Cks Confers Specificity to Phosphorylation-Dependent Cdk Signaling Pathways

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Supplementary Material

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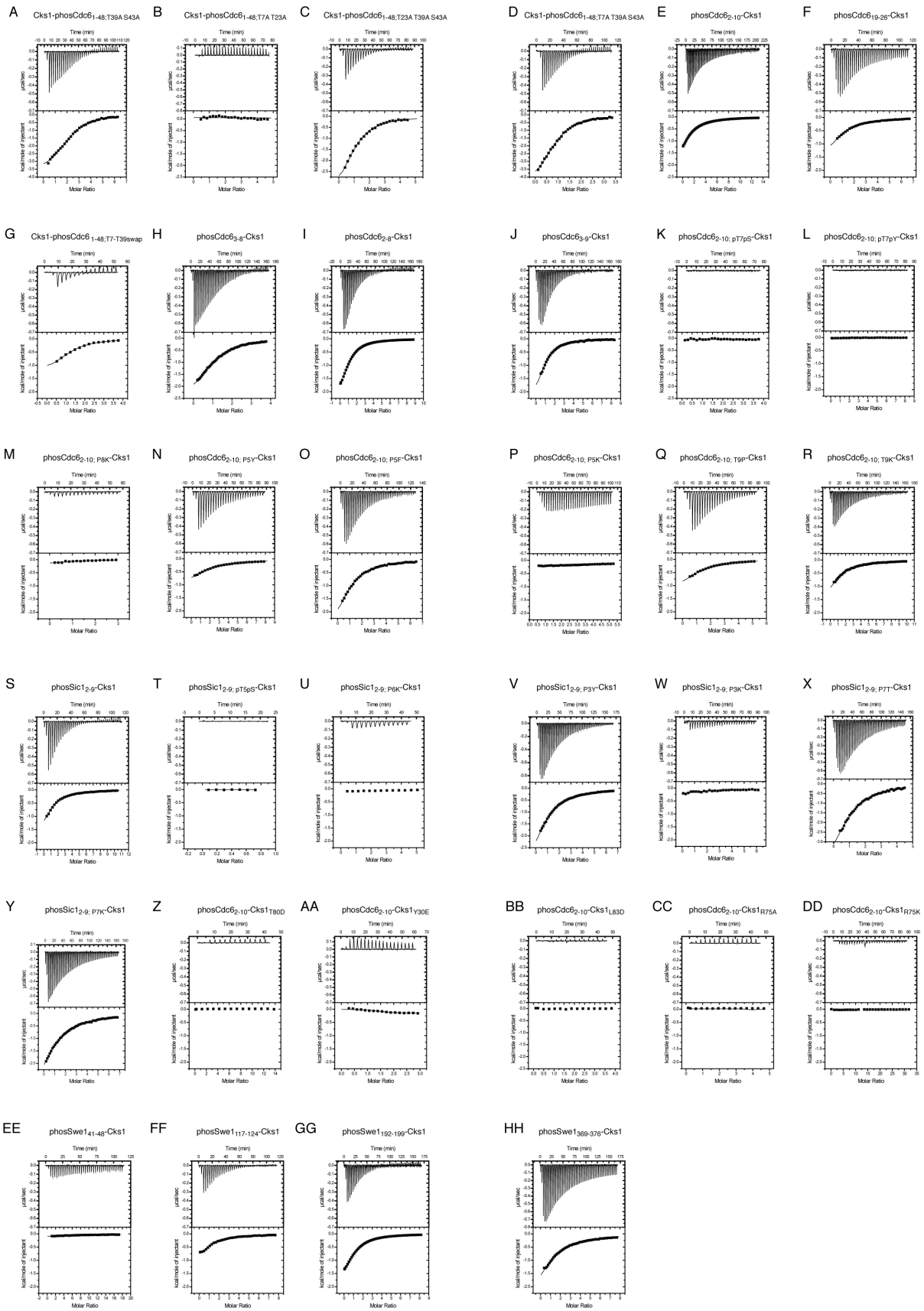

Supplementary Figure 1. Representative isothermal titration calorimetry data.

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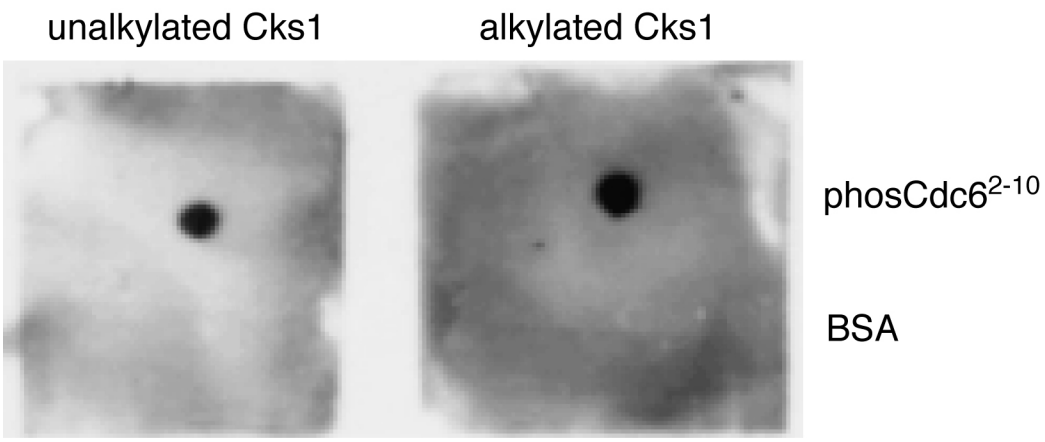
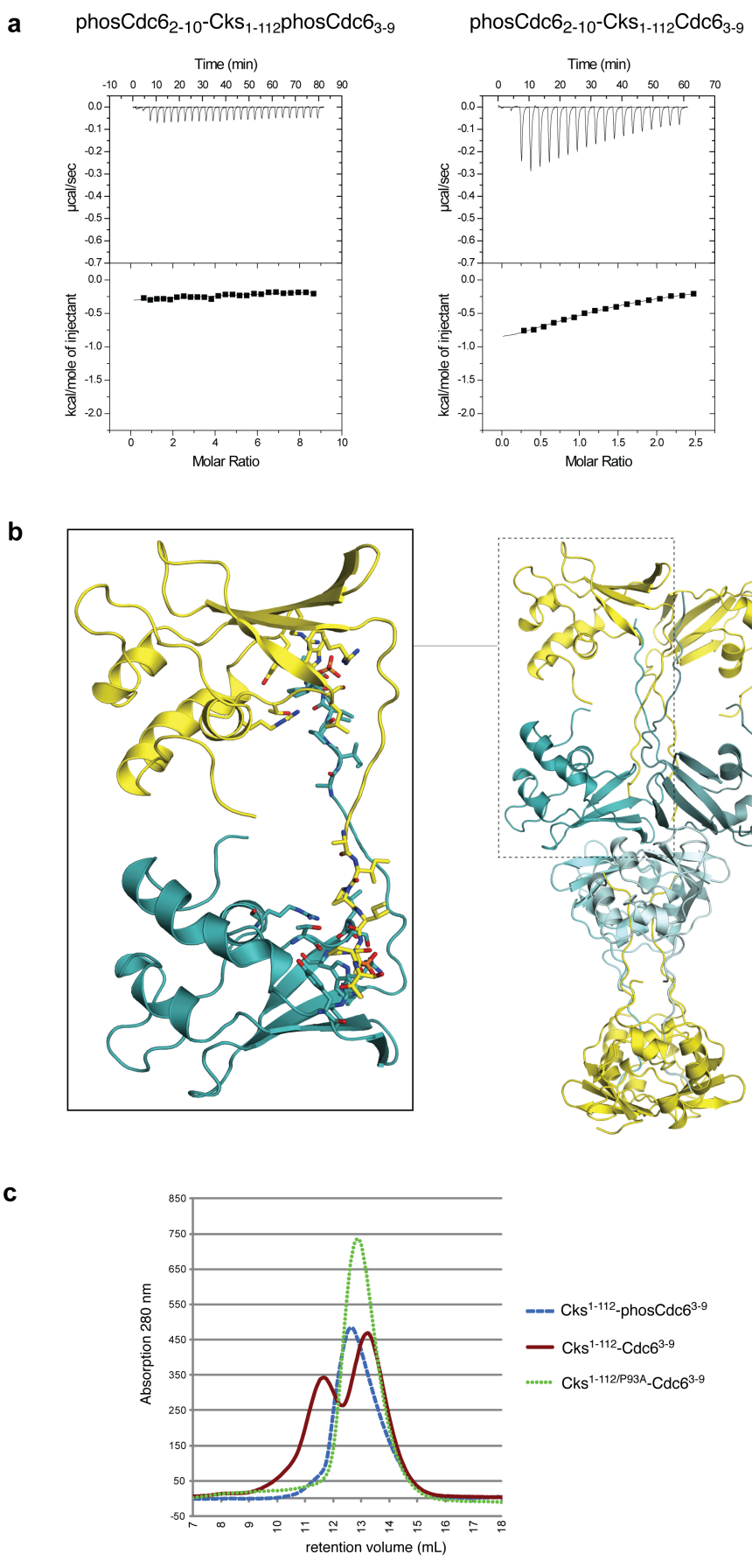
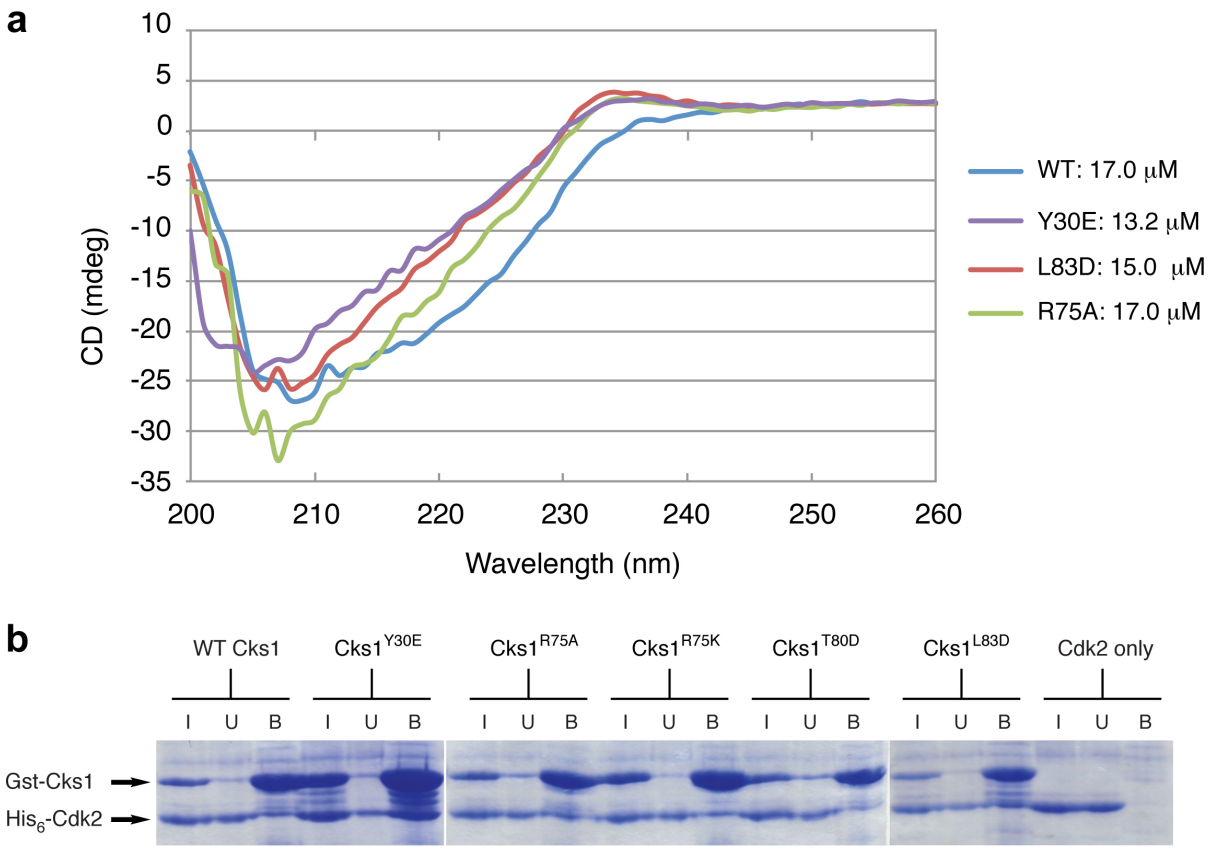


Supplementary Figure 2. Alkylation of Cks1 does not abrogate binding of phosphopeptides. The wild-type phosCdc6$_{2-9}$ peptide was probed with Cks1 and alkylated Cks1 as described for Figure 2a. Alkylation, which was confirmed by mass spectrometry, occurs on a single cysteine that is distal to the phosphate-binding pocket.


a
phosCdc6$_{2-10}$-Cks$_{1-112}$phosCdc6$_{3-9}$
phosCdc6$_{2-10}$-Cks$_{1-112}$Cdc6$_{3-9}$
Time (min)
µcal/sec
kcal/mole of injectant
Molar Ratio
b
c
Absorption 280 nm
retention volume (mL)
Cks$^{1-112}$-phosCdc6$^{3-9}$
Cks$^{1-112}$-Cdc6$^{3-9}$
Cks$^{1-112/P93A}$-Cdc6$^{3-9}$

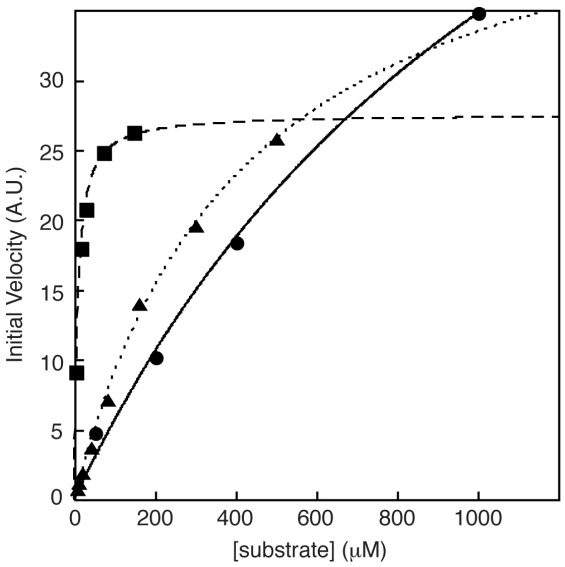
Supplementary Figure 3.

Domain swapping and binding properties of the Cdc6-Cks1 fusion construct. (a) ITC data demonstrate that the phosCdc6$_{3-9}$-Cks1$_{1-112}$ fusion protein does not bind the phosphorylated Cdc6 peptide *in trans* (left), while the unphosphorylated fusion binds the Cdc6 peptide similar to wild-type Cks1 (right). These measurements demonstrate that the phosCdc6 sequence in the fusion can bind Cks1 *in cis* at the appropriate site and exclude the added peptide. (b) In the crystal structure, the fusion undergoes domain swapping in which the phosCdc6 sequence at the C-terminus of one fusion molecule extends to bind the cationic pocket site on a different Cks1 molecule, related by crystallographic symmetry. Four molecules (cyan) are observed in the asymmetric unit. Unlike in several other Cks crystals[34,35], there is no evidence that Cks1$_{1-112}$-phosCdc6$_{3-9}$ forms domain-swapped dimers by exchanging strand 4, although the electron density for the loop preceding strand 4 is weak. Instead, dimers are formed between a molecule in the asymmetric unit (cyan) and its crystallographic symmetry mate (yellow) by the swapping of appended phosCdc6$_{3-9}$ sequences. (c) Superdex 75 gel filtration analysis demonstrates that the phosphorylated fusion behaves as a monomer in gel filtration. The unphosphorylated fusion (maroon line) elutes from gel filtration as a double peak, consistent with a monomer-dimer equilibrium in solution and suggesting that the construct undergoes domain swapping similar to Cks1. The phosphorylated phosCdc6$_{3-9}$-Cks1$_{1-112}$ (blue dashes) elutes as a single peak and in the same volume as the monomer-promoting mutant Cdc6$_{3-9}$-Cks1$_{1-112\ P93A}$ (green dots)[55]. We conclude that phosCdc6$_{3-9}$-Cks1$_{1-112}$ fusion protein is a monomer in solution. It is likely that the phosphorylated Cdc6 sequence binds to Cks1 *in cis*, which disrupts the domain-swapped dimerization that occurs through strand 4 in the wild-type protein *in vitro* at high concentrations.



Supplementary Figure 4.

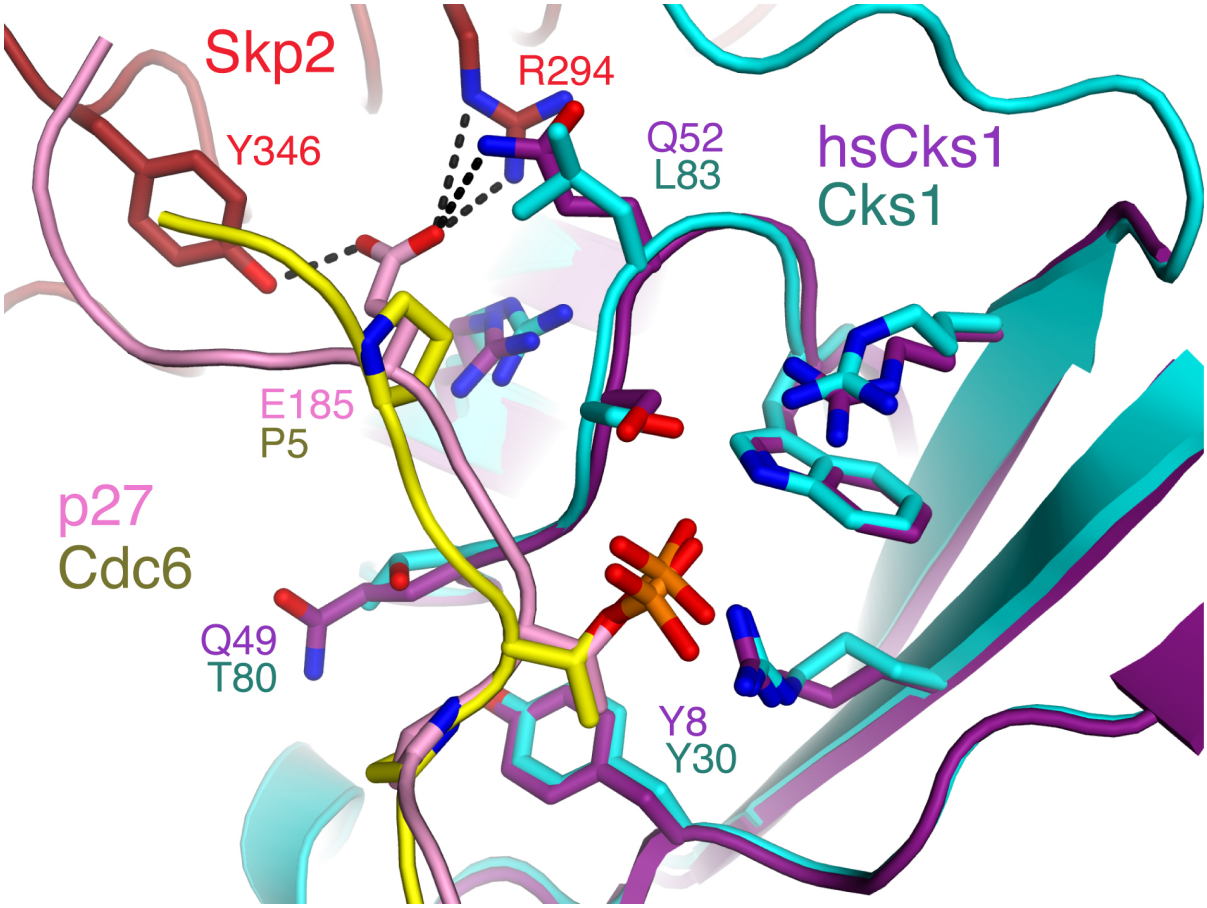
Mutations to Cks that disrupt consensus sequence binding do not disrupt folding or Cdk binding. (a) Circular dichroism spectra of wild-type and mutant Cks1 proteins at a concentration of ~10 μM were obtained as previously described[55]. The spectra demonstrate that the mutations do not disrupt proper folding of Cks1. (b) Binding of Cks1 consensus site mutants to recombinant human Cdk2. The Cks-Cdk interface is highly conserved, and budding yeast Cks1 binds Cdk2 with micromolar affinity[55,56]. We used Cdk2 to facilitate the *in vitro* binding experiment with recombinant proteins. We mixed 12.5 μM purified His_6-Cdk2 with 12.5 μM of the indicated GST-tagged Cks1 protein in a buffer containing 150 mM NaCl, 25mM Tris, and 1 mM DTT (pH 8). The 1 mL binding reaction was mixed with 100 μL GS4B-Sepharose beads and the precipitated beads were washed with binding buffer and eluted with SDS-PAGE sample loading buffer. Input (I), flow-through unbound (U), and bound (B) protein fractions were run on SDS-PAGE and stained with Coomassie Blue. Wild-type and consensus site mutants all bind Cdk2 similarly, which is consistent with the structural observation that the phosphopeptide and Cdk binding interfaces are on opposite faces of Cks (Figure 3a).



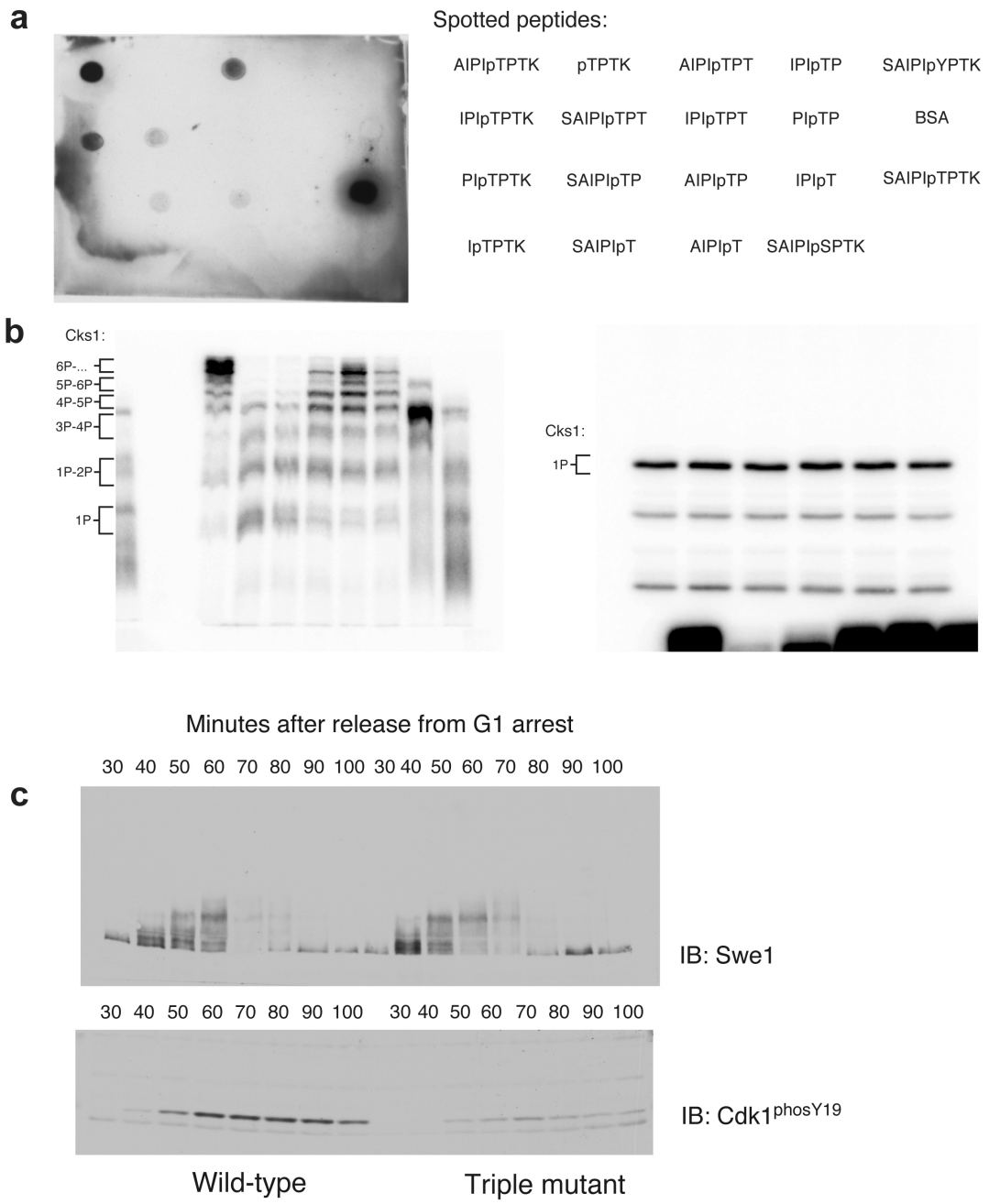
WT Cks1:

Substrate peptide	K_M (μM)	V_{max} (A.U.)	k_{cat} (min^{-1})	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)
● H1	1300 ± 400	80 ± 20	256*	0.2 ± 0.1
▲ Sic1ΔC-T5	420 ± 40	47 ± 3	150 ± 40	0.4 ± 0.1
■ Cdc6 WT	8.3 ± 0.5	27 ± 4	120 ± 30	14 ± 6

Supplementary Figure 5. Comparison in Cdk kinase reaction of unprimed to primed substrate. Kinase reactions are carried out as described in Figure 4 with wild-type Cks. Both the H1 peptide and Sic1ΔC-T5 construct contain single phosphoacceptor sites. In the Sic1ΔC-T5 construct, all the phosphoacceptor sites except T5 are mutated. The Cdc6 peptide contains phosphorylated T7 and a phosphoacceptor site at T23. The k_{cat} for the reaction with the H1 peptide (marked with *) was previously determined[9]. Absolute k_{cat} values reported here and in Figure 4 were calculated by comparison of the V_{max} value in each experiment to the V_{max} determined here for the H1 peptide.



Supplementary Figure 6. Comparison of the phosCdc6-Cks1 structure determined here with the structure of phosp27-hsCks1-Skp2 (PDB code: 2AST)[37].



Supplementary Figure 7. Uncropped blots and audioradiograms for key data shown in the main text. Data are shown for the experiments in (a) Figure 2a, (b) Figure 4a, and (c) Figure 6.

References

54. Balog, E.R. et al. The structure of a monomeric mutant Cks protein reveals multiple functions for a conserved hinge-region proline. *J Mol Biol* 411, 520-8 (2011).
55. Richardson, H.E., Stueland, C.S., Thomas, J., Russell, P. & Reed, S.I. Human cDNAs encoding homologs of the small p34Cdc28/Cdc2-associated protein of Saccharomyces cerevisiae and Schizosaccharomyces pombe. *Genes Dev* 4, 1332-44 (1990).

Supplementary Table 1
Binding affinities of Cks1 to phosphorylated Cdk substrate peptides. Data are shown in the indicated Supplementary Figure 1 panel.

Peptide	Sequence	Cks1 K_d (μM)	Supplementary Figure
phosCdc6$^{2-10}$	SAIPI(pT)PTK	130 ± 60	1E
phosCdc6$^{19-26}$	APAp(T)PPR	140 ± 30	1F
phosCdc6$^{3-8}$	AIPI(pT)P	81 ± 6	1H
phosCdc6$^{2-8}$	SAIPI(pT)P	62 ± 2	1I
phosCdc6$^{3-9}$	AIPI(pT)PT	61 ± 9	1J
phosCdc6$^{2-10;\ pT7pS}$	SAIPI(pS)PTK	no heat	1K
phosCdc6$^{2-10;\ pT7pY}$	SAIPI(pY)PTK	no heat	1L
phosCdc6$^{2-10;\ P8K}$	AIPI(pT)KTK	no heat	1M
phosCdc6$^{2-10;\ P5Y}$	AIYI(pT)PTK	190 ± 1	1N
phosCdc6$^{2-10;\ P5F}$	AIFI(pT)PTK	50 ± 30	1O
phosCdc6$^{2-10;\ P5K}$	AIKI(pT)PTK	no heat	1P
phosCdc6$^{2-10;\ T9P}$	AIPI(pT)PPK	129 ± 8	1Q
phosCdc6$^{2-10;\ T9K}$	AIPI(pT)PKK	158 ± 4	1R
phosSic1$^{2-9}$	TPS(pT)PPRS	95 ± 8	1S
phosSic1$^{2-9;\ pT5pS}$	TPS(pS)PPRS	no heat	1T
phosSic1$^{2-9;\ P6K}$	TPS(pT)KPRS	no heat	1U
phosSic1$^{2-9;\ P3Y}$	TYS(pT)PPRS	90 ± 50	1V
phosSic1$^{2-9;\ P3K}$	TKS(pT)PPRS	no heat	1W
phosSic1$^{2-9;\ P7T}$	TPS(pT)PTRS	70 ± 20	1X
phosSic1$^{2-9;\ P7K}$	TPS(pT)PKRS	120 ± 20	1Y
phosSwe1$^{41-48}$	IGGS(pT)PTN	no heat	1EE
phosSwe1$^{117-124}$	ESVT(pT)PIT	170 ± 90	1FF
phosSwe1$^{192-199}$	RIPE(pT)PVK	60 ± 10	1GG
phosSwe1$^{369-376}$	EEIS(pT)PTR	160 ± 70	1HH