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Cks confers specificity to phosphorylation-dependent CDK signaling pathways

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Cks is an evolutionarily conserved protein that regulates cyclin-dependent kinase (CDK) activity. Clarifying the underlying mechanisms and cellular contexts of Cks function is critical because Cks is essential for proper cell growth, and its overexpression has been linked to cancer. We observe that budding-yeast Cks associates with select phosphorylated sequences in cell cycle–regulatory proteins. We characterize the molecular interactions responsible for this specificity and demonstrate that Cks enhances CDK activity in response to specific priming phosphosites. Identification of the binding consensus sequence allows us to identify putative Cks-directed CDK substrates and binding partners. We characterize new Cks-binding sites in the mitotic regulator Wee1 and discover a new role for Cks in regulating CDK activity at mitotic entry. Together, our results portray Cks as a multifunctional phosphoadaptor that serves as a specificity factor for CDK activity.

Protein kinases must recognize their proper target substrates and regulators from among a large number of proteins in the cell[1]. How specificity is achieved is a critical question, given the prominent role of phosphorylation in signal transduction and the misregulation of kinase activity in disease[2]. In the cell cycle, CDKs process signals that lead to cell division[3]. Hundreds of CDK substrates have been identified in proteomic screens, and CDK phosphorylation alters the location, interactions, stability and activity of these target proteins[4,5]. A deregulated cell cycle is a hallmark of cancer, thus emphasizing the need for tight coordination of CDK activity[6]. Still, many questions remain regarding how regulatory proteins recognize CDKs and how CDKs discriminate among substrates to phosphorylate them in the appropriate order and at the appropriate times in the cell cycle.

The CDK complex is composed of the kinase subunit, the cyclin subunit and Cks. The active site of the kinase recognizes a minimum consensus sequence of (S/T)P and an optimal consensus of (S/T)PX(R/K)[7]. In addition to activating the kinase domain, the cyclin subunit binds docking sequences present in some substrates and confers specificity[8–10]. Although Cks is essential for viability, and its deregulated expression correlates with tumorigenesis and poor cancer prognosis, its particular molecular functions have been less clear[11–17]. Genetic analysis has shown that Cks genes regulate cell growth and division[11,12,15]. In addition to binding CDK and influencing kinase function, Cks has been implicated in other cellular processes such as transcription and the degradation of the CDK inhibitor p27 (refs. 18–21).

Several studies have suggested that Cks associates with phosphorylated cell cycle–regulator proteins and has a role in CDK multisite phosphorylation. Multisite phosphorylation is critical for producing proper signaling output[22–27], because it influences properties such as sensitivity and switch-like behavior and permits integration of a large number of inputs to produce diverse outputs[28–31]. Entry into mitosis, for example, is a switch-like transition, and multisite phosphorylation of the mitotic regulators Wee1 and Cdc25 is critical for this behavior[23,24,26,32,33]. Similarly, multisite phosphorylation of Sic1 in budding yeast generates an ultrasensitive response for S-phase entry[22,25]. Different signaling behaviors are generated by differences in enzyme mechanism such as degree of cooperativity and processivity of substrate processing[30]. Therefore, uncovering mechanistic details of how CDK acts on multisite substrates is important for understanding the molecular origins of highly complex, vastly tunable signaling through phosphorylation.

A role for Cks in binding phosphorylated substrates was postulated after structures of Cks revealed a conserved cationic pocket that weakly binds free phosphate and other anions[34,35]. When Cks is bound to CDK, structural modeling suggests that this cationic pocket is part of a continuous surface including the CDK active site and cyclin[36]. In the specific context of the Skp2–Skp1–Cullin ubiquitin ligase, human Cks1 binds phosphorylated p27 to stimulate its ubiquitylation and degradation[37]. From these structural insights, it has been proposed that Cks binds CDK substrates primed by initial phosphorylation and facilitates further phosphorylation of the primed substrate. This hypothesis is supported by experiments that show that phosphorylation of cell cycle–regulatory proteins is reduced when Cks is immunodepleted from *Xenopus* egg extracts[38]. We found that semiprocessive phosphorylation of the G1-S regulator Sic1 depends on an intact Cks cationic pocket[25]. However, it has not been clarified whether the stimulatory role of Cks on CDK activity relies on

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specific priming sites or whether any site can prime the multisite phosphorylation reaction.

We demonstrate here that Cks recognizes specific phosphorylated sequences in CDK substrates, uncovering a new mechanism for CDK substrate targeting, CDK interactions with its regulators and signaling kinetics. We identify a Cks-binding consensus sequence and characterize the complex with a phosphopeptide–Cks crystal structure. Cks interaction with the consensus sequence is required for efficient multisite phosphorylation of a subset of CDK substrates and for budding-yeast viability. Using the Cks consensus sequence as a search motif, we identify new Cks-binding proteins and demonstrate a critical role for Cks in facilitating Wee1 regulation of CDK at mitotic entry.

RESULTS

Cks binds to specific phosphorylated sites in CDK substrates

A number of phosphorylation-dependent CDK–substrate complexes have been reported[23,39], and we and others have demonstrated that Cks binds to phosphorylated sequences in intrinsically disordered domains of several CDK substrates[25,40]. To test whether Cks binds specific phosphorylated sites, we examined the affinity of *Saccharomyces cerevisiae* Cks (Cks1) for the N-terminal domain of the replication-licensing factor Cdc6. As determined by isothermal titration calorimetry (ITC), Cks1 binds to phosphorylated (phos) $Cdc6_{1–48}$ with $K_d = 9.8 \pm 0.2$ μM, whereas no detectable heat was measured for the unphosphorylated protein (**Fig. 1a**). The presence of two distinct and independent Cks1-binding sites in $phosCdc6_{1–48}$ is supported by the stoichiometry parameter; the data fit to a one-site model with $n \approx 2$, thus indicating that two molecules of Cks1 bind noncooperatively to each phosphorylated Cdc6 molecule.

We then mutated different combinations of phosphoacceptor sites and found that Cks1 binds to $Cdc6_{1–48}$ phosphorylated only at T7 and T23 ($Cdc6_{1–48;T39A\ S43A}$) with a similar affinity and stoichiometry as fully phosphorylated $Cdc6_{1–48}$ (**Fig. 1b** and **Supplementary Fig. 1**). In contrast, we observed no detectable binding signal with $Cdc6_{1–48}$ phosphorylated only at T39 and S43. Cdc6 constructs in which only T7 ($Cdc6_{1–48;T23A\ T39A\ S43A}$) or T23 ($Cdc6_{1–48;T7A\ T39A\ S43A}$) are phosphorylated bind with similar affinity as for the wild-type protein but have stoichiometries of $n \approx 1$. We also tested binding of Cks1 to synthetic peptides, $phosCdc_{2–10}$ and $phosCdc6_{19–26}$, which contain only phosphorylated T7 or T23 respectively. Each bound to Cks1 with an affinity about ten-fold less than that of phosphorylated $Cdc_{1–48}$. A $Cdc6_{1–48}$ construct in which T7 and T23 are mutated to alanine and the sequence surrounding T39 is replaced with the T7 sequence ($Cdc6_{1–48;T7\text{-}T39swap}$) binds Cks1 with wild-type affinity. We conclude that the majority of the cohesive interactions are contained within short sequences including the phosphothreonines and suggest that the peptides bind with slightly lower affinity, owing to a greater entropy penalty. Together, these data demonstrate that T7 or T23 phosphorylation is necessary and sufficient for Cks1 association and that Cks1 has binding requirements beyond the minimum phosphorylated (S/T)P CDK site.

We tested the minimum sequence around T7 required for binding Cks1, using a dot-blot assay. We cross-linked synthetic phosphopeptides to BSA, spotted them onto a PVDF membrane and then incubated the membrane with hexahistidine-tagged Cks1 and probed it with an anti-hexahistidine antibody (**Fig. 2a**). We found that the heptapeptides AIPIpTPT and IPIpTPTK (p, phospho-) were sufficient for Cks1 binding. ITC measurements confirmed that AIPI(pT)P, SAIPI(pT)P and AIPI(pT)PT bind Cks1 with similar affinity as does $phosCdc6_{2–10}$ (**Supplementary Table 1**). We substituted phosphoserine and phosphotyrosine for phosphothreonine and observed no binding to Cks1 in both the dot-blot and ITC assays.

In order to identify the Cdc6 sequence determinants of Cks affinity, we used SPOT arrays with peptides directly synthesized on a membrane (**Fig. 2b**). Every spot contains a version of the $phosCdc6_{3–10}$

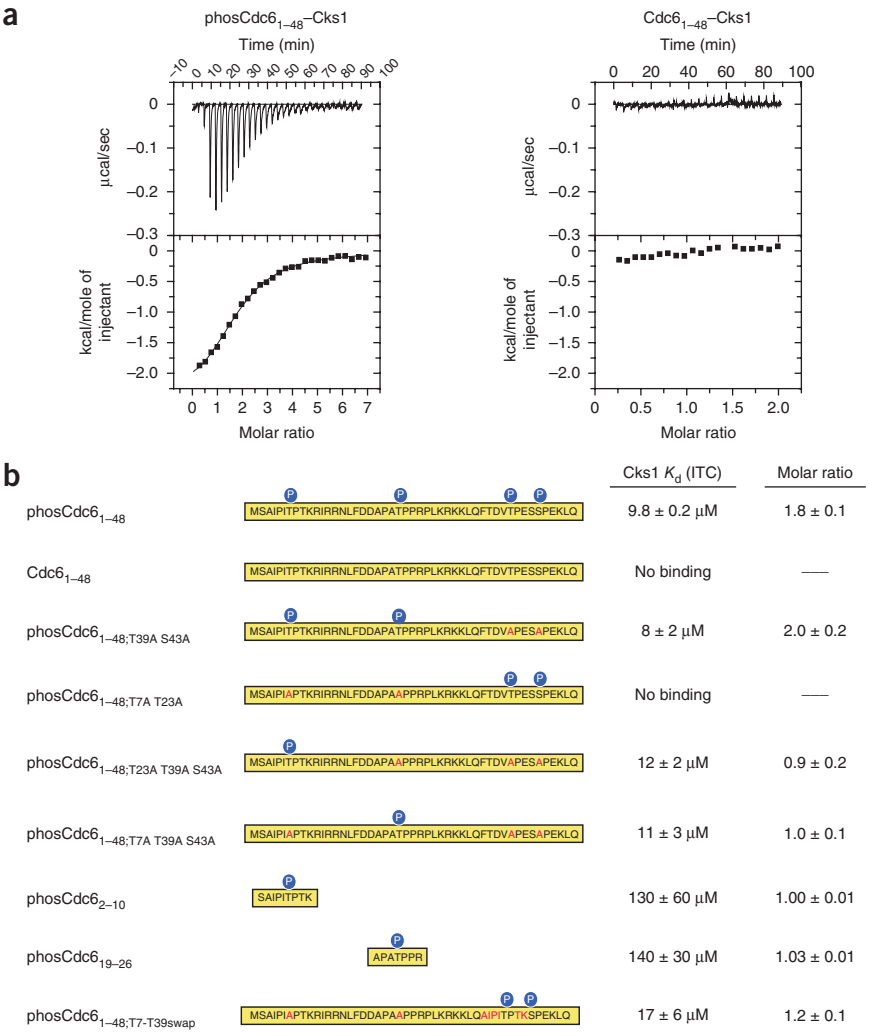

Figure 1 Cks1 binds specific phosphorylated CDK sites in the N-terminal domain of Cdc6. (**a**) ITC measurements of affinity of purified $Cdc6_{1–48}$, phosphorylated with recombinant CDK, for Cks. Left, $phosCdc6_{1–48}$ binding to Cks1 with $K_d = 9.8 \pm 0.2$ μM. Right, titration with unphosphorylated $Cdc6_{1–48}$. (**b**) ITC measurements with phosphorylated $Cdc6_{1–48}$ constructs and synthetic peptides. Sequence mutations are highlighted in red within each construct. Additional ITC data curves are shown in **Supplementary Figure 1a–g**.

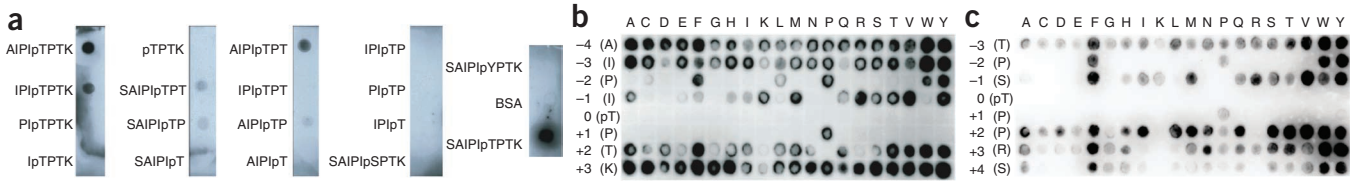

Figure 2 Sequence requirements for Cks1 binding. (**a**) Dot-blot peptide experiment to determine the minimum length required for association with Cks1. Indicated peptides conjugated to BSA, spotted onto PVDF membrane, incubated with histidine-tagged Cks1 and probed with an anti-histidine antibody are shown. The full blot for this experiment is shown in **Supplementary Figure 7a**. (**b**) Scanning mutagenesis SPOT peptide array based on the $Cdc6_{3–10}$ AIPIpTPTK peptide. Peptides containing the indicated single amino acid substitution synthesized onto the membrane and probed for Cks1 binding as in **a** are shown. (**c**) As in **b**, except the array is constructed from a phosphorylated $Sic1_{2–9}$ peptide.

sequence in which a single substitution is made at each position with each of the 20 amino acids. The array was probed as described above, except Cks1 was alkylated to prevent the formation of disulfide linkages (**Supplementary Fig. 2**). We observed that no residue is tolerated at the pT position (0 position), including the phosphomimetics glutamate and aspartate, and that proline is required in the +1 position. These results indicate that all Cks1-binding sequences contain the phosphorylated CDK consensus site TP. Cks1 also displays a marked propensity for binding to peptides with a bulky hydrophobic residue in the −2 position. The array demonstrates that any residue is tolerated in the −4, −3, +2 and +3 positions, although a basic residue or proline seems to be disfavored in the +2 position. There appears to be a varied preference in the −1 position; however, there is no clear correlation between the chemical properties of the tolerated side chains.

In order to corroborate that these observations are general for Cks1 binding and are not influenced by subtle effects of the specific $Cdc6_{3–10}$ sequence context, we conducted the array based on a sequence in Sic1 (**Fig. 2c**). Sic1 T5 is a preferred CDK-phosphorylation site that serves as a priming site for Cks-dependent phosphorylation[25]. Probing a $phosSic1_{2–9}$ array for Cks1 binding, we again found a requirement for phosphothreonine and proline in the 0 and +1 positions, respectively, a preference for a bulky hydrophobic residue in the −2 position and a disfavoring of a positive residue in the +2 position.

We performed ITC with Cks1 and $phosCdc6_{2–10}$ peptides to confirm and quantify the SPOT array results (**Supplementary Table 1**). Replacement of the phosphothreonine for a different phosphorylated side chain (pT7pS and pT7pY) or substitution of the +1 proline (P8K) results in loss of detectable heat. Substitutions of aromatic residues for the proline in the −2 position either have little effect or slightly increase binding affinity relative to that of wild type, whereas substitution of a charged lysine at the −2 position (P5K) results in no detectable heat. The +2 position in the SPOT array disfavored a positive charge or proline. However, by ITC, we found that substitution of a proline (T9P) or a lysine (T9K) for the +2 threonine results in a binding affinity similar to that of wild-type peptide.

Three CDK sites (T5, T33 and T45) in the Sic N-terminal domain ($Sic1_{1–215}$, 'Sic1ΔC') facilitate Cks binding[10], and these sites are known to promote priming-dependent phosphorylation[25]. T5 and T45 contain a proline in the −2 position, and their phosphorylation promotes higher-affinity binding to Cks compared to that of the binding promoted by phosphorylation of T33, which contains a glutamate in the −2 position[10]. We measured affinity for wild type and substituted $phosSic1_{2–9}$ peptides (**Supplementary Table 1**). $PhosSic1_{2–9}$ binds with a slightly weaker affinity (K_d = 95 ± 8 μM) than does the entire phosphorylated Sic1 N terminus and phosSic1ΔC that contains only T5 as a phosphoacceptor site (K_d = 11 ± 3 μM and K_d = 20 ± 10 μM, respectively[10,25]). As in the case of Cdc6, this observation suggests an interaction between the immediate sequence surrounding T5 and Cks1. The affinities of $phosSic1_{2–9}$ peptides with single amino acid substitutions show the same trend as for the Cdc6 peptides (**Supplementary Table 1**). The ITC measurements are also consistent with the SPOT array data, with the exception that a positive lysine is tolerated in the +2 position in the ITC measurements.

Structural basis of Cks binding specificity

We next determined the 2.9-Å crystal structure of a Cks1–phosCdc6 complex to understand the molecular determinants of the specificity observed in our binding experiments (**Table 1** and **Fig. 3**). Crystals were grown from a fusion-protein construct, phosphorylated after purification, in which the $Cdc6_{3–9}$ sequence was appended to the C terminus of $Cks1_{1–112}$ (**Supplementary Fig. 3**). The Cdc6 sequence binds across a surface of Cks1 that is distal to the CDK-binding site and is made up of one face of the Cks1 β-sheet and helix 2 (**Fig. 3a**). The phosphate on the Cdc6 T7 side chain is bound to the previously described cationic pocket (**Fig. 3b**)[34,35]. The Cdc6 T7 γ-methyl makes van der Waals contacts with Y30 and R42 in Cks1; this explains the specificity for phosphothreonine over phosphoserine. Cdc6 P8, which

Table 1 Data collection and refinement statistics

	$phosCdc6_{3–9}$–$Cks1_{1–112}$
Data collection	
Space group	$P4_12_12$
Cell dimensions	
a, *b*, *c* (Å)	84.7, 84.7, 239.9
α, β, γ (°)	90, 90, 90
Resolution (Å)	60.0–2.9 (3.06–2.90)
R_{merge}	0.0463 (0.2598)
$I / \sigma I$	12.65 (3.57)
Completeness (%)	99.79 (99.90)
Redundancy	10.0 (9.9)
Refinement	
Resolution (Å)	58.1–2.9
No. reflections	20,140
R_{work} / R_{free}	24.5% / 28.1%
No. atoms	3,852
Protein	3,800
Water	52
Average *B* factors	56.5
Protein	47.60
Water	52.30
r.m.s. deviations	
Bond lengths (Å)	0.011
Bond angles (°)	1.39

One crystal was used. Values in parentheses are for highest-resolution shell.

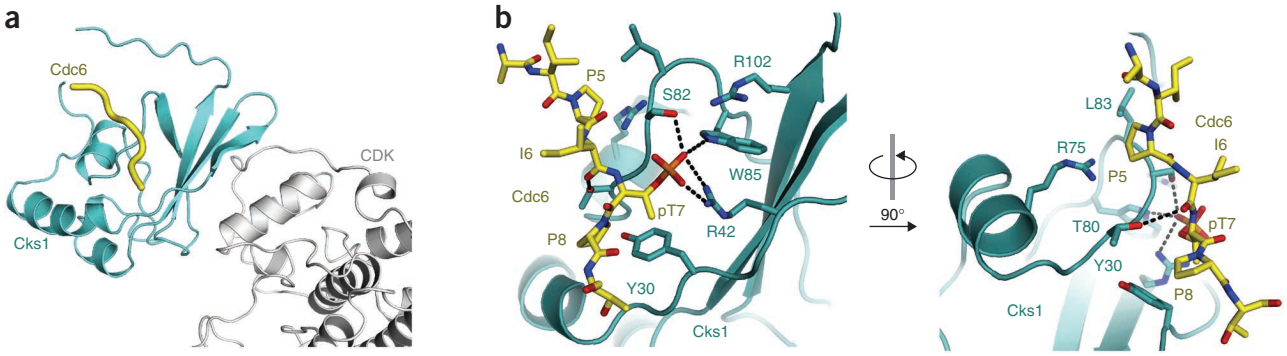

Figure 3 Crystal structure of a phosCdc6$_{3-9}$–Cks1$_{1-112}$ complex. (**a**) Ternary complex including CDK showing the phosphorylated substrate-binding site in Cks distal from CDK. The model was created by alignment of Cks1 from the structure solved here and hsCks1 in the hsCks1–Cdk2 complex (PDB 1BUH[36]). (**b**) Close-up views of the phosCdc6$_{3-9}$–binding site in Cks1.

is in the +1 position, fits into a pocket formed by both Y30 and T80 in Cks1. That no other hydrophobic residues are tolerated in this +1 position suggests that geometry constraints in the Cdc6 backbone are important for proline specificity. The dihedral angles adopted by the +1 proline ($\Phi \approx -80$, $\psi \approx -160$) to contact Y30 and T80 are relatively disfavored by other residues. The loss of affinity upon proline substitution probably arises from the compromise of maintaining the side chain–Cks1 contacts and adopting such strained torsion angles. The γ-hydroxyl of Cks1 T80 acts as a hydrogen-bond donor to the backbone carbonyl of I6 (−1 position) in Cdc6. Although there is no clear correlation among the chemical properties of the tolerated amino acids at the −1 position in the array data (**Fig. 2b**,**c**), this hydrogen bond may select for particular backbone dihedral preferences that best facilitate its formation.

A second pocket formed by Cks L83 and R75 fits Cdc6 P5 (the −2 position). The distances to the −2 proline side chain explain the preference for a large hydrophobic residue in this position. The presence of R75, which is highly conserved in Cks proteins, is also consistent with the observed preference for aromatic residues in the −2 position of the binding consensus sequence (**Fig. 2b**,**c**), because the guanidinium group is capable of making cation-π interactions with aromatic side chains[41]. There are few interactions in the crystal structure between Cks1 and residues in the +2, +3 and −3 positions in the Cdc6 peptide, results consistent with our observation that any amino acid type can substitute there.

We verified the importance of the molecular interactions observed in the crystal structure of the Cks1–phosCdc6 complex with single amino acid mutations in Cks1, purifying Y30E, R75A, R75K, T80D and L83D Cks1 mutants and testing them for binding to phosCdc6 by ITC. The structure shows that Y30 and T80 are important for binding the +1 proline, whereas R75 and L83 form the pocket to accommodate the −2 proline. All the mutants were folded properly, as determined by CD (**Supplementary Fig. 4a**). As predicted by the structure, mutations at these positions inhibited binding to the phosCdc6$_{2-10}$ peptide in the calorimetry assay; in each case, no heat was detected (**Supplementary Fig. 1z–dd**).

Priming-dependent CDK activity requires Cks-substrate binding

We next explored the implications of Cks-substrate docking for its function in stimulating CDK activity toward cell-cycle substrates. We previously found that priming phosphorylation of Sic1 facilitates semiprocessive phosphorylation of critical phosphodegrons, and the requirement for an intact Cks1 cationic pocket demonstrated the importance of Cks-dependent kinetics for CDK signaling[25]. The structural details of the Cks–substrate complex observed here allowed us to test specifically the effect of disrupting this complex on CDK kinetics. We tested whether Cks1 mutations that abolish interactions with substrate residues inhibit multisite phosphorylation (**Fig. 4**). As previously described[25], we added recombinant Cks1 to Cdk1–Clb5 purified from budding yeast and used the enzyme to phosphorylate the noninhibitory truncation Sic1ΔC in a kinase assay. In the reaction with wild-type Cks1, hyperphosphorylated species are present within 8 min and are the dominant product (**Fig. 4a**). Previous substrate-competition experiments established that the rapid accumulation of these hyperphosphorylated forms is due to a semiprocessive mechanism[25]. In contrast, hyperphosphorylated Sic1ΔC does not appear in the same reaction time upon addition of Cks1 with mutations to the phosphate-binding pocket ('+pocket', R33E S82E R102A) or a Y30E mutation. Stronger bands corresponding to hypophosphorylated forms are instead present. Use of R75A, T80D and L83D mutants also results in considerable loss of hyperphosphorylated Sic1ΔC, and intermediate forms containing fewer phosphates are the dominant product. All the mutants are capable of forming a complex with CDK (**Supplementary Fig. 4b**). In reactions with a Sic1ΔC containing a single phosphorylation site (i.e., all sites mutated to alanine except T5), the mutants behave similarly to wild-type Cks1 (**Fig. 4a**). This control reaction demonstrates that Cks1 mutations that disrupt substrate docking have no effect on the catalytic activity of the kinase toward a single phosphoacceptor site but instead result in defects specifically in the mechanism of multisite phosphorylation.

To demonstrate directly the function of Cks in priming-dependent phosphorylation, we assayed kinase activity on primed substrates (**Fig. 4b**,**c**). We synthesized a Cdc6$_{2-29}$ peptide with T7 as a phosphothreonine. We then measured the steady-state kinetics of phosphate incorporation at T23 in the presence of the phosphorylated T7. The K_m for the kinase reaction using Cdk1–Clb5 and wild-type Cks1 is less than that measured for a generic single-site substrate or Sic1 with a single T5 phosphoacceptor site available (**Supplementary Fig. 5**). Conversely, the absolute k_{cat} value for the wild-type reaction, calculated in these experiments by comparison of V_{max} to the published absolute k_{cat} for the H1 peptide[9], is similar to the k_{cat} for reactions with single-site substrates. We tested, in similar reactions, Cks1 that contains mutations in the consensus sequence–binding residues (**Fig. 4b**). These reactions all proceed with K_m values greater than those for reactions with wild-type protein. As observed in the Sic1ΔC multisite phosphorylation assay, mutations in the cationic pocket or Y30 have the largest effect on K_m, whereas the T80D, L83D and R75A mutations all show clear but more modest effects. These data demonstrate that Cks1 residues critical for

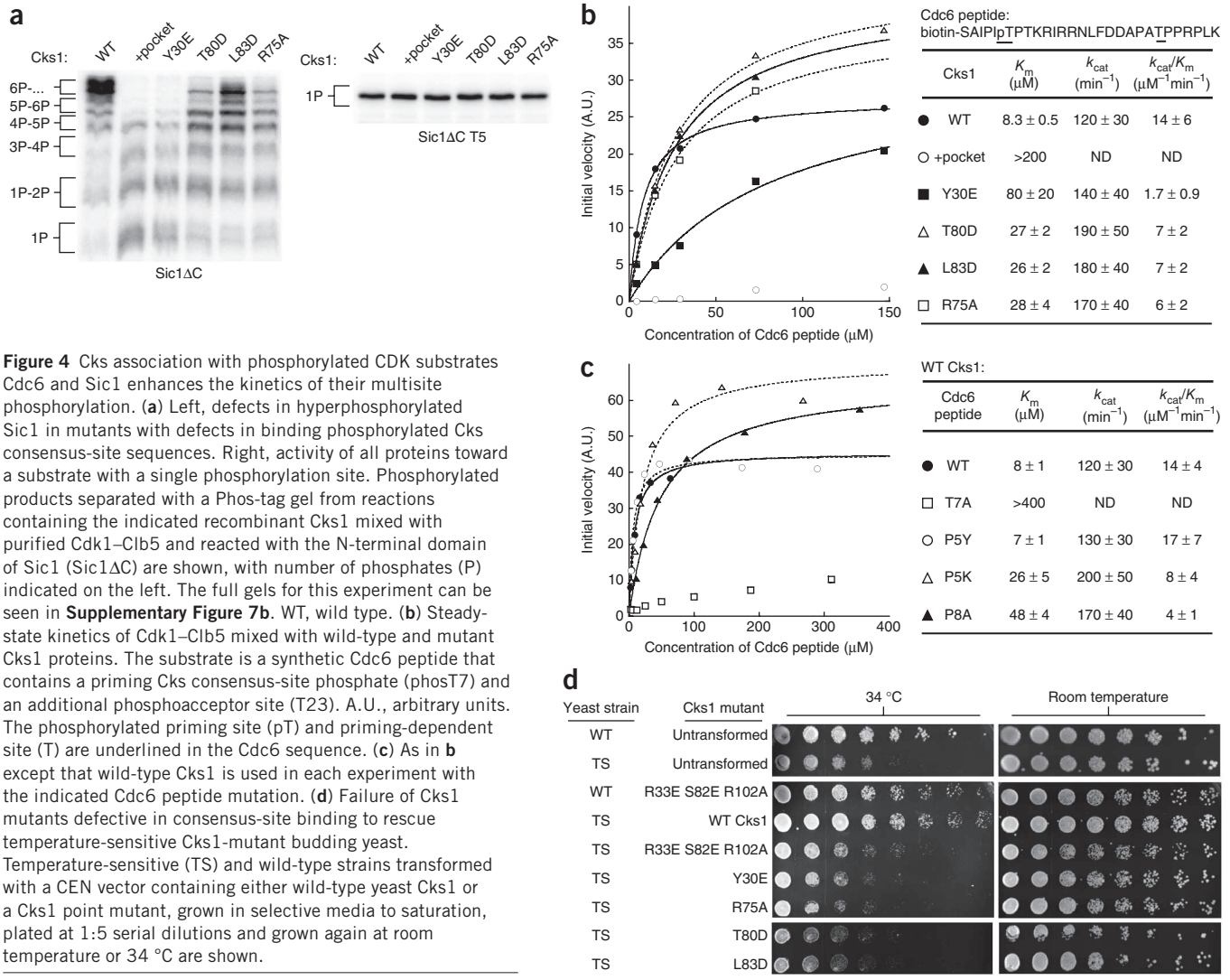

Figure 4 Cks association with phosphorylated CDK substrates Cdc6 and Sic1 enhances the kinetics of their multisite phosphorylation. (**a**) Left, defects in hyperphosphorylated Sic1 in mutants with defects in binding phosphorylated Cks consensus-site sequences. Right, activity of all proteins toward a substrate with a single phosphorylation site. Phosphorylated products separated with a Phos-tag gel from reactions containing the indicated recombinant Cks1 mixed with purified Cdk1–Clb5 and reacted with the N-terminal domain of Sic1 (Sic1ΔC) are shown, with number of phosphates (P) indicated on the left. The full gels for this experiment can be seen in **Supplementary Figure 7b**. WT, wild type. (**b**) Steady-state kinetics of Cdk1–Clb5 mixed with wild-type and mutant Cks1 proteins. The substrate is a synthetic Cdc6 peptide that contains a priming Cks consensus-site phosphate (phosT7) and an additional phosphoacceptor site (T23). A.U., arbitrary units. The phosphorylated priming site (pT) and priming-dependent site (T) are underlined in the Cdc6 sequence. (**c**) As in **b** except that wild-type Cks1 is used in each experiment with the indicated Cdc6 peptide mutation. (**d**) Failure of Cks1 mutants defective in consensus-site binding to rescue temperature-sensitive Cks1-mutant budding yeast. Temperature-sensitive (TS) and wild-type strains transformed with a CEN vector containing either wild-type yeast Cks1 or a Cks1 point mutant, grown in selective media to saturation, plated at 1:5 serial dilutions and grown again at room temperature or 34 °C are shown.

binding phosphorylated substrates are required for the K_m decrease that results from priming in a multisite kinase reaction.

We also assayed the importance of the Cks-binding consensus sequence in the substrate. We synthesized Cdc6 peptides with mutations in the phosphoacceptor site or in surrounding consensus residues and used them in kinase reactions with wild-type Cks1 (**Fig. 4c**). Mutation of the priming phosphothreonine to an alanine (T7A) leads to a K_m increase for the reaction measuring phosphate incorporation at T23. Mutation of the proline in the −2 position of the consensus site to a tyrosine (P5Y) does not change the K_m from the wild-type reaction, whereas mutation to a lysine (P5K) increases the K_m by three-fold, results consistent with our binding measurements. Mutation of the +1 proline (P8A) results in a six-fold increase in K_m. These results together further establish the importance of the Cks-substrate association for enhancing CDK multisite kinetics and demonstrate that a binding consensus, and not solely a phosphate, directs the priming-dependent reaction.

To demonstrate the importance of Cks1 association with specific priming-site sequences *in vivo*, we examined the viability of Cks1 mutants that are defective in consensus-sequence binding in budding yeast (**Fig. 4d**). We identified a mutation (L98S) in Cks1 that has temperature sensitivity at 34 °C. At the restrictive temperature, growth is impaired, as described previously[12], but is rescued by wild-type Cks1 expressed from a CEN vector and under control of the alcohol dehydrogenase promoter. In contrast, expression of a phosphate binding–pocket mutant or the consensus sequence–binding mutants fails to rescue the temperature sensitivity.

The Cks-binding consensus is present in a subset of CDK substrates

To find CDK substrates that associate with Cks, we considered the sequence (F/I/L/P/V/W/Y)XTP, which contains the minimum requirements for binding Cks1, on the basis of our data. We inputted this sequence into the Yeast Genome Database Pattern Matching tool (http://www.yeastgenome.org/cgi-bin/PATMATCH/nph-patmatch/) and found that 3,263 sequences match the consensus; these sequences are found in 2,100 different open reading frames (ORFs) (**Fig. 5a**). 269 of the 2,100 ORFs are known CDK substrates, as determined in at least one of the two global CDK-substrate identification studies[4,5]. There are 522 putative Cks-binding sequences within these 269 ORFs (listed in **Supplementary Table 2**).

In order to filter and characterize better the properties of Cks targets, we further analyzed the 522 putative Cks-binding sites found in CDK substrates (**Fig. 5**). We first searched for evidence validating that the sites are phosphorylated by CDK or other kinases. 48 of the 522 sites were identified as CDK-phosphorylation sites in an MS-based proteomics screen to identify CDK substrates (**Fig. 5a**)[5], and 42 other

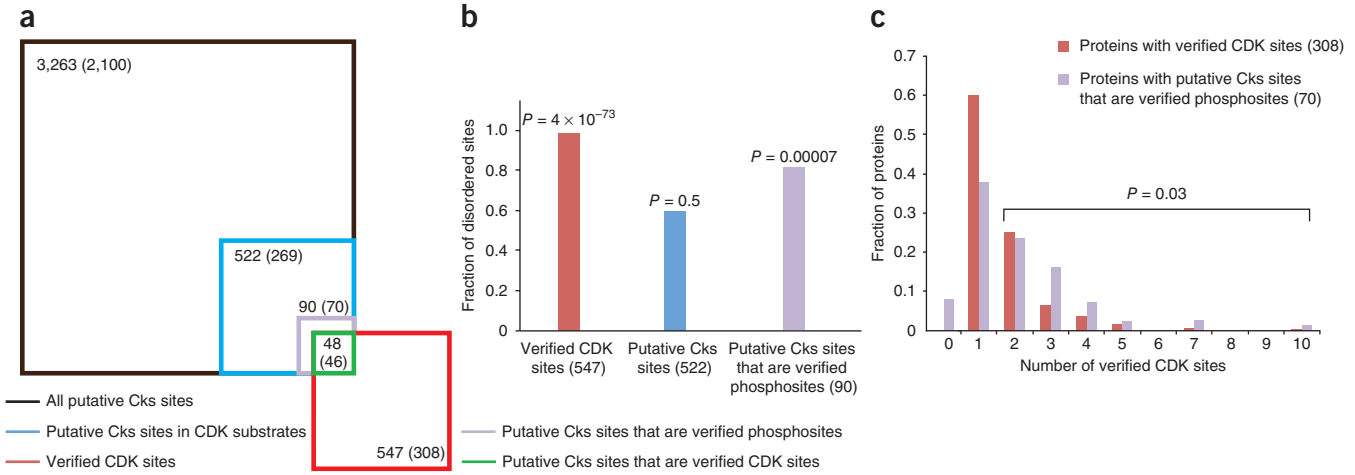

Figure 5 Sequence context of putative Cks binding sites. (**a**) Venn diagram showing in each set the number of sequences that match the minimal Cks consensus sequence (F/I/L/P/V/W/Y-XTP) and the number of proteins containing those sequences (in parentheses). The set of all verified CDK sites is taken from an MS-based screen[5]. Square areas are proportional to the number of sites. (**b**) Fraction of sites found in disordered protein sequences. *P* values are calculated by χ^2 test, assuming an expected distribution equivalent to that found for all (S/T)P sites in the yeast proteome (60.6% disordered)[5]. In the χ^2 test, $n = 2$ from the fact that a site could be found in either an ordered or disordered part of a protein. (**c**) Multiplicity of CDK sites in CDK and putative Cks-directed substrates. Bars show the number of CDK sites verified in the MS screen[5] for all CDK substrates found in that screen (red) and for substrates that contain putative Cks sequences that are also verified phosphorylation sites (purple). The substrates that contain zero verified CDK sites in the MS screen contain Cks sequences that are phosphorylated according to the PhosphoGRID database. The *P* value is calculated for a distribution between either zero or one CDK sites or more than one CDK site (i.e., $n = 2$, with n describing the number of categories into which the data were binned). The χ^2 test was used, assuming an expected distribution for the Cks-containing proteins that is equivalent to the distribution for all verified CDK sites in the MS screen.

sites were identified as phosphorylation sites in the PhosphoGRID database (www.phosphogrid.org/). These 90 Cks consensus sites that correspond to validated phosphorylation sites (highlighted in **Supplementary Table 2**) are found in 70 different ORFs.

CDK sites tend to be found in regions of proteins that lack structure[5,42]. We analyzed whether the Cks consensus sites are in sequences that are likely to be structured or disordered, using a web-based disorder prediction tool[43]. We found that 309 of the 522 putative Cks sites (59%) are in regions of likely disorder, whereas 73 of the 90 (81%) Cks sites that are verified phosphorylation sites are found in likely disordered regions (**Fig. 5b**). When all putative Cks sites are considered, there is no significant preference for disordered sequences; however, the statistics for the subset of Cks sites corresponding to verified phosphorylation sites do suggest that Cks preferentially binds unstructured sequences. The disorder frequency of Cks sites is less than the disorder frequency (99%) of all verified CDK sites in the MS screen[5]. It may be that Cks sites are more likely to be found in predicted structured regions because of the hydrophobic amino acid in the −2 position.

Considering the role of Cks in stimulating multisite phosphorylation kinetics, we predicted and found that Cks sites preferentially occur in substrates that contain multiple CDK sites. Putative Cks sites that are verified phosphorylation sites occur in substrates with more than one CDK site at a frequency that is greater than expected from the multiplicity distribution of all CDK substrates found in the MS screen (**Fig. 5c**)[5].

Cks mediates Cdk1-directed Wee1 activity at mitotic entry

In examining the list of putative Cks binding partners, we found the potential docking association of Cks1 with budding yeast Wee1 (called Swe1) to be of particular interest because Swe1 is known to form a phosphorylation-dependent complex with Cdk1 during mitotic entry. This complex is required for efficient inhibitory phosphorylation of Cdk1 by Swe1 on Y19 (refs. 23,32,33). Formation of the complex requires phosphorylation of eight consensus sites in Wee1 by Cdk1 (ref. 33). Similar mechanisms are likely to act in vertebrate cells[32,44,45]. We hypothesized that Cks docking to specific phosphorylated consensus sites mediates the Cdk1–Wee1 complex, and we tested this idea with mutational analysis of Swe1 in budding yeast.

Of the eight consensus Cdk1 phosphosites in Swe1, there are four phosphothreonines: T45, T121, T196 and T373. All except T45 possess a bulky hydrophobic residue in the −2 position, and T196 and T373 were previously found to have an important role in Cdk1-complex formation in budding yeast[33]. Although neither the precise sequences surrounding the sites nor their location in the primary sequence is conserved in higher eukaryotes, most Swe1 orthologs contain three or four putative Cks-binding sites. We tested Cks1 binding to each of these phosphopeptides by ITC and found, as predicted, that all except T45 bound Cks1 with an affinity similar to that between phosCdc6$_{2-10}$ and Cks1 (**Supplementary Table 1**). To assay the importance of the Cks-binding sites *in vivo*, we constructed a yeast strain in which the three consensus threonines in

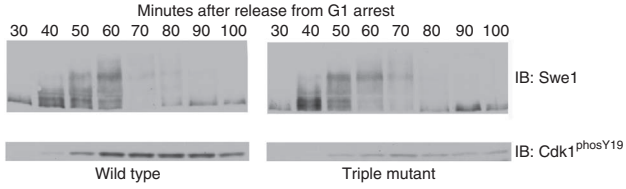

Figure 6 Cks consensus sites in Wee1 are required for Cdk1 inhibitory phosphorylation. Immunoblots (IB) probing Swe1 and phosphorylation on Cdk1 Y19 in yeast expressing wild-type (left) or triple-mutant (T121S T196S T373S; right) Wee1 (Swe1). Time course after release from a G1 arrest is shown. Exposures of the full gels for this experiment are shown in **Supplementary Figure 7c**.

Swe1 were mutated to serines (T121S T196S T373S; 'triple mutant') and in which Cdk1 phosphorylation of Swe1 is preserved, but Cks association is inhibited. We observed no considerable difference in the phosphorylation pattern of mutant and wild-type Swe1 (**Fig. 6**) after release from G1 arrest, results suggesting that the Cks consensus sites do not have a role in Swe1 hyperphosphorylation *in vivo*. The triple-mutant cells do show a defect in phosphorylation of Cdk1 on Y19 (**Fig. 6**). The decreased Cdk1 phosphorylation is not due to reduced Swe1, because the total amount of Swe1 and its phosphorylation state are consistent between mutant and wild-type cells (**Fig. 6**). We conclude that the Cks consensus sites are critical for Swe1 activity toward Cdk1. That hyperphosphorylation of Swe1 appears to be normal in the mutant suggests that Cks1 binding does not influence multisite phosphorylation in this context. Rather, Cks1 mediates formation of the complex in response to phosphorylation of the Cks1 consensus site, and this in turn allows Swe1 to phosphorylate Cdk1 efficiently on its inhibitory site.

DISCUSSION

Although it has been postulated that Cks stimulates CDK activity by binding primed substrates[3,13], little evidence has been provided for this model, and the association of Cks with phosphorylated substrates has not been well characterized. We demonstrate here that Cks binds phosphorylated CDK substrates in a sequence-dependent manner and find that efficient hyperphosphorylation of substrates depends on recognition of sequence elements that surround the phosphate. Our results support a model in which Cks binds specific priming sites to facilitate phosphorylation at other sites (**Fig. 7a**). Other data suggest that Cks1 orients substrates for CDK phosphorylation at sites that are C terminal to the Cks1-binding site[10]. Characterization of the Cks-binding consensus sequence has permitted identification of a large set of CDK substrates (269 proteins containing 522 Cks sites) that may use the Cks-dependent hyperphosphorylation mechanism. Considering that a smaller subset of putative Cks-binding sites contains sites for which phosphorylation has been directly observed (90 sites in 70 proteins), we anticipate that the actual number of Cks-interacting substrates in the cell is more modest. It remains an open question how many real Cks-directed CDK substrates exist, and it will be interesting to explore the different cell-cycle contexts in which Cks contributes to the desired signaling response.

The observation that only a subset of CDK sites have the required surrounding sequence for Cks binding has important implications for the understanding of multisite phosphorylation in cell-cycle regulation. The data presented here and in the accompanying paper demonstrate that CDK phosphoacceptor sites differentially influence the kinetics of phosphate incorporation at other sites[10]. This regulatory mechanism can be used to control whether and when sites with critical outputs are phosphorylated. For example, in Sic1, phosphorylation at specific sites required for ubiquitylation and degradation requires priming phosphorylation at the Cks consensus site[10]. It has also been shown recently that different phosphorylation events in the retinoblastoma protein induce distinct conformational changes[27], and this motivates the need for unique control of phosphorylation at distinct sites. This notion that specific phosphorylation sites have distinct roles in tuning signaling outputs differs from other models for the mechanism of multisite phosphorylation in the cell cycle[22,24], in which phosphorylation sites have generic roles.

Our data suggest that the influence of Cks on CDK-substrate phosphorylation kinetics depends on whether Cks-binding consensus sites are phosphorylated early or late in the multisite reaction. In the case that a Cks-binding site is phosphorylated early, phosphorylation triggers Cks binding to enhance phosphorylation of the remaining sites (**Fig. 7a**). The consensus sequence for optimal Cks1 affinity contains a CDK-acceptor site (TP), and this is consistent with a self-priming mechanism. We note that other kinases could phosphorylate the Cks consensus site, including MAP kinases, which also recognize TP[46]. The scenario depicted in **Figure 7a** is true for Sic1. Two optimal CDK sites that act as Cks-dependent priming (T5 and T33) sites are required for semiprocessive phosphorylation of Sic1 and its degradation[10,25]. Cdc25 phosphorylation also uses the priming mechanism; mutation of Cks consensus sites abrogates cooperativity in the phosphorylation kinetics[26]. Alternatively, if Cks consensus sites are phosphorylated late in the multisite reaction, CDK phosphorylation proceeds without or with limited Cks-induced stimulation for that particular substrate (**Fig. 7b**). Swe1 (Wee1) may be an example of such a substrate. We observe no effect on Swe1 hyperphosphorylation *in vivo* when the Cks consensus is disrupted, and it has been proposed that the best Cks consensus sites (T196 and T373) are phosphorylated late[33].

We found that the Cks consensus site in budding-yeast Wee1 is required for its ability to phosphorylate CDK on the inhibitory Y19 site. This observation demonstrates a new role for Cks in regulation of CDK activity through influencing post-translational modifications and explains one mechanism by which Cks regulates mitotic entry. It also emphasizes that the cellular role of Cks extends beyond its capacity to stimulate CDK kinetics. Cks mediates phosphorylation-dependent interactions between CDK and its own regulators (**Fig. 7b**). The Wee1–Cdk1 complex is a second example of this function, the first example being the binding of human Cks1 (hsCks1) to phosphorylated p27 (phosp27) within a ubiquitin ligase[18,19].

The structure of hsCks1 bound to phosp27 in the context of the Skp2–Skp1–Cullin ligase was previously solved[37], and comparison with the phosCdc6–Cks1 structure here indicates several similarities (**Supplementary Fig. 6**). The location of the phosphothreonine phosphate and γ-methyl groups and contacts with the +1 proline are equivalent. One notable difference is the presence of a glutamate in the −2 position in phosp27, which is not preferred in the

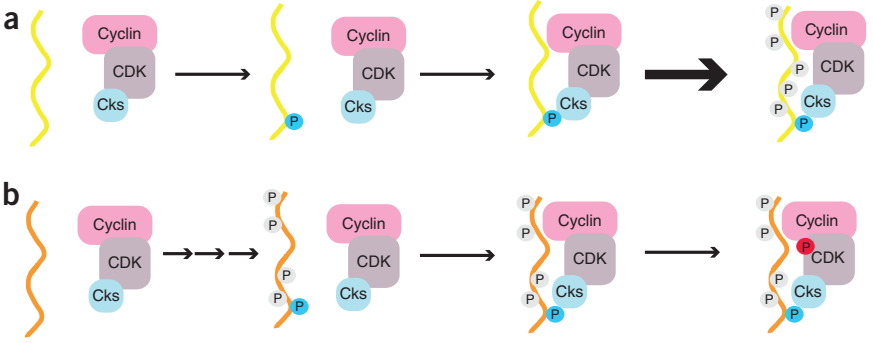

Figure 7 Cks is a specificity factor that mediates CDK-substrate association for multiple functions. (**a**) Cks binds substrates phosphorylated at specific consensus sites (blue phosphate (P)), and the Cks–CDK–substrate complex stimulates further phosphorylation. The stimulatory effect requires that the Cks consensus site be phosphorylated early in the reaction, as is true in Sic1. (**b**) Cks acts as an adaptor that targets CDK to its regulators. In the case of Wee1, association with Cks consensus sites induces inhibitory phosphorylation in CDK (red phosphate).

Cks1-binding consensus site. In the phosp27–hsCks1 ligase structure, this phos27 E185 hydrogen-bonds with R294 and Y346 of Skp2 and Q52 of hsCks1. In Cks1, L83 is in the equivalent position as Q52. This difference explains why glutamate in the −2 position facilitates the phosp27-hsCks1 interaction in the context of ubiquitylation, whereas a hydrophobic residue is required for general Cks1 binding to phosphorylated targets. HsCks2 has a leucine at the hsCks1 Q52 position, similarly to Cks1, the fission-yeast ortholog suc1 and the frog ortholog p9. Our observations suggest that hsCks2, like its more distant orthologs, has a general role in CDK binding to its substrates and regulators, whereas Cks1 has evolved to gain its specific function as an adaptor for the Skp2 SCF ligase. Further dissection of the functional differences between the human Cks paralogs will be important for understanding the role of Cks in tumorigenesis and for its use as a cancer diagnostic.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors for the $Cks1_{1-112}$–$phosCdc6_{3-9}$ fusion protein have been deposited in the Protein Data Bank under accession code 4LPA.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

D.A.M., E.R.M.B., M.K., R.L., D.R.K., M.L. and S.M.R. designed the study. D.A.M., E.R.M.B., M.K., R.L., M.V.M. and A.H. performed experiments. All authors analyzed data. D.A.M., E.R.M.B. and S.M.R. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

1. Ubersax, J.A. & Ferrell, J.E. Jr. Mechanisms of specificity in protein phosphorylation. *Nat. Rev. Mol. Cell Biol.* **8**, 530–541 (2007).
2. Brognard, J. & Hunter, T. Protein kinase signaling networks in cancer. *Curr. Opin. Genet. Dev.* **21**, 4–11 (2011).
3. Morgan, D.O. *The Cell Cycle: Principles of Control* (New Science Press, 2007).
4. Ubersax, J.A. *et al.* Targets of the cyclin-dependent kinase Cdk1. *Nature* **425**, 859–864 (2003).
5. Holt, L.J. *et al.* Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. *Science* **325**, 1682–1686 (2009).
6. Sherr, C.J. Cancer cell cycles. *Science* **274**, 1672–1677 (1996).
7. Loog, M. & Morgan, D.O. Cyclin specificity in the phosphorylation of cyclin-dependent kinase substrates. *Nature* **434**, 104–108 (2005).
8. Schulman, B.A., Lindstrom, D.L. & Harlow, E. Substrate recruitment to cyclin-dependent kinase 2 by a multipurpose docking site on cyclin A. *Proc. Natl. Acad. Sci. USA* **95**, 10453–10458 (1998).
9. Kõivomägi, M. *et al.* Dynamics of Cdk1 substrate specificity during the cell cycle. *Mol. Cell* **42**, 610–623 (2011).
10. Koivomagi, M. *et al.* Multisite phosphorylation networks as signal processors for Cdk1. *Nat. Struct. Mol. Biol.* **20**, doi:10.1038/nsmb.2706 (3 November 2013).
11. Hayles, J., Aves, S. & Nurse, P. suc1 is an essential gene involved in both the cell cycle and growth in fission yeast. *EMBO J.* **5**, 3373–3379 (1986).
12. Tang, Y. & Reed, S.I. The Cdk-associated protein Cks1 functions both in G1 and G2 in *Saccharomyces cerevisiae*. *Genes Dev.* **7**, 822–832 (1993).
13. Pines, J. Cell cycle: reaching for a role for the Cks proteins. *Curr. Biol.* **6**, 1399–1402 (1996).
14. Shapira, M. *et al.* Alterations in the expression of the cell cycle regulatory protein cyclin kinase subunit 1 in colorectal carcinoma. *Cancer* **100**, 1615–1621 (2004).
15. Martinsson-Ahlzén, H.S. *et al.* Cyclin-dependent kinase-associated proteins Cks1 and Cks2 are essential during early embryogenesis and for cell cycle progression in somatic cells. *Mol. Cell Biol.* **28**, 5698–5709 (2008).
16. Lan, Y. *et al.* Aberrant expression of Cks1 and Cks2 contributes to prostate tumorigenesis by promoting proliferation and inhibiting programmed cell death. *Int. J. Cancer* **123**, 543–551 (2008).
17. Westbrook, L. *et al.* High Cks1 expression in transgenic and carcinogen-initiated mammary tumors is not always accompanied by reduction in p27Kip1. *Int. J. Oncol.* **34**, 1425–1431 (2009).
18. Ganoth, D. *et al.* The cell-cycle regulatory protein Cks1 is required for SCF^{Skp2}-mediated ubiquitinylation of p27. *Nat. Cell Biol.* **3**, 321–324 (2001).
19. Spruck, C. *et al.* A CDK-independent function of mammalian Cks1: targeting of SCF^{Skp2} to the CDK inhibitor p27Kip1. *Mol. Cell* **7**, 639–650 (2001).
20. Morris, M.C. *et al.* Cks1-dependent proteasome recruitment and activation of CDC20 transcription in budding yeast. *Nature* **423**, 1009–1013 (2003).
21. Yu, V.P., Baskerville, C., Grunenfelder, B. & Reed, S.I. A kinase-independent function of Cks1 and Cdk1 in regulation of transcription. *Mol. Cell* **17**, 145–151 (2005).
22. Nash, P. *et al.* Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. *Nature* **414**, 514–521 (2001).
23. Harvey, S.L., Charlet, A., Haas, W., Gygi, S.P. & Kellogg, D.R. Cdk1-dependent regulation of the mitotic inhibitor Wee1. *Cell* **122**, 407–420 (2005).
24. Kim, S.Y. & Ferrell, J.E. Jr. Substrate competition as a source of ultrasensitivity in the inactivation of Wee1. *Cell* **128**, 1133–1145 (2007).
25. Kõivomägi, M. *et al.* Cascades of multisite phosphorylation control Sic1 destruction at the onset of S phase. *Nature* **480**, 128–131 (2011).
26. Trunnell, N.B., Poon, A.C., Kim, S.Y. & Ferrell, J.E. Jr. Ultrasensitivity in the Regulation of Cdc25C by Cdk1. *Mol. Cell* **41**, 263–274 (2011).
27. Burke, J.R., Hura, G.L. & Rubin, S.M. Structures of inactive retinoblastoma protein reveal multiple mechanisms for cell cycle control. *Genes Dev.* **26**, 1156–1166 (2012).
28. Goldbeter, A. & Koshland, D.E. Jr. An amplified sensitivity arising from covalent modification in biological systems. *Proc. Natl. Acad. Sci. USA* **78**, 6840–6844 (1981).
29. Ferrell, J.E. Jr. Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. *Trends Biochem. Sci.* **21**, 460–466 (1996).
30. Salazar, C. & Hofer, T. Multisite protein phosphorylation: from molecular mechanisms to kinetic models. *FEBS J.* **276**, 3177–3198 (2009).
31. Thomson, M. & Gunawardena, J. Unlimited multistability in multisite phosphorylation systems. *Nature* **460**, 274–277 (2009).
32. Deibler, R.W. & Kirschner, M.W. Quantitative reconstitution of mitotic CDK1 activation in somatic cell extracts. *Mol. Cell* **37**, 753–767 (2010).
33. Harvey, S.L. *et al.* A phosphatase threshold sets the level of Cdk1 activity in early mitosis in budding yeast. *Mol. Biol. Cell* **22**, 3595–3608 (2011).
34. Arvai, A.S., Bourne, Y., Hickey, M.J. & Tainer, J.A. Crystal structure of the human cell cycle protein CksHs1: single domain fold with similarity to kinase N-lobe domain. *J. Mol. Biol.* **249**, 835–842 (1995).
35. Bourne, Y. *et al.* Crystal structure of the cell cycle-regulatory protein suc1 reveals a β-hinge conformational switch. *Proc. Natl. Acad. Sci. USA* **92**, 10232–10236 (1995).
36. Bourne, Y. *et al.* Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1. *Cell* **84**, 863–874 (1996).
37. Hao, B. *et al.* Structural basis of the Cks1-dependent recognition of $p27^{Kip1}$ by the SCF^{Skp2} ubiquitin ligase. *Mol. Cell* **20**, 9–19 (2005).
38. Patra, D., Wang, S.X., Kumagai, A. & Dunphy, W.G. The *Xenopus* Suc1/Cks protein promotes the phosphorylation of G_2/M regulators. *J. Biol. Chem.* **274**, 36839–36842 (1999).
39. Mimura, S., Seki, T., Tanaka, S. & Diffley, J.F. Phosphorylation-dependent binding of mitotic cyclins to Cdc6 contributes to DNA replication control. *Nature* **431**, 1118–1123 (2004).
40. Odaert, B. *et al.* Solution NMR study of the monomeric form of p13suc1 protein sheds light on the hinge region determining the affinity for a phosphorylated substrate. *J. Biol. Chem.* **277**, 12375–12381 (2002).
41. Crowley, P.B. & Golovin, A. Cation-π interactions in protein-protein interfaces. *Proteins* **59**, 231–239 (2005).
42. Tyanova, S., Cox, J., Olsen, J., Mann, M. & Frishman, D. Phosphorylation variation during the cell cycle scales with structural propensities of proteins. *PLOS Comput. Biol.* **9**, e1002842 (2013).
43. Ishida, T. & Kinoshita, K. PrDOS: prediction of disordered protein regions from amino acid sequence. *Nucleic Acids Res.* **35**, W460–W464 (2007).
44. Mueller, P.R., Coleman, T.R. & Dunphy, W.G. Cell cycle regulation of a *Xenopus* Wee1-like kinase. *Mol. Biol. Cell* **6**, 119–134 (1995).
45. Tang, Z., Coleman, T.R. & Dunphy, W.G. Two distinct mechanisms for negative regulation of the Wee1 protein kinase. *EMBO J.* **12**, 3427–3436 (1993).
46. Mukhopadhyay, N.K. *et al.* An array of insulin-activated, proline-directed serine/threonine protein kinases phosphorylate the p70 S6 kinase. *J. Biol. Chem.* **267**, 3325–3335 (1992).

ONLINE METHODS

Protein expression and purification. Wild-type full-length *S. cerevisiae* Cks1 was either expressed untagged or tagged as indicated from a pET vector in *E. coli*. The untagged wild-type protein and the Cks_{1-112}–phos$Cdc6_{3-9}$ fusion used for crystallization were purified by ion-exchange chromatography and size-exclusion chromatography, and the hexahistidine-tagged protein was purified with nickel-NTA chromatography and subsequent ion-exchange chromatography. The GST-tagged protein was purified with glutathione Sepharose resin and subsequently by ion-exchange chromatography. The GST tag was then cleaved by GST-tagged TEV protease, and the GST-TEV and cleaved GST tag were separated from Cks1 with glutathione Sepharose. The *S. cerevisiae* $Cdc6_{1-48}$ peptides were all expressed as hexahistidine fusion constructs and purified as described for His-tagged Cks. Cdk6–CycV or Cdk2–CycA were used as previously described to phosphorylate purified proteins for calorimetry and crystallization[47]. Quantitative phosphate incorporation was verified by electrospray MS in all cases, with the exception that for wild-type $Cdc6_{1-48}$, we could at most achieve a maximum of three phosphates added and determined that our sample was a mixture of peptides containing three out of four sites phosphorylated. This mixture is consistent with our observation of a stoichiometry of slightly less than $n = 2$.

For kinase assays, N-terminally His-tagged recombinant Sic1ΔC (aa 1–215) protein was purified by cobalt affinity chromatography. Clb5–CDK purified with the TAP-tag method was used as described previously[4]. Wild-type Cks1 and Cks1 +pocket mutant (R33E S82E R102A) used in kinetics assays were purified as described[48].

Peptide SPOT-binding assays. Synthetic phosphopeptides were resuspended in PBS, pH 7.4, to a concentration of 1.8 mM and covalently coupled to 1 mg/ml BSA by stirring with an equal volume of 0.2% glutaraldehyde at room temperature. Reactions were quenched after 1 h with an equal volume of 1M glycine, and samples were dialyzed against 25 mM Tris-HCl, pH 7.4, and 150 mM NaCl. Small volumes (0.5–3 µl) of peptide samples were spotted onto methanol-activated PVDF membrane. Positional scanning peptide arrays were synthesized on amino-PEG cellulose membranes by the MIT Biopolymers Laboratory. Blocked membranes were probed with 2 µM hexahistidine-tagged Cks, detected with HRP-His Probe (Santa Cruz, sc-8036; validation provided on manufacturers website) and developed with SuperSignal West Dura ECL reagents (Thermo Scientific).

Crystallization and structure determination. After phosphorylation, the fusion protein was prepared for crystallization by elution from a Superdex 75 (GE Healthcare) column in a buffer containing 25 mM Tris, 150 mM NaCl, and 1 mM DTT at a concentration of 40 mg/mL. Proteins were crystallized by sitting-drop vapor diffusion at 20 °C. Crystals grew for 2 weeks in a solution containing 400 mM potassium sodium tartrate and 0.1 M MES, pH 5.5, and were harvested and flash frozen in the same solution with 30% glycerol.

Data were collected at λ = 1.0332Å, 100 K on Beamline 23-ID-D at the Advanced Photon Source, Argonne National Laboratory. Diffraction spots were integrated with Mosflm[49] and scaled with SCALE-IT[50]. Phases were solved by molecular replacement with PHASER[51]. ScCks1 (PDB 1QB3) was used as a search model. The initial model was rebuilt with Coot[52], and the Cdc6 peptide was added to electron density visible in the previously described phosphate-binding pocket. The resulting model was refined with Phenix[53]. Several rounds of position refinement with simulated annealing and individual temperature-factor refinement with default restraints were applied. Final Ramachandran statistics were as follows: outliers, 0.2%; allowed, 6.8%; favored, 93.0%.

Isothermal titration calorimetry. ITC was performed with the MicroCal VP-ITC calorimeter. Cks1 was dialyzed overnight before the assay in a buffer containing 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM β-mercaptoethanol, and 0.01% NaN_3. In most cases, the synthetic phosphopeptides were dialyzed for up to 5 d in the same buffer. $Cdc6_{1-48}$ was buffer-exchanged into ITC buffer over Superdex75 on the day of ITC. For experiments with synthetic peptides, phosphopeptides at concentrations of 2–6 mM were titrated into Cks1 at a concentration of ~50–100 µM. For experiments with $Cdc6_{1-48}$, Cks1 at a concentration of ~0.5–1 mM was titrated into $Cdc6_{1-48}$ at a concentration of ~30–80 µM. Data were analyzed with the Origin calorimetry software package, assuming a one-site binding model. Experiments were repeated two to four times, and the reported error is the s.d. of each set of measurements.

Kinetic assays. The general composition of the assay mixture was as follows: 50 mM HEPES, 180 mM NaCl, 5 mM $MgCl_2$, 0.2 mg/ml BSA, 2% glycerol, 2 mM EGTA, 500 nM Cks1 and 500 µM ATP (with added [γ-^{32}P]ATP (Perkin Elmer)), pH 7.4. 20 mM imidazole was also added in experiments with protein substrate. Around 1–5 nM of purified Cdk1–Clb5 kinase complex was used. Reactions were initiated by addition of preincubation mixture and [γ-^{32}P]ATP to the substrate protein or peptide. Aliquots were collected at least at two different time points, and the reaction was stopped by addition of the SDS-PAGE sample buffer (in the case of proteins) or 2.5 M guanidine hydrochloride (in the case of biotin-labeled peptides). For quantitative phosphorylation assays, the initial velocity conditions were defined as a substrate turnover ranging up to 20% of the total turnover in assays with biotinylated peptides and as 5% in assays with protein substrate. For the assay with different Cks1 mutants, purified kinase complex was preincubated for 45 min with Cks1 proteins. The composition of the preincubation mixture was as follows: 50 mM HEPES, 150 mM NaCl, 5 mM MgCl2, 0.4 mg/ml BSA, 500 µM ATP and 1 mM Cks1, pH 7.4. For capturing biotinylated peptides, SAM2 Biotin Capture Membrane (Promega) was used according to the instructions given by the manufacturer. 10% SDS-PAGE with added Phos-tag reagent (FMS Laboratory NARD Institute) was used to separate different Sic1ΔC phosphoforms[54]. Sic1ΔC concentrations were kept in the range of 1–3 µM, several fold below the estimated K_m value.

Budding-yeast temperature-sensitivity rescue experiment. A temperature-sensitive Cks1-mutant yeast strain (TS) was created with error-prone PCR and homologous recombination in the wild-type *S. cerevisiae* strain DK186 (WT). The TS strain (containing a L98S mutation) and wild-type strains were transformed with the CEN vector pRS315 containing either wild-type yeast Cks1 or a Cks1 point mutant and grown in selective media to saturation. The transformants were then spot-plated at 1:5 serial dilutions on selective media and grown at room temperature or 34 °C. All Cks1 expression from the CEN vector was under control of the yeast alcohol dehydrogenase promoter (pADH).

Wee1 and Cdk1 phosphorylation in budding yeast. A strain in which the endogenous SWE1 gene was replaced by Swe1 T121S T196S T373S was generated by transformation of a strain carrying *wee1*Δ::*URA3* with the full-length *wee1* T121S T196S T373S allele excised from a plasmid. Loss of the *URA3* gene was selected for by plating on FOA and was confirmed by PCR and western blot. A control strain was also created by integration of the wild-type copy of the SWE1 gene from a plasmid at the SWE1 locus. The time course and western blots were performed as previously described[33]. For Swe1 western blots, electrophoresis was performed on a 10% polyacrylamide gel until a 66.5-kD marker ran to the bottom of the gel. Western blots were transferred for 90 min at 800 mA at 4 °C in a transfer tank in a buffer containing 20 mM Tris base, 150 mM glycine, and 20% methanol. Blots were first probed overnight at 4 °C with P-cdc2 (Tyr15 9111L; Cell Signaling Technology, dilution 1:5,000; validation on manufacturer website) and affinity-purified rabbit polyclonal antibodies raised against a Swe1 peptide (dilution 1:1,000; validation in ref. 23). Blots were then probed with an HRP-conjugated donkey anti-rabbit secondary antibody (GE Healthcare, dilution 1:5,000; validation provided on manufacturer website).

Original images of autoradiographs and blots used in this study can be found in **Supplementary Figure 7**.

47. Burke, J.R., Deshong, A.J., Pelton, J.G. & Rubin, S.M. Phosphorylation-induced conformational changes in the retinoblastoma protein inhibit E2F transactivation domain binding. *J. Biol. Chem.* **285**, 16286–16293 (2010).
48. Reynard, G.J., Reynolds, W., Verma, R. & Deshaies, R.J. Cks1 is required for G_1 cyclin-cyclin-dependent kinase activity in budding yeast. *Mol. Cell Biol.* **20**, 5858–5864 (2000).
49. Leslie, A.G. The integration of macromolecular diffraction data. *Acta Crystallogr. D Biol. Crystallogr.* **62**, 48–57 (2006).
50. Howell, P.L. & Smith, G.D. Identification of heavy-atom derivatives by normal probability methods. *J. Appl. Cryst.* **25**, 81–86 (1992).
51. McCoy, A.J. *et al.* Phaser crystallographic software. *J. Appl. Crystallogr.* **40**, 658–674 (2007).
52. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132 (2004).
53. Adams, P.D. *et al.* PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 213–221 (2010).
54. Kinoshita, E., Kinoshita-Kikuta, E., Takiyama, K. & Koike, T. Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol. Cell Proteomics* **5**, 749–757 (2006).