Supporting Information

Klebba et al. 10.1073/pnas.1417967112

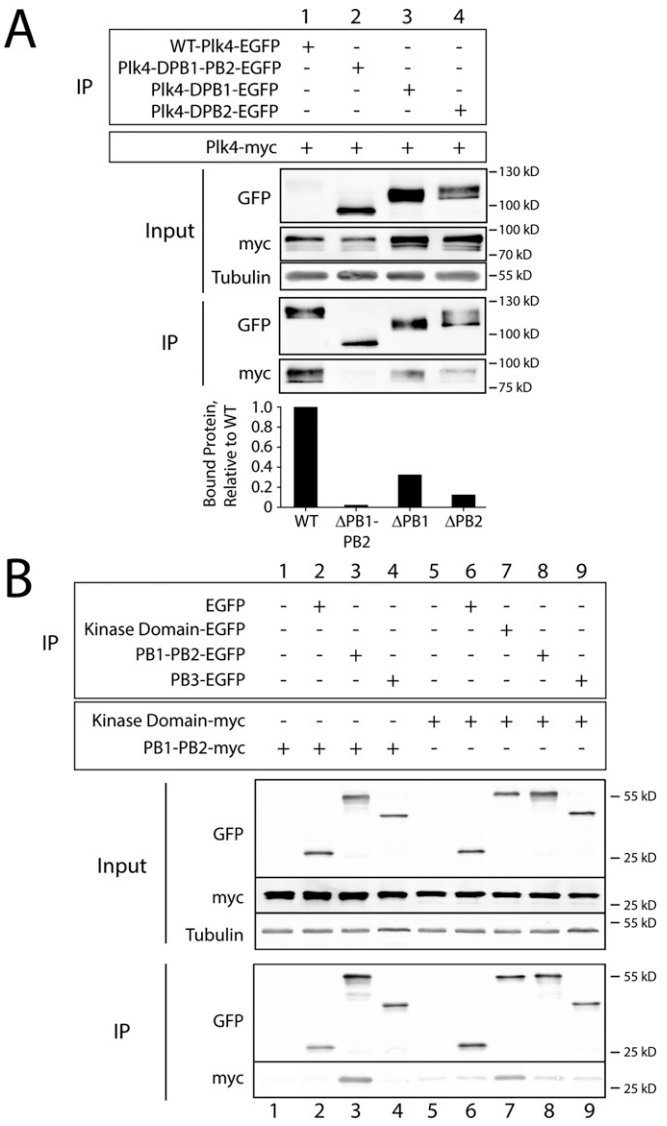

Fig. S1. PB1–PB2 is necessary and sufficient for homodimerization. (*A*) PB1 and PB2 are critical for Plk4 homodimerization. Anti-GFP IPs were prepared from lysates of S2 cells transiently coexpressing the indicated inducible EGFP- and WT-myc–tagged Plk4 constructs. The PB3 domain was present in all constructs. Blots of the input lysates and IPs were probed for α-tubulin, GFP, and myc. Amounts of copurifying Plk4-myc recovered in each GFP IP were measured by densitometry of the immunoblots, normalized to the measured amount of Plk4-EGFP in the same IP sample, and then graphed relative to the normalized amount of WT-Plk4-myc recovered in the WT-Plk4-EGFP IP in lane 1. (*B*) PB1–PB2 is sufficient for dimerization. Anti-GFP IPs were prepared from lysates of S2 cells transiently coexpressing the indicated inducible EGFP- and myc-tagged Plk4 constructs containing EGFP alone, a kinase domain, PB1–PB2, or PB3. Blots of the input lysates and IPs were probed for α-tubulin, GFP, and myc. PB1–PB2 interacts strongly with itself (lane 3) but not with PB3 (lane 4). We note that Plk4 kinase domain associates with itself (lane 7) and also binds the GBP-coated beads (lane 5), explaining its presence in the PB1–PB2 and PB3 IPs (lanes 8 and 9).

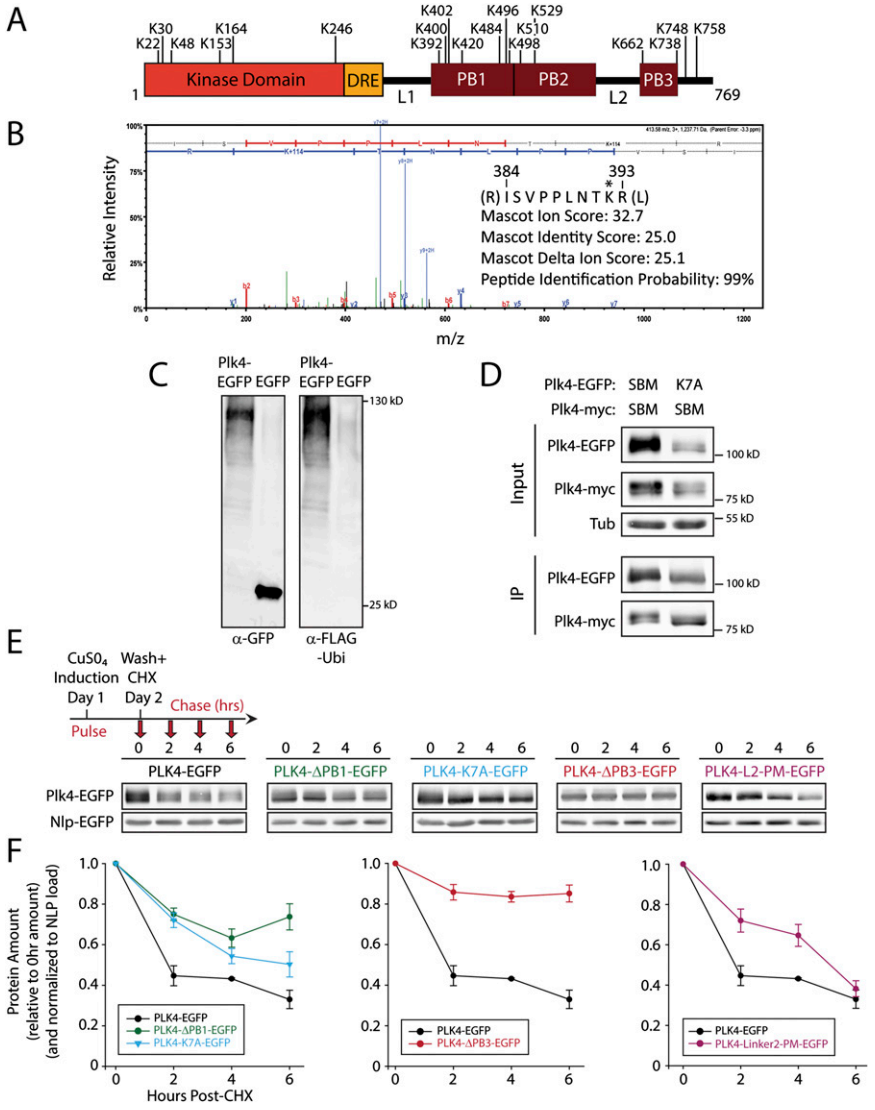

Fig. S2. Trypsinized Plk4 bears numerous Gly–Gly modifications (Ubi remnants), many of which are found in PB1. (*A*) An immunoprecipitated sample of WT-Plk4-EGFP from S2 cell lysates was resolved on SDS/PAGE, processed (including trypsinization) for MS analysis, and then analyzed by MS/MS to identify Gly–Gly–modified residues. From 97% coverage of full-length Plk4 (which included all Lys resides), 19 Gly–Gly–modified Lys were identified; their positions are indicated in the linear map depicting the Plk4 domain structure. Seven of the Gly–Gly–modified Lys reside in PB1. (*B*) Samples of Plk4 were prepared for MS analysis from in vivo ubiquitinated Plk4-EGFP immunoprecipitated from S2 cell lysates. Immunoprecipitated proteins were resolved by SDS/PAGE, and the band corresponding to Plk4-EGFP was cut from the gel, processed, and then analyzed by MS/MS. An example of a mass spectrum is shown. The sequence of the PB1 peptide is shown in bold; the Gly–Gly–labeled Lys residue (K392) is indicated by an asterisk. (*C*) 3xFLAG-tagged Ubi specifically labels Plk4-EGFP but not EGFP. Anti-GFP immunoprecipitates of lysates prepared from S2 cells transiently expressing 3xFLAG-Ubi and either EGFP or Plk4-EGFP were probed with anti-GFP and FLAG antibodies. (*D*) The K7A mutation in PB1 does not prevent Plk4 homodimerization. Anti-GFP IPs were prepared from lysates of S2 cells transiently co-expressing the indicated inducible EGFP- and myc-tagged Plk4 constructs. Blots of the input lysates and IPs were probed for α-tubulin, GFP, and myc. Non-degradable SBM-Plk4 was used in these IPs because it is abundant and easily detected. (*E*) Measurements of Plk4 mutations that suppress protein turnover. S2 cells cotransfected with Plk4-EGFP or the indicated Plk4 mutant were induced to express for 24 h and then were treated with fresh medium and CHX to inhibit protein translation. Immunoblots of cell lysates were probed for GFP. Plk4-EGFP protein turnover was analyzed by densitometry of the anti-GFP immunoblots over a 6-h time course. Cotransfected Nlp-EGFP was used as a loading control. (*F*) Graphs show relative Plk4-EGFP levels normalized against Nlp-EGFP over the 6-h time course for three experiments. Error bars show SEM.

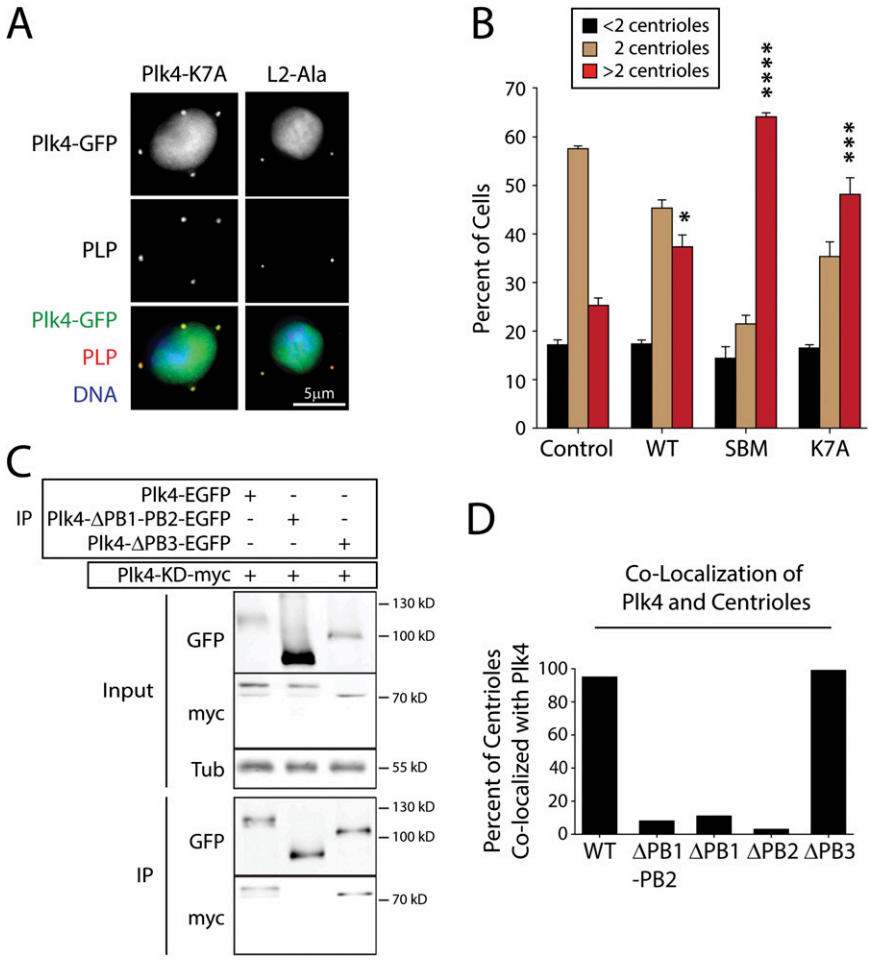

Fig. S3. Plk4-K7A localizes to centrioles and induces centriole amplification. Unlike Plk4–ΔPB1–PB2, Plk4-ΔPB3 binds KD-Plk4 and localizes to centrioles. (*A*) S2 cells coexpressing the indicated Plk4-EGFP construct (green) and the transfection marker Nlp-EGFP (green nuclei) were immunostained for PLP to mark centrioles (red). DNA is shown as blue. Both Plk4 mutants K7A and L2-PM localize to centrioles in interphase cells. (*B*) Transfected S2 cells were induced to express Plk4-EGFP constructs for 3 d, then were immunostained for PLP, and their centrioles were counted. Nlp-EGFP–transfected cells were used as control. Each bar shows the average percentage of cells containing the indicated number of centrioles ($n = 300$ cells from each of three experiments). Asterisks mark significant differences (relative to control) for comparisons mentioned in the text. Error bars indicate SEM. Centriole amplification (an increase in the percentage of cells with more than two centrioles) occurs in cells expressing WT-Plk4 ($P = 0.01$), SBM ($P < 0.0001$), and K7A ($P = 0.0003$). (*C*) Anti-GFP IPs were prepared from lysates of S2 cells transiently co-overexpressing the indicated inducible EGFP- and myc-tagged Plk4 constructs. Blots of the input lysates and IPs were probed for α-tubulin, GFP, and myc. (*D*) Graph shows the percent of centrioles (labeled with the centriolar marker PLP) that colocalize with the indicated Plk4-GFP protein. Colocalization was scored in cells that were clearly GFP positive (100 cells and >245 total centrioles were examined for each construct).

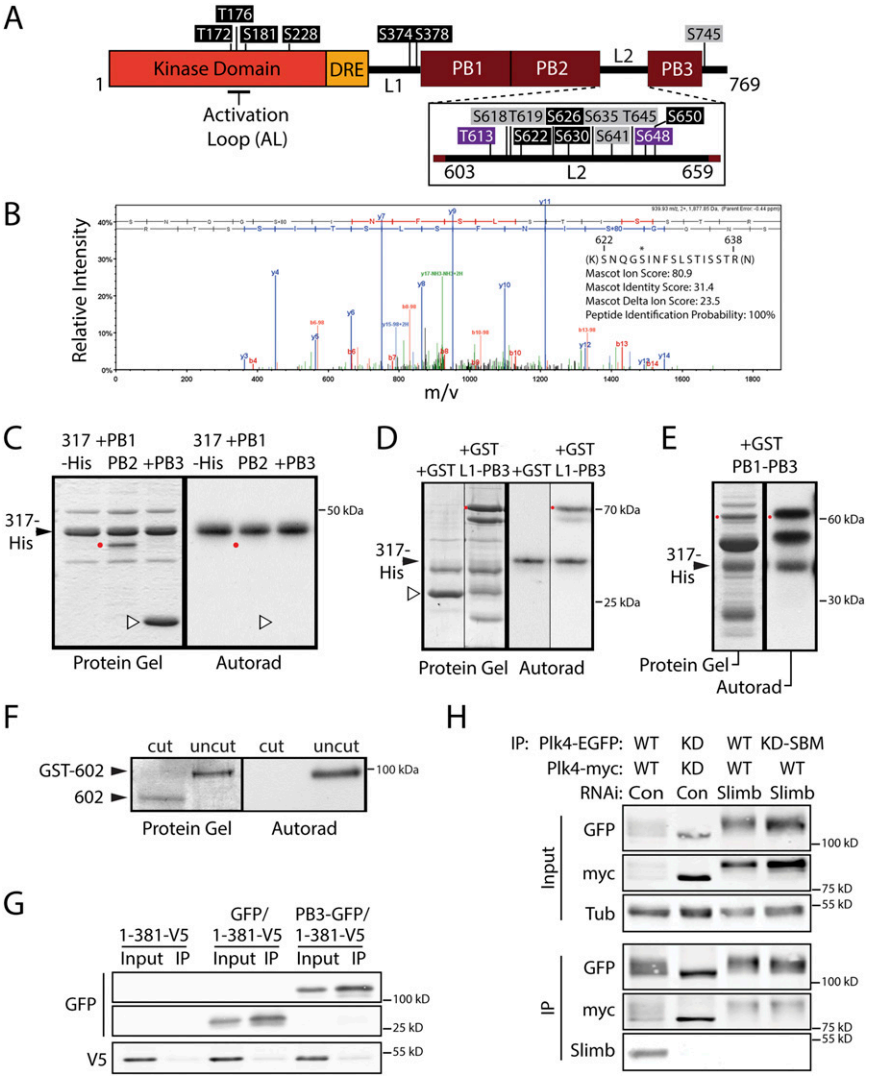

Fig. S4. Plk4 extensively autophosphorylates numerous residues within the linker and loop regions but not in its Polo boxes. (*A*) Immunoprecipitation of WT-Plk4-EGFP from S2 cell lysates was subjected to MS/MS to identify phosphorylated Ser and Thr residues. From 97% coverage of full-length Plk4, MS identified 16 in vivo Ser/Thr phosphorylated residues, not including residues within the DRE that were described previously (1). To prepare the in vitro samples for MS analysis, bacterially expressed and purified Plk4–DRE-His_6 containing only the kinase domain and DRE (amino acids 1–317) or GST-1–602 (Fig. 5*A*) were incubated with or without MgATP and either were analyzed alone (KD forms of both proteins were analyzed also) or were mixed with various GST- or His_6-tagged downstream regions. Proteins then were resolved by SDS/PAGE, and phosphorylated bands were cut from the gel, processed, and analyzed by MS/MS. The positions of the phospho-Ser/Thr residues are indicated in the linear map depicting the Plk4 domain structure. Phosphorylated residues observed only in in vivo samples are highlighted in gray; those observed only in vitro are highlighted in purple; and those identified in both samples are highlighted in black. (*B*) An example of a mass spectrum obtained from the analysis of in vivo phosphorylated Plk4. The sequence of the peptide is shown; the phosphorylated residue (S626) is indicated by an asterisk. (*C*–*E*) In vitro kinase assays of purified His_6-tagged Plk4 kinase domain + DRE (amino acids 1–317; denoted as "317-His") mixed with various Plk4 fusion proteins. The Coomassie-stained SDS/PAGE protein gels and their corresponding autoradiographs are shown. (*C*) 317-His autophosphorylates (lane 1, black arrowhead) but does not phosphorylate purified His_6-tagged PB1–PB2 (lane 2, red circle) or PB3 (lane 3, white arrowhead) proteins. (*D*) 317-His does not phosphorylate purified GST protein alone (white arrowhead) but does phosphorylate GST-L1–PB3 (amino acids 318–741) (red circle). (*E*) 317-His also phosphorylates GST-PB1–PB3 protein (amino acids 382–741) (red circle). Both GST-L1–PB3 and GST-PB1–PB3 displayed some proteolysis and were phosphorylated. (*F*) Proteolytic removal of GST from Plk4 1–602 inhibits kinase activity. In vitro kinase assays of purified Plk4–GST-602 (containing L1 but not L2 or PB3) (uncut) compared with the same protein in which GST was removed using PreScission Protease (cut; GE Healthcare). The Coomassie-stained SDS/PAGE protein gels and their corresponding autoradiographs are shown. (*G*) Plk4 1–381-V5 (containing kinase domain-DRE-L1) does not coimmunoprecipitate with PB3-EGFP. Anti-GFP IPs were prepared from lysates of S2 cells transiently co-overexpressing the inducible PB3-EGFP and 1–381-V5–tagged Plk4 constructs. (*H*) Plk4 kinase activity promotes dimer separation. Anti-GFP IPs were prepared from lysates of S2 cells transiently co-overexpressing the indicated inducible EGFP- and myc-tagged Plk4 constructs and RNAi-treated with control or Slimb dsRNA for 5 d. Blots of the input lysates and IPs were probed for α-tubulin, Slimb, GFP, and myc. The greatest recovery of coprecipitated Plk4-myc is observed when two KD-Plk4 proteins are coexpressed (lane 2), indicating that dimer association is most stable in absence of kinase activity. Dimerization was not influenced by turnover of the constructs, because Slimb depletion dramatically stabilized Plk4 levels but had no qualitative effect on the amount of Plk4-myc recovered in the IP (lanes 1 and 3).

1. Klebba JE, et al. (2013) Polo-like kinase 4 autodestructs by generating its Slimb-binding phosphodegron. *Curr Biol* 23(22):2255–2261.

Table S1. In vivo ubiquitination sites of *Drosophila* Plk4

Modified Lys	Peptide sequence	Mascot ion score	Mascot identity score	Mascot Δ ion score	Peptide identification probability, %
K22	(R)AFGETIEDYEVQHLLG**K**GGFATVYK(A)	70.1	32.1	17.2	100
K30	(R)AFGETIEDYEV**Q**HLLGKGGFATVY**K**AR(C)	88.2	30.6	24.0	100
K48	(K)**K**LIQGTGLTNR(V)	27.9	25.0	21.8	94
K153	(R)E**M**HV**K**IADFGLATQLK(R)	31.8	28.9	23.0	98
K164	(K)IADFGLATQL**K**RPDER(H)	28.1	28.8	20.9	94
K246	(K)VV**M**SEYI**M**PAHLSYEA**Q**DLI**NK**LLK(K)	52.7	30.1	6.4	100
K246	(K)VV**M**SEYI**M**PAHLSYEAQDLIN**K**LLK(K)	47.5	30.0	5.1	100
K246	(K)VV**M**SEYI**M**PAHLSYEAQDLI**NK**LLK(K)	29.9	33.1	8.2	92
K392	(R)ISVPPLNT**K**R(L)*	32.7	25.0	25.1	99
K400	(R)Y**K**TK**N**AI**M**SILR(N)	26.4	25.7	3.1	96
K400	(R)Y**K**TKNAI**M**SILR(N)	24.1	25.3	4.9	94
K402	(K)T**K**NAI**M**SILR(N)	25.6	26.2	16.6	94
K402	(K)T**K**NAI**M**SILR(N)	21.3	26.0	4.9	85
K420	(R)NGEVVLEFL**K**FRPTYNEDR(I)	40.2	31.4	35.2	99
K484	(K)**K**YIYGAR(F)	24.1	25.0	8.1	91
K496	(R)FVGLV**K**SK(T)	37.0	25.0	14.5	100
K498	(K)S**K**TPKVTYFSTLGK(C)	30.9	26.8	3.9	98
K510	(K)VTYFSTLG**KC**QL**M**ET**M**TDFEIR(F)	42.3	34.4	31.3	99
K529	(R)FYSGA**K**LLK(T)	20.8	25.0	13.6	82
K662	(R)SN**M**LAAHQNIPI**K**R(I)	35.6	28.5	21.9	99
K738	(K)L**K**TAPLLGSGR(K)	35.2	25.0	27.6	99
K748	(R)**K**TDYNNA**M**TPK(T)	58.8	28.9	46.8	100
K758	(K)TDYNNA**M**TP**K**TTTPYYNR(M)	41.7	33.0	35.9	99

Transgenic full-length Plk4-EGFP was affinity-purified from lysates of transfected S2 cells, resolved by SDS/PAGE, processed (e.g., trypsinized) for MS, and then analyzed by MS/MS (*Materials and Methods*). Multiple experiments were analyzed, and their combined results cover 97% of the Plk4 sequence. Gly–Gly modifications are indicated by a +114 Da mass increase and are the remnants of trypsinized ubiquitin modifications. Only Lys were observed to be Gly–Gly modified. In the table, the modified residues of recovered peptides are underlined and in bold font; all Lys modifications are Gly–Gly. Shaded rows refer to residues within PB1 of Plk4. Note that not all recovered peptides are listed. Mascot parameters were obtained from Scaffold 4.2.1 (Proteome Software).
*The spectrum of this fragment (K392) is shown in Fig. S2*B*.

Table S2. Phosphorylation sites of the kinase, L1, L2, and C-terminal domains of *Drosophila* Plk4

Modified residue	Peptide sequence	Mascot ion score	Mascot identity score	Mascot Δ ion score	Peptide identification probability, %
Phosphorylation modifications observed in vivo					
T172	(R) HM**T**MCGTPNYISPEVVSR (T)	31.1	31.1	10.0	95
T176	(R) HMTMCG**T**PNYISPEVVSR (T)	27.3	30.6	1.7	89
S181	(R) HMTMCGTPNYI**S**PEVVSR (T)	58.8	31.1	18.1	100
S228	(K) VVM**S**EYIMPAHLSYEAQDLINK (L)	39.7	34.1	21.6	99
S374	(K) SSAFCMEAGNVPN**S**KQASLKEDR (I)	29.9	33.8	7.4	88
S378	(K) SSAFCMEAGNVPNSKQA**S**LKEDR (I)	40.3	33.5	7.2	99
S378	(K) QA**S**LKEDRISVPPLNTK (R)	24.8	29.9	17.9	84
S618	(R) DTTNIAF**S**TPKSNQGSINFSLSTISSTR (N)	52.8	34.1	0.0	100
T619	(R) DTTNIAFS**T**PKSNQGSINFSLSTISSTR (N)	50.7	34.2	0.4	100
S622	(K) **S**NQGSINFSLSTISSTR (N)	86.2	34.2	26.2	100
S626	(K) SNQG**S**INFSLSTISSTR (N) *	80.9	31.4	23.5	100
S630	(K) SNQGSINF**S**LSTISSTR (N)	82.9	31.4	15.8	100
S635	(K) SNQGSINFSLSTI**S**STR (N)	55.3	31.4	13.2	100
S635	(K) SNQGSINFSLSTI**S**STR (N)	47.3	31.4	8.0	100
S641	(R) NT**S**DFGTNCSR (S)	43.4	25.0	13.8	100
T645	(R) NTSDFG**T**NCSR (S)	34.1	25.0	2.8	98
S648	(R) NTSDFGTNC**S**RSNMLAAHQNIPIKR (I)	45.8	34.1	4.1	95
S650	(R) **S**NMLAAHQNIPIKR (I)	27.1	30.4	0.0	89
Phosphorylation modifications observed in vitro					
T172	(R) HM**T**MCGTPNYISPEVVSR (T)	72.9	39.4	45.8	100
T176	(R) HMTMCG**T**PNYISPEVVSR (T)	57.6	39.6	1.2	100
T176	(R) HMTMCG**T**PNYISPEVVSR (T)	56.4	39.5	1.1	100
S181	(R) HMTMCGTPNYI**S**PEVVSR (T)	51.0	39.2	17.0	100
S228	(K) VVM**S**EYIMPAHLSYEAQDLINK (L)	56.9	40.8	24.2	100
S374	(K) SSAFCMEAGNVPN**S**KQASLKEDR (I)	63.3	41.1	10.1	99
S378	(K) SSAFCMEAGNVPNSKQA**S**LKEDR (I)	53.4	41.4	17.2	99
S374, S378	(K) SSAFCMEAGNVPNSKQA**S**LKEDR (I)	34.0	40.4	18.4	92
T613	(R) LDGLRDT**T**NIAFSTPK (S)	41.5	30.9	2.5	96
S622	(K) **S**NQGSINFSLSTISSTR (N)	52.5	31.5	0.0	100
S626	(K) SNQG**S**INFSLSTISSTR (N)	70.4	31.2	12.1	100
S630	(K) SNQGSINF**S**LSTISSTR (N)	81.2	31.2	15.2	100
S648	(R) NTSDFGTNC**S**RSNMLAAHQNIPIKR (I)	45.8	34.1	4.1	95
S648	(R) NTSDFGTNC**S**RSNMLAAHQNIPIKR (I)	42.4	34.0	1.3	93

To identify in vivo phosphorylated residues, transgenic full-length Plk4-EGFP was affinity-purified from lysates of transfected S2 cells, resolved by SDS/PAGE, processed (e.g., trypsinized) for mass spectrometry, and then analyzed by MS/MS (*Materials and Methods*). Four in vivo experiments were analyzed; their combined results cover 97% of the Plk4 sequence. To identify in vitro phosphorylated residues, bacterially expressed and purified Plk4 domains were incubated with purified and active Plk4 kinase domain and then were similarly processed and analyzed by MS/MS. The phosphorylated Ser or Thr residues of recovered peptides are underlined and in bold font. Note that not all recovered peptides are listed. Mascot parameters were obtained from Scaffold 4.2.1 (Proteome Software).

*The spectrum of this fragment is shown in Fig. S4*B*.