

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 Plk1-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 Plk1 inhibitor, and inhibitor-sensitive phosphorylation sites were identified by mass spectrometry.

Rat-PLK1 Mouse_PLK1 human_PLK1 Elegans_PLK1 POLO_DROME CDC5 PLO1_SCHPO	MNAAAKAGKLARAPADLGKGGVPGDAVPG MNAAAKAGKLARAPADLGKGGVPGDAVPG MSAAVTAGKLARAPADPGKAGVPGDAAPG
Rat-PLK1	APVAAPLAKEIPEVLVDPRSRQQYVRGRFLGKGGFAKCFEISDSDTKEVFPGKIVPKSLL
Mouse_PLK1	APVAAPLAKEIPEVLVDPRSRRQYVRGRFLGKGGFAKCFEISDADTKEVFAGKIVPKSLL
human_PLK1	APAAAPPAKEIPEVLVDPRSRRRYVRGRFLGKGGFAKCFEISDADTKEVFAGKIVPKSLL
Elegans_PLK1	PEVPALIADKDRGTYYEKGRFLGKGGFAKCYELTNRATREVVAGKVVPKSML
POLO_DROME	TDIPDRLVDINQRKTYKRMFFGKGGFAKCYEIIDVETDDVFAGKIVSKLM
CDC5	REKLSALCKTPPSLIKTRGKDYHRGHFLGEGGFARCFQIKD-DSGEIFAAKTVAKASI
PLO1_SCHPO	KSKASRLCFTPPTNLHNNKKNIFYTRYDCIGEGGFARCFRVKD-NYGNIYAAKVIAKRSL
Rat-PLK1 Mouse_PLK1 human_PLK1 Elegans_PLK1 POLO_DROME CDC5 PLO1_SCHPO	LKPHQKEKMSMETSIHRSLEHQHVVGFHGFFEDSDFVFVVLELCRRRSLLELHKRRKALT LKPHQKEKMSMEISIHRSLAHQHVVGFHDFFEDSDFVFVVLELCRRRSLLELHKRRKALT LKPHQREKMSMEISIHRSLAHQHVVGFHGFFEDNDFVFVVLELCRRRSLLELHKRRKALT VKQYQRDKMTQEVQIHRELGHINIVKLFNFFEDNLNVYITLELCARRSLMELHKRRKAVT IKHNQKEKTAQEITIHRSLNHPNIVKFHNYFEDSQNIYIVLELCKKRSMMELHKRRKSIT KSEKTRKKLLSEIQIHKSMSHPNIVGFIDCFEDDSNVYILLEICPNGSLMELLKRRKVLT QNDKTKLKLFGEIKVHQSMSHPNIVGFIDCFEDSTNIYLILELCEHKSLMELLRKRKQLT -2
Rat-PLK1	EPEARYYLRQIVLGCQYLHRNQVIHRDLKLGNLFLNEDLEVKIGDFGLATKVEYEGERKK
Mouse_PLK1	EPEARYYLRQIVLGCQYLHRNQVIHRDLKLGNLFLNEDLEVKIGDFGLATKVEYEGERKK
human_PLK1	EPEARYYLRQIVLGCQYLHRNRVIHRDLKLGNLFLNEDLEVKIGDFGLATKVEYDGERKK
Elegans_PLK1	EPEARYFTHQIVDGVLYLHDLNIIHRDMKLGNLFLNDDLVVKIGDFGLATTVNGD - ERKK
POLO_DROME	EFECRYYIYQIIQGVKYLHDNRIIHRDLKLGNLFLNDLLHVKIGDFGLATTVNGD - ERKK
CDC5	EPEVRFFTTQICGAIKYMHSRRVIHRDLKLGNIFFDSNYNLKIGDFGLAAVLANESERKY
PLO1_SCHPO	EPEVRFFTTQICGALKYMHKKRVIHRDLKLGNIMLDESNNVKIGDFGLAALLMDDEERKM
Rat-PLK1 Mouse_PLK1 human_PLK1 Elegans_PLK1 POLO_DROME CDC5 PLO1_SCHPO	TLCGTPNYIAPEVLS KKGHSFEVDVWSIGCIMYTLLVGKPPFETSCLKETYLRIKKNE TLCGTPNYIAPEVLS KKGHSFEVDVWSIGCIMYTLLVGKPPFETSCLKETYLRIKKNE TLCGTPNYIAPEVLS KKGHSFEVDVWSIGCIMYTLLVGKPPFETSCLKETYLRIKKNE TLCGTPNYIAPEVLN KAGHSFEVDIWAVGCILYILLFGQPPFETSCLKETYLRIKKNE TLCGTPNYIAPEULT KKGHSFEVDIWSIGCVMYTLLVGQPPFETKTLKDTYSKIKKCE TICGTPNYIAPEULT KKGHSFEVDIWSIGCVMYTLLVGQPPFETKTLKDTYSKIKKCE TICGTPNYIAPEVLMGKHSGHSFEVDIWSLGVMLYALLIGKPPFQARDVNTIYERIKCRD TICGTPNYIAPEILFNSKEGHSFEVDIWSAGVVMYALLIGKPPFQDKEVKTIYRKIKANS
Rat-PLK1	YSIPKHINPVAASLIQKMLQTDPAARPTIHELLNDEFFTSGYIPARLPITCLTIPPRF
Mouse_PLK1	YSIPKHINPVAASLIQKMLQTDPTARPTIHELLNDEFFTSGYIPARLPITCLTIPPRF
human_PLK1	YSIPKHINPVAASLIQKMLQTDPTARPTINELLNDEFFTSGYIPARLPITCLTIPPRF
Elegans_PLK1	YTIPSIATQPAASLIRKMLDPEPTRRPTAKQVQRDGFFKSGFMPTRLPVSCLTMVPKF
POLO_DROME	YRVPSYLRKPAADMVIAMLQPNPESRPAIGQLLNFEFLKGSKVPMFLPSSCLTMAPRI
CDC5	FSFPRDKPISDEGKILIRDILSLDPIERPSLTEIMDYVWFR-GTFPPSIPSTVMSEAPNF
PLO1_SCHPO	YSFPSNVDISAEAKDLISSLLTHDPSIRPSIDDIVDHEFFHTGYMASTLPDEILHSMPIW
Rat-PLK1 Mouse_PLK1 human_PLK1 Elegans_PLK1 POLO_DROME CDC5 PLO1_SCHPO	SIAPSSLDPSNRKP SIAPSSLDPSNRKP GGHETSMMEENVAPRGVDARSQRPLN GSNDTIEDSMHRKPLMEMN EDIPEEQSLVNFKDCMEKSLLLESMSSDKIQRQKRDYISSIKSSIDKLEEYHQNR PSSQ <mark>S</mark> KSSFQRNLDFVASASGVGFGNSAGVEKNKPYALRTDEVDNDRILPSVLSPRDRVN
Rat-PLK1	LTVLNKGVENPLPDRPREKEEPVVRETNEAIECHLSDLLQQ
Mouse_PLK1	LKVLNKGVENPLPDRPREKEEPVVRETNEAIECHLSDLLQQ
human_PLK1	LTVLNKGLENPLPERPREKEEPVVRETGEVVDCHLSDMLQQ
Elegans_PLK1	GRAGLSALPQHIVSNNADRERAQQQAAEATFREPEDAYLSQLFHQ
POLO_DROME	GIRPDDTRLESTFLKANLHDAITASAQVCRHSEDYRSDIESLYQQ
CDC5	PFLPHSLSPGGTKQKYKEVVDIEAQRRLNDLAREARIRRAQQAVLRKELIATSTNVIKSE
FLO1 SCHPO	PVMKIGPETKPVPSKLSTALHAARKSTDGSLGSRVKVLREESQSFVPTKSAVTEQVEPIQ

Figure S3. Conservation of specificity-determining residues in Polo-like kinase 1. A sequence alignment of the kinase domains of Plk1 orthologs from Rattus norwegicus, 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.

Rat_AurB Mouse_AurB human_AurB Elegans_AurB AURKB_DROME ARK1_SCHPO IFL1 consensus	MAQKENVYPI MAQKENAYPI MAQKENSYPI MTLSRAKHAI -MSDSKLADSLNCLSV MQRNSLVNIKLNANSI maqk-n	VPYGSKTSQSGLNTLPQF VPYGSKTSQSGLNTLSQF VPYGRQTAPSGLSTLPQF STPSTTANPGRQQLLRI PSKKTTTRPNTSRINKPY	VLRKEPAVTPAQALMNRSNSQSTA VLRKEPATTSALALVNRFNSQSTA VLRKEPVTPSALVLMSRSNVQPTA RNHLPHLLAKVPEEHQEPIK AVSNQRQVNNVSLANGKENKRTSNSKF RISHSPQQRNPNSKIPSPVREKLNRLP -1p1-1m-r-n-qst
Rat_AurB Mouse_AurB human_AurB Elegans_AurB AURKB_DROME ARK1_SCHPO IPL1	VPGOKLTENK APGOKLAENKS APGOKVMEN KGGK NMCLKMMSHD NSSLRKIEEP VNNKKFLDMESSKIP	GATALQGS QGSTASQGS SSGTPDJ 	QSRQPFTIDNFEIGRPLGKGKFGNVYL QNKQPFTIDNFEIGRPLGKGKFGNVYL LTRH-FTIDDFEIGRPLGKGKFGNVYL FTINDFEIGRPLGKGKFGSVYL GQPYDWSPRDFEMGAHLGRGKFGRVYL PQWREFHIGMFEIGKPLGKGKFGRVYL PKFKSL <mark>SLDD</mark> FELGKKLGKGKFGKVYC
Rat_AurB Mouse_AurB human_AurB Elegans_AurB AURKB_DROME ARK1_SCHPO IPL1	AREKKSRFIVALKILI AREKKSRFIVALKILI AREKKSHFIVALKVLI ARTKTGHFHVAIKVLI ARERHSHYLVAMKVMI AKEKKTGFIVALKTLI VRHRSTGYICALKVMI -3	FKSQIEKEGVEHQLRREI FKSQIEKEGVEHQLRREI FKSQISGGVEHQLRREI FKSQLISGGVEHQLRREI FKEELRKGCVQRQVLREI HK <mark>S</mark> ELVQSKIEKQVRREI SKEE <mark>I</mark> I <mark>K</mark> YNLQKQF <mark>RRE</mark> I	EIQAHLKHPNILQLYNYFYDQQRIYLI EIQAHLKHPNILQLYNYFYDQQRIYLI EIQAHLHHPNILRLYNYFYDRRIYLI EIQSHLNHPNIIKLYTYFWDAKKIYLV EIQSRLKHPHILRLLTWFHDESRIYLA EIQSNLRHKNILRLYGHFHDEKRIYLI EIQTSLNHPNLTKSYGYFHDEKRVYLL -2
Rat_AurB Mouse_AurB human_AurB Elegans_AurB AURKB_DROME ARK1_SCHPO IPL1	LEYAPRGELYKELQ LEYAPRGELYKELQ LEYAPRGELYKELQ LEYAPGGEMYKQLT LEIASEGELFKHLRG/ LEFAGRGELYQHLR MEYLVNGEMYKLLR +1	-KSGTFDEQRTATIMEEI -KSRTFDEQRTATIMEEI -KSCTFDEQRTATIMEEI -VSKRFSEPTAAKYMYEI APNHRFDEPRSAKYTYQ -RAKFSEEVASKYIFQ -LHGPFNDILASDYIYQ +1 +1	SDALMYCHKKKVIHRDIKPENLLLGLQ SDALTYCHKKKVIHRDIKPENLLLGLQ ADALMYCHGKKVIHRDIKPENLLLGLK ADALSYCHKNVIHRDIKPENILLGSQ ANALNYCHLNNVIHRDIKPENILLTST ANALSYLHKKHVIHRDIKPENILLGID ANALDYMHKKNIIHRDIKPENILLGFN -2
Rat_AurB Mouse_AurB human_AurB Elegans_AurB AURKB_DROME ARK1_SCHPO IPL1	GELKIADFGWSVHAP GELKIADFGWSVHAP GELKIADFGWSVHAP GELKIGDFGWSVHAP DDLKLADFGWSAHTP GEIKLSDFGWSVHAP NVIKLTDFGWSIINP	-SLRRKTMCGTLDYLPPE -SLRRKTMCGTLDYLPPE -SLRRKTMCGTLDYLPPE -SNRRQTMCGTMDYLPPE -NNKRRTLCGTLDYLPPE -SNRRTTLCGTLDYLPPE PENRRKTVCGTIDYLSPE	MIEGRMHNEMVDLWCIGVLCYELMVGN MIEGRMHNEMVDLWCIGVLCYELMVGN MIEGRMHNEKVDLWCIGVLCYELLVGN MVNGADHSDAVDLWAIGVLCYEFLVGK MVDGNSYDDSVDQWCLGILCYEFVVGC MVEGKHTEKVDLWSLGVLTYEFLVGA MVESREYDHTIDAWALGVLAFELLTGA
Rat_AurB Mouse_AurB human_AurB Elegans_AurB AURKB_DROME ARK1_SCHPO IPL1	PPFESP <mark>S-HS</mark> ETYRR PPFESP <mark>S-HS</mark> ETYRR PPFESAS-HNETYRR PPFEHED-Q <mark>S</mark> KTYAA PPFESNS-TESTYSK PPFEDM <mark>SGHS</mark> ATYKR PPFEEEM-KDTTYKR	IVKVDLKFPSSMPLGAKI IVKVDLKFPSSVPSGAQI IVKVDLKFPASVPMGAQI IKAARFTYPDSVKKGARI IRRMEISYPSHLSKGCK IA <mark>KVDLKIPSFVP</mark> PDARI IAALDIKMPSNISQDAQI	LISKLLKHNPSQRLPLEQVSAHPWVR- LISKLLKHNPWQRLPLAEVAAHPWVR- LISKLLRHNPSERLPLAQVSAHPWVR- LIGRLLVVDPKARCTLEQVKEHYWIQG LIGGLLRKESKGRITLVDVMTHYWVKA LISRLLQHNPEKRMSLEQVMRHPWIV- LILKLLKYDPKDRMRLGDVKMHPWIL-
Rat_AurB Mouse_AurB human_AurB Elegans_AurB AURKB_DROME ARK1_SCHPO IPL1	ANSRRVLI ANSRRVLI MMEAKIRAEKQQKIEI GMAERELQLQKRERG KYKDSWTI RNKPFWI	PPSAL PPSAL PPSALQSVA KEASLRNH- KENTARN RK <mark>S</mark> SESS ENKRL	

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

A sequence alignment of Aurora B orthologs from Rattus norwegicus, 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.

Mouse_NEK2 Rat-NEK2 human_NEK2 Xenopus_NEK2 Zebrafish_NEK2 FIN1_SCHPO KIN3 Mouse_NEK2	
Rat-NEK2 human_NEK2 Xenopus_NEK2 Zebrafish_NEK2 FIN1_SCHPO KIN3	MTEVEKQMLVSEVNLLRELKHPNIVRYYDRIIDRTNTTLYIVMEYCEGGDLASVITKGTK MTEAEKQMLVSEVNLLRELKHPNIVRYYDRIIDRTNTTLYIVMEYCEGGDLASVITKGTK MTEAEKQMLVSEVNLLRELKHPNIVRYYDRIIDRTNTTLYIVMEYCEGGDLASLIAKCTK MAEGEKQMLVSEVNLLRELKHPNIVRYHDRIIDRTNTTLYIVMEYCEGGDLASLINRSIK ITRQEKQYIADEVNILRNLKHPNIVQYCGEELNRSAQVINLYMEYCGHGDLANLIQRYKE MNSKERQQLIAECSILSQLKHENIVEFYNWDFDEQKEVLYLYMEYCSRGDLSQMIKHYKQ -3
Mouse_NEK2	DRQYLEEEFVLRVMTQLTLALKECHRRSDGGHTVLHRDLKPANV
Rat-NEK2	DRQYLEEEFVLRVMTQLTLALKECHRRSDGGHTVLHRDLKPANV
human_NEK2	ERQYLDEEFVLRVMTQLTLALKECHRRSDGGHTVLHRDLKPANV
Xenopus_NEK2	ERQYLEEDFILRMFCQLALALKDCHKRSDGGHTVLHRDLKPANI
Zebrafish_NEK2	DKRYLEEEFILRVMAQLSLALKECHGRSNGSSTVLHRDLKPANI
FIN1_SCHPO	EKKRFTEQEVLKFFTQLLLALYRCHYGENAPACDSQWPREIFHPKQSVLHRDIKPANI
KIN3	EHKYIPEKIVWGILAQLLTALYKCHYGVELPTLTTIYDRMKPPVKGKNIVIHRDLKPGNI
Mouse_NEK2 Rat-NEK2 human_NEK2 Xenopus_NEK2 Zebrafish_NEK2 FIN1_SCHPO KIN3	FLDSKHN VKLGDFGLARILNH FLDSKHN VKLGDFGLARILNH FLDGKQN VKLGDFGLARILNH FLDGKQN VKLGDFGLARILNH FLDAKNN VKLGDFGLARILNH FLDAKQN VKLGDFGLARILHH FLDENNS VKLGDFGLARILNH FLSYDDSDYNINEQVDGHEEVNSNYYRDHRVNSGKRGSPMDYSQVVVKLGDFGLAKSLET +1 +1 +1 -2
Mouse_NEK2	DTSFAKTFVGTPYYMSPEQMSCLSYNEKSDIWSLG-CLLYELCALMPPFTAFNQKELAGK
Rat-NEK2	DTSFAKTFVGTPYYMSPEQISRLSYNEKSDIWSLG-CLLYELCALMPPFTAFNQKELAGK
human_NEK2	DTSFAKTFVGTPYYMSPEQMNRMSYNEKSDIWSLG-CLLYELCALMPPFTAFSQKELAGK
Xenopus_NEK2	DSSFAKTFVGTPYYMSPEQMNRMSYNEKSDIWSLG-CLLYELCALSPPFTAYNQKELAEK
Zebrafish_NEK2	DTSFAKTFVGTPYYMSPEQMNRMSYNEKSDIWSLG-CLLYELCALSPPFTAYNQTELARK
FIN1_SCHPO	TRVFTQSYVGTPYYMSPEIIRSSPYSAKSDVWALG-CVIFEICMLTHPFEGRSYLELQRN
KIN3	SIQFATTYVGTPYYMSPEVLMDQPYSPLSDIWSLG-CVIFEMCSLHPPFQAKNYLELQTK
Mouse_NEK2	IREGRFRRIPYRYSDGLNDLITRMLNLKDYHRPSVEEILESPLIADLVAEEQRRNLERRG
Rat-NEK2	IREGRFRRIPYRYSDGLNDLITRMLNLKDYHRPSVEEILESPLIADLVAEEQRRNLERRG
human_NEK2	IREGKFRRIPYRYSDELNEIITRMLNLKDYHRPSVEEILENPLIADLVADEQRRNLERRG
Xenopus_NEK2	IREGRFRRIPYRYSEELNQVITNMLHLKDYLRPSIEEILQHHLLAEFVREEQKK-TEKKV
Zebrafish_NEK2	IREGRFRRIPYRYSDELNTLLSKMLNLKDYLRPSVESILQNGLISGYVALEQKRLQEKQR
FIN1_SCHPO	ICQGNLSCWDHHYSDDVFLLIRHCLEVNSDLRPTTYQLLRSPILSDIRSKLESERVVLE-
KIN3	IKNGKCDTVPEYYSRGLNAIIHSMIDVNLRTRPSTFELLQDIQIRTARKSLQLERFER
Mouse_NEK2	RRSGEPSKLPDS-SPVLSELKLKERQLQDREQALRAREDILEQKERELCIRER
Rat-NEK2	RRSGEPSKLQDS-SPVLSELKLKERQLQDRERALRAREDSLEQKERELCIRER
human_NEK2	RQLGEPEKSQDS-SPVLSELKLKEIQLQERERALKAREERLEQKEQELCVRER
Xenopus_NEK2	WKATEQERLSTP-DPVPSELRLKEQQLQSRERALKEREDRLEQRERELCVRER
Zebrafish_NEK2	RRSDEAEQPKHPESPLLAELRLKEQILGEREQALKEREQRLEQREQELCVRER
FIN1_SCHPO	-QSDLLHKKHQMLIQLENDLQFREQRLSARESELENVIASRLAQREEILRRELEKQLRDM
KIN3	KLLDYENELTNIEKILEKQAIEYERELSQLKEQFTQAVEERAREV
Mouse_NEK2 Rat-NEK2 human_NEK2 Xenopus_NEK2 Zebrafish_NEK2 FIN1_SCHPO KIN3	LAEDK

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

A sequence alignment of the kinase domains of Nek2 orthologs from Mus musculus, Rattus norwegicus, 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 ider	ntified	Phosphopeptide		
Gene	Purif	Accession	Name(s)	Sequence	Mascot	
PDS5A [Hs]	IP	O60216	RAD21, Scc1, Kleisin- a [Hs]	SLNQS#RVEEITMR	36.3	
Mad2I1 [Mm]	LAP-TAP	Q6IBB1	MAD2L1BP, p31 ^{comet} [Hs]	STQEPLNAS#EAFCPR	46.0	
Bub1 [Mm]	LAP-TAP	O08901	Bub1 [Mm]	RCVNQS#VHEFMPQ	44.0	

 Table S2. Statistical significance of co-occurance of Scansite kinase substrate site predictions

 within proteins.
 Numbers indicate p-value for enrichment of co-occurance.

	Kinase 2							
Kinasa 1	Aurora A&B		Cdk1/cyclin B		Nek2		Pik1	
NIIIdse I	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		