

Figure S1. Experimental approach for determining protein kinase motifs using Positional Scanning Oriented Peptide Library Screening (PS-OPLS). See Materials and Methods section for additional details. The blot for Cdk1/cyclin B is shown as an example. The arrowhead indicates a particularly strongly selected residue in the Ser/Thr+1 position.

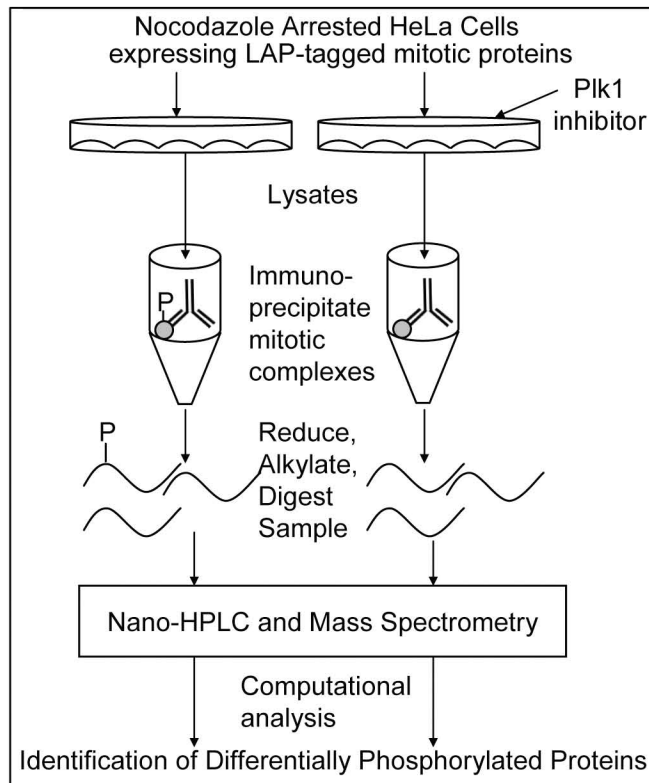


Figure S2. An experimental strategy for the identification of PIK1-dependent substrate sites.

Endogenous human proteins, or LAP-tagged murine proteins expressed as BAC TransgenOmics, in HeLa cells were isolated from mitotic extracts in the absence or presence of the PIK1 inhibitor, and inhibitor-sensitive phosphorylation sites were identified by mass spectrometry.

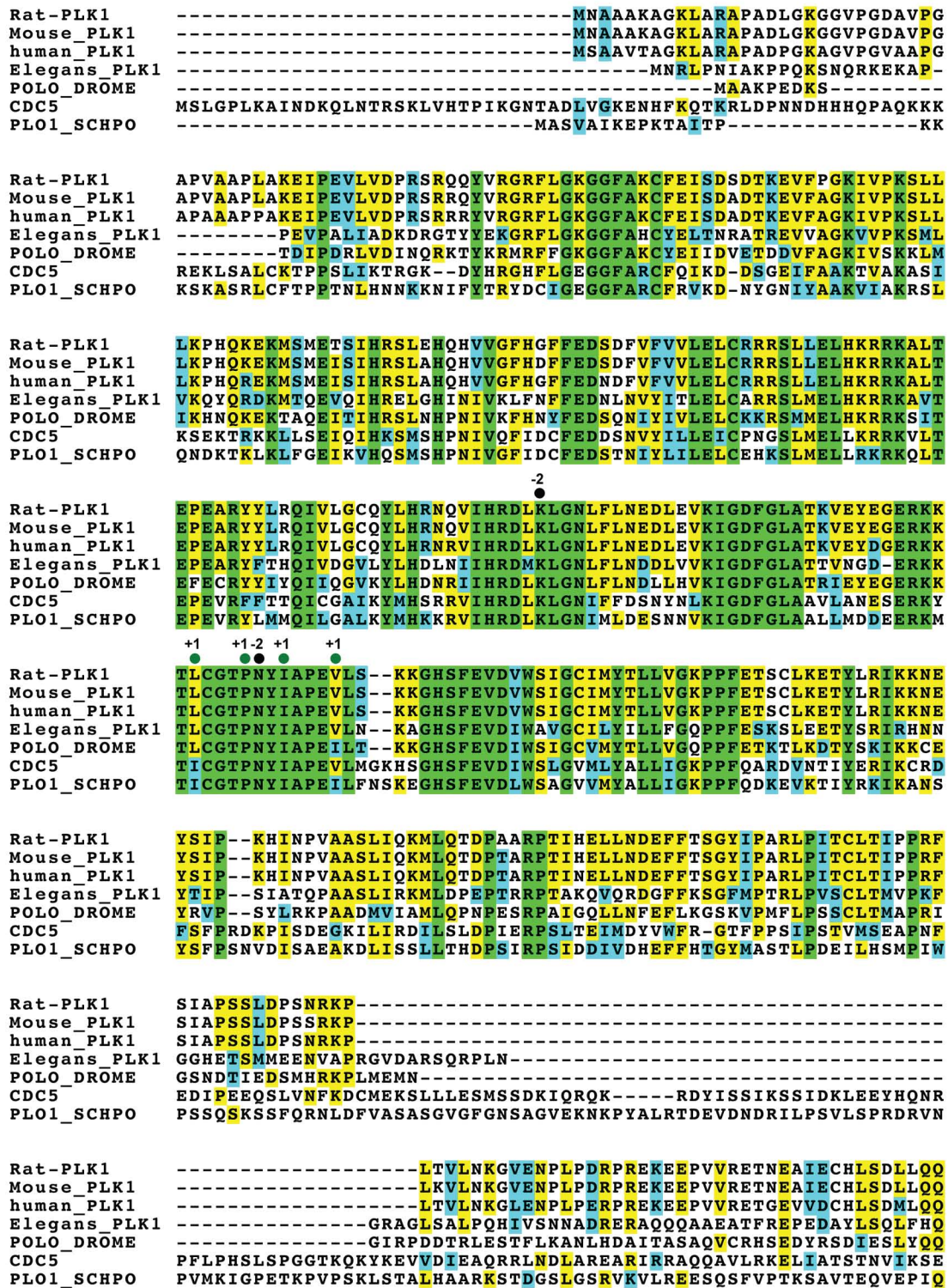


Figure S3. Conservation of specificity-determining residues in Polo-like kinase 1. A sequence alignment of the kinase domains of Plk1 orthologs from *Rattus norvegicus*, *Mus musculus*, *Homo sapiens*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. Residues implicated in selection of specific amino acids at the indicated positions within the Plk1 substrate motif on the basis of structural modeling are indicated by filled circles. The number above each circle indicates the motif position with which the indicated residue interacts.


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Rat_AurB      -----MAQKENVYPWPYGSKTSQSGLNLTLPQRVLRKEPAVTPAQALMNRNSNSQSTA---
Mouse_AurB   -----MAQKENAYPWPYGSKTSQSGLNLTLSQRVLRKEPATTSAALVNRFNNSQSTA---
human_AurB   -----MAQKENSYWPYGRQTAPSGLSTLPQRVLRKEPVTPSALVLMRSRNVQPTA---
Elegans_AurB -----MENK-----PPVINLPEKETVNTPO---
AURKB_DROME -----MTLSRAKHAN-----RNHLPHLLAKVPEEHQPEIK---
ARK1_SCHPO   -MSDSKLDADSLNCLSVSTPSTTANPGRQQLRLAVSNQRQVNNVSLANGKENKRTSNSKF
IPL1         MQRNSLVNIKLNANSPSKKTTRPNTSRINKPWRISSHSPQQRNPNSKI PSPVREKLNRLP
consensus    -----maqk-n-----s-t--g--l--l--p-----l-lm-r-n-qst----

Rat_AurB      VFGQKLTENK-----GATALQGSQSRQPFTIDNFEIGRPLGKGGKFGNVYL
Mouse_AurB   APGQKLAENKS-----QGSTASQGSQNKQFTIDNFEIGRPLGKGGKFGNVYL
human_AurB   APGQKVMEN-----SSGTPDILTRH-FTIDDFEIGRPLGKGGKFGNVYL
Elegans_AurB KGGK-----FTINDFEIGRPLGKGGKFGNVYL
AURKB_DROME NMCLKMMSHD-----AYGQPYDWSPRDFEMGAHLGRGKFGFRVYL
ARK1_SCHPO   NSSLRKIEEP-----IAGVPSSAGPQWREFHIGMFEIGKPLGKGGKFGFRVYL
IPL1         VNNKFLDMESSKI PSPIRKATSSKMIHENKKLPKFKSLSLDDFELGKGLGKGGKFGKVVY

Rat_AurB      AREKKS RFIVALKILFKSQIEKEGVEHQLRREIEIQAHLKHPNIIQLYNYFYDQQRYYLI
Mouse_AurB   AREKKS RFIVALKILFKSQIEKEGVEHQLRREIEIQAHLKHPNIIQLYNYFYDQQRYYLI
human_AurB   AREKKS HFIVALKVLFKSQLISGGVEHQLRREIEIQAHLHHPNIIQLYNYFYDRRRIYLI
Elegans_AurB ARTKTGFHFVAIKVLFKSQLISGGVEHQLRREIEIQSHLNHPNIIKLYTYFWDACKIYLV
AURKB_DROME ARERHSHYLVAMKVMFKEELRKGCVQRQVLRREIEIQSRLKHPHILRLTWFFHDESRIYLA
ARK1_SCHPO   AKEKKTGFIVALKTLHKSELVQSKIEKQVRREIEIQSNLRHKNILRLYGFHFDEKRIYLI
IPL1         VRRHSTGYICALKVMKEEIIKYNLQKQFRREVEIQTSLNHPNLTKSYGYFHFDEKRVYLL

Rat_AurB      LEYAPRGELYKELQ--KSGTFDEQRTATIMEELSDALMYCHKKKVIHRDIKPENLLLGLO
Mouse_AurB   LEYAPRGELYKELQ--KSRTFDEQRTATIMEELSDALTYCHKKKVIHRDIKPENLLLGLO
human_AurB   LEYAPRGELYKELQ--KSCTFDEQRTATIMEELADALMYCHGKKVIHRDIKPENLLLGLO
Elegans_AurB LEYAPGGEMFKQLT--VSKRFSEPTAAKYMYEIAADALSYCHRKNVIHRDIKPENLLIGSQ
AURKB_DROME LEIASEGELFKHLRGAPNHRFDEPRSAKYTYQVANALNYCHLNVIHRDIKPENILLTST
ARK1_SCHPO   LEFAGRGELYQHLR--RAKRFSEEVASKYIFQMANALSYLHKKHVIHRDIKPENILLGID
IPL1         MEYLVNGEMYKLLR--LHGPFNDILASDYIYQIANALDYMHKKNIHRDIKPENILIGFN

Rat_AurB      GELKIADFGWSVHAP-SLRRKTMCGTLDYLPPEMIEGRMHNEMVDLWCIGVLCYELMVGN
Mouse_AurB   GELKIADFGWSVHAP-SLRRKTMCGTLDYLPPEMIEGRMHNEMVDLWCIGVLCYELMVGN
human_AurB   GELKIADFGWSVHAP-SLRRKTMCGTLDYLPPEMIEGRMHNEKVDLWCIGVLCYELLVGN
Elegans_AurB GELKIGDFGWSVHAP-SNKRQTMCGTMDYLPPEMVNGADHSDAVDLWAIGVLCYEFVVGK
AURKB_DROME DDLKLADFGWSAHTP--NNKRRTLCGTLDYLPPEMVDGNSYDSDVDQWCLGILCYEFVVGK
ARK1_SCHPO   GEIKLSDFGWSVHAP-SNRRRTLCGTLDYLPPEMVEGKEHTEKVDLWLSGLVLTYEFLVGA
IPL1         NVIKLTDGWSIINPEENRRKTVCGTIDYLSPEMVESREYDHTIDAWALGVLAFFELLTGA

Rat_AurB      PPFESPS-HSETYRRIVKVDLKFSSMPLGAKDLISKLLKHNPSQRLPLEQVSAHPWVR-
Mouse_AurB   PPFESPS-HSETYRRIVKVDLKFSSVSPGAQDLISKLLKHNPWQRLPLAEVAHPWVR-
human_AurB   PPFESAS-HNETYRRIVKVDLKFPAVPMGAQDLISKLLRHNPSERLPLAEVSAHPWVR-
Elegans_AurB PPFEHED-QSKTYAAIKAAFTYPSVKKGARDLIGRLLVVDPKARCTLEQVMSKHYWVIGQ
AURKB_DROME PPFESNS-TESTYSKIRMEISYPSHLSKGCKELIGLLRKESKGRITLVDVMTHYVWKA
ARK1_SCHPO   PPFEDMSGHSATYKRIAKVDLKI PSFVPPDARDLISRLLQHNPEKRMSELEQVMRHPWIV-
IPL1         PPFEEEM-KDTTYKRI AALDIKMPSNISQDAQDLILKLLKYDPKDRMRLGDVVMKHPWIL-

Rat_AurB      -----ANSRRVLPPSAL-----
Mouse_AurB   -----ANSRRVLPPSAL-----
human_AurB   -----ANSRRVLPPSALQSV-----
Elegans_AurB MMEAKIRAEKQOKIEKEASLRNH-----
AURKB_DROME GMAERELQLOKRERKENTARN-----
ARK1_SCHPO   -----KYKDSWTRKSSSS-----
IPL1         -----RNKPFWENKRL-----

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Figure S4. Conservation of specificity-determining residues in Aurora B.

A sequence alignment of Aurora B orthologs from *Rattus norvegicus*, *Mus musculus*, *Homo sapiens*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae*. Residues implicated in motif selection are indicated as described in Figure S3.

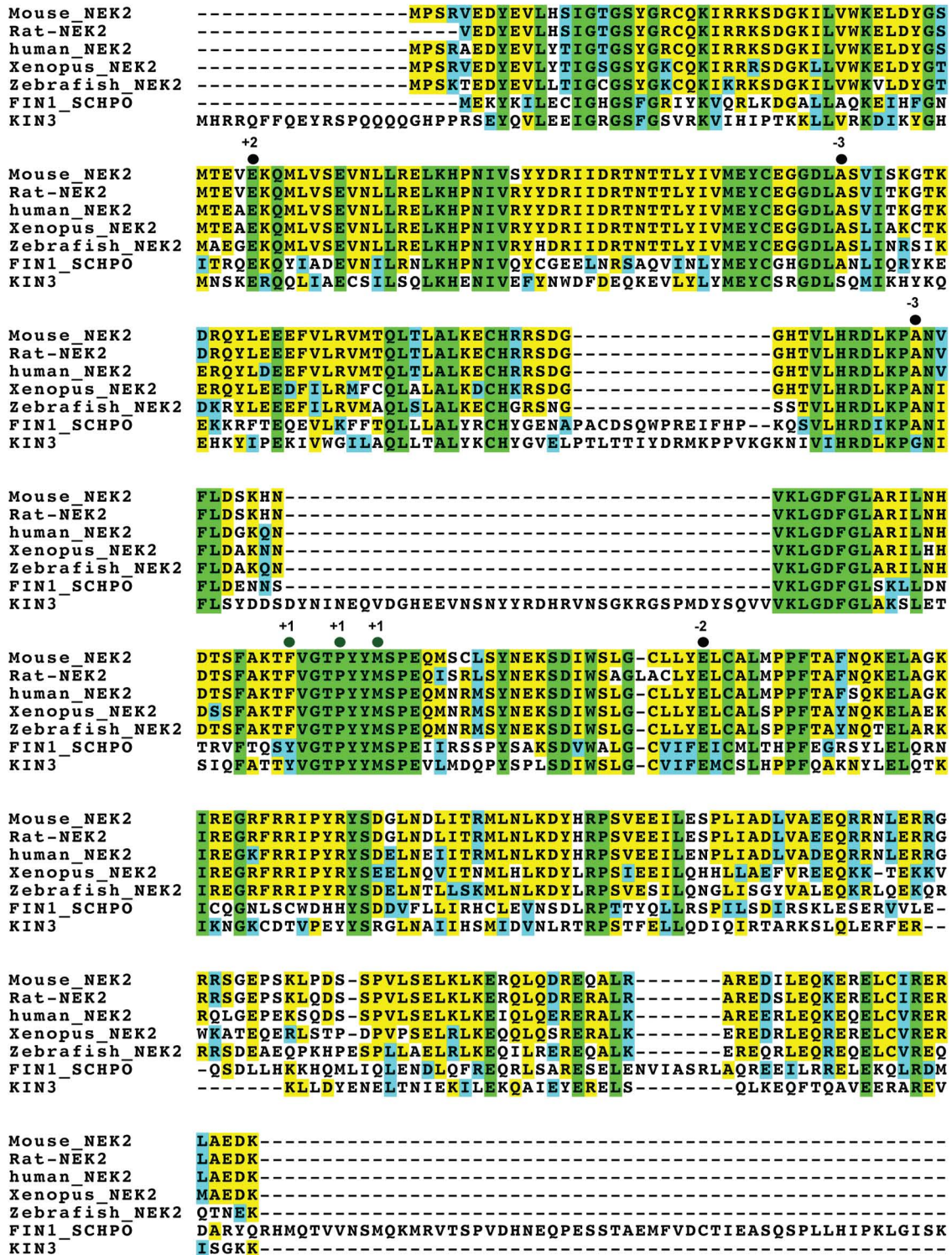


Figure S5. Conservation of specificity-determining residues in Nek2.

A sequence alignment of the kinase domains of Nek2 orthologs from *Mus musculus*, *Rattus norvegicus*, *Homo sapiens*, *Xenopus laevis*, *Danio rerio*, *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae*. Residues implicated in motif selection are indicated as described in Figure S3.

Table S1. Characterization of Plk1-dependent phosphorylation sites. Species are indicated as follows: [Hs], Homo sapiens; [Mm], Mus musculus. Purif, purification method; IP, immunoprecipitation; LAP-TAP, tandem affinity purification using the LAP tag; Mascot, Mascot ions score; S#, phosphoserine.

Bait		Protein identified		Phosphopeptide	
Gene	Purif	Accession	Name(s)	Sequence	Mascot
PDS5A [Hs]	IP	O60216	RAD21, Scc1, Kleisin- α [Hs]	SLNQS#RVEEITMR	36.3
Mad2l1 [Mm]	LAP-TAP	Q6IBB1	MAD2L1BP, p31 ^{comet} [Hs]	STQEPLNAS#EAF CPR	46.0
Bub1 [Mm]	LAP-TAP	O08901	Bub1 [Mm]	RCVNQS#VHEFMPQ	44.0

Table S2. Statistical significance of co-occurrence of Scansite kinase substrate site predictions within proteins. Numbers indicate p-value for enrichment of co-occurrence.

Kinase 1	Kinase 2							
	Aurora A&B		Cdk1/cyclin B		Nek2		Plk1	
	Whole Proteome	Nuclear Proteome	Whole Proteome	Nuclear Proteome	Whole Proteome	Nuclear Proteome	Whole Proteome	Nuclear Proteome
Aurora A&B			4.50E-53	1.53E-12	5.34E-31	2.16E-10	3.56E-20	8.01E-06
Cdk1/cyclin B	4.50E-53	1.53E-12			1.25E-31	2.96E-04	4.83E-22	2.80E-06
Nek2	5.34E-31	2.16E-10	1.25E-31	2.96E-04			1.44E-32	2.83E-08
Plk1	3.56E-20	8.01E-06	4.83E-22	2.80E-06	1.44E-32	2.83E-08		