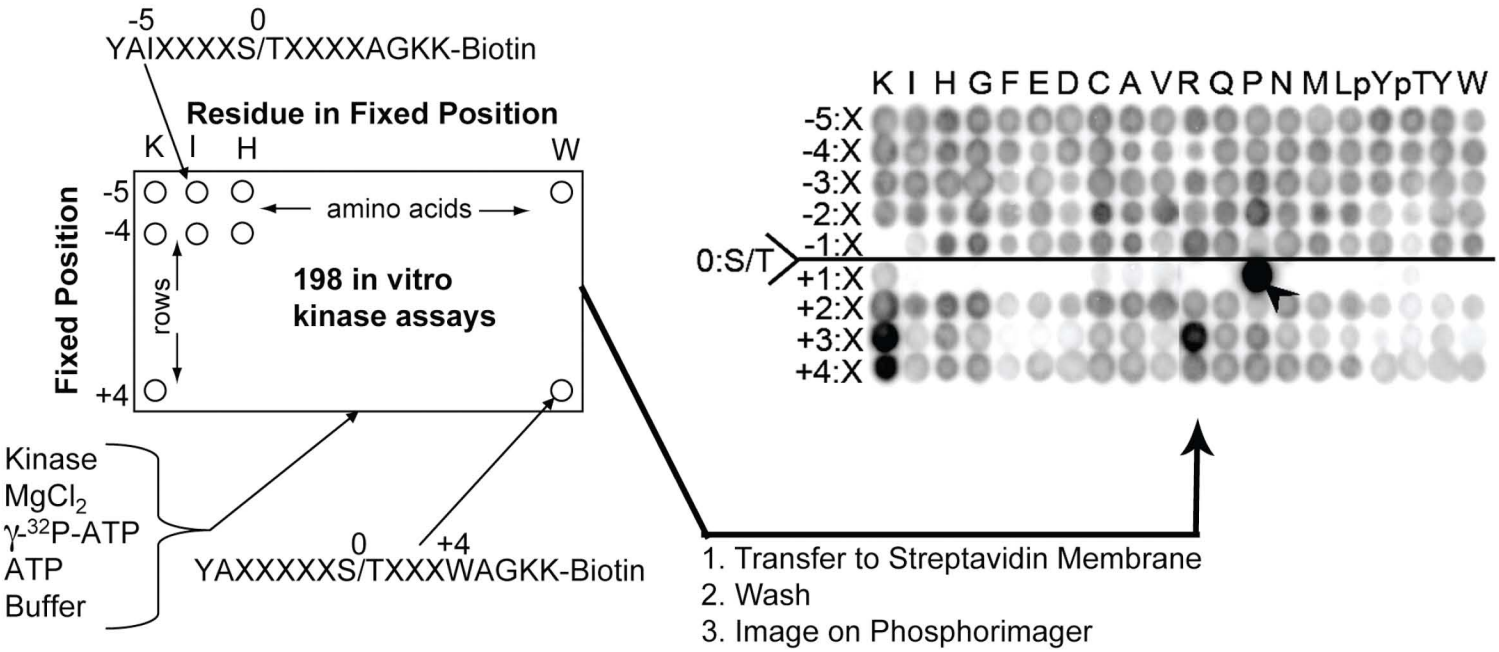

Figure S1. Experimental approach for determining protein kinase motifs using Positional Scanning Oriented Peptide Library Screening (PS-OPLS). See Materials and Methods section for additional details. The blot for Cdk1/cyclin B is shown as an example. The arrowhead indicates a particularly strongly selected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

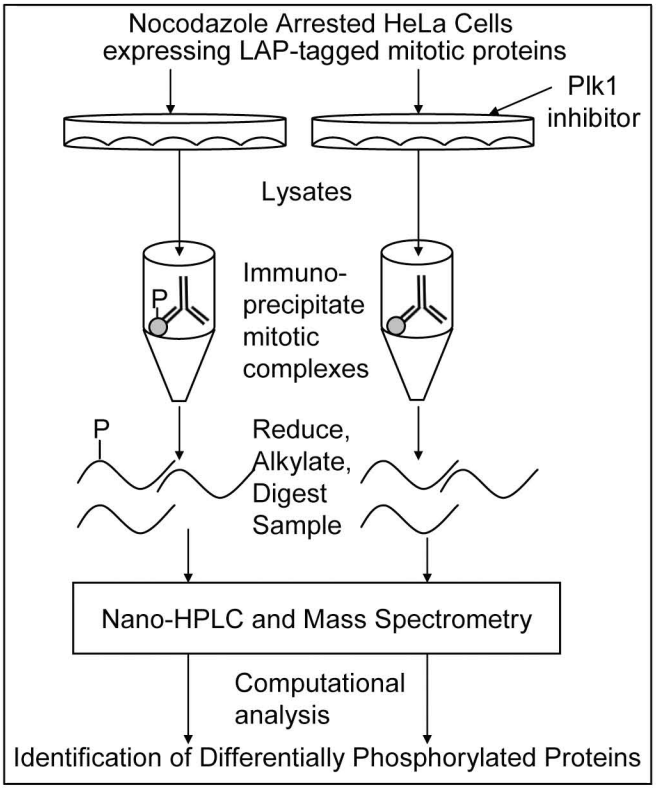

Figure S2. An experimental strategy for the identification of Plk1-dependent substrate sites. Endogenous human proteins, or LAP-tagged murine proteins expressed as BAC TransgenOmics, in HeLa cells were isolated from mitotic extracts in the absence or presence of the Plk1 inhibitor, and inhibitor-sensitive phosphorylation sites were identified by mass spectrometry.

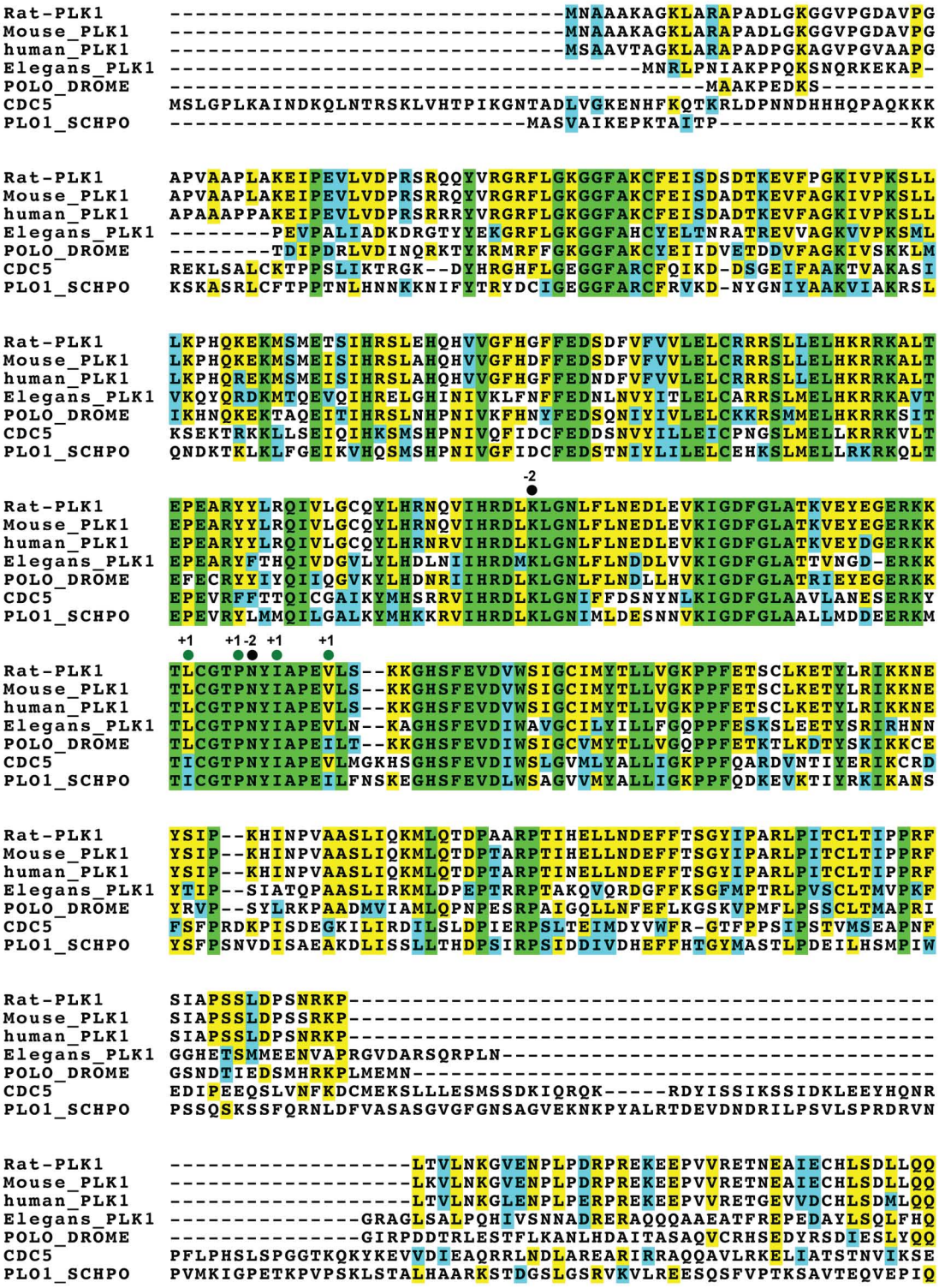

Figure S3. Conservation of specificity-determining residues in Polo-like kinase 1. A sequence alignment of the kinase domains of Plk1 orthologs from Rattus norwegicus, Mus musculus, Homo sapiens, Caenorhabditis elegans, Drosophila melanogaster, Saccharomyces cerevisiae, and Schizosaccharomyces pombe. 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.

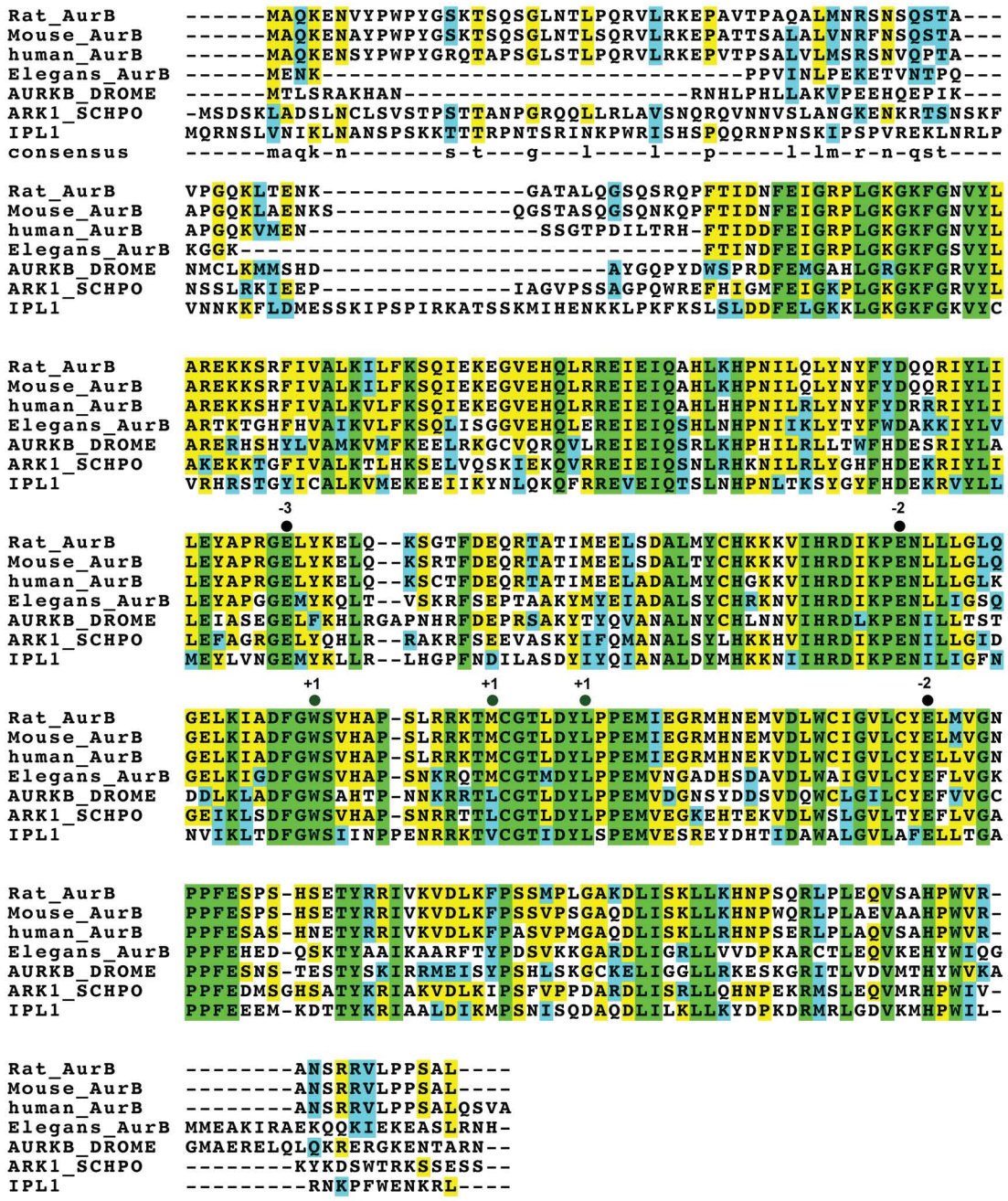

Figure S4. Conservation of specificity-determining residues in Aurora B.
A sequence alignment of Aurora B orthologs from Rattus norwegicus, Mus musculus, Homo sapiens, Caenorhabditis elegans, Drosophila melanogaster, Schizosaccharomyces pombe, and Saccharomyces cerevisiae. Residues implicated in motif selection are indicated as described in Figure S3.

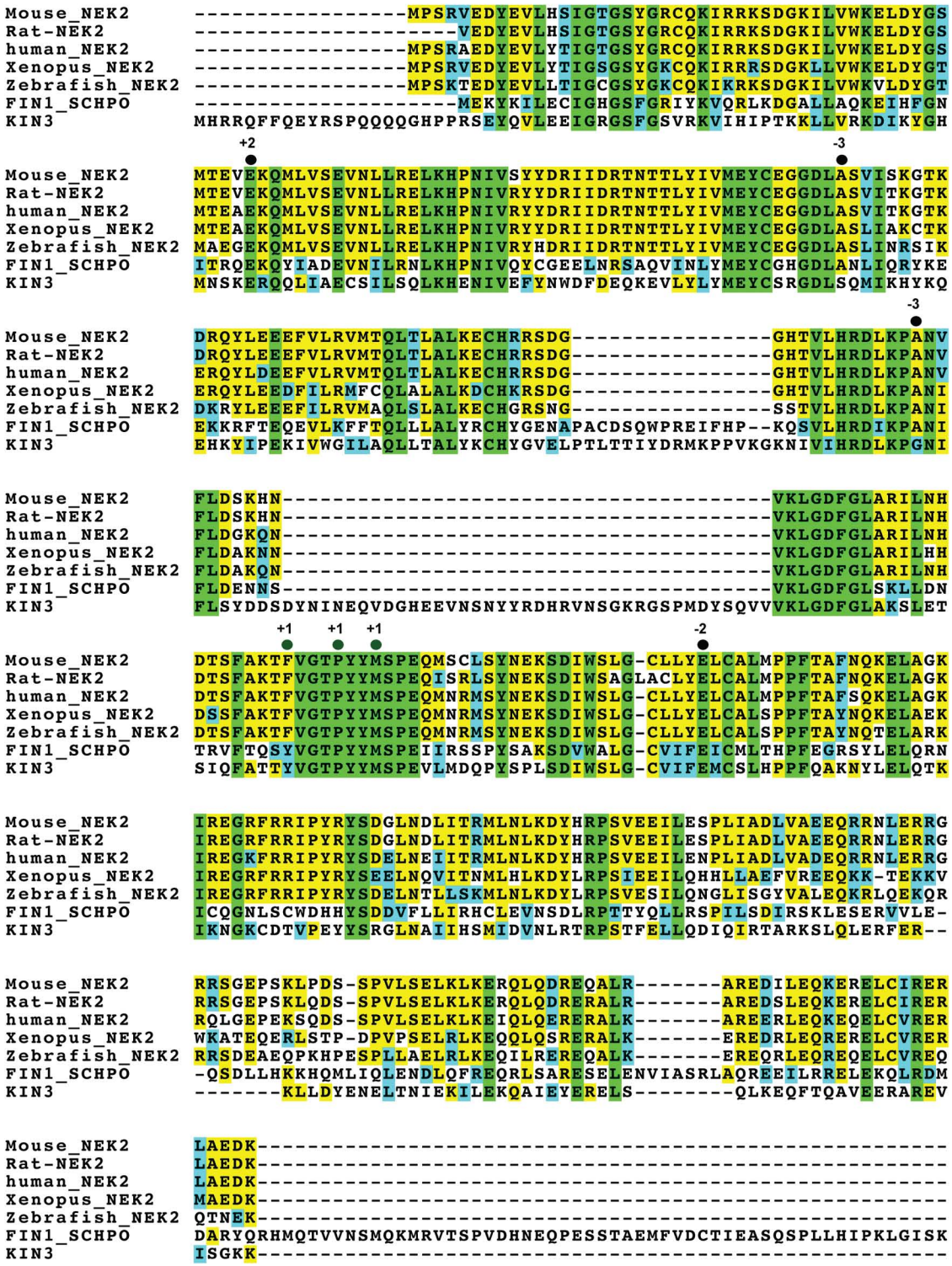

Figure S5. Conservation of specificity-determining residues in Nek2.
A sequence alignment of the kinase domains of Nek2 orthologs from Mus musculus, Rattus norwegicus, Homo sapiens, Xenopus laevis, Danio rerio, Schizosaccharomyces pombe, and Saccharomyces cerevisiae. Residues implicated in motif selection 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 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#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 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		