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Report

The SIN Kinase Sid2 Regulates Cytoplasmic Retention of the *S. pombe* Cdc14-like Phosphatase Clp1

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Summary

Cdc14-family phosphatases play a conserved role in promoting mitotic exit and cytokinesis by dephosphorylating substrates of cyclin-dependent kinase (Cdk). Cdc14-family phosphatases have been best studied in yeast (for review, see [1, 2]), where budding yeast Cdc14 and its fission yeast homolog Clp1 are regulated partly by their localization; both proteins are thought to be sequestered in the nucleolus in interphase. Cdc14 and Clp1 are released from the nucleolus in mitosis, and in late mitosis conserved signaling pathways termed the mitotic exit network (MEN) and the septation initiation network (SIN) keeps Cdc14 and Clp1, respectively, out of the nucleolus through an unknown mechanism [3–6]. Here we show that the most downstream SIN component, the Ndr-family kinase Sid2, maintains Clp1 in the cytoplasm in late mitosis by phosphorylating Clp1 directly and thereby creating binding sites for the 14-3-3 protein Rad24. Mutation of the Sid2 phosphorylation sites on Clp1 disrupts the Clp1-Rad24 interaction and causes Clp1 to return prematurely to the nucleolus during cytokinesis. Loss of Clp1 from the cytoplasm in telophase renders cells sensitive to perturbation of the actomyosin ring but does not affect other Clp1 functions. Because all components of this pathway are conserved, this might be a broadly conserved mechanism for regulation of Cdc14-family phosphatases.

Results and Discussion

Rad24 Binding to Clp1 Depends on Sid2 Phosphorylation of Clp1

Despite considerable work on the budding yeast mitotic exit network (MEN) and the fission yeast septation initiation network (SIN), the key question of how each pathway acts to keep its respective Cdc14-family phosphatase out of the nucleolus has remained unknown. Previous studies showed that in late mitosis the SIN maintains Clp1 in the cytoplasm until cytokinesis is completed by regulating the nuclear shuttling of Clp1, perhaps through the action of the 14-3-3 protein Rad24 [7, 8]. Binding of Rad24 to Clp1 depends on the most downstream SIN kinase Sid2 [7]. 14-3-3 proteins are known to bind phosphopeptides, particularly the RXXpS motif [9], and RXXpS matches the predicted consensus phosphorylation site for Sid2 family kinases [10]. Because Rad24 is restricted to the cytoplasm, we hypothesized that Sid2 phosphorylation of Clp1 might allow Rad24 to bind to and retain Clp1 in the cytoplasm. Therefore, we tested whether Sid2 could phosphorylate Clp1 directly and whether Sid2 phosphorylation of Clp1 created binding sites for the 14-3-3 protein Rad24. We found that Sid2 kinase purified by tandem affinity purification (TAP) from yeast cells was capable of directly phosphorylating bacterially produced Clp1 (Figure 1A). Furthermore, Clp1 only bound Rad24 when it had been prephosphorylated by Sid2 kinase (Figure 1B).

To ascertain the significance of Clp1 phosphorylation by Sid2 in vivo, we sought to identify and mutate Clp1 sites phosphorylated by Sid2. Phosphoamino acid analysis of in vitro phosphorylated Clp1 showed that it was phosphorylated exclusively on serine residues (Figure 1C). In-vitro-phosphorylated Clp1 was analyzed by two-dimensional phosphopeptide mapping, which identified six major tryptic peptides and a number of less abundant spots (Figure 1D). Analysis of in-vitro-phosphorylated Clp1 by mass spectrometry identified five Clp1 phosphorylation sites that were all within the C-terminal half (Figure 1E). Analysis of Clp1 purified from yeast cells by mass spectrometry identified the same five sites (Figure S1). Mutation of the five sites to alanine (Clp1-5A) significantly reduced the overall levels of Clp1 phosphorylation in vitro (Figure 1F, lane 3) and eliminated five of the six major tryptic phosphopeptides (Figure 1D). Through a combination of mutagenesis of additional sites, subsequent in vitro phosphorylation, and two-dimensional phosphopeptide analyses, we identified serine 493 as the last remaining site of significant phosphorylation. Mutation of S493 in addition to the previously identified five sites (Clp1-6A) eliminated the last major phosphopeptide and caused almost complete elimination of phosphorylation of Clp1 by Sid2 in vitro (Figure 1F, lane 4, and data not shown). Mutation of any site singly, including S493, did not cause a major reduction in Clp1 phosphorylation in vitro or binding to Rad24 in vitro (data not shown), suggesting that no single site is crucial. Bacterially expressed Clp1-6A retained wild-type in vitro phosphatase activity, suggesting that the mutations did not grossly affect the structure of the protein (Figure 1G). All six sites of phosphorylation fit the consensus RXXS motif predicted for Sid2-family kinases [10]. Mutation of an additional single RXXS motif at amino acid 499 (Clp1-7A) did not cause further reduction of the overall level of phosphorylation (Figure 1F, lane 2) and resulted in reduced in vitro phosphatase activity of recombinant Clp1 (Figure 1G); therefore, we did not pursue it further.

Loss of Sid2 Phosphorylation Sites in Clp1 Causes Premature Return of Clp1 to the Nucleolus in Late Mitosis and Failure of Rad24 Binding

To determine the role of Clp1 phosphorylation by Sid2, we integrated Clp1-6A-GFP into the *clp1*$^{+}$ locus such that it was expressed from the endogenous promoter and was the only expressed copy of *clp1* in the cell (Supplemental Experimental Procedures). The level of Clp1-6A protein was similar to that of

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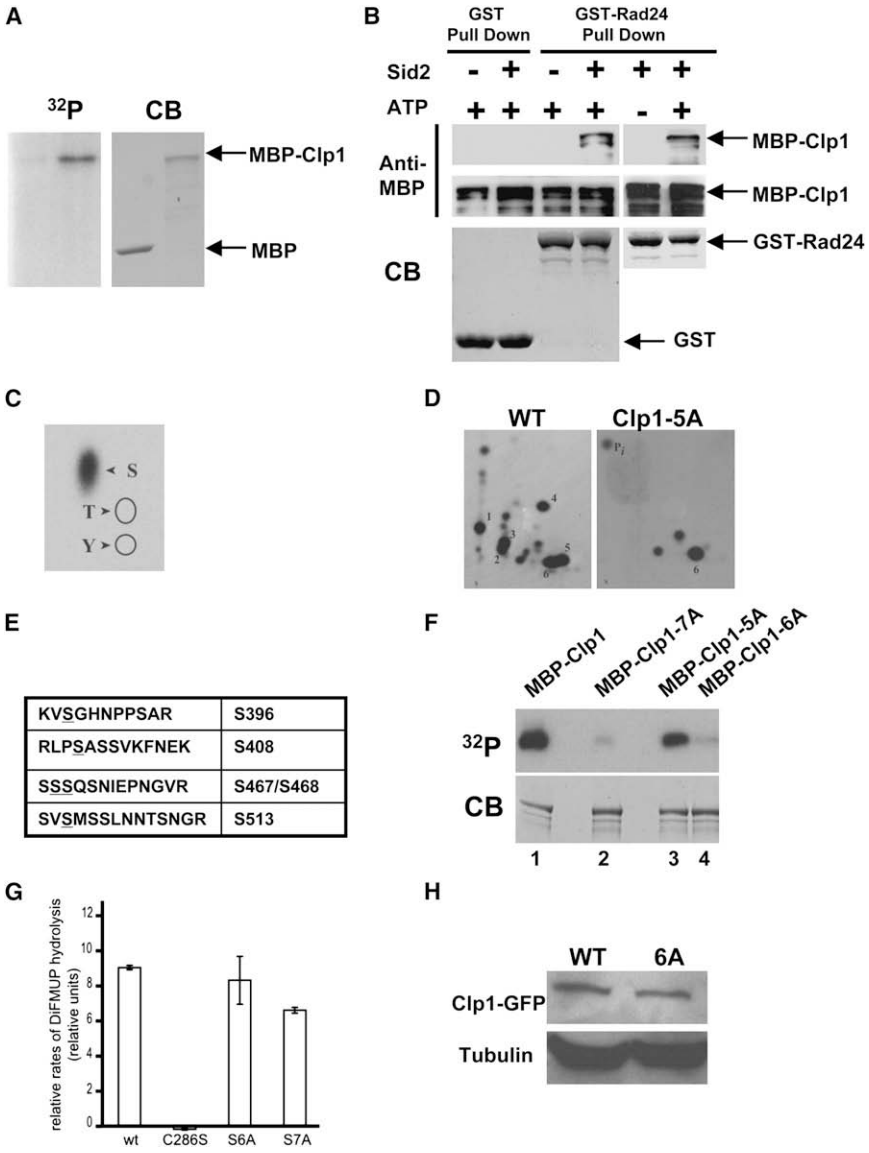


Figure 1. Sid2 Phosphorylation of Clp1 Promotes binding of Rad24 (14-3-3) to Clp1 In Vitro

(A) In vitro kinase assays [23] were performed with Sid2 kinase complexes from TAP (tandem-affinity purification) eluates from *S. pombe* cells and bacterially expressed MBP-Clp1. Protein labeled by γ-^{32}P was detected with a Phospho Imager (Molecular Dynamics), and the gel was stained with Coomassie Blue (CB) as a loading control.

(B) MBP-Clp1 was preincubated with Sid2 kinase in the presence or absence of unlabeled ATP and then incubated with bacterial lysates expressing GST or GST-Rad24. Glutathione sepharose resin was added, and the precipitates were detected by immunoblot with anti-MBP antiserum (New England BioLabs).

(C) Phosphoamino acid analysis of MBP-Clp1 phosphorylated by Sid2 kinase. The positions of the phospho-threonine and phospho-tyrosine standards are indicated by circles.

(D) Phospho-tryptic peptide analysis of MBP-Clp1 and MBP-Clp1-5A phosphorylated by Sid2 kinase. The positions of six major phosphopeptides are numbered. The position of the origin was indicated with an "x." The anode is on the left.

(E) Sites where Clp1 was phosphorylated by Sid2 kinase in vitro as identified by mass spectrometry are listed.

(F) MBP-Clp1, MBP-Clp1-5A, MBP-Clp1-6A, and MBP-Clp1-7A were purified from bacterial lysates and phosphorylated with Sid2 kinase purified by Myc antibody from *cdc16-116 sid2-13Myc* cells.

(G) The phosphatase activity of MBP-Clp1, MBP-Clp1-C286S (phosphatase inactive mutant), MBP-Clp1-6A, and MBP-Clp1-7A was determined from their ability to hydrolyze DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate) [24]. Reactions were performed in triplicate for standard error analysis. Data are representative of two independent experiments.

(H) Cell lysates of *clp1-GFP* and *clp1-6A-GFP* were prepared in NP-40 buffer (Supplemental Experimental Procedures). The Clp1-GFP and tubulin protein levels were determined by immunoblot with GFP (Santa Cruz Biotechnology) and TAT1 antibodies.

wild-type Clp1 (Figure 1H and data not shown). Like wild-type Clp1-GFP, Clp1-6A-GFP localized in interphase to the spindle pole body (SPB) and nucleolus, was released from the nucleolus as cells entered mitosis, and localized to the kinetochores and actomyosin ring in early mitosis (Figure S2A). In anaphase cells, Clp1-6A-GFP localized to the spindle, and it often appeared somewhat brighter than wild-type Clp-GFP (Figure 2A). In contrast, in anaphase the Clp1-6A-GFP actomyosin ring signal began to diminish with respect to wild-type Clp1-GFP (Figure S2A). In telophase cells, where the spindle has broken down but cells have not completed cytokinesis, wild-type Clp1 remained out of the nucleolus in the cytoplasm and appeared faintly at the contractile ring until cytokinesis was completed. In contrast, Clp1-6A appeared to return to the nucleolus prematurely and was observed only faintly if at all in the contractile ring (Figures S2A and S2B). To examine the timing of Clp1-6A release more carefully, we performed time-lapse analysis of Clp1-GFP and Clp1-6A-GFP cells expressing a marker for the actomyosin ring (Rlc1-GFP) (Figure 2A and Movies S1 and S2) and quantified the nucleolar/cytoplasmic ratios of the GFP signal (Figure S2C). This analysis showed that Clp1-6A reaccumulated in the nucleolus as soon as the spindle broke down prior to constriction of the actomyosin ring (Figure 2A [30 min], and Figure S2C). In fact, Clp1-6A most likely begins to accumulate in the nucleolus at anaphase onset because there are already significantly higher levels in the nucleolus at the time of spindle breakdown (Figure S2C). In contrast, wild-type Clp1 did not reaccumulate in the nucleolus until 75 min after the actomyosin ring had finished constriction and disappeared (Figure 2A [95 min] and Figure S2C).

When cytokinesis is perturbed by low doses of the actin-depolymerizing drug Latrunculin B, Clp1 remains cytoplasmic during the resulting cytokinesis delay (Figure 2B). In contrast, Clp1-6A returns to the nucleolus (Figure 2B and Figure S3A). This relocalization is similar to the behavior of wild-type Clp1 in SIN mutants [6]. Interestingly, unlike *clp1*Δ cells, and like wild-type cells, *clp1-6A* cells halt further rounds of nuclear division when cytokinesis is delayed by Latrunculin B treatment and remain in a binucleate state with interphase microtubules

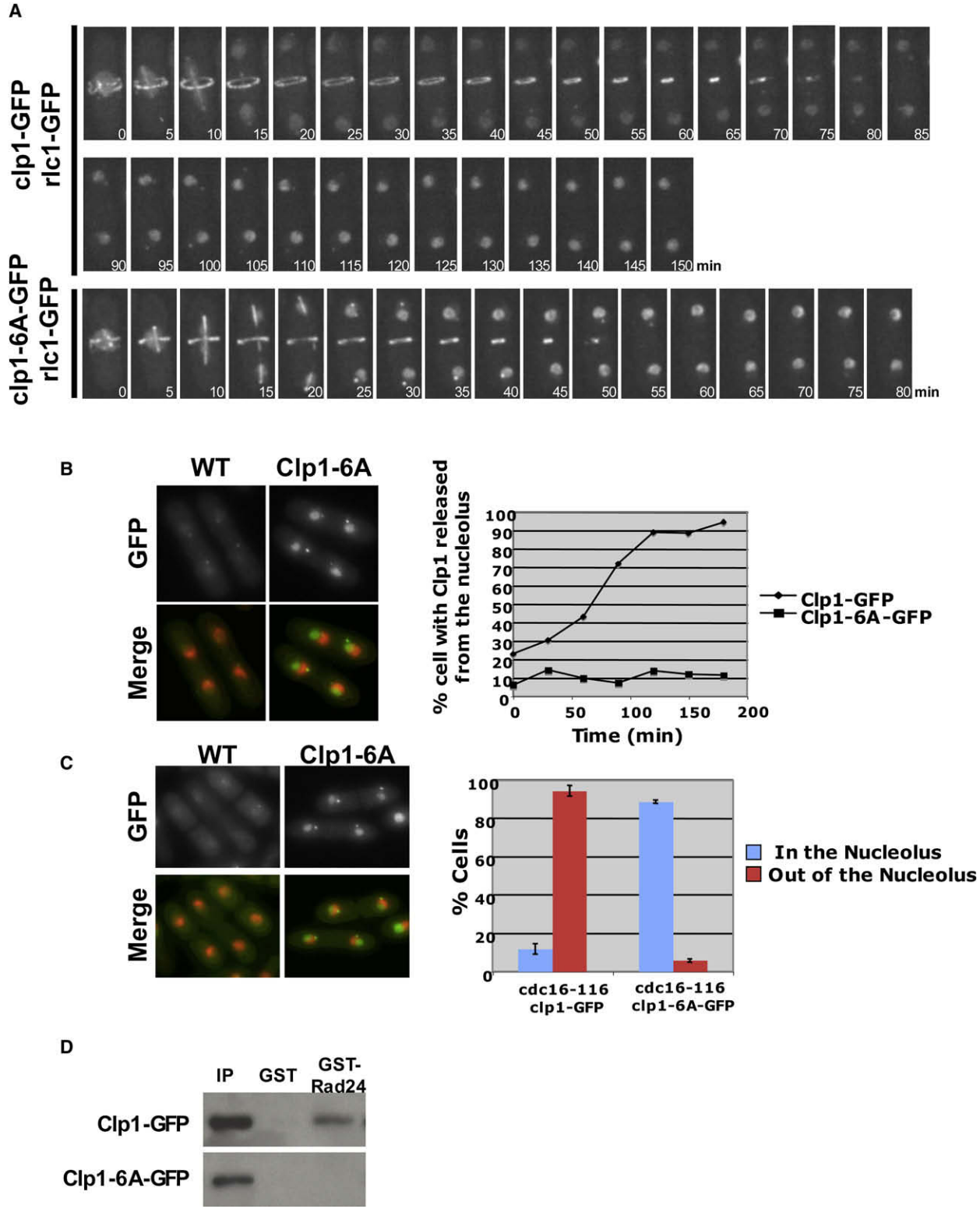


Figure 2. The *clp1-6A* Mutation Disrupts SIN Regulation of Clp1 Nucleolar Localization

(A) Time-lapse images of *clp1-GFP* and *clp1-6A-GFP* cells both expressing Rlc1-GFP as an actomyosin-ring marker were collected every 5 min, with a spinning-disc confocal microscope. Ten stacks of images were captured for each time point, and there was a step size of 0.55 μm between focal planes. Nucleolar-to-cytoplasmic ratios were calculated and are shown in Figure S2C.

(B) *clp1-GFP* and *clp1-6A-GFP* cells were grown to mid-log phase and treated with 4 μM Latrunculin B (Sigma). Cells were collected every 30 min and subjected to methanol fixation and DAPI staining (shown in red). Localization of Clp1-GFP and Clp1-6A-GFP are shown after 180 min (left panel). Cells with nucleolar or dispersed GFP localization were quantified over time (right panel).

(Figures S3B and S3C). Similarly, when the cytokinesis checkpoint is activated with the *cps1-191* mutant defective in septum assembly and actomyosin-ring constriction [11], *clp1-6A cps1-191* mutant cells arrest like *cps1-191* single mutant cells at restrictive temperature as binucleates with active SIN, interphase microtubules, and actomyosin rings. Together, these results indicate that *clp1-6A* cells have an intact cytokinesis checkpoint (Figure S4).

We previously showed that when the SIN is constitutively activated in telophase by inactivation of a component of its GTPase-activating protein, Cdc16, cells undergo repeated rounds of cytokinesis and Clp1 persists in the cytoplasm once it is released from the nucleolus in the first mitosis [6]. However, constitutive activation of the SIN in telophase is unable to keep Clp1-6A in the cytoplasm, and the mutant protein returns to the nucleolus (Figure 2C). Interestingly Clp1-6A-GFP appears to localize more strongly to the SPB than the wild-type protein in both *cdc16* cells and cells arrested by the cytokinesis checkpoint (Figures 2B and 2C). The fact that the SIN is active in both situations suggests that it might antagonize both nucleolar and SPB localization of Clp1.

We also expected that loss of Sid2 phosphorylation sites on Clp1 would disrupt binding of Rad24 to Clp1. To test this hypothesis, we examined whether bacterially produced GST-Rad24 would bind to Clp1-6A from yeast lysate. Unlike wild-type Clp1, Clp1-6A failed to bind to Rad24 (Figure 2D and Figure S5) suggesting that the cause of premature return of Clp1-6A to the nucleolus might be loss of Rad24 binding. To try to make a phosphomimetic Clp1 version that bound Rad24 independently of Sid2, we mutated the six Sid2 phosphorylation sites on Clp1 to asparate residues to generate *clp1-6D*. However, Clp1-6D did not bind Rad24 (data not shown), suggesting that aspartic acid residues cannot substitute in Clp1 for phosphorylated serines for 14-3-3 binding. The *clp1-6D* cells also displayed a general loss-of-function phenotype (data not shown), indicating that the asparate mutations caused defects in the structure of the protein; therefore, this mutant was not analyzed further (data not shown).

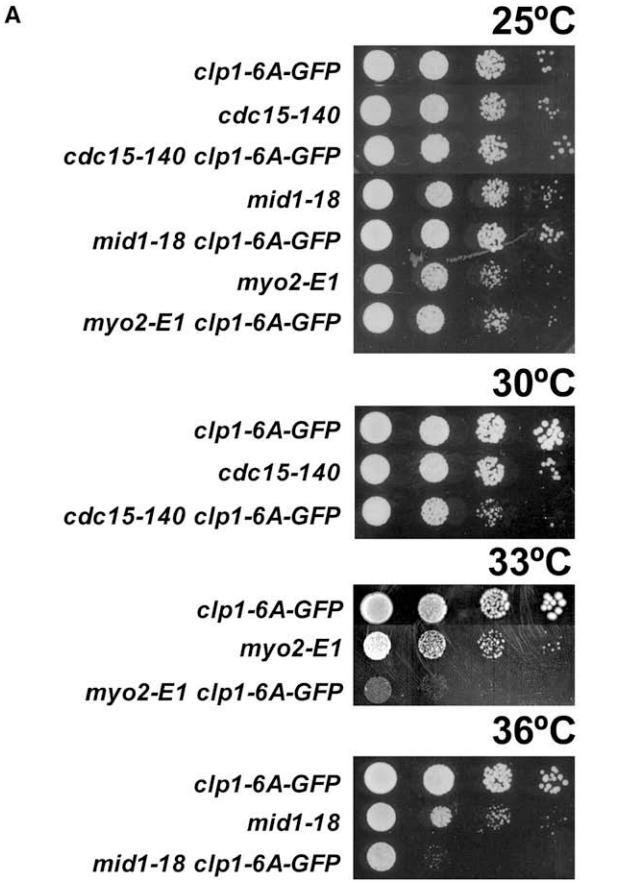
Absence of Clp1 from the Cytoplasm Causes Defects in Cytokinesis

The *clp1-6A* mutant allowed us to test the function of SIN-mediated retention of Clp1 in the cytoplasm during telophase. We assayed whether the *clp1-6A* strain displayed any of the defects found in *clp1*Δ cells. Clp1 has roles in chromosome segregation, cytokinesis, the cytokinesis checkpoint, and regulation of cell size [5, 6, 12, 13]. Unlike *clp1*Δ, the *clp1-6A* mutation does not have negative interactions with mutations in genes, such as *dis1,* that are involved in chromosome segregation (Figure S6A, and data not shown). Clp1 negatively regulates Cdc25, explaining both why *clp1*Δ cells have a reduced cell size and why overexpression of Clp1 causes a block in mitotic entry and cell elongation [5, 6, 14, 15]. Clp1-6A presumably is able to regulate Cdc25 normally because *clp1-6A* cells have a wild-type cell size and overexpression of Clp1-6A, like wild-type Clp1, blocks mitotic entry (Figures S6B and S6C). As shown earlier, *clp1-6A* is also wild-type for the cytokinesis checkpoint. It has been previously shown that the main function of Clp1 in the cytokinesis checkpoint is to promote SIN activity [12]. Consistent with this, *clp1-6A*, unlike *clp1*Δ, did not show any negative interactions with the SIN mutants *sid1-239, sid4-A1, cdc11-136, sid2-250, cdc14-118, spg1-B8,* or *mob1-R4* (data not shown). However, we did find that *clp1-6A* is sensitive to perturbations of the actomyosin ring; it is sensitive to low doses of the actin inhibitor Latrunculin B and has negative genetic interactions with several mutations affecting actomyosin-ring assembly and cytokinesis (Figure 3). In particular, *clp1-6A* cells had negative interactions with the actomyosin-ring-assembly mutants *cdc15-140*, *mid1-18*, and *myo2-E1*, and the double mutants showed synthetic growth defects at semipermissive temperatures (Figure 3A). Examination of double-mutant cells in liquid culture at semipermissive temperatures showed enhanced cytokinetic defects (Figure S7). For example, after 8 hr at 30°C, both *myo2-E1* and *clp1-6A myo2-E1* cells showed single nuclei separated by relatively complete but misformed septa. In contrast, *clp1*Δ *myo2-E1* cells, which lack the cytokinesis checkpoint, have only occasional partial septa and are highly multinucleate (Figure S8). However, the *myo2-E1* single mutant, unlike *clp1-6A myo2-E1*, was able to complete cytokinesis: There were a significant number of mononucleate cells and fewer tetranucleate cells (Figure S7). Overall, these results suggest that maintenance of Clp1 in the cytoplasm is important for completion of cytokinesis when the cell-division apparatus is perturbed.

Previous studies showed that both the SIN and Clp1 are required for a cytokinesis checkpoint that, in response to perturbation of the cell-division apparatus, halts further cell-cycle progression until cytokinesis can be completed [6, 11, 12]. When the cell-division apparatus is perturbed, Clp1 is required for maintaining SIN activity, and the SIN is required for keeping Clp1 in the cytoplasm [6, 12]. Based on these results, it was proposed that the SIN and Clp1 act in a positive-feedback loop whereby they promote each others' activity: The SIN promotes cytoplasmic retention of Clp1, and cytoplasmic Clp1 acts to maintain SIN activity [6, 12]. However, we found that cytoplasmic retention of Clp1 is not required for maintaining SIN activity and halting cell-cycle progression when the actomyosin ring is damaged (Figures S3 and S4), but it is required for completing cytokinesis (Figure 3), presumably because it maintains the cell-division apparatus. This is consistent with recent results showing that an inability to target Clp1 to the actomyosin ring causes similar cytokinetic defects when the ring is perturbed but does not cause cytokinesis checkpoint defects [16].

Although many studies have shown that the SIN and MEN regulate the conserved phosphatases Clp1 and Cdc14, respectively, to keep them out of the nucleolus during late mitosis, the mechanism has been unclear. Here we show that the most downstream SIN kinase, Sid2, phosphorylates Clp1 to promote binding of the 14-3-3 protein Rad24 (Figure 4). Binding to Rad24 results in cytoplasmic retention

(C) *clp1-GFP* and *clp1-6A-GFP* in a *cdc16-116* temperature-sensitive background were cultured to mid-log phase at 25°C, then shifted to 36°C for 2 hr. The cells were subjected to methanol fixation and DAPI staining (shown in red). Quantification of nucleolar or dispersed localization of Clp1-GFP and Clp1-6A-GFP was scored in binucleate septated *cdc16-116* cells. Error bars represent the standard deviation.
(D) Protein lysates prepared from *clp1-GFP* and *clp1-6A-GFP* cells grown at 30°C were split three ways. Clp1 was immunoprecipitated from one sample (IP) with a mouse monoclonal GFP antibody (Molecular Probes), and the other two samples were mixed with bacterially produced GST (GST) or GST-Rad24 (GST-Rad24) (Supplemental Experimental Procedures). The complexes were precipitated with glutathione sepharose resin and probed, along with the immunoprecipitated sample, by immunoblot with GFP antibodies.



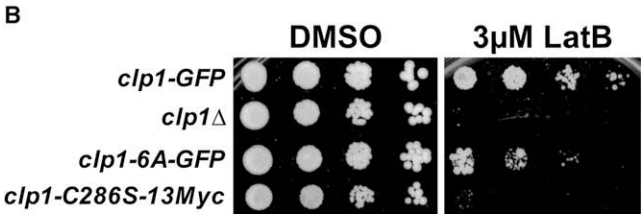


Figure 3. Functional Analysis of Clp1-6A in Cytokinesis

(A) *clp1-6A-GFP* in different actomyosin-ring mutant backgrounds (*cdc15-140*, *myo2-E1*, and *mid1-18*) were grown to mid-log phase, spotted on YE plates in 10-fold serial dilutions, and incubated at 25°C, 30°C, 33°C, and 36°C, as indicated.

(B) *clp1-GFP, clp1*Δ, *clp1-6A-GFP*, and *clp1-C286S-13Myc* were grown to mid-log phase and spotted in 10-fold serial dilutions on YE plates containing 3 μM LatB or DMSO (solvent control). The plates were incubated at 30°C for 3 days.

of Clp1. A recent study showed that the Cds1 kinase phosphorylates Clp1 on similar residues to promote cytoplasmic retention of Clp1 in response to blocks in DNA replication [17], suggesting that the same mechanism could be used by multiple inputs to regulate Clp1. In addition, the Sid2 homolog in animal cells, the Lats1/2 tumor suppressor, might regulate targets by using a similar strategy. Lats1/2 phosphorylates the oncogene YAP1, causing it to bind a 14-3-3 protein and be retained in the cytoplasm [18–22]. Given that mammalian cells have at least two Cdc14 homologs, it is tempting to speculate that they too may be regulated through Lats1/2 phosphorylation and 14-3-3 binding, as we observe in yeast.

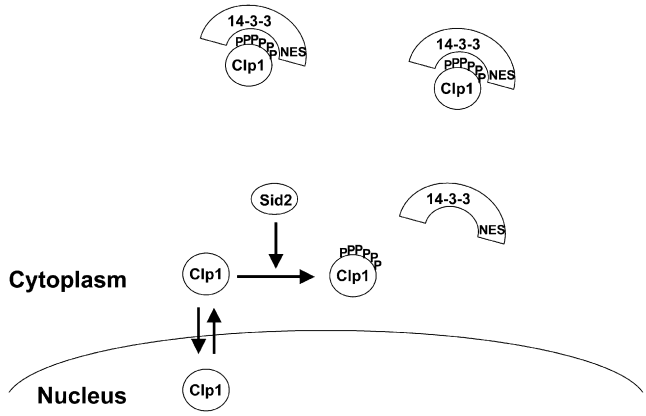


Figure 4. A Model of Clp1 Regulation by Sid2 Kinase and Rad24

Supplemental Data

Supplemental data include Supplemental Experimental Procedures, eight figures, and two movies and are available with this article online at http://www.current-biology.com/supplemental/S0960-9822(08)01241-4.

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