was significantly enriched on proteins at both the centrosome and spindle apparatus, whereas the Nek2 motif was enriched on proteins at the centrosome, but not on the spindle subproteome, as might be expected for a centrosomal kinase. The Aurora motif was not significantly enriched at the centrosome or the spindle, which may not be surprising because there are many basophilic kinases with similar motifs, for example, PKA and protein kinase G (PKG), that control processes other than mitosis. The enrichment of potential substrates at subcellular structures where Cdk1, Plk1 and Nek2 localize during mitosis supports the intuitive idea that substrates are likely to undergo extensive phosphorylation in regions of the cell where the local concentration of the kinase is highest.

Whereas motifs for individual kinases appeared to be significantly enriched in the subcellular proteomes appropriate to those kinases, we wondered about the prevalence of co-occurence of motifs for multiple kinases in the same protein. We found that in both the entire proteome and the nuclear subproteome, the co-occurrence of motifs for all pairs of mitotic kinases was significantly enriched (see Materials and Methods, table S2), indicating perhaps that different sites on the same protein are commonly phosphorylated by distinct kinases in the normal course of mitosis. Because the sites phosphorylated by Cdk1, Plk1, and the different Aurora kinases are mutually exclusive, this observation is consistent with the co-localization of multiple mitotic kinases at centrosomes, kinetochores, or along the mitotic spindle, or various combinations thereof. For Nek2, motif co-occurrence suggests that some substrates may move from the inner centrosome to other mitotic structures in order to be phosphorylated by the appropriate kinase at the appropriate time.

Discussion

Mitosis is an intricate and highly regulated process in which the temporal order of events is controlled by the action of specific protein kinases, including the master regulator Cdk1 and the other major mitotic kinases, Aurora A, Aurora B, Nek2, and Plk1. Proper progression through mitosis requires that these kinases phosphorylate specific sites on specific proteins in an organized and presumably non-overlapping manner. We used PS-OPLS, which in contrast to studies using amino acid substitutions within a single peptide corresponding to a known substrate phosphorylation site, allows every amino acid within each position in a sequence motif to be tested independently of the rest of the sequence. The method therefore identifies not only amino acids that are positively selected in each position, but also those that are disfavored or deselected in each position. Subsequent kinetic studies with individual peptides verified the PS-OPLS motifs, and provided insights into the relative role of flanking residues in peptide binding or turnover, or both, at the kinase active site.

The Cdk1/cyclin B motif revealed by PS-OPLS expands upon the generally accepted S/T-P-X-R/K consensus motif to now include a Lys in the Ser/Thr+4 position. This expanded motif explains ~25% of the reported Cdk1 substrates detected with a chemical genetics screen for Cdk1/cyclin B in HeLa cell extracts (57). A mass spectrometry-based screen for mitotic phosphoproteins that used a weighted bioinformatics analysis to determine abundant mitotic phosphorylation motifs showed many phosphorylation sites containing the sequence S/T-P-X-X-K, leading the authors to propose that this constituted a substrate motif for a new mitotic kinase that had not yet been identified (104). Our PS-OPLS results suggest that this motif matches that of human Cdk1/cyclin B.

Our structural modeling results for Cdk1/cyclin B suggested that selection for Lys in the Ser/Thr+4 position in the motif arises from a pair of acidic residues within cyclin B both of which are conserved in mammals, worms, and frogs, and one of which is conserved in flies. No such acidic residues, however, are found in the corresponding positions of Clb2, the budding yeast mitotic cyclin, suggesting that selection for basic residues in this position of the phosphorylation motif is unique to metazoans. A mass spectrometry screen of in vivo Cdk1/Clb2 substrates did not reveal enrichment for Lys in the Ser/Thr+4 position of the mapped phosphorylation sites in substrates from *S. cerevesiae* (105), which contrasts with what was observed for Cdk1/cyclin B substrates in human cells (49).

The Plk1 motif identified by PS-OPLS revealed strong selectivity for Asn, in addition to Asp and Glu, in the Ser/Thr-2 position. We identified phosphopeptides containing Asn in the Ser/Thr-2 position from three proteins, Scc1, Bub1, and p31^{comet}, for which phosphorylation in nocodozole-treated cells was abrogated by Plk1 inhibition. Scc1 is a subunit of the cohesin complex that prevents chromosome segregation until it is cleaved by separase at the metaphase-anaphase transition (106). Although Scc1 is phosphorylated at multiple sites by Plk1, which enhances the cleavage of Scc1 by separase, all the phosphorylation sites had not been mapped (106). The location of the phosphorylation site at Ser^{138} that we found is near one of the separase cleavage sites and may increase Scc1 cleavage. Phosphorylation of Bub1 by Cdk1 recruits Plk1 to kinetochores in vivo and enhances Plk1 phosphorylation of Bub1 in vitro, although the Plk1 phosphorylation sites on Bub1 were not known (97). We identified Ser^{399} as a Plk1-dependent Bub1 phosphorylation site in vivo that matches the Asn-containing Plk1 motif, although we do not know if this site (or those on Scc1 and p31^{comet}) is a direct or indirect Plk1 target. Maintenance of the spindle checkpoint until attachment of all of the chromosome kinetochores involves an interaction between Mad2 and Cdc20 (107). p31^{comet} binds Mad2 causing Mad2 to release Cdc20 (108). Although Plk1 is required for the recruitment of Mad2 to the kinetochore for the spindle assembly checkpoint, the Plk1-dependent phosphorylation of p31^{comet} suggests Plk1 may have other roles as well (26). A mass-spectrometry screen for Plk1 substrates on the early mitotic spindle identified many spindle-associated proteins with Plk1-dependent phosphorylation sites containing Asn in the Ser/Thr-2 position, providing independent validation of our PS-OPLS results (98).

The strong similarity in optimal phosphorylation motifs that we observed between Aurora A and B is not surprising because these kinases are similar in their primary sequences, and the functions of both kinases are performed by a single kinase in yeast (109). PS-OPLS motif determination for Aurora A and B suggested that Arg in the Ser/Thr-2 position is likely to be an important determinant for substrate selection, and that Lys is a poor substitute for Arg despite their similarity in charge. Although this finding agrees well with most mapped Aurora A and B substrates (Fig. 4C, D), some mapped sites contain a Lys in the Ser/Thr-2 position, including the regulatory Thr^{210} site on the activation loop of Plk1 that is phosphorylated by Aurora A during mitotic entry (78, 110). Phosphorylation of Thr^{210} by Aurora A during release from a DNA damage checkpoint requires the formation of a complex between Aurora A and the G2/M regulatory protein hBora (78, 110), suggesting

that a targeting subunit (hBora), rather than intrinsic kinase selectivity, may be important for phosphorylation of this particular site.

The Aurora A motif that we determined is consistent with previous reports (74, 111). Both of those studies revealed the importance of an Arg residue in the Ser/Thr-2 position, and the Ser/Thr+1 hydrophobic residue within single peptide substrates, as well as the strong negative selectivity for a Pro residue in the Ser/Thr+1 position. The optimal phosphorylation motif revealed by PS-OPLS screening of Aurora B has some differences from the consensus motif [R/K]-X-[S/T]-[I/L/V] derived from ten phosphorylation sites on kinetochore proteins that are phosphorylated by the yeast Aurora ortholog Ipl1 (109) and from a K-R-S-[S/T]-S motif mapped in the evolutionarily conserved Aurora B substrate INCENP (72, 76), likely because these prior motifs were based on a small number of phosphorylated peptides rather than an global assessment of site preferences provided by PS-OPLS.

The motif that we identified for Nek2 is the first reported and revealed strong negative selection against Pro in the Ser/Thr+1 position, similar to what we observed for Plk1 and the Aurora kinases, indicating that Nek2 will not phosphorylate sites targeted by Cdk1 and vice versa. Direct comparison of the Plk1, Aurora A, Aurora B, and Nek2 motifs (Fig. 6A, B) revealed strong mutual exclusivity of Aurora A and Plk1 substrates on the basis of amino acid residues in the Ser/Thr-2 position. In contrast, the strongest positive selection within the Nek2 motif was in the Ser/Thr-3 and Ser/Thr+2 positions. The optimal peptide for Nek2 contained an Arg in the Ser/Thr-2 position, which was also present in the Aurora kinase motif, and optimal Nek2 peptides were phosphorylated by Aurora B (Fig. 7A). Nek2 may also phosphorylate some of the same sequences as Plk1, if such sequences had hydrophobic amino acids in the Ser/Thr-3 position to satisfy the Nek2 motif. However, the optimal Plk1 peptide, which contained a His in this position, was a poor Nek2 substrate in vitro.

The positions in the Nek2 motif showing the strongest negative selection, aside from Ser/Thr+1 Pro, were acidic residues and phosphoThr residues in the Ser/Thr-3, +1, and +2 positions, suggesting that Nek2 phosphorylation sites should be mutually exclusive with sites phosphorylated by casein kinase 1 (CK1), which shows a strong selection for acidic or phosphoSer/Thr residues in the Ser/Thr-3 position of its substrates (112). The association of CK1δ with centrioles at the centrosomal core is mediated by the scaffolding protein AKAP450 (113, 114) and we predict that motif exclusivity between Nek2 and CK1δ may establish distinct phoshorylation sites for these kinases on centriolar substrates.

For the mitotic kinases that we studied, the motif data and structural modeling results suggest an evolutionarily conserved mode of mitotic kinase substrate targeting in which the presence or absence of a Pro in the Ser/Thr+1 position functions as a binary switch allowing phosphorylation of a site by either Plk1, Aurora A, Aurora B, or Nek2 if a residue other than proline is present in this position, or by Cdk1 if a Pro is present. This proline specificity has been suggested as a mode of regulation between Cdk1 and Aurora A and between the CMGC proline-directed kinase group, of which Cdk1 is a member, and the basophilic CAMK and AGC kinase groups, to which Aurora A and Aurora B are closely related but are not members (74, 115, 116).

Integrating the motif and colocalization data suggests that specificity of phosphorylation by these kinases is enforced by a combination of non-overlapping localizations and both positively and negatively selected sequence motifs. Each kinase possesses a positively selected motif, and also displays an ‘anti-motif’ whereby the motifs of other kinases are specifically disfavored. Plk1 and the Aurora kinases do not phosphorylate the same sites despite overlapping localizations because their motifs are mutually exclusive. Despite partially overlapping motifs, Nek2 and Plk1 and Nek2 and the Aurora kinases would not

phosphorylate the same sites because of non-overlapping localizations, because Nek2 localizes to the proximal centrioles within the core of the centrosome, whereas Aurora A and Plk1 are localized at the periphery of the pericentriolar material (36), Thus, our data suggest that kinases exist in two functionally orthogonal spaces: 'localization space' and 'motif space' (Fig. 8C). 'Localization space' reflects all of the subcellular locales where the kinase can reside. 'Motif space' contains the optimal sequence motifs of all S/T kinases. In this context, each major mitotic kinase overlaps with every other kinase in, at most, one of space, either localization or motif space. For example, Cdk1 overlaps all of the other kinases in 'localization space', but not in 'motif space' and Nek2 overlaps Plk1 and Aurora Kinases in 'motif space', but not in localization spaces'. Thus, the combination of positive and negative amino acid motif selection and spatial exclusivity that we observe appears to underlie the cooperative nature of mitotic kinase signaling. This potentially provides both a systems-level mechanism for regulating substrate phosphorylation, and a coordinated evolutionary pressure to maintain discriminatory substrate motifs and localizations for major mitotic kinases and their substrates.

Materials and Methods

Kinase Protein Production and Purification

Recombinant full-length human wild-type C-terminally His_6-tagged Aurora A, *Xenopus laevis* wild-type Aurora B (residues 60-361):INCENP (residues 790-847) complex, and full-length human T175A Nek2A proteins were purified from *Escherichia coli* as described previously (88, 117, 118). Nucleotide sequence encoding the kinase domain of human Plk1 (amino acids 38-346) was cloned as an N-terminally His_6-tagged fusion construct into a modified version of the pET28a (Novagen) bacterial expression vector. The resulting fusion protein contains an N-terminal His_6-tag followed by maltose binding protein (MBP) and a Tev protease cleavage site between MBP and the kinase domain. A Thr^{210} mutation to Asp was introduced with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) to increase specific activity. The protein was expressed in *E. coli* Rosetta 2 (Novagen) cells and purified by Ni-NTA affinity chromatography The kinase domain was further purified by affinity chromatography on amylose beads, cleaved with Tev protease, re-applied to Ni-NTA to remove the cleaved His_6-MBP tag, and finally purified by gel filtration on a Superose 12 column. The Cdk1/Cyclin B kinase complex, which consisted of active human recombinant full-length C-terminally His_6-tagged Cdk1 and glutathione-*S*-transferase (GST)-tagged human full-length cyclin B, was purchased from Millipore.

Phosphorylation Motif Determination by Peptide Library Array Screening

PS-OPLS was performed following Hutti *et al.* (42). Briefly, solution-phase kinase reactions were performed in parallel on 198 separate biotinylated, partially degenerate oriented peptide libraries (Anaspec, Inc) arrayed in a 384 well microtiter plate in a 20 row X 9 column format (fig. S1). Each peptide library contains a C-terminal biotin tag, a equal mixture of serine and threonine at the orienting phospho-acceptor residue, a single second fixed amino acid located between the Ser/Thr-5 and Ser/Thr+4 positions, and a mixture of amino acids at all other positions. Individual libraries contain any of the 18 non-Ser/Thr natural amino acids, as well as phosphothreonine or phosphotyrosine, in the second fixed position, corresponding to the 20 rows. Scanning down each column of the array moves the position of the fixed amino acid from Ser/Thr-5 to Ser/Thr+4 relative to the fixed phospho-acceptor residue. As an example, the peptide library with Lys fixed in the Ser/Thr-4 position has the following sequence: Y-A-X-**K**-X-X-X-**S/T**-X-X-X-X-A-G-K-K-biotin, where amino acids are represented in 1-letter code, and X is an equal mixture of all 17 natural amino acids excluding Cys, Ser, and Thr to prevent oxidation effects and eliminate secondary phosphorylation events. S/T denotes a 1:1 mixture of Ser and Thr. Kinase reactions were

performed at 30°C in a total volume of 16 μL containing 31.25 μM peptide library, 100 μM ATP, and 200 μCi of [^{32}P]-γ-ATP, in 150 mM NaCl (500 mM NaCl for Nek2 kinase reactions), 10 mM $MgCl_2$, 1 mM DTT, 0.1% Tween 20, and 50 mM Tris, pH 7.5. For Aurora A and Aurora B, reactions were performed for 6 hours with 0.25 and 0.792 μg of protein per reaction, respectively. For Nek2, reactions were performed for 8 hours with 2.4 μg of protein per reaction. For Plk1, reactions were performed for 3 hours with 0.5 μg of protein per reaction. For Cdk1/Cyclin B, reactions were performed for 4 hours with 0.08 μg of protein complex per reaction. Following incubation, 2 μL of each reaction were simultaneously transferred to a Streptavidin-coated membrane (Promega SAM^2 biotin capture membrane) using a 384-slot pin replicator (VP Scientific). The membrane was washed three times with 140 mM NaCl, 0.1% SDS, 10 mM Tris, pH 7.4, three times with 2 M NaCl, twice with 2 M NaCl containing 1% H_3PO_4, and once with water. The extent of peptide library phosphorylation was determined by imaging the membrane with a phosphorimager (Molecular Dynamics).

In Vitro Kinase Assays

Kinase assays for kinetic parameter determination were performed at 30°C in 90 μL of kinase reaction buffer (50 mM Tris, pH 7.5, 150 mM NaCl [500 mM NaCl for Nek2 assays], 10 mM $MgCl_2$, 100 μM ATP, 9 μCi [^{32}P]-γ-ATP, and 1 mM DTT). Each reaction contained 0.003 μg of Aurora B:INCENP complex, 3.6 μg of T175A Nek2 protein, or 0.054 μg of T210D Plk1 kinase domain. The sequences of the optimal substrate peptides for Aurora B, Nek2, and Plk1 were ARRHSMGWAYKKKK, WFRMSIRGGYKKKK, GHDTSFYWAAYKKKK, respectively. These optimal peptides were determined by taking the most highly selected amino acid from each position, Ser/Thr-4 to Ser/Thr+3, of the PS-OPLS blot and using Ser as the phospho-acceptor residue, at position 0. A C-terminal tyrosine was added to each peptide to allow determination of concentration of peptide solutions by UV spectrophotometry, and four C-terminal lysines were appended to increase solubility and electrostatic interaction with phosphocellulose paper. Additional peptides with single amino acid changes from the optimal peptides were as indicated in Figs. 3B, 4B, and 5B. Concentrations of peptides were as indicated. 5 μL of each reaction were spotted on phosphocellulose at 0, 3, 6, 9, 12, and 15 minutes in duplicate. The phosphocellulose paper was washed 4 times with 0.5% phosphoric acid, transferred to vials containing scintillation cocktail and counted. From these kinase assays, K_m, V_{max}, and V_{max}/K_m values were determined by curve fitting assuming Michaelis-Menten kinetics. For each concentration of peptide, care was taken to ensure that less than 5% of the total substrate was phosphorylated and that the reaction rate was linear with respect to time.

Kinase assays for comparison of activity of each kinase against the optimal peptides of each kinase were performed at 30°C in 25 μL of kinase reaction buffer. Each reaction contained 0.008 μg of Aurora B:INCENP complex, 1 μg of T175A Nek2 protein, or 0.046 μg of T210D Plk1 kinase domain. Amounts of each kinase were chosen so that reactions of each kinase with its optimal peptide would yield approximately equivalent amounts of ^{32}P-radiolabel incorporation. Concentrations of optimal peptide were set to the K_m of the kinase in the reaction. Reactions were performed in triplicate and 5 μL of each reaction was spotted on phosphocellulose at 0 minutes and 1 hour. The phosphocellulose paper was washed 4 times with 0.5% phosphoric acid, and analyzed by scintillation counting.

Mass Spectrometry Identification of Plk1-Dependent Mitotic Phosphorylation Sites containing Asn in the Ser/Thr-2 Position

HeLa cells were cultured 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). In some cases, cells were transfected with LAP-tagged murine bait

proteins (Mad2l1 or Bub1) and selected with 500 μg/ml G418 (Gibco). Cells were arrested in mitosis by addition of 100 μg/ml nocodazole for 18 hr prior to harvesting. For mitotic cells in which the activity of endogenous Plk1 was inhibited, nocodazole-arrested cells were further incubated with 250 nM of BI4834 (a gift from Boehringer Ingelheim, Vienna, Austria) for an additional 2 hrs. Cell pellets were harvested, snap-frozen in liquid nitrogen and stored at −80°C. Thawed pellets were resuspended in one pellet-volume of extract buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl, 5 mM EDTA, 20 mM β-glycerophosphate, 10 mM NaF, 10% glycerol, and 0.1% NP-40) containing 1 μM okadaic acid, 0.2 mM $NaVO_4$, 1 mM DTT, 0.1 mM PMSF and 10 μg/ml each of leupeptin, pepstatin and chymostatin, dounce homogenized, and clarified by centrifugation at 14,000 rpm for 15 min. Mitotic cohesin and kinetochore protein complexes were immunoprecipitated with antibodies against the endogenous cohesin regulator PDS5A or tandem affinity purified for LAP-tagged Mad2l1 and Bub1, respectively as described (91, 119). Following extensive washing, bound proteins were eluted using 0.2 M glycine pH 2.0, neutralized, digested in solution using trypsin or subtilisin, and analyzed by MS.

Digests were analysed using a UltiMate 3000 Nano-LC system (Dionex Benelux, Amsterdam, The Netherlands) equipped with a trap column for sample desalting and concentration. Samples were loaded onto an analytical C18 column (PepMap C18, 75 μm ID × 150 mm length, 3 μm particle size, 100 Å pore size, Dionex) for separation. Mobile phase A contained 5% acetonitrile and 0.1% formic acid, while mobile phase B contained 80% acetonitrile and 0.08% formic acid. Following a 10 min wash in 0.1% TFA, peptides were eluted using a linear gradient from 20% to 50% mobile phase B in 180 min at a flow rate of 300 nl/min. 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, equipped with a nano-electrospray ionization (ESI) source. 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 performed in the linear ion trap using the multi-stage activation (MSA) method of Schroeder et al. (120). MS RAW files were analysed by database searching using Mascot (121) for phosphopeptides containing Asn in the Ser/Thr-2 position. 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 modifications. Mass tolerances of the parent ion and the fragments were set to 10 ppm and 0.80 Da, respectively. Each mass spectrum of an Asn-containing phosphopeptide identified by MASCOT was then manually inspected and annotated.

Structural Modeling

The X-ray crystal structures of Cdk2/cyclin B (PDB identifier 2JGZ), and the kinase domains of Aurora B in complex with an INCENP fragment (PDB identifier 2BFX), Nek2 (PDB identifier 2JAV), and human Plk1 (PDB identifier 2OU7) were used as base models. For the Plk1 model, the activation loop segment was modeled based on the corresponding region from the zebrafish crystal structure (PDB identifier 3D5W). For the Plk1 and Aurora B models, peptides were manually docked into the substrate binding cleft based on the structure of the substrate GSK3-beta peptide in the active site cleft of Akt (PDB identifier 106L). For the Cdk1 model, the structure of CyclinA/Cdk2 containing a substrate peptide in the active site cleft (PDB identifier 2CCI) was used for manually docking the peptide, whereas for Nek2, the peptide structure from the PKA catalytic subunit:AMP-AlF_4^-:substrate peptide co-complex was used for docking. Molecular surface representations of the Aurora B, Cdk1, Nek2, and Plk1 active sites were created using

PyMOL and shaded by electrostatic potential using surface projections of charge calculated with DelPhi software (122).

Centrosome and Spindle Enrichment Bioinformatic Analysis

We obtained a list of proteins associated with the centrosome from Andersen *et al.* (102) and a list of spindle proteins from Sauer *et al.* (103). Sequences for these proteins were downloaded directly from NCBI Entrez Protein using NCBI Entrez Utilities (http://www.ncbi.nlm.nih.gov/entrez/query/static/eutils_help.html). The human proteome was downloaded as version 3.23 of the file ipi.HUMAN.fasta from the International Protein Index (http://www.ebi.ac.uk/IPI/). We generated a list of the amino acid sequences surrounding each Ser or Thr residue within the sequences of each protein in each of these three data sources. The human proteome yielded 1,850,231 unique sites from 66,619 proteins. The centrosome data and spindle data yielded 35,425 unique sites from 524 proteins and 34,909 unique sites from 277 proteins, respectively. For each motif of interest (Fig. 8B), statistical significance of enrichment of that motif in the centrosomal or spindle proteomes relative to the full proteome was calculated using the hypergeometric distribution:

$$P = \sum_{i=k}^{\min(n,m)} \left[\frac{\binom{m}{i}\binom{N-m}{n-i}}{\binom{N}{n}} \right]$$

where N is the number of Ser/Thr sites in the human proteome, n is the number of Ser/Thr sites in the centrosomal or spindle proteome, m is the number of motif sites in the human proteome, and k is the number of motif sites in the centrosomal or spindle proteome. This corresponds to the probability of seeing as many instances or more of the motif by chance as are seen in the centrosomal or spindle proteome if a dataset of the same size as the centrosomal or spindle proteome is drawn at random from the human proteome.

Kinase Phosphorylation Motif Logos

Phosphorylation content of individual peptide libraries in the PS-OPLS blots was quantified by phosphorimage analysis using ImageQuant 5.2 software (Molecular Dynamics). Background correction was performed by subtracting an adjacent region of the blot of the same size that did not overlap with any of the phosphorylated library spot positions. Sequence logos were generated from the $\log_2$ values of the intensities of background corrected, normalized peptide library spots using POSTSCRIPT files generated by Visual Basic code adapted from Stephen Shaw's original PSSM logo code (123), which contains POSTSCRIPT code adapted from Tom Schneider's MAKELOGO (version 8.69, http://www.bio.cam.ac.uk cgi-bin seqlogo logo.cgi) (124).

Enrichment of Multiple Kinase Motifs in the Same Protein

Predicted high-stringency sites on proteins for CyclinB/Cdk1, Plk1, Aurora A and B, and Nek2 were generated from PS-OPLS results using Scansite (http://scansite.mit.edu) (125). Sites for Aurora Kinase A and B were combined into a single list, as were sites for each of the two Cdk1/cyclinB motifs (one for Ser/Thr+3 R/K and one for Ser/Thr+4 K). Predicted sites were identified by scanning all human proteins from SWISS-PROT (version 42.7), or limited to those containing "nucleus" or "nuclear" in the description or keywords fields. For each pair of kinases, the statistical significance of co-occurance was calculated according to a hypergeometric distribution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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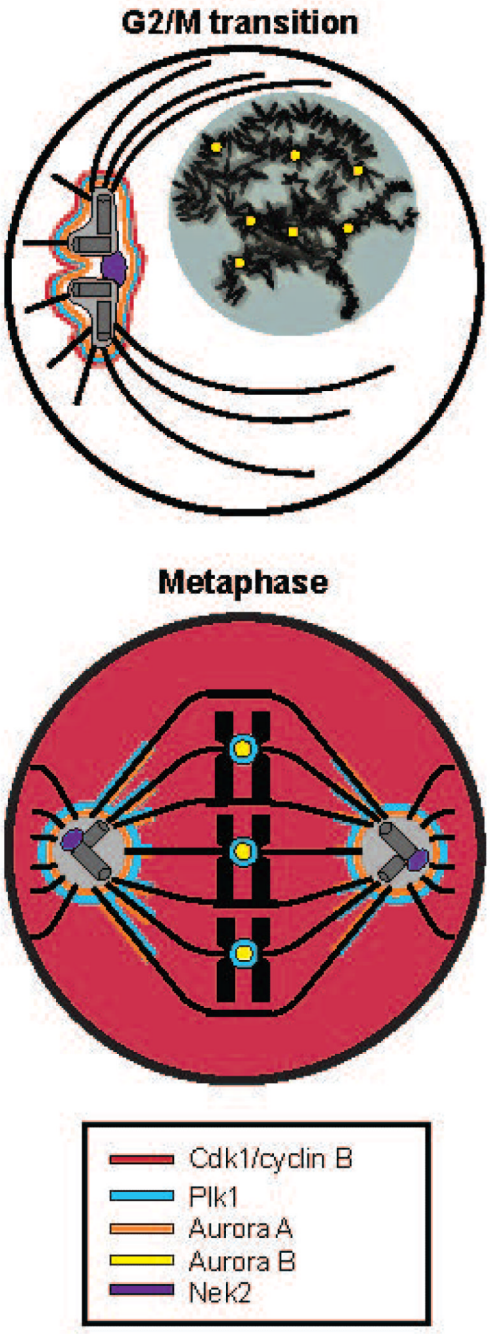
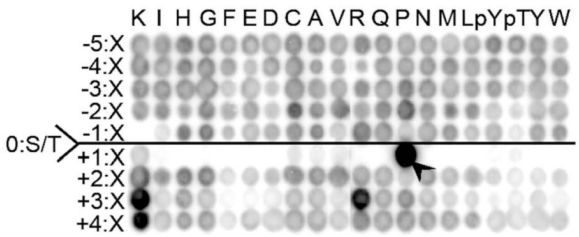


Fig. 1. Major mitotic kinases have both discrete and overlapping subcellular localizations in mitotic cells

Schematic representation showing the localizations of Cdk1/Cyclin B, Aurora A, Aurora B, Plk1, and Nek2 are indicated at the G2/M transition or early prophase (top) and in metaphase (bottom). In the top panel, a magnified representation of the centrosome with nucleated microtubules is shown, illustrating Nek2 localization near the proximal centrioles in the centrosomal core. The nucleus is shaded grey, containing duplicated but uncondensed chromosomes (black). In the bottom panel, condensed chromosomes are shown aligned on the metaphase plate, with their kinetochores indicated by central co-axial circles, attached to spindle microtubules.

A.



B.

Substrate Protein	Mapped Phosphorylation Site
	pS/pT-P-x-R/K motif
Lamin A	STPL***pS***$_{22}$ PT**R**I
Lamin B	TTPL***pS***$_{23}$ PT**R**L
DNA Ligase 1	DEAL***pS***$_{76}$ PA**K**G
KI-67	EMFK***pT***$_{761}$PV**K**E
Nucleophosmin	NYEG***pS***$_{70}$ PI**K**V
	pS/pT-P-x-x-K motif
ERp57 PDI	TIYF***pS***$_{456}$PAN**K**
NSBP1	LVPV***pT***$_{31}$ PEV**K**
OGFR	PEPL***pS***$_{378}$PKE**K**
Stathmin	ELIL***pS***$_{23}$ PRS**K**
Treacle	PLGK***pS***$_{506}$PQV**K**PAS
	pS/pT-P-x-R/K-K motif
Histone H1E	PAEK***pT***$_{17}$ PV**KK**
NIPA	EVPS***pS***$_{395}$ PL**RK**
Nucleolin	ALVA***pT***$_{83}$ PG**KK**
Nup358	PLAS***pS***$_{2251}$PV**RK**
PP1-RS12A	ATPT***pS***$_{409}$ PI**KK**

C.

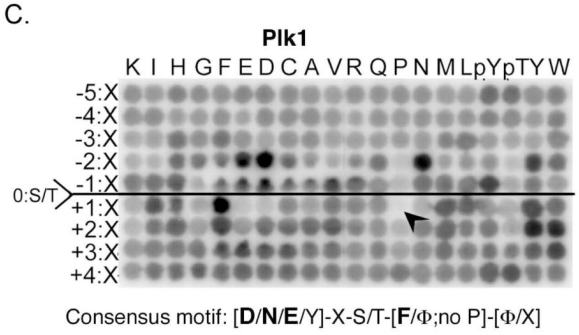
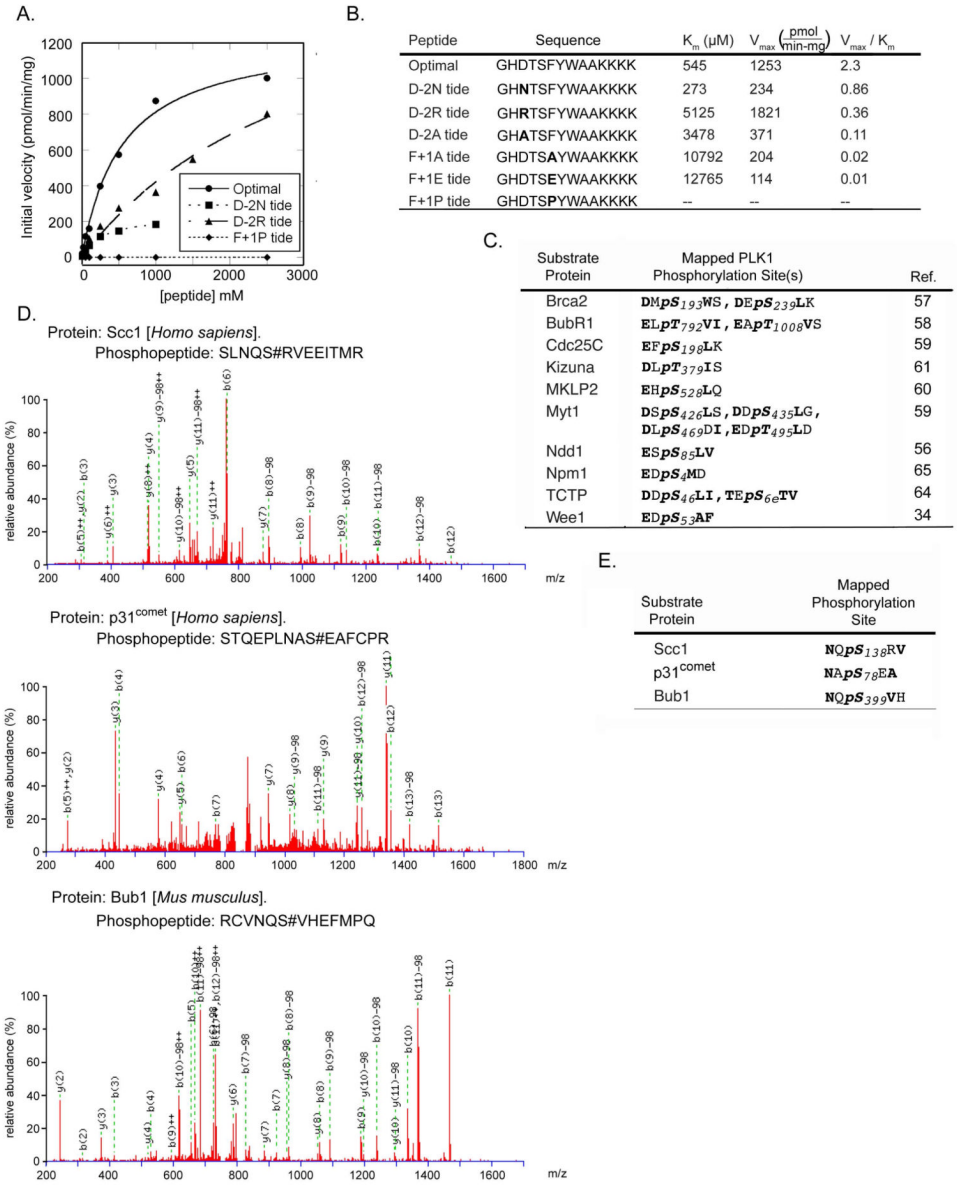


Fig. 2. Phosphorylation motifs for Cdk1/cyclin B and Plk1

(A) The substrate motif for Cdk1/cyclin B reveals near exclusive selection for Pro in the Ser/Thr+1 position, strong selectivity for Arg and Lys in the Ser/Thr+3 position, and Lys in the Ser/Thr+4 position. **(B)** Comparison of the expanded Cdk1/cyclin B motif with mapped substrate sites (49). Bold represents residues that distinguish the submotifs. (**C**) The substrate motif for Plk1 reveals selection for Asp, Asn, or Glu in the Ser/Thr-2 position and hydrophobic amino acids (Φ) in the Ser/Thr+1 position, and discrimination against Pro in the Ser/Thr+1 position.



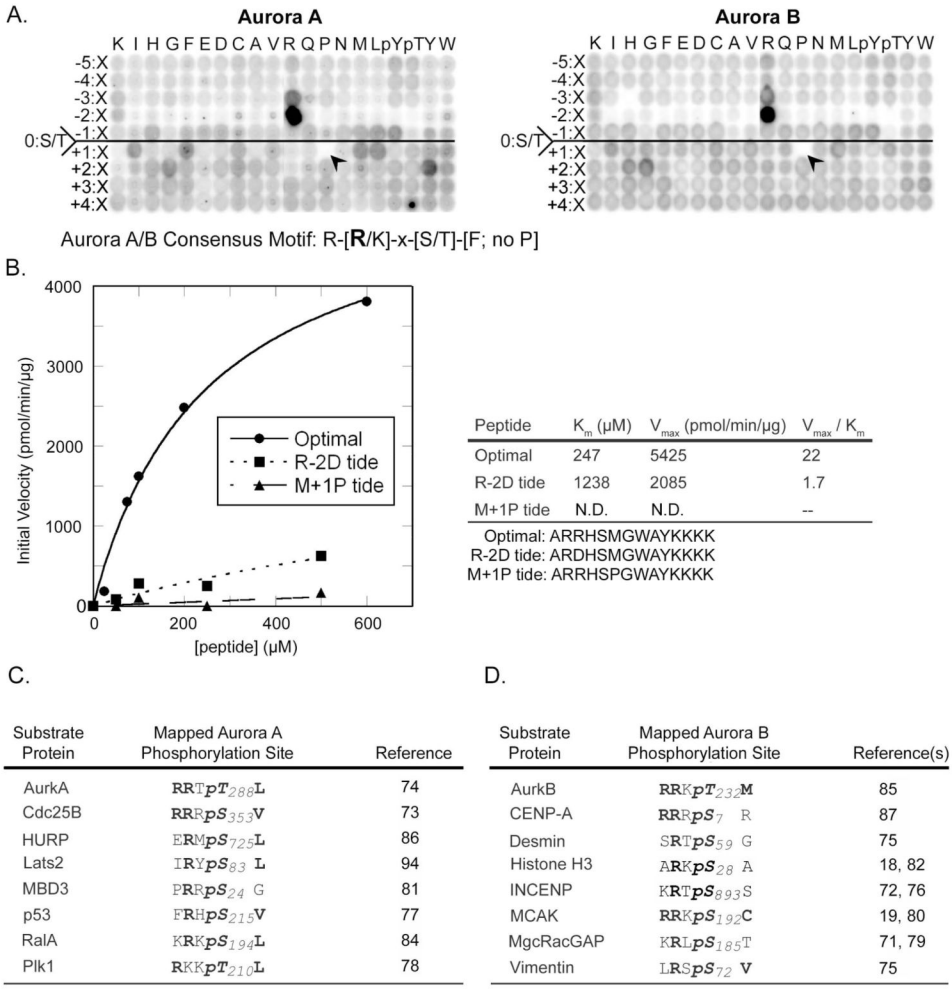
Peptide	Sequence	K_m (μM)	V_{max} (pmol/min-mg)	V_{max} / K_m
Optimal	GHDTSFYWAAKKKK	545	1253	2.3
D-2N tide	GH**N**TSFYWAAKKKK	273	234	0.86
D-2R tide	GH**R**TSFYWAAKKKK	5125	1821	0.36
D-2A tide	GH**A**TSFYWAAKKKK	3478	371	0.11
F+1A tide	GHDTS**A**YWAAKKKK	10792	204	0.02
F+1E tide	GHDTS**E**YWAAKKKK	12765	114	0.01
F+1P tide	GHDTS**P**YWAAKKKK	--	--	--

Substrate Protein	Mapped PLK1 Phosphorylation Site(s)	Ref.
Brca2	DM***pS***$_{193}$WS, DE***pS***$_{239}$LK	57
BubR1	EL***pT***$_{792}$VI, EA***pT***$_{1008}$VS	58
Cdc25C	EF***pS***$_{198}$LK	59
Kizuna	DL***pT***$_{379}$IS	61
MKLP2	EH***pS***$_{528}$LQ	60
Myt1	DS***pS***$_{426}$LS, DD***pS***$_{435}$LG, DL***pS***$_{469}$DI, ED***pT***$_{495}$LD	59
Ndd1	ES***pS***$_{85}$LV	56
Npm1	ED***pS***$_{4}$MD	65
TCTP	DD***pS***$_{46}$LI, TE***pS***$_{6e}$TV	64
Wee1	ED***pS***$_{53}$AF	34

Substrate Protein	Mapped Phosphorylation Site
Scc1	NQ***pS***$_{138}$RV
p31^{comet}	NA***pS***$_{78}$EA
Bub1	NQ***pS***$_{399}$VH

Fig. 3. Kinetics of Plk1 phosphorylation and identification of an expanded consensus motif

(A) Kinetics of peptide phosphorylation by the Plk1 T210D kinase domain using an optimal substrate peptide and peptides with single amino acid substitutions at the indicated positions. Peptide sequences are shown in panel B. **(B)** Kinetic parameters (K_m, V_{max}, and V_{max}/K_m ratio) for the phosphorylation of the indicated peptides by Plk1 were determined by fitting the data to the Michaelis-Menten equation. Bold residues in the sequences represent the ones substituted. (**C**) The substrate specificity motif of Plk1 matches previously mapped Plk1 phosphorylation sites, which contain Asp or Glu in the Ser/Thr-2 and hydrophobic amino acids in the Ser/Thr+1 position. Bold represents motif-distinguishing residues. **(D)** Annotated MS/MS spectra for the phosphopeptides containing three Plk1 inhibitor-sensitive phosphosites containing Asn in the Ser/Thr-2 position. S# in the phosphopeptide sequence represents phosphoserine. (**E**) Sequence motifs of the sites mapped from the data shown in panel D.



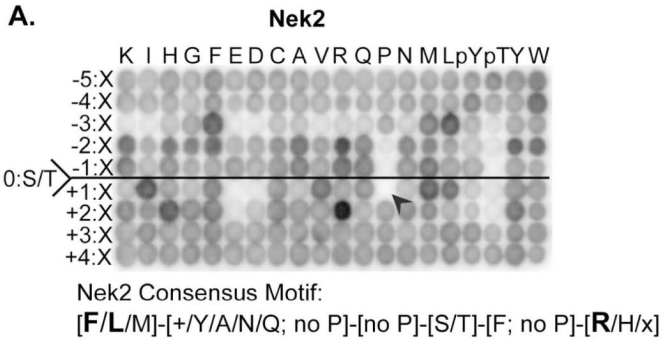
Peptide	K_m (µM)	V_{max} (pmol/min/µg)	V_{max} / K_m
Optimal	247	5425	22
R-2D tide	1238	2085	1.7
M+1P tide	N.D.	N.D.	--

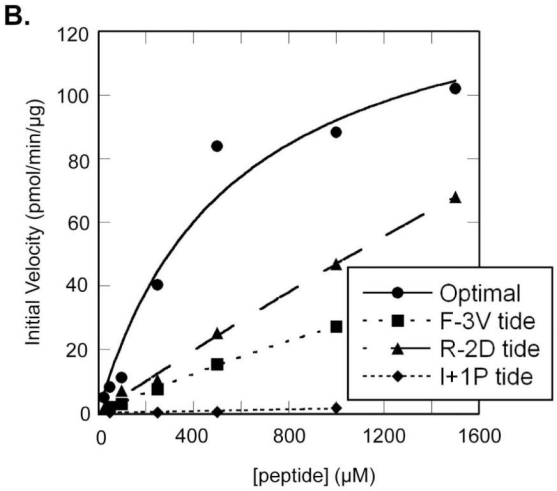
Substrate Protein	Mapped Aurora A Phosphorylation Site	Reference
AurkA	RRT*pT*$_{288}$L	74
Cdc25B	RRR*pS*$_{353}$V	73
HURP	ERM*pS*$_{725}$L	86
Lats2	IRY*pS*$_{83}$ L	94
MBD3	PRR*pS*$_{24}$ G	81
p53	FRH*pS*$_{215}$V	77
RalA	KRK*pS*$_{194}$L	84
Plk1	RKK*pT*$_{210}$L	78

Substrate Protein	Mapped Aurora B Phosphorylation Site	Reference(s)
AurkB	RRK*pT*$_{232}$M	85
CENP-A	RRR*pS*$_{7}$ R	87
Desmin	SRT*pS*$_{59}$ G	75
Histone H3	ARK*pS*$_{28}$ A	18, 82
INCENP	KRT*pS*$_{893}$S	72, 76
MCAK	RRK*pS*$_{192}$C	19, 80
MgcRacGAP	KRL*pS*$_{185}$T	71, 79
Vimentin	LRS*pS*$_{72}$ V	75

Fig. 4. Phosphorylation motif selectivity of Aurora A and B

(A) PS-OPLS blots of Aurora A (left) and Aurora B:INCENP complex (right) reveal positively and negatively selected residues for motifs recognized by these kinases. The arrowheads identify the complete selectivity against Pro at position Ser/Thr+1. **(B)** Peptide phosphorylation and determination of kinetic parameters (K_m, V_{max}, and V_{max}/K_m ratio) for reactions of Aurora B:INCENP with the optimal substrate peptide and peptides with single amino acid substitutions. A graph of reaction data fitted to the Michaelis-Menten equation for peptides is indicated on the left, and a table of kinetic parameters is shown on the right. R-2D tide and M+1P tide sequences are indicated below the table with the substituted positions shown in bold. **(C)** Previously published phosphorylation sites on mapped substrates of Aurora A (left) and Aurora B (right) conform to the optimal phosphorylation motif determined by PS-OPLS. Bold represents motif selected residues.





Peptide	Sequence	K_m (µM)	V_{max} (pmol/min/µg)	V_{max} / K_m
Optimal	WFRMSIRGGYKKKK	554	143	0.26
F-3V tide	W**V**RMSIRGGYKKKK	4597	153	0.033
R-2D tide	WF**D**MSIRGGYKKKK	13477	677	0.050
I+1P tide	WFRMS**P**RGGYKKKK	N.D.	N.D.	—

C.

Substrate Protein	Mapped Nek2 Phosphorylation Site	Reference
Nek2	**FA**K***pT***$_{175}$**F**V	88
Hec1	**FA**L***pS***$_{165}$KS	89

Fig. 5. Phosphorylation motif selectivity for Nek2

(A) PS-OPLS screening reveals that Nek2 strongly selects a subset of hydrophobic amino acids in the Ser/Thr-3 and the Ser/Thr+1 positions and Arg in the Ser/Thr+2 position, but strongly discriminates against Pro in the Ser/Thr+1 position. Additional selection against acidic residues in the Ser/Thr-3, Ser/Thr+1 and +2 positions is also observed, as is positive selection for certain residues in the Ser/Thr-2 position. Within the indicated motif, + denotes basic residues, Φ denotes hydrophobic residues, and Ψ denotes aromatic residues. **(B)** Peptide phosphorylation and determination of kinetic parameters (K_m, V_{max}, and V_{max}/K_m ratio) for reactions of Nek2 with the optimal substrate peptide and peptides with single amino acid substitutions. A graph of reaction data fitted to the Michaelis-Menten equation for peptides is indicated on the top, and a table of kinetic parameters is shown on the bottom. F-3V tide, R-2D tide, and I+1P tide, sequences are indicated in the table with the substituted positions relative to the optimal peptide shown in bold. **(C)** Previously published mapped

Nek2 phosphorylation sites are in reasonable agreement with the Nek2 PS-OPLS phosphorylation motif. Bold represents motif selected residues.

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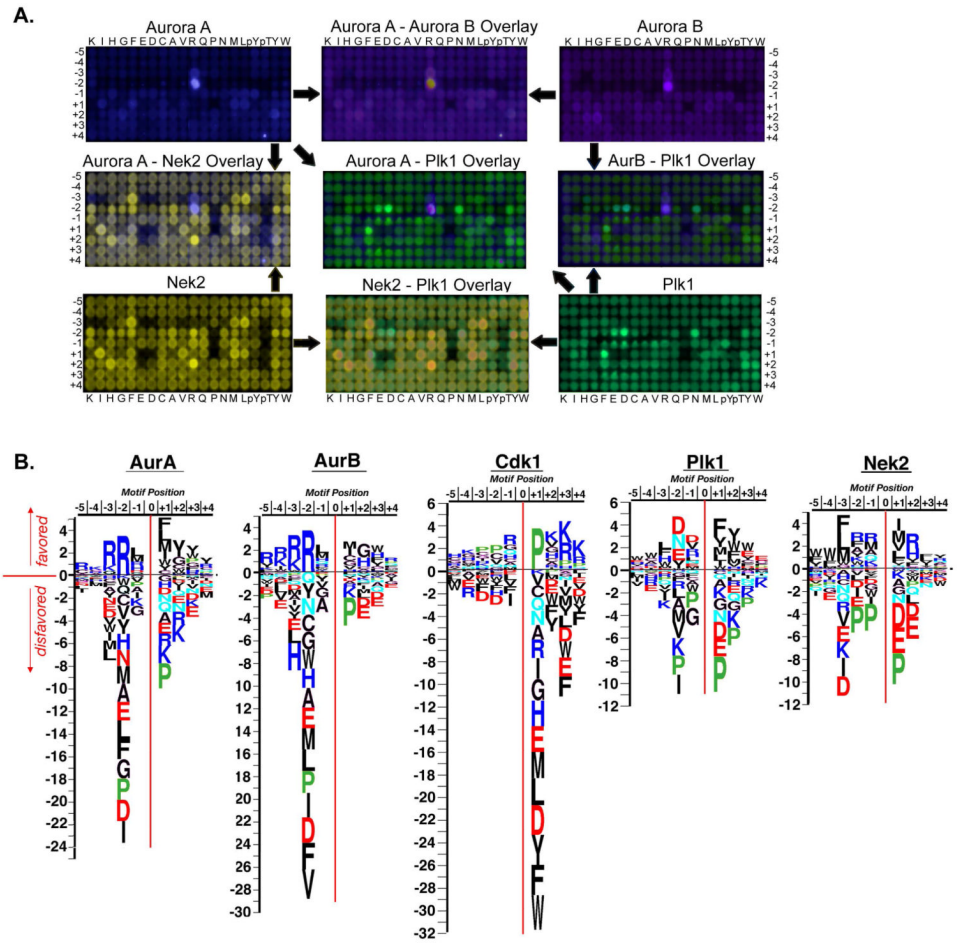

Fig. 6. Exclusivity of phosphorylation motifs for major mitotic kinases

(A) PS-OPLS blots for Plk1, Aurora A, Aurora B, and Nek2 are pseudocolored and superimposed. Note the near identity of the Aurora A and B motifs in the merged view, and the distinct lack of superposition of the Aurora A/B and Plk1 motifs, as revealed by the largely distinct green and purple spots. The Nek2 motif shows substantial overlap with that of Plk1 and Aurora at multiple positions. **(B)** Spot intensities from the PS-OPLS blots were quantified and used to generate motif logos for each of the major mitotic kinases. Basic residues are colored blue, acidic residues red, amide side chain-containing residues cyan, Pro green, and the remaining residues in black. AurA, Aurora A; AurB, Aurora B.

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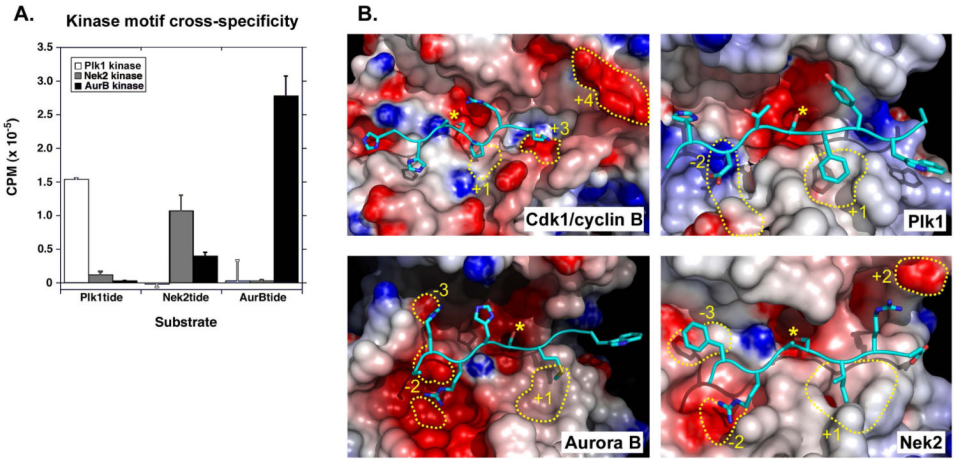
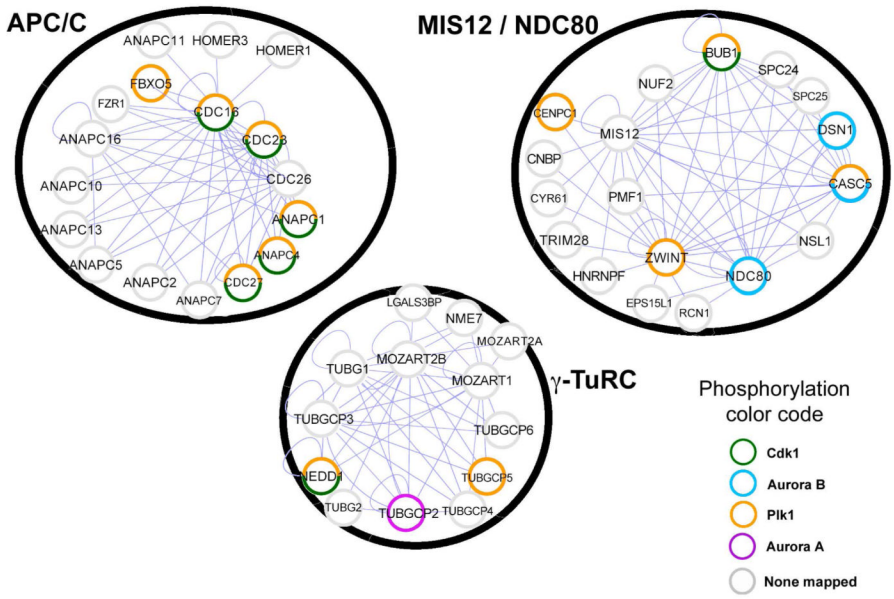


Fig. 7. Structural basis for kinase motif selectivity and exclusivity

(A) Optimal peptides for Plk1 (Plk1tide), Nek2 (Nek2tide), and Aurora B (AurBtide) were used as substrates in phosphorylation reactions containing the indicated kinases. The amount of radioactivity incorporated into each peptide after a one-hour incubation is indicated, showing mean values and standard deviations from triplicate measurements. **(B)** Modeled structures of kinases bound to optimal substrates (Cdk1/cyclin B:HHASPRK; Plk1:HDTSFYWA; Aurora B:RRHSMGW; Nek2:FRASIR). The molecular surfaces corresponding to the active site regions for each kinase are shown with red and blue regions indicating negative and positive electrostatic potentials, respectively. Active site features important for substrate selectivity are circled with yellow dotted lines and numbered relative to the position of the phospho-acceptor. Peptide substrates are shown with cartoon rendering for the backbone and stick rendering for relevant side chains. The phosphoacceptor Ser residues are indicated with yellow asterisks. For Cdk1, the +1 region corresponds to Val^{164} of Cdk1, the +3 region corresponds to $pThr^{160}$ of Cdk1, and the +4 region corresponds to Glu^{265} and Asp^{268} of cyclin B. For Plk1, the −2 regions correspond to Lys^{178} and Asn^{216}, and the +1 region corresponds to Leu^{211}, Pro^{215}, Ile^{218} and Val^{222}. For Aurora B, the −3 region corresponds to Glu^{177}, the −2 regions correspond to Glu^{220} and Glu^{281}, and the +1 region corresponds to Trp^{237}, Met^{249} and Leu^{256}. For Nek2, the −3 region corresponds to Ala^{95} and Ala^{145}, the −2 region corresponds to Glu^{208}, and the +1 region corresponds to Phe^{176}, Pro^{180} and Met^{183}.

A.



B.

Kinase	Motif	Enrichment Signficance Score Centrosome	Spindle
Cdk1/cyclin B	[S/T]-P-X-[R/K]	0.65	1.06×10^{-20}
	[S/T]-P-X-[R/K]-K	0.34	1.69×10^{-5}
	[S/T]-P-X-X-K	0.09	6.40×10^{-22}
Plk1	[D/N/E]-X-[S/T]-[I/L/M/V/F/W/Y]	2.50×10^{-11}	2.83×10^{-4}
Nek2	[F/M/L]-[!P]-[!P]-[S/T]-[I/L/M/V]	5.22×10^{-3}	1.0

C.

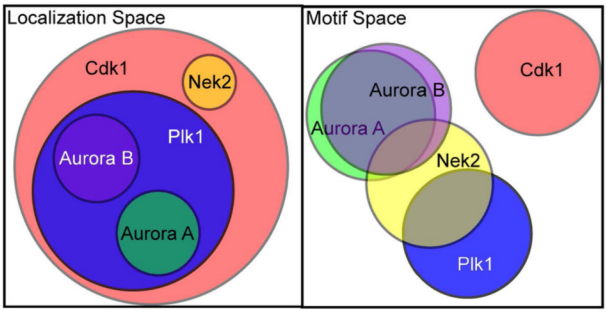


Fig. 8. Orthogonal mitotic kinase motif and localization 'spaces' provide substrate site specificity

(A) A selection of mitotic protein complexes identified by Hutchins *et al.* (122) are shown within black circles and annotated based on the literature for the presence of mapped mitotic phosphorylation sites by the indicated kinases. Note that multiple mitotic kinases phosphorylate discrete sites within single complexes. Blue lines indicate direct intra-cluster interactions. **(B)** Subproteomes associated with the spindle and centrosome were scored for the occurrence of the Cdk1, Nek2 and Plk1 motifs and compared to the expected number of sites at those locations based on the entire proteome. The enrichment significance score indicates the statistical significance of overenrichment based on a hypergeometric probability distribution. **(C)** Mitotic kinase functionality is represented by Venn diagrams of 'localization space' and 'motif space'. In 'localization space', each circle represents the total subcellular locales available to that kinase. In 'motif space', each circle represents the

sequences that can be phosphorylated by the kinase. In this representation, a major mitotic kinase can overlap with every other kinase in at most one of these two spaces.

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