Multisite phosphorylation networks as signal processors for Cdk1

Mardo Kõivomägi1, Mihkel Õrd1, Anna Iofik1, Ervin Valk1, Rainis Venta1, Ilona Faustova1, Rait Kivi1, Eva Rose M Balog2, Seth M Rubin3 & Mart Loog1

CDKs are the master regulators of the eukaryotic cell cycle1. CDKs catalyze an ordered phosphorylation of hundreds of targets that trigger a sequence of coordinated molecular events that drive the cell through the stages of cell division2–5. Several individual CDK-driven switches have been identified6, but understanding of CDK as a general coordinator of the entire process of cell division is not clear.

The existing model states that as levels of CDK activity rise during the cell cycle, specific molecular events are executed at each of a series of activity thresholds7–11. This model requires that substrates are phosphorylated with a wide range of efficiencies, such that very good substrates are phosphorylated early, at a low CDK activity threshold, whereas poor substrates are phosphorylated only when higher CDK activity is achieved later in the cell cycle. CDK targets contain both optimal consensus phosphorylation motifs (S/T-P-x-K/R)12,13 and suboptimal consensus sites (S/T-P) that are phosphorylated much less efficiently12,14. Therefore, a mechanism in which optimal motifs are used for the ‘early’ switches and suboptimal motifs for the ‘later’ switches is conceivable. However, if the system relied entirely on consensus motifs, led us to a hypothesis that the relative positions of phosphorylation sites and cyclin-dependent docking motifs are used.

Cyclin specificity in substrate recognition has provided some insight into the mechanism by which CDKs coordinate cell-cycle events18–22. In budding yeast, the intrinsic activities of different cyclin–Cdk1 complexes increase during the cycle, and the low activity of early complexes is compensated by cyclin-dependent docking sites14,23. Docking interactions enhance the abrupt phosphorylation of selected early targets in late-G1 and early-S phases without interference from later targets. Although this specificity allows early cyclins to efficiently phosphorylate early targets, the early cyclins cannot drive the later stages of the cell cycle in budding yeast24. The difference in cyclin specificities and the overall modulation of CDK activity due to its own regulatory phosphorylations provide a coarse mechanism to separate early and late events, but this still fails to explain how relatively small changes in CDK activity can trigger the ordered sequence of discrete events observed on a finer time scale.

The complexity of CDK function is further increased by the multisite nature of most of its targets. The input CDK signal is often processed through several phosphorylation steps to yield a multiphosphorylated output state of the target25–30. Recently, we demonstrated that a network of Cdk1 phosphorylation sites in budding-yeast Sic1 is phosphorylated in semiprocessive cascades22. The output of the cascade is the phosphorylation of two diphosphodegrons that direct Sic1 to degradation through the Skp1–Cul1–F box (SCF) and proteasome pathway31,32. The degron phosphorylation sites are not specific enough to be efficiently phosphorylated by Cdk1 directly because they contain suboptimal sets of recognition elements. Instead, the suboptimal degron sites become phosphorylated because of docking-dependent amplification of Cdk1 specificity through sequential steps of priming phosphorylations and interactions with the phosphoadaptor subunit Cks1.

These semiprocessive cascades, whose net phosphorylation depends on parameters other than consensus motifs, led us to a hypothesis that the relative positions of phosphorylation sites and cyclin-dependent

1Institute of Technology, University of Tartu, Tartu, Estonia. 2Department of Molecular, Cell, and Developmental Biology, University of California, Santa Cruz, Santa Cruz, California, USA. 3Department of Chemistry and Biochemistry, University of California, Santa Cruz, Santa Cruz, California, USA. Correspondence should be addressed to M.L. (mart.loog@ut.ee).

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docking sites within multisite clusters govern the net phosphorylation rate through the cascades and thereby can generate thresholds for phosphorylation of functional sites at different CDK activity levels. Variation of these parameters provides a wide range of possibilities for the differential outputs in response to changes of CDK input strengths.

The central element defining the output signal strength of these multisite processor systems is the phosphoadaptor Cks1. Cks1 was first discovered in Schizosaccharomyces pombe as a high-copy suppressor of a defective Cdk1 mutant\(^3\). In budding yeast, Cks1 is essential for both G1-S and G2-M transitions\(^34,35\) and associates with cyclin–Cdk1 complexes at close to stoichiometric ratios in vivo\(^36\). An in vitro study with the Xenopus version of Cks1 demonstrated its possible role in promoting the multisite phosphorylation of the CDK regulators Wee1, Myt1 and Cdc25, as well as the APC\(^37,38\), whereas in budding yeast the G1-specific complex Cln1,2–Cdk1 requires the Cks1 subunit for activity\(^39\). However, the function of Cks1 as a phosphoadaptor protein in the mechanism of CDK-driven switches has been largely overlooked. Also, recent large-scale proteomic screens have identified hundreds of CDK targets\(^2,4\), but these studies have not touched over the level of complexity that the multisite nature of these targets may present for the mechanism of CDK signal processing.

In the present study, we set out to analyze the biochemical parameters that control Cdk1 signal flux through multisite phosphorylation networks. We explored how different combinations of these parameters influence the net output of the signal, and we analyzed the mechanism of Cks1-dependent multiphosphorylation cascades and how processivity can be used for differential amplification of output signals. We propose a new mechanism on the basis of these data that provides a solution to the question of how accumulating CDK activity triggers the correct timing and sequence of cell-cycle events.

**RESULTS**

**Cks1-dependent multisite phosphorylation of Cdk1 targets**

Most known Cdk1 targets contain multiple phosphorylation sites, which tend to be clustered in regions of predicted disorder\(^3\). To obtain an overview of the importance of Cks1 in promoting multisite phosphorylation cascades in different targets, we analyzed a set of targets in phosphorylation assays involving either wild-type Cks1 (Cks1wt) or Cks1 with a mutated phosphate-binding pocket (Cks1mut)\(^22,40\). The autoradiographs of the phosphorylation patterns reveal that there are wide variations in Cks1 dependence among the targets (Fig. 1a). In some cases, the appearance of multiphosphorylated patterns is highly dependent on Cks1. In other cases, phosphorylation is not affected by Cks1. Also, there are targets that display intermediate effects, thus suggesting that only a subset of sites is enhanced by Cks1. We also found differences among cyclin–Cdk1 complexes: for example, phosphorylation of the G1 transcriptional regulator Whi5 by G1-specific Cln2–Cdk1 compared to M phase–specific Clb2–Cdk1 complexes. Notably, Cks1 had little effect on the phosphorylation of a substrate containing a single Cdk1 site (T5-Sic1ΔC; Fig. 1b).

The observed differences indicate that the multisite networks may have functionally different patterns. What are the parameters that determine Cdk1 activity through the networks? The specificity of cyclin–Cdk1 complexes is controlled at three different levels: first by the active site specificity of Cdk1, second by cyclin-specific docking interactions and third by the specificity of Cks1 (Fig. 1c). Using a noninhibitory form of Sic1 (Sic1ΔC) and other Cdk1 targets, we investigated how these three factors control the Cdk1-dependent phosphorylation of multisite networks.

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**Figure 1** Cks1-dependent multisite phosphorylation of Cdk1 targets. (a) Demonstration of Cks1-dependent accumulation of multiphosphorylated forms in selected targets of Cdk1. The kinase assays used purified Cln2–, Clb5– and Clb2–Cdk1 complexes preincubated with either wild-type Cks1 or Cks1mut (phosphopocket mutant). The radioactively labeled multiphosphorylated forms separated by Phos-tag SDS PAGE are shown. The number of optimal and suboptimal consensus motifs, together with an indication of whether the sites have serine or threonine residue as the phosphoacceptor, are indicated below the panels. (b) Phosphorylation assays of a substrate construct containing a single Cdk1 consensus phosphorylation site (T5-Sic1ΔC) and a standard substrate histone H1. (c) Schematic diagram indicating three pockets in the cyclin–Cdk1–Cks1 complex whose local site specificity and positioning geometry could potentially control the phosphorylation dynamics of multisite targets. Uncropped autoradiography scans are in Supplementary Figure 8.
Cks1 binds phosphothreonines but not phosphoserines

One source of variation among the targets in Figure 1 is the ratio of threonines to serines as phosphoacceptor residues. Interestingly, we noticed that the targets that do not contain Cks1 dependence (Bop3, Sld2ΔC and Ypr174) had exclusively serine residues in the phosphorylation-site positions of the optimal consensus motifs (S-P-x-K/R). Therefore, we questioned whether Cks1 may prefer phosphothreonines over phosphoserines. Of the nine CDK consensus motifs in Sic1, five are threonines, and four are serines (Fig. 2a).

The N-terminal residues T2, T5 and T33 were shown previously to serve as phosphodocking sites for Cdk1, thereby promoting fast phosphorylation of the C-terminal phosphodegrons. To test whether serines are equally able to mediate Cks1-dependent phosphorylation, we replaced the threonines in the CDK consensus sites of Sic1 with serines (Supplementary Fig. 1a). This result together with the analysis of the protein levels and phosphorylation shifts (Supplementary Fig. 2a,b) indicate that Cdk1 is not able to phosphorylate the all-serine form of Sic1 to a sufficient level to cause its proper degradation. It is most likely that inviability is caused by weak binding of phosphoserine sites to Cks1 because no phosphoserine versus phosphothreonine specificity has been observed for SCF–Cdc4 phosphodegrons.

Additionally, a single serine substitution in the crucial priming site at position 33 caused a partial loss of viability (Fig. 2d). Thus, it is possible to disrupt the docking connections of the Cks1-dependent cascade by replacing threonines in CDK sites with serines.

To directly confirm the inability of a serine to prime a single Cks1-dependent phosphorylation step, we constructed a version of Sic1ΔC containing only two sites: the optimal Cdk1-target site T33 (or S33) served as a priming site, and a suboptimal site, T48, served as a secondary site. Indeed, the threonine was required for the accumulation of the doubly phosphorylated species (Fig. 2c).

Cells overexpressing the all-serine form of Sic1 are inviable, unlike cells overexpressing wild-type Sic1. This result together with the analysis of the protein levels and phosphorylation shifts (Supplementary Fig. 2a,b) indicate that Cdk1 is not able to phosphorylate the all-serine form of Sic1 to a sufficient level to cause its proper degradation. It is most likely that inviability is caused by weak binding of phosphoserine sites to Cks1 because no phosphoserine versus phosphothreonine specificity has been observed for SCF–Cdc4 phosphodegrons. Additionally, a single serine substitution in the crucial priming site at position 33 caused a partial loss of viability (Fig. 2d). Thus, it is possible to disrupt the docking connections of the Cks1-dependent cascade by replacing threonines in CDK sites with serines. The resulting construct follows a phosphorylation mode in which phosphorylation of one site is not dependent on previous phosphorylation of the other. In fact, such a random distributive mode was the basis of an earlier model of Sic1 phosphorylation, and these results provide an additional argument supporting the semiprocessive Cks1-dependent cascade model.
with purified proteins. We produced stoichiometrically phosphorylated versions of Sic1ΔC containing single phosphorylation sites. The data obtained further confirmed the exclusive preference of phosphothreonine over phosphoserine in Cks1 binding (Fig. 2e and Supplementary Table 1).

Finally, we demonstrated the requirement for threonine as the crucial priming residue in vivo. The replacement of the threonine at the crucial N-terminal priming site T3 in Sic1 resulted in a severe reduction in the multiphosphorylated forms of Sic1ΔC-based substrate constructs containing five serine consensus motifs (Fig. 2f and Supplementary Fig. 2c).

To confirm that the effects observed in the threonine-to-serine mutations in the Sic1 constructs were reflecting a general phenomenon and were not specific for the Sic1 protein, we investigated another multisite target, Srl3. Similarly, the mutation of threonines to serines in the CDK consensus sites suppressed the Cks1 dependence of Srl3 (Supplementary Fig. 2d). Moreover, the mutation of threonines to serines in the CDK consensus sites suppressed the Cks1 dependence of Srl3 (Supplementary Fig. 2d).

We also performed a set of positional variations by introducing basic and hydrophobic residues around the T33 site. We found that a proline residue at position −2 relative to phosphothreonine enhanced the interaction of the phosphoepitope with Cks1 (Supplementary Table 1, Supplementary Fig. 3 and ref. 42).

Site positions affect the rate of the Cks1-dependent step
The second parameter that is likely to control signal flux through multisite cascades is the distance and relative positioning between the priming site and the secondary phosphorylation site. To analyze the impact of this parameter on the rate of Cks1-dependent phosphorylation steps, we created a series of Sic1ΔC-based substrate constructs containing two phosphorylation sites at different distances from each other. Due to its intrinsically disordered nature, the Sic1 polypeptide is an excellent system to study such distance requirements. By varying the distance, we aimed to measure the optimal distances between the phosphorylation sites in vivo by western blotting analysis of phosphorylation shifts in Sic1ΔC-derived constructs after the release of cells from α-factor arrest. (g) Distance dependence in phosphorylation of Cnn1 by Cln2–Cdk1. Numbers indicate location of a secondary phosphorylation site based on site T42, introduced at different distances from the potential priming site T3. (h) Distance dependence in phosphorylation of Far1 by Clb5–Cdk1, analyzed by MS. Numbers indicate location of a segment containing a secondary phosphorylation site, introduced at different positions relative to the potential priming site T3 (Supplementary Fig. 4e–g and Supplementary Table 2). Asterisk indicates that the +14 phosphopeptide of the Cks1wt incubation was not detected; therefore the Cks1wt/Cks1mut ratio is large but not precisely measurable. (i) Scheme explaining the optimal distance requirements and the N-to-C-directionality in the Cks1-dependent phosphorylation step. Uncropped scans are in Supplementary Figure 8.

**Figure 3** Analysis of the influence of distance between the priming phosphorylation site and the secondary phosphorylation site. (a) Scheme explaining the positional variation of the secondary site along the Sic1 polypeptide. (b) Autoradiographs of Phos-tag gels showing phosphorylation of constructs with varied distances between the priming site (T33) and the secondary site by Clb5–Cdk1. The lane at left panel shows the construct containing only the priming site T33. (c–e) Quantified profiles of relative accumulation of doubly phosphorylated forms at different distances between the priming sites and the secondary sites, as catalyzed by Clb5–Cdk1 (e), Clb2–Cdk1 (d) and Cln2–Cdk1 (e). AU, arbitrary units. (f) Demonstration of the requirement of optimal distances between the phosphorylation sites in vivo by western blotting analysis of phosphorylation shifts in Sic1ΔC-derived constructs after the release of cells from α-factor arrest. (g) Distance dependence in phosphorylation of Cnn1 by Clb5–Cdk1. Numbers indicate location of a secondary phosphorylation site based on site T42, introduced at different distances from the potential priming site T3. (h) Distance dependence in phosphorylation of Far1 by Clb5–Cdk1, analyzed by MS. Numbers indicate location of a segment containing a secondary phosphorylation site, introduced at different positions relative to the potential priming site T3 (Supplementary Fig. 4e–g and Supplementary Table 2). Asterisk indicates that the +14 phosphopeptide of the Cks1wt incubation was not detected; therefore the Cks1wt/Cks1mut ratio is large but not precisely measurable. (i) Scheme explaining the optimal distance requirements and the N-to-C-directionality in the Cks1-dependent phosphorylation step. Uncropped scans are in Supplementary Figure 8.
We constructed a series of Sic1 proteins carrying the site T33, which contains the optimal CDK consensus motif (TPQK), as the primary phosphorylation site. The secondary site was a short sequence bearing a suboptimal CDK motif (TPQA), which was placed at various distances from T33 (Online Methods). The priming site T33 was efficiently phosphorylated by cyclin–Cdks1, whereas the suboptimal motif used in the secondary site was not phosphorylated (Supplementary Fig. 1f). Therefore, when doubly phosphorylated species were detected, the sequence of the two-step cascade was always primary phosphorylation of T33 first, then phosphorylation of the secondary site (Fig. 3a and Supplementary Fig. 1f).

We observed a distance dependence in the rates of Cks1-dependent secondary steps (Fig. 3b). A surprisingly sharp change in rate was present in the step from 10 to 12 aa from the T33 priming site: a distance of 10 aa yielded no secondary phosphorylation, whereas a distance of 12 aa showed very rapid accumulation of the doubly phosphorylated form. This drastic distance cutoff was similar for all three cyclin–Cdks1 complexes tested (Fig. 3c–e), thus indicating that the Cks1–Cdks1 module has a similar architecture and functional capability that does not depend on cyclin specificity. In all three complexes tested, a sharp peak value of 12–16 aa in the secondary phosphorylation rates was followed by rapid decline around the distance of 20–30 aa downstream from the priming site.

A surprising feature of the Cks1-dependent step was that the cascade operates exclusively in the N-to-C direction (Fig. 3c–e). We did not observe any docking-enhanced secondary phosphorylation even when the priming site T33 was moved further downstream, to yield distances of −40, −60 and −80 aa (Fig. 3c–e).

Interestingly, the relatively sharp distance optimum was broadened when the Cks1 specificity was improved by introduction of a proline at position −2 from the T33 priming site (Fig. 3c–e). This suggests that the negative effect of above-optimal distances can be compensated by improved binding of the phosphorylated site to Cks1. The improved Cks1 specificity, however, did not improve activity with a distance shorter than the 10-aa minimal cutoff. Additionally, it is possible to increase the effective distance window for secondary phosphorylation by introducing the optimal CDK consensus site in the secondary site (Supplementary Fig. 4a–e). We also confirmed that the distance relationship was not specific for the particular sequence context downstream from the site T33. For this, we created a control set of similar constructs with the priming site T5 bearing an optimal motif PSTPPR (Supplementary Fig. 4d).

To test whether the distances between sites are also important for multisite phosphorylation in vivo, we varied the positions of phosphorylation sites in a nondestructible version of Sic1ΔC and analyzed phosphorylation by western blotting (as in Fig. 2f). We made one artificial substrate with five phosphorylation sites in their original positions but lacking the intermediate sites T33 and T45 to remove the possibility for Cks1-dependent docking steps. We compared this substrate to a construct that was identical except that three serine Cdks1 sites were repositioned to be 16 aa apart (Fig. 3f) (d16-Sic1ΔC). These constructs contain a single optimal priming site, T5, whereas the other sites were serine-based suboptimal sites. The first construct, which has sites either N terminal of T5 or greater than 64 aa downstream, showed almost no change in phosphorylation profiles after entry into S phase. In contrast, the construct with the sites repositioned at accessible distances from the T5 priming site displayed mobility shifts of singly and doubly phosphorylated species. The phosphorylation pattern of this construct fits well within the distance relationship observed in the case of the optimal priming site containing a proline in position −2 (Fig. 3c–e and Supplementary Fig. 4d). The sites at distances of +16 and +32 are within the range of relatively fast rates, whereas the longer distances apparently fail to gain any enhancement from Cks1-dependent docking.

Next we analyzed the distance requirements for the Cks1-dependent phosphorylation of two additional representative Cdks1 targets. First, we varied the distance between a potential priming site and a secondary site in the kinetochore protein Cnn1 (Fig. 3g). The obtained activity profile closely matched the one obtained from the detailed approach with the Sic1-based constructs.

Second, to analyze the distance requirement between a single pair of sites in the context of many other sites in a multisite target, we focused on the priming site in the pheromone pathway. Again, the obtained distance profile closely resembled that obtained with the Sic1-based constructs (Fig. 3h and Supplementary Table 2).

These experiments reveal a strikingly sharp minimal distance cutoff and strict N-to-C-directionality both in vitro and in vivo. From this, we conclude that the underlying mechanism involves simultaneous binding of the phosphorylated priming site to the Cks1 pocket and the secondary site to the active site pocket of Cdks1 (Fig. 3i).

Distances between phosphorylation sites are critical in vivo

To confirm that the distances between sites encode biological information in multisite networks, we performed a series of viability assays (as in Fig. 2d). To increase the sensitivity of the critical distance variations, we used a version of Sic1 with a minimally viable set of five phosphorylation sites (Fig. 4a). Surprisingly, moving the priming site T33 in these constructs by only 2 aa upstream or downstream caused inviability. Apparently, in these constructs a docking distance that perfectly fits the optimum of 12–16 aa (Fig. 3c–e) is required for efficient phosphorylation of both sites of the diphosphodegron (T45 and T48). One of the acceptor sites of the diphosphodegron
is a ‘non-CDK’ consensus site (T48) whose phosphorylation could be even more sensitive to the docking distance as compared with that of the consensus sites. We also moved the position of the diphosphodegron T45–T48 by 10 aa in a version of Sic1 containing all physiological sites. Similarly, this moderate shift of the position caused a severe reduction of viability (Fig. 4b). These data indicate that site positioning in multisite networks in Cdk1 targets is not random but involves critical distances between primer and acceptor sites that must fit the distance between the Cks1 phosphate-binding pocket and the active site of Cdk1.

**Cln2 directs phosphorylation to a specific C-to-N distance**

Next we studied the effect of altering the distance of phosphorylation sites from cyclin-specific docking sites. In Sic1, there are two Cln2-specific docking sites (RLX)18–20,45–50 and a single Cln2-specific docking motif (LLPP)14,46,51. We analyzed constructs containing only one of the RXL motifs and the LLPP motif (Fig. 5a). The position of an optimal CDK consensus motif based on site T5 was varied over a wide range in both C- and N-terminal directions relatively to the fixed positions of the docking sites. We tested phosphorylation of these constructs with wild-type Cln5–Cdk1 or Cln2–Cdk1 as well as with kinase complexes with a mutated hydrophobic-patch docking site on cyclin (hpm)18,19 and plotted the distance profiles as ratios of the phosphorylation rates obtained with wild-type and hpm kinase complexes (Fig. 5b,c). For Cln2, we observed a striking increase in docking-dependent phosphorylation rate when the phosphory acceptor site was placed at various distances from the Cln2 docking site RTL and Cln2 docking site LLPP. (b) Specificity profiles obtained from substrate constructs with different distances from the RXL motif, plotted as a ratio of phosphorylation rates of the wild-type and hpm version of the Cln5–Cdk1 complex. (c) Analogous specificity profile as in b, plotted for Cln2–Cdk1. (d) Analogous specificity profile as in b, plotted for Cln2–Cdk1 assayed in the absence and in the presence of the competitor peptide containing the cyclin-docking motif LLPP. (e) Schematic representation of cyclin–Cdk1–Cks1 complex showing the arrangement of the three pockets that are key for processing of Cdk1 signal through different multisite phosphorylation networks. The model was created by superimposition of domains from crystal structures (PDB 1BUH152, 2CCI146 and 4LPA47) solved in the presence of the relevant substrate peptide bound to the pocket. (f) Structural model of the cyclin–CDK–Cks1 complex showing the positioning of the three key substrate pockets.
Figure 6 Analysis of the processivity of multiphotosphorylation. (a) A mechanism in which the phosphorylation of a priming site is followed by the phosphorylation of the secondary site without dissociation of the substrate from the enzyme. (b) Scheme of the substrate construct T5-T33-Sic1AC. (c) Time courses showing early stages of the accumulation of doubly phosphorylated forms, separated by Phos-tag. Asterisk indicates unidentified phosphoforms. (d,e) Graphs showing the relative accumulation patterns of doubly phosphorylated forms in the experiments exemplified in c. The y axis (multiphotosphorylation factor, XMP) shows the ratio of 2P/(1P + 2P), where 2P and 1P are the quantified amounts of the doubly and singly (pT5-T33-Sic1AC) phosphorylated species, respectively. The y intercepts are the estimates of the processivity factors, representing the immediate (without a lag period) appearance of doubly phosphorylated forms (Supplementary Fig. 6). (f) A scheme for the second major processive mechanism. The phosphorylated priming site, when docked to the Cks1 pocket, can processively phosphorylate several secondary sites without dissociation. (g–j) Analysis of mechanism II with the construct T33-S69-S76-S80-Sic1AC (g), in which T33 is a priming site, with wild-type (i) or mutant (j) Cks1 complexes (quantification in h). The full time course was used to facilitate broader analysis of the phosphorylated forms. The intercepts in h provide the estimates for the processivity factors at each step. pS76/pS80 indicates the position of comigrating forms with a single phosphate, phosphorylated at site S76 or S80. Uncropped scans are in Supplementary Figure 8.

Directionality of the peptides in the crystal structures. As shown, a continuous polypeptide chain can be modeled that has an N-terminal Cks1-docking phosphate, a middle phosphoacceptor site and a C-terminal RxLxF cyclin-docking sequence. Under the assumption of a substrate in an extended conformation, the minimum sequence lengths between these elements roughly correspond to the distance requirements found in our experiments.

Processivity of Cks1-dependent multisite phosphorylation

Our observations suggest that multisite phosphorylation of many Cdk1 substrates depends on multiple interactions between the substrate and kinase. It is likely that these interactions enable processive or semiprocessive attachment of multiple phosphates during a single substrate-binding event (Supplementary Fig. 5).

In the case of Cks1-dependent cascades, there are two mechanisms by which the processive step could take place. The first mechanism would allow the phosphorylated priming site to withdraw from the active site of Cdk1 and subsequently reassociate with the phosphate-binding pocket of Cks1 without dissociation from the enzyme (Fig. 6a). Support from cyclin-docking sites would be required to maintain the complex during such a displacement. To explore the existence of such a mechanism, we used a Sic1AC-based construct containing a pair of phosphorylation sites (Fig. 6b). We measured the accumulation of singly and doubly phosphorylated forms, which were resolved in Phos-tag gels (Fig. 6c). Interestingly, we observed a considerable degree of processivity when either Clb5– or Cln2–Cdk1 was used, as the relative abundance of phosphorylated forms was high and constant at the early stages of the reaction (Fig. 6c–e).

Additionally, in the same time window, we did not see any change in the ratios of doubly and singly phosphorylated species at different enzyme concentrations, a result that further confirms the semiprocessivity (Fig. 6d,e and Supplementary Figs. 6 and 7a–d). Explanation of the analysis is presented by the simulations of a mathematical model (Supplementary Fig. 6). The processivity factors obtained for the Clb5 complex suggest that after the phosphorylation of the first site there is about a 40% chance to add the secondary phosphate without dissociation. Intriguingly, in the case of the hpm version of Clb5, the appearance of the doubly phosphorylated form was not immediate, and the apparent processivity factor was very low (Fig. 6d). These data suggest that support from the cyclin-docking site is necessary for processivity (as depicted in Fig. 6a). In addition, the Clb5–dependent reaction lost its processivity when Cks1mut was used in the assay (Fig. 6c,d and Supplementary Fig. 7c,d). Furthermore, whereas Cln2–Cdk1 showed only a slightly lower level of processivity compared with that of Clb5–Cdk1, the mitotic Clb2–Cdk1 complex showed almost no processivity. This finding correlates with the weak hydrophobic-patch docking of Clb2 (Fig. 5c). Thus, the earlier cyclin complexes show higher processivity because they can use cyclin-docking sites.

The second possible mechanism for Cks1-dependent processivity is the sequential addition of phosphates without dissociation of the primed phosphate from Cks1 (Fig. 6f). To analyze this mechanism, we used a construct containing T33 as a priming site and a triple-serine cluster S69-S76-S80 as secondary sites (Fig. 6g). These experiments showed that this type of processivity exists as well. The processivity factors at subsequent steps showed a 20–40%
probability of continuing with the next phosphorylation step without dissociation (Fig. 6h–j).

Thus, the degree of processivity may be an additional factor that can differentiate the output signals in different Cdk1 substrates. We tested this possibility on two additional physiological targets: Whi5 and Fin1. Our results (Supplementary Fig. 7e–j) suggest that various multisite networks can provide different degrees of processivity for each cyclin–CDK complex, illustrating the potential for broad dynamic range in the processing of Cdk1 signals by different substrates.

Multisite clusters form Cks1-dependent docking networks
To detail the role of Cks1 in other multisite targets, we analyzed the phosphorylation of Stb1, Ndd1 and Swi5, using a quantitative MS-based approach (Fig. 7). The Cln2-specific target Stb1 showed a number of Cks1 docking connections that are in agreement with the N-to-C directionality rule, threonine versus serine specificity and the distance rules. Similarly, the Clb2–Cdk1 target Ndd1 showed Cks1-dependent phosphorylation enhancements that can be explained by the network rules. Finally, the threonine versus serine rule was well exemplified in Swi5, which contains eight serine-based optimal CDK

![Figure 7](image-url)

**Figure 7** Cks1 differentially stimulates the phosphorylation of various sites in Stb1, Ndd1 and Swi5. (a–l) Quantitative MS analysis of relative abundance of proteolytic phosphopeptides in kinase reactions containing purified cyclin–Cdk1 complexes with either Cks1wt or Cks1mut and recombinant Stb1, Ndd1 and Swi5. Example spectra (a,b,e,f,i,j) show the relative phosphorylation intensity of various phosphopeptides in kinase reactions with Cks1wt or Cks1mut ([16O] and [18O], respectively). Reactions were supplemented with [16O]ATP or [18O]ATP as indicated, to allow detection of the phosphopeptides by the isotope shift (further experimental details in Online Methods). Diagrams outlining the relative patterns of phosphopeptides obtained in the reactions with Cks1wt and Cks1mut (c,g,k) and outlining Cks1-dependent docking networks in Stb1, Ndd1 and Swi5 (d,h,l) are shown.
sites, with no threonine-based optimal priming sites at required distances. None of these SP sites showed defects in phosphorylation with the Cks1mut, whereas Cks1 did enhance phosphorylation at sites downstream of suboptimal threonine-based sites. In conclusion, the phosphorylation of these three representative substrates followed the rules of Cks1-dependent phosphorylation derived from the Sic1 model experiments (detailed interpretation of the MS experiments in Supplementary Tables 3 and 4; Supplementary Note).

DISCUSSION
In this study, we analyzed parameters that control the phosphorylation of multisite targets of Cdk1. We propose that the ability of Cdk1 to produce the multiphosphorylated output form of its targets depends on the overall spatial pattern of the multisite cluster, the distances between the sites, the direction of docking connections, the composition of the network with respect to serine versus threonine residues, the Cks1 phosphoepitope specificity and the processivity at each step.

These mechanisms may have broad importance in cell-cycle regulation, because the majority of CDK targets contain clusters of multiple phosphorylation sites within regions that are predicted to be intrinsically disordered. Within these clusters, many potential Cks1-dependent docking connections can be predicted. Among the 74 in vivo proven Cdk1 targets listed in a recent review, 53 contain potential docking connections between pairs of sites (data not shown). Altogether, the number of possible docking connections in these targets was 151, and 75% of these pairs of sites were located in the same disordered region of the substrate.

It is important to note that besides the parameters controlling the cyclin–Cdk1–Cks1–dependent phosphorylation of multisite clusters, there is also a possibility that dephosphorylation specificity contributes to the timing of CDK-dependent phosphorylation, with earlier targets being more resistant to phosphatases. Gradually changing phosphatase specificity has been shown to have a role in ordered dephosphorylation of Cdk1 targets in mitotic exit by Cdc14 (ref. 53). However, it is not clear whether the same is true for phosphatases countering the rising Cdk1 activity before mitotic exit. Instead, a phosphatase was shown to have highly specific interaction with a mammalian early Cdk target, the pRB protein. Indeed, different phosphatase specificity at each step would further increase the possibility of the networks.

Similar principles of stepwise modulation of the output signal of Cdk1 targets can be extended to other kinases that use phosphorylated CDK sites as priming sites (for example, GSK, Cdc7 and Cdc5), as demonstrated recently. Future studies of the mechanisms of multisite processor systems will, we hope, uncover the intricate complexity of Cdk1 switches and finally provide a full understanding of the general mechanism of the Cdk-controlled cell-cycle clock.

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

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AUTHOR CONTRIBUTIONS
M.K., M.O., A.I., E.V., R.V., I.F. and R.K. designed and performed the experiments. The ITC experiments were performed by E.R.M.B., and the structural model was constructed by S.M.R.; M.L. coordinated the project and wrote the manuscript with assistance from S.M.R., M.K. and M.O.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Protein purification. TAP purification of cyclin-Cdk1 complexes (Cib5– and Cib2–Cdk1) was performed as described previously2,5, with C-terminally TAP-tagged cyclin constructs cloned into 2-micron vectors and overexpressed from the GALI promoter (strains and plasmids in Supplementary Tables 5 and 6). For purification of 3HA-tagged Cln2–Cdk1, a yeast strain was used (a kind gift from D. Kellogg, UCSC) with the GALI promoter introduced along with the N-terminal 3HA tag in the chromosomal locus of the CLN2 gene. The overexpressed 3HA-tagged Cln2–Cdk1 complex was purified as previously described58, by immunosaffinity chromatography with a rabbit polyclonal antibody against the HA epitope (custom made by Labas, Estonia). N-terminally His6-tagged recombinant Sic1ΔC constructs and His6-tagged substrates in Figure 1 were purified by standard cobalt affinity chromatography, with 200 mM imidazole used for elution. GST-tagged substrates used in the kinase assay in Figure 1 were purified on glutathione agarose columns. For Cdk1 constructs, used in MS analysis, contained GST-His6 tags and were purified by affinity chromatography by GST tag.

Kinase assays. For the phosphorylation assays of Sic1ΔC constructs, substrate concentrations were kept in the range of 0.2–5 μM (in the linear [S] versus ν range, several times below the estimated Km value). About 0.1–10 nM of purified kinase complex was used, reaction aliquots were taken at two or more time points, and the reaction was stopped with an SDS-PAGE sample buffer. The basal composition of the assay mixture contained 50 mM HEPES, pH 7.4, 180 mM NaCl, 5 mM MgCl2, 20 mM imidazole, 0.1 mg/ml 2HA peptide, 2% glycerol, 2 mM EGTA, 0.2 mg/ml BSA, 500 nM Cks1, and 500 μM ATP (with added [γ-32P]ATP (PerkinElmer)). For the phosphorylation assay with mutant Cks1 (Cks1mut), purified kinase complexes were preincubated for 45 min with Cks1wt or Cks1mut to compensate for differences in the amounts of Cks1 already present in the preparations. The optimal working concentration for Cks1 (500 nM) was based on optimizations described previously22. The general composition of the preincubation mixture was 50 mM HEPES, pH 7.4, 180 mM NaCl, 5 mM MgCl2, 20 mM imidazole, 0.1 mg/ml 2HA peptide, 2% glycerol, 2 mM EGTA, 0.2 mg/ml BSA, 500 nM Cks1, and 50 μM ATP (with added [γ-32P]ATP (PerkinElmer)). For the phosphorylation assay with mutant Cks1 (Cks1mut), purified kinase complexes were preincubated for 45 min with Cks1wt or Cks1mut to compensate for differences in the amounts of Cks1 already present in the preparations. The optimal working concentration for Cks1 (500 nM) was based on optimizations described previously22. The general composition of the preincubation mixture was 50 mM HEPES, pH 7.4, 180 mM NaCl, 5 mM MgCl2, 20 mM imidazole, 0.1 mg/ml 2HA peptide, 2% glycerol, 2 mM EGTA, 0.2 mg/ml BSA, 500 nM Cks1, and 50 μM ATP (with added [γ-32P]ATP (PerkinElmer)).

Supplementary Figure 4d, e were performed under conditions below 10–30% of initial substrate turnover. The phosphorylation of the substrate was followed in a conventional kinase assay, and singly and doubly phosphorylated species were resolved with Phos-tag SDS-PAGE and quantified by ImageQuant. The quantified values expressing the docking efficiencies (Fig. 3c–e) were calculated as the ratio of observed fraction of doubly phosphorylated form and the sum of observed fraction of doubly phosphorylated form and the form with a single phosphate. The obtained value was divided by enzyme concentration. In this way, quantitative and comparable indicators showing the ability of Cks1-dependent docking steps were obtained. The determination of real kinetic constants in this system would become complicated, owing to the mixed mechanism of the two-step process. The reaction aliquot was taken at two consecutive time points (i.e., 8 and 16 min), and the average value for the docking efficiency of the two time points was calculated. For processivity analysis, lower enzyme concentrations were used to minimize the impact of the distributive/cooperative mechanism in the formation of the multiphosphorylated form(s). The range of enzyme concentration for observation of purely processive process was chosen so that the difference in relative ratios of multiply phosphorylated forms at two different enzyme concentrations was lower than 20%. The processivity factors were calculated similarly to docking efficiencies, except that the obtained value was not divided by enzyme concentration. For all the phosphorylation assays presented in the paper, the concentration of the Phos-tag in 10% SDS-PAGE was 100 μM for Sic1, Fin1 and Ypr174c, or 20 μM for Sic1, Whi5, Nrd1, Bop3, Xpl1, Hcm1, Orc6, Cdc6, Cin1, Sld2(1–185 aa N-terminal fragment), Ndd1, Plm2, Yox1 and 100 μM Phos-tag reagent. 7% SDS-PAGE supplemented with 20 μM Phos-tag reagent was used for Far1 protein.

Isothermal calorimetry. Dissociation constants were obtained with ITC binding experiments. Recombinant His6-Sic1ΔC–based constructs were phosphorylated with purified Cib2–Cdk1 (no Csk1 was added). Stoichiometric phosphorylation was confirmed by the phosphorylation shift in Phos-tag SDS-PAGE. Phosphorylated constructs were repurified on chelating chromatography as described above, with the exception that the elution was performed with 10 mM EDTA. Purified proteins were dialyzed overnight at 4 °C in 25 mM Tris, pH 8.0, and 150 mM NaCl. Csk1 at a concentration of 0.2–1 μM was titrated into a 20–90 μM solution of phosphoSic1. Experiments were performed at 25 °C with a VP-ITC instrument (Microcal). When binding was detected, experiments were performed in duplicate. Binding constants were calculated by averaging of the Ks, and the error is the s.d. of the Ks.

Western blotting and viability assays. For western blotting experiments, versions of Sic1ΔC-SHA were cloned into vector pRS315 and constitutively expressed under the ADH1 promoter. The cells were treated for 2.5 h with 1 μg/ml α-factor and released from the arrest by removal of the α-factor by washing. The cells were lysed by bead-beating in lysis buffer containing urea. Blotting of Phos-tag SDS-PAGE gels was performed with a dry system iBlot (Invitrogen). Covance HA.11 Clone 16B12 monoclonal antibody (1:500) (cat. no. MMS-101P) and HRP-conjugated anti-mouse antibody (1:7,500) from Labs, Estonia (cat. no. 001PC GAM-HRP) were used for detection of HA-tagged proteins by western blot. For the viability assays, the Sic1ΔC versions were cloned into the pRS413 vector under the control of GALI promoter. Uncropped images of gels are shown in Supplementary Figure 8.

Sic1- and Cnn1-based substrate constructs. For studies of docking distances, we used substrate constructs based on a noninhibitory version of Sic1 with truncation of the inhibitory domain at position 216–284 (Sic1ΔC). For the constructs used for experiments presented in Figure 3b–e, the sequence motif QATPQAAQQ (based on the surroundings of T33 with the lysines, prolines and serines mutated to alanines) was introduced in positions 8, 13, 23, 39, 41, 43, 45, 47, 49, 51, 53, 65, 75, 85, 95 and 105 (the number designates the position of phosphoacceptor threonine). The priming site T33 was left unchanged in this set of the constructs. At the positions of other physiological phosphorylation sites, the double alanine replacements were introduced at the S/TP motifs in case of all Sic1ΔC constructs. In order to analyze the potential N-terminally directed docking-enhanced phosphorylation steps over longer distances, the T33 motif (QKTQPKPSQ) was replaced into positions 47, 67, and 87, and the secondary site motif was introduced at position 8. For studies of distance requirements of cyclin-dependent docking sites, the substrate constructs contained only a single RXL motif (positions 89–91), and the other motifs were mutated to alanines as described previously34. The Clb2 non-specific LLPP motif (in positions 137–140) was left intact. In these constructs, the sequence motif PSTPPRSG based on the site T5 was introduced at positions 25, 45, 67, 69, 73, 77, 81, 103, 115, 135 and 155. In case of the construct used in Supplementary Figure 4h, the QATPQAAQQ phosphorylation site was introduced at position 45. The substrate constructs used in the western blotting experiments in Figure 2f were based on a noninhibitory version of Sic1 containing a 3HA tag in its C terminus (Sic1ΔC-3HA). The sequence fragments AMSPSA, LTSHPQA, and QRSPPS based on the primary structure surrounding the physiological sites T2, T76, and S80 (with the exceptions that the lysine in position +3 from the site S76 was mutated to alanine, and the threonine in position T2 was mutated to serine), were replaced into positions 21, 37, and 53 (indicating the new positions of the phosphoacceptor residue). The sites T5 and S69 were left in their original positions. These replacements yielded constructs with five CDK consensus sites with 16 aa intervals between the sites. For testing serine as the priming site, the TSS mutation was introduced. In case of these constructs, the RXL motifs and the LLPP motif were mutated to alanines as described in ref. 22, owing to the observed slight RXL-LLPP-dependent preference of serine residues over the threonine in the phosphorylation efficiencies in vivo. In the western blotting experiment in Figure 5a, the construct with the five physiological sites in their original positions was made by mutation of the sites T33, T45, T173, and S191 to alanines; the site T2 to serine; and the lysine in position +3 from the site S76 to alanine. The construct with the changed distances between the sites was made by similar rearrangements of sequence motifs as described above for the constructs used in Figure 2f. In Sic1ΔC-based constructs used in western blotting experiments in Supplementary Figure 2c, the phosphorylation sites T2, T48, S80, T173 and S191 were mutated to alanines. Additionally, the lysine in position +3
from the site T33, the proline in position −2 from the site T45 and the arginine in position +3 from the site S76 were mutated to alanines. The priming site T5 was left unchanged or mutated to serine (Sic1∆C-S(5)PXK constructs). The RXL (positions 89–91 and 114–116) and VLLPP (positions 137–140) docking motifs were left unchanged or mutated to alanines (rxl-vllpp mutant constructs). For the western blotting experiments in Supplementary Figure 4j, the constructs with suboptimal sites had similar positioning of the sequence fragments as did the constructs with equal distance interval shown in Figures 2f and 3f, with the exception that the arginine in position +3 from the phosphorylation site T5 or S5 was mutated to alanine. For the Cnn1 constructs used in studies of docking distances in Figure 3h, the sequence motif of RNTPGY (based on the surroundings of T42) was introduced in positions 8, 12, 16, 16 and 39. In these constructs, the CDK phosphorylation site T21 was mutated to alanine.

Quantitative mass spectrometry. To quantitatively determine how Cks1 stimulates phosphorylation of different Stb1, Ndd1, Swi5, and Far1 phosphorylation sites, equal amounts of Stb1, Ndd1, Swi5 or Far1 proteins were phosphorylated by Cln2− (Stb1), Clb5− (Far1) or Clb2−Cdk1 (Ndd1, Swi5) supplemented with normal isotopic ATP ([16O]ATP) or heavy ATP ([18O]ATP) (Cambridge Isotope Laboratories). Kinase assays were performed to achieve 20–30% of total substrate turnover. Aliquots from reactions with Cks1wt and Cks1mut were pooled together in a 1:1 ratio in SDS-PAGE sample buffer. The proteins were separated by 10% SDS-PAGE, the gels were stained with Coomassie brilliant blue G-250 (Sigma), and protein bands were excised from the stained gels. Trypsin/P (20 ng µl−1) was used for in-gel digestion of proteins, and peptides were purified with C18 StageTips. Peptides were separated by Agilent 1200 series nano-flow system (Agilent Technologies) connected to an LTQ Orbitrap classic mass spectrometer (Thermo Electron) equipped with a nanoelectrospray ion source (Proxeon). Purified peptides were loaded on a fused silica emitter (75 µm × 150 mm) (Proxeon) packed in house with Reprosil-Pur C18-AQ 3 µm particles (Dr. Maisch HPLC GmbH). Peptides were separated with 30 min 3–40% B gradient (A, 0.5% acetic acid; B, 0.5% acetic acid/80% acetonitrile) at a flow rate of 200 nl/min, and eluted peptides were sprayed directly into an LTQ Orbitrap mass spectrometer with a spray voltage of 2.2 kV. The MS scan range was m/z 300–1,800, and the top five precursor ions were selected for subsequent MS/MS scans. A lock mass was used for the LTQ-Orbitrap to obtain constant mass accuracy during the gradient analysis.

Peptides were identified with the Mascot 2.3 (http://www.matrixscience.com/) search engine (Supplementary Tables 2 and 3). Peptide mass tolerance of 7 p.p.m. and fragment ion mass tolerance of 0.6 Da were used. Two missed cleavage sites for Trypsin/P were allowed. The carboxymethylation of cysteine was set as fixed modification. The oxidation of the methionine and the phosphorylation of serine and threonine were set as variable modifications. The relative intensities of phosphorylated peptides (Cks1wt/Cks1mut) were standardized by the intensity of the phosphorylated peptide containing the most N-terminal optimal Cdk1 phosphorylation site (S72 of Stb1, T179/T183 of Ndd1 and S225 of Swi5). Two independent phosphorylation experiments were performed, and the peptide intensities from at least three retention times in each experiment were quantified and used for calculation of the Cks1wt/Cks1mut effect.

Search criteria for potential docking connections in physiological targets. Criteria were a distance of 12–32 aa toward the N-terminus for any TP motif or a distance of 12–52 aa toward the N-terminus for the optimal Cks1-binding motif (F/I/L/P/V/W/YxTP82). Yeast Genome Pattern Matching (http://www.yeastgenome.org/cgi-bin/PATMATCH/nph-patmatch/) was used for the search of docking connections, and PSIPRED v3.3 (http://bioinf.cs.ucl.ac.uk/psipred/) was used for the prediction of disordered regions in the targets.

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