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# Global Analysis of Cdc14 Phosphatase Reveals Diverse Roles in Mitotic Processes*[S]

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**Cdc14 phosphatase regulates multiple events during anaphase and is essential for mitotic exit in budding yeast. Cdc14 is regulated in both a spatial and temporal manner. It is sequestered in the nucleolus for most of the cell cycle by the nucleolar protein Net1 and is released into the nucleus and cytoplasm during anaphase. To identify novel binding partners of Cdc14, we used affinity purification of Cdc14 and mass spectrometric analysis of interacting proteins from strains in which Cdc14 localization or catalytic activity was altered. To alter Cdc14 localization, we used a strain deleted for *NET1*, which causes full release of Cdc14 from the nucleolus. To alter Cdc14 activity, we generated mutations in the active site of Cdc14 (C283S or D253A), which allow binding of substrates, but not dephosphorylation, by Cdc14. Using this strategy, we identified new interactors of Cdc14, including multiple proteins involved in mitotic events. A subset of these proteins displayed increased affinity for catalytically inactive mutants of Cdc14 compared with the wild-type version, suggesting they are likely substrates of Cdc14. We have also shown that several of the novel Cdc14-interacting proteins, including Kar9 (a protein that orients the mitotic spindle) and Bni1 and Bnr1 (formins that nucleate actin cables and may be important for actomyosin ring contraction) are specifically dephosphorylated by Cdc14 *in vitro* and *in vivo*. Our findings suggest the dephosphorylation of the formins may be important for their observed localization change during exit from mitosis and indicate that Cdc14 targets proteins involved in wide-ranging mitotic events.**

Cdc14 is a serine/threonine phosphatase that is essential for mitosis. Cdc14 promotes inactivation of cyclin-dependent kinase (Cdk1 in budding yeast) during exit from mitosis by dephosphorylating three important substrates: Cdh1, which activates the $APC^{Cdh1}$ for the degradation of mitotic cyclins, Swi5, which up-regulates the Cdk1 inhibitor Sic1, and Sic1, which prevents its ubiquitin-dependent degradation (1–3). Cdc14 is regulated during the cell cycle by subcellular localization. The nucleolar protein, Net1, sequesters Cdc14 in the nucleolus and likely maintains it in an inactive state, until anaphase (4–6). Cdc14 is then released from the nucleolus by two signaling pathways, the Cdc14 early anaphase release (FEAR) network and the mitotic exit network (MEN)[5] (4, 5, 7). Sequestration of Cdc14 prevents cells from exiting mitosis before the anaphase spindle is properly aligned along the mother-bud axis (8) and prevents unscheduled dephosphorylation of replication proteins during S phase (9).

Whereas the specific substrates of Cdc14 that are critical for the decline in Cdk1 activity that accompanies mitosis have been well established, additional targets that are important for other mitotic events are less well characterized. Similar to Cdk1, Cdc14 has a preference for phospho-Ser/Thr-Pro motifs (10), suggesting that Cdc14 may directly reverse some Cdk1-dependent phosphorylation events. Cdc14 has been shown to dephosphorylate multiple substrates, including Sli15, Ask1, Fin1, and Ase1, which are important for stabilization and extension of the anaphase spindle (11–14). Cdc14 is also important for the segregation of repetitive ribosomal DNA and telomeric regions of chromosomes during anaphase (15–17), although the relevant target(s) for these processes have not been fully elucidated. In addition, Cdc14 has a role in nuclear positioning and proper segregation of replicated DNA to both the daughter and mother cells (18). In the absence of FEAR network-released Cdc14, nuclei move to the daughter cell. This suggests that Cdc14 targets proteins that generate forces pulling nuclei into mother cells in late anaphase. Finally, FEAR network-released Cdc14 dephosphorylates the MEN component, Cdc15 (19), indicating that Cdc14 assists in activation of MEN for sustained Cdc14 release and subsequent mitotic exit (20).

Cdc14 has at least an indirect role in cytokinesis, since inactivation of Cdk1 is required for cells to undergo cell division. However, it is still not clear if Cdc14 also directly promotes cytokinesis by dephosphorylating specific substrates. There is some evidence that Cdc14 directly influences cytokinesis, independent of its role in Cdk1 inactivation. In cells where Cdk1 activity is ectopically inactivated without Cdc14 release from the nucleolus, the actomyosin ring shows defects in contraction and cell separation (21, 22). Interestingly,

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[5] The abbreviations used are: MEN, mitotic exit network; PTP, protein tyrosine phosphatase; NES, nuclear export sequence.

Cdc14 localizes to the bud neck in late mitosis, and cells carrying Cdc14 with a mutated nuclear export sequence (NES) fail to localize Cdc14 to the bud neck or contract the actomyosin ring at restrictive temperatures (23). Thus, it is likely that Cdc14 has cytoplasmic targets at the bud neck whose dephosphorylation is essential for cytokinesis.

We initiated studies to identify and characterize novel Cdc14 substrates that are important for mitosis and cytokinesis. Prior research has revealed that immunopurification and mass spectrometric (MS) analysis of Cdc14 from asynchronous budding yeast yielded the known Cdc14 substrate, Sli15, as well as additional proteins associated with cell cycle control (24). We extended these immunopurification and MS studies, initially using mutants that affect Cdc14 localization to distinguish between proteins that associate with nucleolar (sequestered) Cdc14 *versus* nuclear and cytoplasmic (released) Cdc14. We then used a catalytically inactive Cdc14 mutant, which binds phosphatase substrates with higher affinity, potentially enriching for Cdc14 substrates. With these methods, we identified many Cdc14-associated proteins, some of which were previously established and some of which have not yet been reported. We have characterized a subset of these interactors as new Cdc14 substrates. Our results indicate that Cdc14 is important for multiple mitotic processes in addition to Cdk1 inactivation.

## EXPERIMENTAL PROCEDURES

*Yeast Strain Construction*—Standard methods were used for mating, tetrad analysis, and transformations. All strains are in the w303 background. A list of strains generated in this study is provided in the supplemental information (supplemental Table S1). Protein A tagging of *NET1*, *BNI1*, *BNR1*, *BUD3*, *MAD1*, *KAR9*, *SPC110*, *SPC42*, and *SLI15* at the C terminus was performed by genomic integration of a DNA sequence that encodes the IgG binding domains of protein A (*PRA*) from *Staphylococcus aureus* and the *HIS3MX* selection marker from *Schizosaccharomyces pombe* (25, 26). Tagging of *NET1*, *BNI1*, *BNR1*, *KAR9*, *SFI1*, *HSL1*, *GIN4*, *DMA2*, *NUD1*, and *SLI15* at the C terminus with six repeats of the HA epitope (HA) was done by genomic integration of a polymerase chain reaction (PCR) product that also contained the *klTRP1* gene, as described previously (27). The constructs, *RS304-GALS-CDC14-FLAG*, *RS304-GALS-CDC14-D253A-FLAG*, *RS304-GALS-CDC14–5GFP*, and *RS304-GALS-CDC14-D253A-5GFP* were integrated by EcoRV digestion, and single integrations were confirmed by Southern blot analysis.

*Immunopurification for Mass Spectrometry*—Immunopurification of Cdc14–5GFP for mass spectrometric analysis was performed as previously described (24). For the Δ*net1 CDC14–5GFP* and *cdc15–2 CDC14–5GFP* strains, 8 g of ground cells were extracted in TBT buffer (20 mM K-HEPES, pH 7.4, 110 mM K-acetate, 0.1% Tween 20, 2 μg/ml DNase I, and 0.5% protease inhibitor mixture (Sigma-Aldrich)) with 0.2% Triton and 200 mM NaCl. Samples were incubated with 4 mg of magnetic beads conjugated to anti-GFP polyclonal antibody for 1 h. For the *GAL1-GFP* and *GALS-CDC14-D253A-5GFP* strains, 8 g of ground cells were extracted in TBT buffer with 0.25% Triton and 250 mM NaCl. Samples were incubated with 12 mg of magnetic beads conjugated to anti-GFP polyclonal antibody for 1 h.

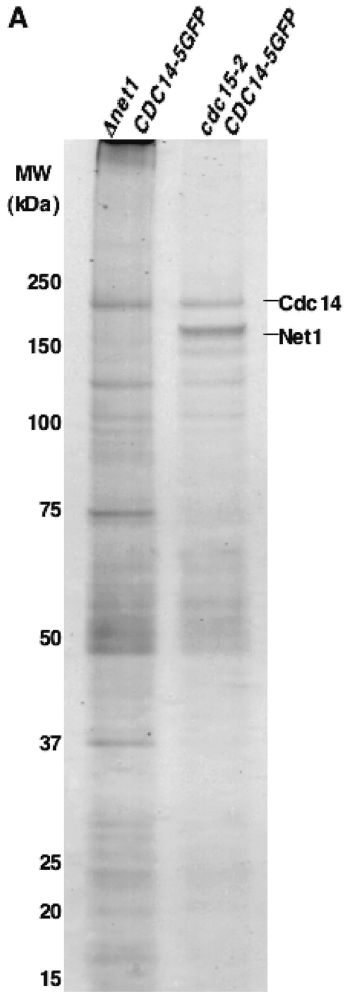
*Protein Extraction, Western Blotting, and Binding Assays*—Protein extraction and immunoblot were performed as previously described (28). To test binding of protein A-tagged proteins to recombinant Cdc14, yeast were extracted in TBT with protease inhibitor mixture and PhosSTOP phosphatase inhibitor mixture (Roche) and incubated for 1 h with glutathione-Sepharose beads coated with 5 μg of GST, GST-Cdc14, GST-Cdc14-C283S, or GST-Cdc14-D253A produced in *Escherichia coli* BL21 cells. The beads were washed six times with TBT buffer and resuspended in Laemmli sample buffer. To test binding of $HA_6$-tagged proteins to galactose-inducible Cdc14-FLAG and Cdc14-D253A-FLAG, yeast strains were induced with 0.3% galactose and then extracted in TBT with protease inhibitor mixture and PhosSTOP phosphatase inhibitor mixture (Roche). Extracts were incubated with M2 anti-FLAG antibody (Sigma) for 1 h and then protein G-agarose beads (Roche) for 1 h. The beads were washed six times with TBT buffer and resuspended in Laemmli sample buffer. Immunoblotting was performed using rabbit anti-HA and rabbit anti-Cdc14 (Santa Cruz Biotechnology).

*In Vitro Phosphatase Assays—In vitro* phosphatase assays were carried out according to Ref. 29. Briefly, $HA_6$-tagged proteins were purified using rabbit anti-HA (Santa Cruz Biotechnology) and protein A-agarose beads (Roche). Beads were washed four times with lysis buffer and twice with phosphatase buffer (25 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 0.1 mg/ml bovine serum albumin). Immunoprecipitates were resuspended in phosphatase buffer containing 2 mM $MnCl_2$ and GST, GST-Cdc14, GST-Cdc14-C283S, GST-Cdc14-D253A, or λ phosphatase (New England Biolabs) in a total volume of 50 μl and incubated for 30 min at 30 °C. Immunoblotting was performed using mouse anti-HA (Covance) and mouse anti-phosphoserine/phosphothreonine (BD Biosciences).

*Formin Localization Studies*—Strains were arrested in metaphase using 2 mM methionine to turn off *MET3-CDC20*. If released from arrest, removal of methionine restored *MET3-CDC20* expression. For galactose induction of either *CDC14* or *ESP1* expression, cells grown in medium containing raffinose and galactose was added to 3% final concentration. Cells were fixed briefly with 4% paraformaldehyde for visualizing fluorescent markers.

## RESULTS

*Identification of Proteins Associated with Released Cdc14 and Sequestered Cdc14*—In a prior study (24), Sli15, an established Cdc14 substrate (11), was identified as a Cdc14 interactor by pulldown of Cdc14-GFP from asynchronous yeast cells and subsequent MS analysis of associated proteins, indicating that phosphatase-substrate interactions can be stable enough to be detected by these methods. However, since Cdc14 is sequestered for most of the cell cycle, it is likely that in this previous analysis a significant population of purified Cdc14 was restricted to the nucleolus. As a result, substrates of Cdc14 that are present in the nucleus and cytoplasm may




B

***Δnet1 CDC14-5GFP***

| Mitosis | Spindle pole body component | Ubiquitination/ Proteasome |
|---|---|---|
| Bni1 | Cdc31 | Cdc53 |
| Bud3 | Nud1 | Rpn2 |
| Ctf18 | Mps2 | |
| Mad1 | Spc29 | **Transport/Sorting** |
| Kar9 | Spc110 | Bmh1 |
| Sgo1 | | Rav1 |
| **DNA repair** | **Chromatin remodeling** | **Sporulation** |
| Rad50 | Itc1 | Spo14 |
| **Autophagy** | Ris1 | **Unknown** |
| Atg20 | Sir1 | Ydr056c |

***cdc15-2 CDC14-5GFP***

| Mitosis | Spindle pole body component | Ubiquitination/ Proteasome |
|---|---|---|
| Bni1 | Cnm67 | Dcn1 |
| Bnr1 | Spc110 | |
| Ctf18 | | |
| Dyn1 | | **Transport/Sorting** |
| Ipl1 | **Chromatin remodeling** | Ret2 |
| Kel1 | Rsc1 | Vps13 |
| Net1 | Set1 | |
| Nis1 | | |
| **DNA repair** | | **Unknown** |
| Rad9 | | Ecm5 |
| Rad50 | | |

FIGURE 1. **Identification of proteins associated with released *versus* sequestered Cdc14.** *A*, released Cdc14–5GFP from Δ*net1 CDC14–5GFP* cells or sequestered Cdc14–5GFP from *cdc15–2 CDC14–5GFP* cells was immunopurified with a polyclonal GFP antibody conjugated to magnetic beads. Eluates were resolved on SDS-PAGE gels and stained with Coomassie Blue. *B*, Cdc14-associated proteins identified from the gel shown in *A* were classified into functional groups.

have escaped detection. To address this, we repeated these pulldowns and MS analysis of associated proteins in cells deleted for the *NET1* gene. We reasoned that if Cdc14 were fully released from the nucleolus, we would enrich for Cdc14 substrates. For comparison, we performed immunopurification of Cdc14 and MS analysis of associated proteins under conditions where Cdc14 is kept sequestered in the nucleolus. To accomplish this, we used a strain in which the gene encoding *CDC15*, an MEN component, is temperature-sensitive (*cdc15–2*). These cells were shifted to the restrictive temperature of 37 °C for 3 h prior to collection and immunopurification of Cdc14. Fig. 1*A* shows Coomassie Blue staining of Cdc14–5GFP-associated proteins under conditions where Cdc14 is released (Δ*net1*) or sequestered (*cdc15–2*). There were substantially more bands in the pulldowns from the Δ*net1* strain than from the *cdc15–2* strain. This result was anticipated, given that Cdc14 is likely to interact with more proteins when it is released into the nucleus and cytoplasm, than when it is sequestered in the nucleolus.

Fig. 1*B* shows a list of proteins, divided into functional categories, which were found to associate with Cdc14 under conditions when Cdc14 is released (left panel, Δ*net1*) *versus* sequestered (right panel, *cdc15–2*). We also found many chaperones, metabolic enzymes and translation proteins, which are highly abundant proteins that are frequent contaminants of many target proteins in this approach (supplemental Table S2). In similar analyses of cell cycle proteins, such proteins were removed from data sets (30); we also excluded these proteins from further analysis.

For released Cdc14, we identified multiple associated proteins that are involved in mitotic events. These included Bni1 and Bud3, which have roles in cytokinesis (31–33), Mad1, which is a component of the spindle assembly checkpoint (34, 35), and Kar9, which orients the mitotic spindle (36–41). Additionally, we identified multiple spindle pole body (SPB) components as Cdc14 interactors under conditions when Cdc14 is fully released. Cdc14 has been shown to transiently associate with the SPB during early anaphase (42), at which time it promotes MEN activation by dephosphorylating SPB-localized Cdc15 (29).

Notably, many of the Cdc14-interacting proteins identified are predicted to be Cdk1 targets (43). Cdc14 has been shown to have a preference for Cdk1 sites (10), and these proteins may represent substrates that are mutually regulated by Cdk1

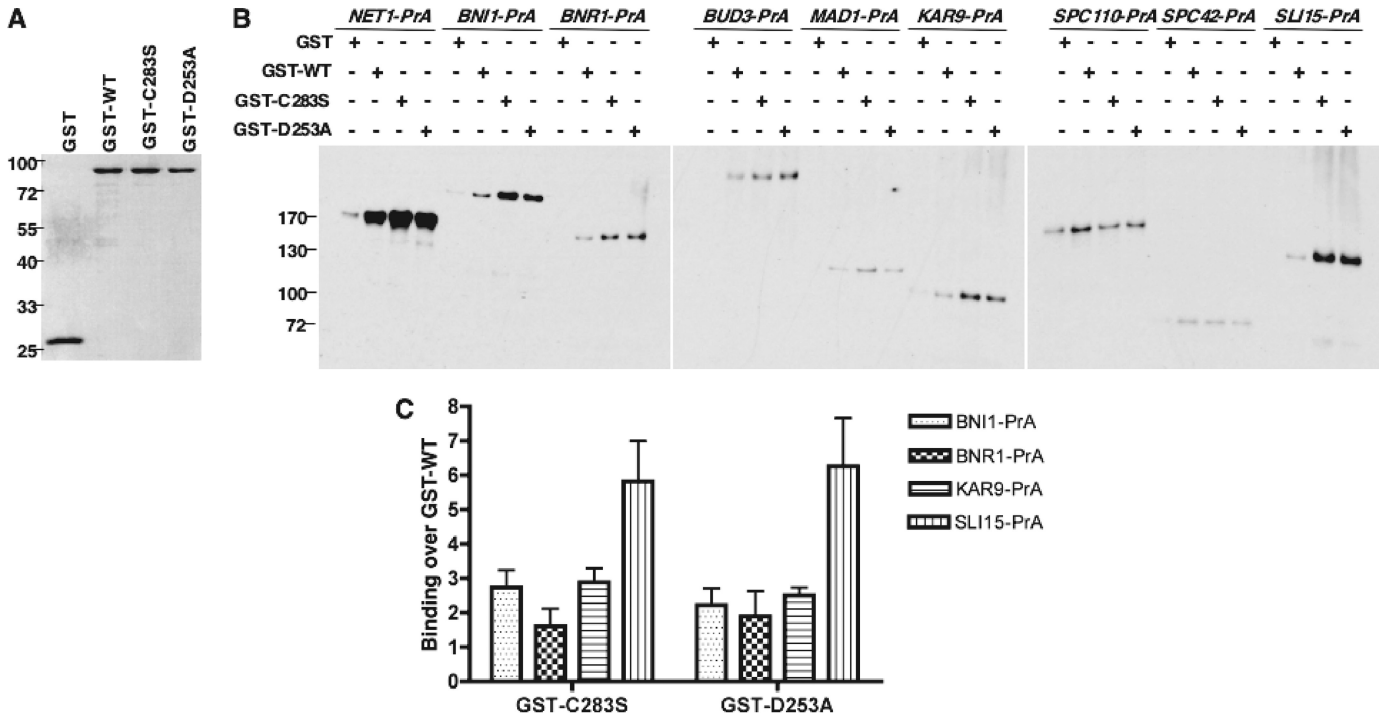



FIGURE 2. **A subset of Cdc14 interactors display enhanced binding to catalytically inactive Cdc14 mutants *in vitro*.** *A*, Sepharose beads were coated with similar amounts of recombinant GST alone, GST-tagged wild-type Cdc14 (GST-WT) or GST-tagged catalytically inactive Cdc14 mutants (GST-C283S and GST-D253A). Eluates were resolved on SDS-PAGE gels and stained with Coomassie Blue. *B*, beads shown in *A* were incubated with extract from the indicated yeast strains. Affinity purified proteins were resolved on SDS-PAGE gels and immunoblotted with a rabbit IgG antibody. *C*, quantification of binding of Bni1-PrA, Bnr1-PrA, Kar9-PrA, and Sli15-PrA to GST-C283S and GST-D253A over binding to GST-WT. Quantification represents three independent experiments.

and Cdc14. Recently, Holt *et al.* (44) presented a global list of *in vivo* Cdk substrates. Of 37 proteins identified as Cdc14 interactors, 10 were identified as *in vivo* Cdk substrates by Holt *et al.* ($p < 0.0001$) (44). This result remained statistically significant even upon inclusion of the likely contaminating highly abundant proteins detected (supplemental Table S2).

Surprisingly, we also identified multiple proteins involved in mitosis that interact with sequestered Cdc14, although the overall gel pattern (Fig. 1) suggested that most of these interactions were occurring at lower stoichiometry. These included Bni1 and the related protein, Bnr1, which are partially redundant functionally (45), as well as Dyn1, a protein that is partially functionally redundant with Kar9 (36). These interactions may occur post-cell lysis, since Cdc14 and these proteins are expected to be spatially separated in *cdc15-2* cells. Post-lysis interaction is a frequent artifact in this procedure (30).

*Catalytically Inactive Mutants of Cdc14 Enhance Binding to a Subset of Cdc14-interacting Proteins*—Two issues complicate confirmation of the association of Cdc14 with potential substrates identified by pulldown and MS analysis. For one, Cdc14 is sequestered in the nucleolus and inhibited by Net1 for the majority of the cell cycle, and therefore the interaction of Cdc14 with substrates occurs only during the brief period of anaphase. In addition, the association of Cdc14 with substrates may be transient, if the substrate is released from the Cdc14 active site immediately following Cdc14-mediated dephosphorylation. To enhance binding of Cdc14 to potential substrates, we employed a strategy that has been used for protein tyrosine phosphatases (PTPs). Conversion of a catalytic residue in human PTP-1B (Asp-181) to alanine creates a "substrate-trapping" form of PTP-1B, which interacts more stably with substrates (46). Mutation of the active site cysteine of PTP-1B has also improved the interaction of these phosphatases with substrates (47). Structural analysis of human Cdc14b reveals that it has a similar architecture to PTP-1B, with Cys and Asp residues of the active site of Cdc14b positioned in an orientation equivalent to those in PTP-1B (10). Notably, these Cys and Asp residues are conserved in budding yeast Cdc14, and mutation of either of these residues renders Cdc14 inactive (48, 49). These data suggest that mutation of either of these sites in Cdc14 could generate a "substrate-trapping" form of Cdc14, similar to that obtained with PTP-1B.

We mutated Cys-283 or Asp-253 to Ser or Ala, respectively, in pGEX-*CDC14* (9) to generate pGEX-*CDC14-C283S* and pGEX-*CDC14-D253A*. Wild-type and mutant versions of Cdc14 were produced in *E. coli* and bound to glutathione-Sepharose. Beads coated with recombinant wild-type or mutant Cdc14 (Fig. 2*A*) were incubated with extract from yeast carrying Protein A-tagged (PrA) versions of potential substrates, identified in Fig. 1, expressed from their endogenous promoters. Fig. 2*B* shows the binding of these potential substrates to wild-type *versus* mutant Cdc14.

Because Net1 and Cdc14 are stable binding partners, we tested the association of Net1-PrA to GST-Cdc14 as a control. Net1 displayed a much stronger interaction with wild-type Cdc14 than any of the substrates tested, an expected result given that Net1 acts as a regulator of Cdc14, binding to it for most of the cell cycle. The binding of Net1 to Cdc14-

C283S or Cdc14-D253A appeared to be slightly increased compared with wild-type Cdc14. This may reflect that Net1 is an *in vitro* substrate of Cdc14, although hypophosphorylated Net1 has been shown to have an increased affinity for Cdc14 (44, 50).

Several of the Cdc14 interactors that we identified in the initial mass spectrometry analysis interacted more strongly with Cdc14-C283S or Cdc14-D253A than with wild-type Cdc14, in particular Bni1, Bnr1, and Kar9 (Fig. 2, *B* and *C*). This suggests that these proteins may be bona fide Cdc14 substrates. In contrast, other proteins tested did not exhibit enhanced binding to the Cdc14 mutants compared with wild type, such as the SPB components, Spc42 and Spc110. This may reflect that Cdc14 binds these substrates but does not dephosphorylate them. Given that Cdc14 has been shown to associate with the SPB during anaphase (42), it is possible that these SPB proteins could serve as a scaffold for Cdc14. Importantly, Sli15, an established Cdc14 dephosphorylation substrate, displayed stronger binding to Cdc14-C283S or Cdc14-D253A than to wild-type Cdc14. Thus, Cdc14-C283S and Cdc14-D253A likely are effective substrate-trapping mutants.

*Immunopurification of Catalytically Inactive Cdc14 May Enrich for Cdc14 Substrates*—Since mutation of residues in the active site of Cdc14 increased the affinity of Cdc14 for the known substrate, Sli15, and potential substrates, Bni1, Bnr1, and Kar9, *in vitro*, we generated yeast strains carrying either wild-type Cdc14 or catalytically inactive Cdc14 to test these interactions *in vivo*. Overexpression of *CDC14* is lethal (2), and we were concerned that expression of catalytically inactive Cdc14 would also be lethal, since it could act as a dominant-negative mutant. Therefore, we generated strains carrying *CDC14* or *CDC14-D253A* under the control of a weakened galactose-inducible *GAL1* promoter (*GALS*) (51), which allowed for regulatable expression. We added a FLAG or 5GFP epitope tag to the C terminus of *CDC14* or *CDC14-D253A* to facilitate subsequent immunopurification experiments. Expression of *GALS-CDC14-D253A* was not lethal, unlike expression of *GALS-CDC14* (data not shown), indicating that catalytically inactive Cdc14 does not act as a dominant-negative mutant. Nevertheless, we saw increased binding of Cdc14-D253A to Sli15 and the potential substrates, Bni1, Bnr1 and Kar9, over wild-type Cdc14 *in vivo* (Fig. 4 and supplemental Fig. S1). Thus, we reasoned that Cdc14-D253A-5GFP could be useful for immunopurification and MS analysis to enrich for substrates of Cdc14.

The *GALS-CDC14-D253A-5GFP* yeast strain was grown under non-inducing conditions and then induced with galactose for 3 h prior to collection and immunopurification of Cdc14. We found this induction time is sufficient for accumulation of Cdc14-D253A-5GFP and release into the nucleus and cytoplasm as determined by fluorescent microscopy analysis. For comparison, we performed the same induction and immunopurification using a yeast strain carrying a plasmid encoding *GAL1-GFP*. Fig. 3*A* shows Coomassie Blue staining of Cdc14-D253A-5GFP-associated proteins. There were substantially fewer bands in pulldowns from the control strain (*GAL1-GFP*), indicating that the associated proteins were likely bound to Cdc14-D253A, rather than to the GFP tag.

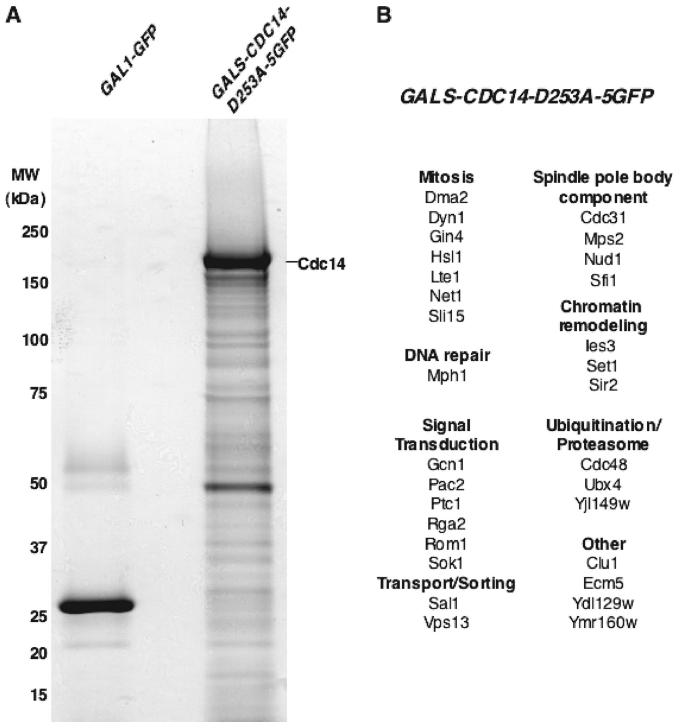



FIGURE 3. **Identification of proteins associated with a "substrate-trapping" Cdc14 mutant.** *A*, GFP alone from a control strain (*GAL1-GFP*) or of "substrate-trapping" Cdc14-D253A-5GFP expressed from a galactose-inducible promoter (*GALS-CDC14-D253A-5GFP*) was immunopurified with a polyclonal GFP antibody conjugated to magnetic beads. Eluates were resolved on SDS-PAGE gels and stained with Coomassie blue. *B*, Cdc14-associated proteins identified from the gel shown in *A* were classified into functional groups.

Fig. 1*B* shows a list of proteins divided into functional categories, which were identified by MS as interactors of Cdc14-D253A. In this experiment, we found additional proteins involved in mitosis. These included Hsl1 and Gin4, which are important for sensing septin organization and initiating the morphogenesis checkpoint (52). We also identified proteins involved in mitotic spindle orientation, Dma2, a protein with a role in spindle positioning (53), and Dyn1, a protein that is partially functionally redundant with Kar9 (36). Lte1, a component of the mitotic exit network, was also identified as a Cdc14-D253A interactor. Interestingly, Cdc14 regulates Lte1 dephosphorylation and redistribution of Lte1 from the bud cortex during mitosis (54). This indicates that probable substrates of Cdc14 can be identified by immunopurification of Cdc14-D253A and subsequent MS analysis. Consistently, Sli15 was also detected using this method. In addition to the SPB components that we found by analysis of Cdc14 interactors in Δ*net1* cells, Cdc31, Mps2 and Nud1 (Fig. 1*B*), Cdc14-D253A pulldown yielded Sfi1. Sfi1 is a component of the SPB half-bridge that is essential for SPB duplication (55, 56) and has a role in SPB separation (57). Notably, mutations of a Cdk1 consensus site in the C terminus of Sfi1 prevents separation of duplicated SPBs (57). This indicates that phosphorylation of Sfi1 is important for SPB separation, and dephosphorylation of Sfi1 may be a step in licensing a new round of SPB duplication.

Using this method, we did not detect some potential Cdc14 substrates that were identified in Fig. 1, such as Bni1, Bnr1, or

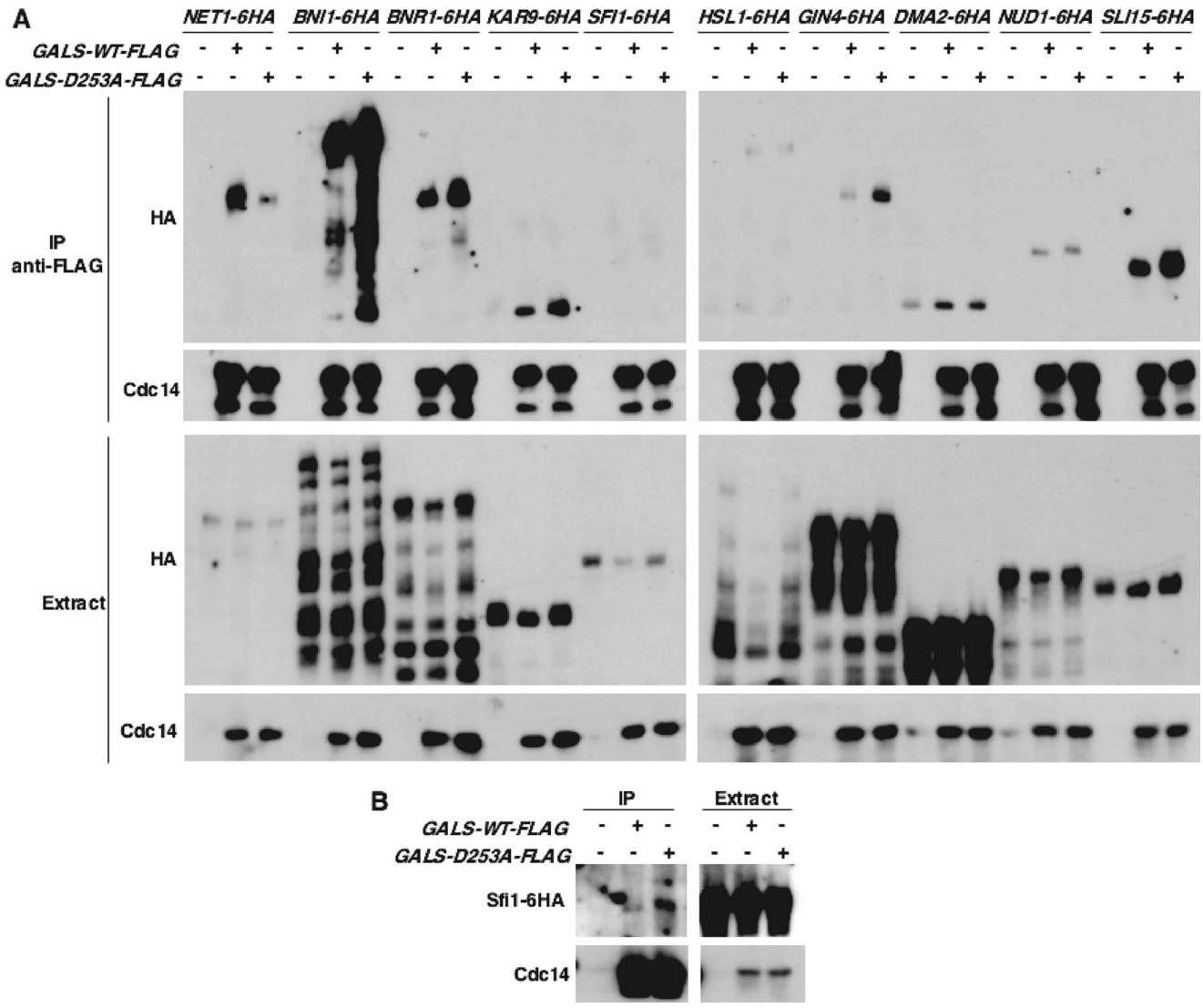



FIGURE 4. **A subset of Cdc14 interactors display enhanced binding to a substrate-trapping Cdc14 mutant *in vivo*.** *A*, immunopurification of FLAG-tagged Cdc14 from strains transiently expressing wild-type Cdc14 (*GALS-WT-FLAG*) or the substrate-trapping Cdc14 mutant (*GALS-D253A-FLAG*) in combination with the indicated $HA_6$-tagged proteins (*upper panels*) and whole cell extract from these strains (*lower panels*). Immunoprecipitates and extracts were resolved on SDS-PAGE gels and immunoblotted with an antibody to the HA tag or to Cdc14. *B*, immunopurifications of FLAG-tagged Cdc14 from strains transiently expressing wild-type Cdc14 (*GALS-WT-FLAG*) or the substrate-trapping Cdc14 mutant (*GALS-D253A-FLAG*) in combination with the indicated Sfi1-$HA_6$ (*left panel*) and whole cell extract from these strains (*right panel*). Lysate was extracted using buffer with additional salt and detergent (100 mM NaCl and 0.1% Triton X-100). Immunoprecipitates and extracts were resolved on SDS-PAGE gels and immunoblotted with an antibody to the HA tag or to Cdc14.

Kar9. Because of the large number of proteins interacting with Cdc14-D253A, this may reflect a lower signal that was not readily distinguished by MS analysis. Additionally, we found a higher number of chaperones, metabolic enzymes and translation proteins associating with Cdc14-D253A than with Cdc14 in Fig. 1 (supplemental Table S3). This is likely due to the fact that Cdc14-D253A-5GFP was overexpressed, while Cdc14–5GFP from Δ*net1* cells was expressed from its endogenous promoter. As with the proteins identified as interactors with Cdc14–5GFP, Cdc14-D253A interactors were enriched in a highly significant manner for proteins identified as *in vivo* Cdk targets (44).

*Some Cdc14 Interactors Have Higher Affinity for the Substrate-trapping Cdc14 Mutants*—We selected a subset of Cdc14 interacting proteins identified by the MS analysis from the pulldown conditions used in Figs. 1 and 3 for further analysis by co-immunoprecipitation experiments, using wild-type or substrate-trapping Cdc14. We generated strains carrying the interactor with a $HA_6$ tag at its C terminus, expressed from its endogenous promoter. These strains were crossed with strains carrying *CDC14* or *CDC14-D253A* under the control of the *GALS* promoter with a FLAG tag at the C terminus. After transient expression of *GALS-CDC14* or *GALS-CDC14-D253A*, Cdc14 was immunoprecipitated with an antibody to the FLAG tag, and binding of the potential interactor was detected by immunoblot using an antibody to the $HA_6$ tag (Fig. 4*A*). Interestingly, there was less Net1 bound to Cdc14-D253A than to wild-type Cdc14. This could be due to the fact that Cdc14-D253A binds more tightly to substrates, and therefore, there is less Cdc14-D253A available to associate with Net1. As described above, Bni1, Bnr1, Kar9, and Sli15 exhibited enhanced binding to Cdc14-D253A compared with

wild-type Cdc14. Of the novel Cdc14-interacting proteins that were identified in Fig. 3, only Gin4 exhibited enhanced binding to Cdc14-D253A compared with wild-type Cdc14.

We anticipate that proteins that bind better to Cdc14-D253A than to wild-type Cdc14 are *bona fide* substrates. However, proteins that do not exhibit enhanced binding could be scaffolds for Cdc14 in addition to being substrates. In such cases, differential binding to wild-type Cdc14 *versus* Cdc14-D253A would not be detected. Alternatively, proteins that bind to Cdc14, but do not exhibit enhanced binding to Cdc14-D253A may instead be regulators of Cdc14. An interesting observation from this experiment is that Hsl1 protein levels appeared to decrease when wild-type Cdc14 is overexpressed (Fig. 4*A*, *bottom panel*). Hsl1 is a substrate of $APC^{Cdh1}$ (58), and Cdc14 activates $APC^{Cdh1}$ by dephosphorylating Cdh1 (2, 3). Thus, hyperactivation of $APC^{Cdh1}$ by overexpressed Cdc14 likely increases Hsl1 degradation.

We were unable to detect Sfi1 in association with either wild-type Cdc14 or Cdc14-D253A (Fig. 4*A*). Because Sfi1 is part of the SPB half-bridge and is embedded in the nuclear envelope, we reasoned that it might be more difficult to extract this protein during cell lysis, and, thus, observe binding to Cdc14. We tested several lysis conditions to improve Sfi1 extraction, and found that increasing the concentration of sodium chloride and Triton X-100 in the lysis buffer allowed us to see an interaction of Sfi1 with wild-type Cdc14, which was slightly enhanced with Cdc14-D253A (Fig. 4*B*).

*Validation of Novel Cdc14 Substrates in Vivo and in Vitro*—We next examined the phosphorylation status of proteins with increased binding to Cdc14-D253A compared with wild-type Cdc14, because these were most likely to represent *bona fide* Cdc14 substrates. Initially, we looked at the mobility of these proteins from yeast overexpressing wild-type Cdc14 or Cdc14-D253A on SDS-PAGE. We found that some of the Cdc14-interacting proteins exhibited clear shifts in the presence of galactose-inducible wild-type Cdc14, but not galactose-inducible Cdc14-D253A, as evidenced by increased mobility (Fig. 5*A*). In particular, Kar9, Bni1, and Bnr1, as well as the characterized substrate Sli15, were less phosphorylated in the presence of *GALS-CDC14.* Sfi1 and Gin4 showed less pronounced shifts but appeared to undergo slight changes in mobility when wild-type Cdc14 was overexpressed. These data indicate that Cdc14 either directly or indirectly regulates dephosphorylation of these proteins *in vivo*.

We then assayed the phosphatase activity of Cdc14 toward Kar9, Bni1, Bnr1, and Sli15 *in vitro*. Kar9, Bni1, Bnr1, and Sli15 were phosphorylated *in vivo* by expression of *GAL-CLB2*, immunoprecipitated, and subjected to *in vitro* phosphatase assays. Kar9, Bni1, and Sli15 were efficiently dephosphorylated by GST-Cdc14, similar to treatment with λ phosphatase, as evidenced by a mobility shift on SDS-PAGE (Fig. 5*B*). Importantly, these proteins were not dephosphorylated when treated with the catalytically inactive Cdc14 mutants (C283S or D253A). We also checked the phosphorylation status of Bni1 following the *in vitro* phosphatase assay using an antibody that recognizes both phosphoserine and phosphothreonine residues. There was a decrease in the amount of phosphorylated Bni1 following treatment with

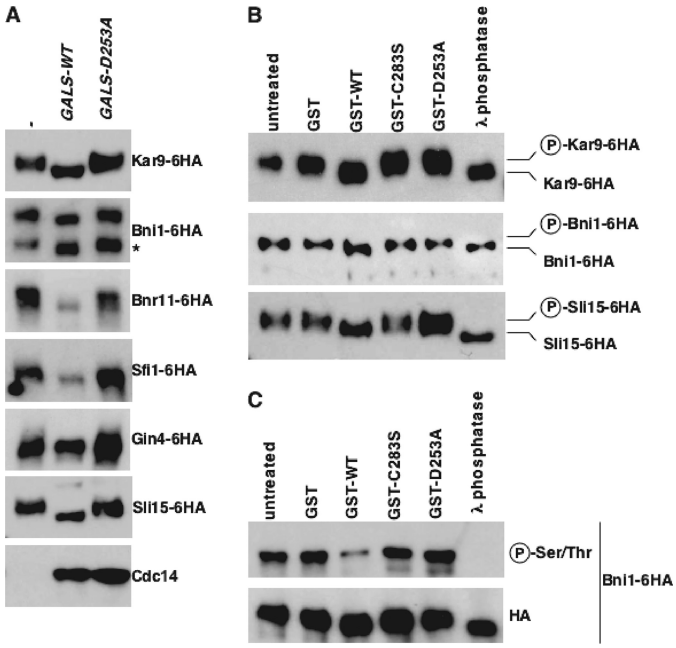



FIGURE 5. **Confirmation of new Cdc14 substrates *in vivo* and *in vitro*.** *A*, immunoblot analysis of the indicated $HA_6$-tagged proteins in the absence of overexpressed Cdc14 (−) or following transient expression of wild-type Cdc14 (*GALS-WT*) or the "substrate-trapping" Cdc14 mutant (*GALS-D253A*). The *asterisk* indicates a Bni1-$HA_6$ degradation product. *B*, *in vitro* phosphatase assay after immunopurification of $HA_6$-tagged proteins following transient expression of *GAL-CLB2*. Purified proteins were incubated with buffer (−), recombinant GST alone (GST), GST-tagged wild-type Cdc14 (GST-WT), GST-tagged catalytically inactive Cdc14 mutants (GST-C283S and GST-D253A), or 800 U λ phosphatase. Immunoprecipitates were analyzed by immunoblotting with an antibody to HA. *C*, *in vitro* phosphatase assay after immunopurification of $HA_6$-tagged Bni1 following transient expression of *GAL-CLB2* as in *B*. Immunoprecipitates were analyzed by immunoblotting with an antibody to phosphoserine/phosphothreonine residues or an antibody to HA.

GST-Cdc14 compared with total protein levels, which did not occur after treatment with the catalytically inactive Cdc14 mutants (Fig. 5*C*). Following treatment with λ phosphatase, no phosphorylated Bni1 was detected. This may reflect the fact that Cdc14 only dephosphorylates a subset of phosphoserine and phosphothreonine residues (Cdk sites), while λ phosphatase dephosphorylates all phosphoserine and phosphothreonine residues. We obtained qualitatively similar results for Bnr1; we have somewhat less confidence in these results because the mobility shift for Bnr1 was less pronounced, and the anti-phospho-serine/threonine assay was quantitatively less reproducible (data not shown).

*Cdc14 Affects Localization of Bni1 and Brn1 in Vivo*—Localization of Bnr1 and Bni1 has been reported to vary during the cell cycle. Here we examine whether Cdk phosphorylation and Cdc14 dephosphorylation of the formins could regulate their localization and, therefore, function.

We first confirmed the timing of formin localization during a synchronized cell cycle (Fig. 6). Cells with Bnr1-GFP or Bni1-GFP were released from a metaphase block and visualized. Bnr1-GFP showed bud neck localization starting at the time of the block and decreasing 20 min after release. In contrast, Bni1-GFP was not present at bud necks during the metaphase arrest but arrived around the time Bnr1-GFP left. The switch between formins at the bud neck was coincident, and

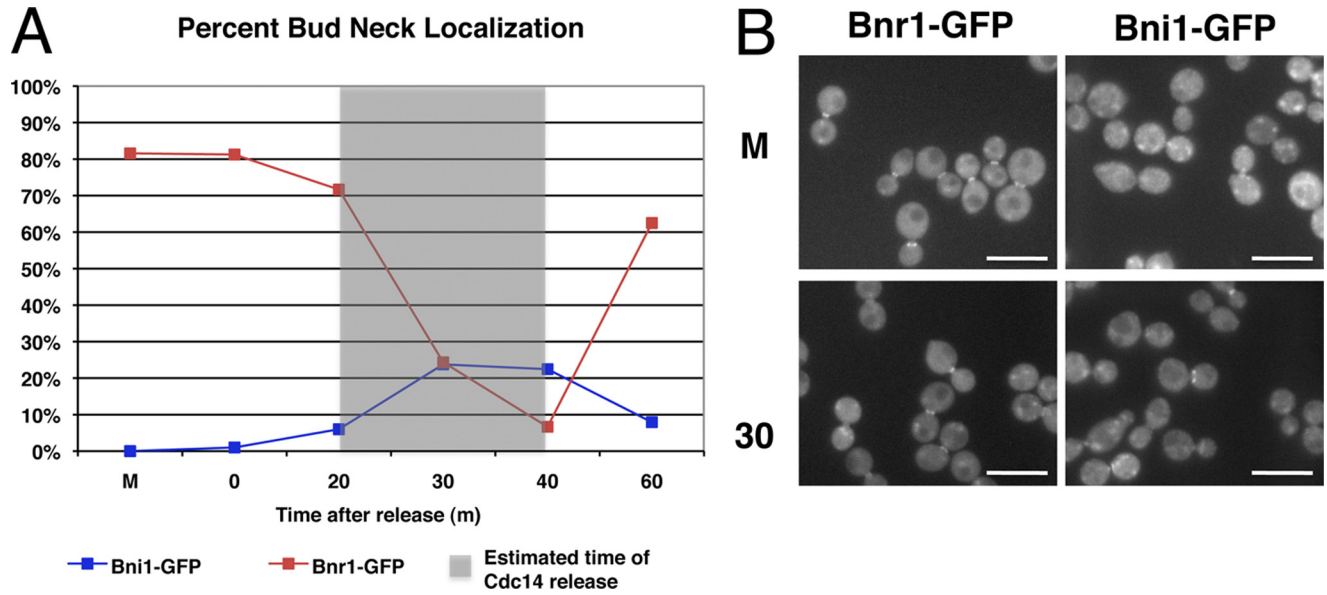



FIGURE 6. **The timing of the switch in formin localization is coincident with Cdc14 release.** *A*, after a metaphase arrest (*M*), cells were released synchronously, and the localization of the formins (Bni1 or Bnr1) at the bud neck was scored. The timing of Bnr1 leaving the bud neck is coincident with Bni1 arriving. This switch is also coincident with the timing of Cdc14 release from the nucleolus during the same protocol, *gray box* (Footnote 7). *B*, representative images from the experiment in *A*. Scale bar is 10 $\mu$m.

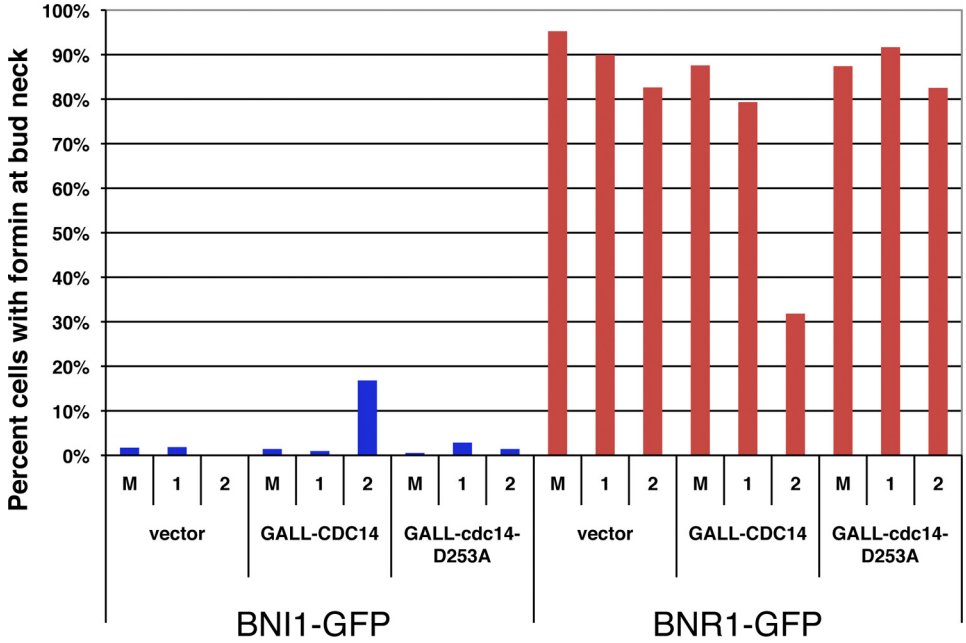



FIGURE 7. **Localization of formins during Cdc14 overexpression at a metaphase arrest.** Strains were arrested in metaphase and then galactose was added to induce the expression of *CDC14* from the plasmids (vector control, wild type *CDC14*, or the catalytically inactive *cdc14D253A* mutant). Presence of the formin at the bud neck is shown during arrest (*M*) and at 1 and 2 h after addition of galactose (1, 2). Whereas results show *CDC14* overexpression caused a relocalization of both formins, further examination showed that the overexpression was also causing exit from mitosis (assayed by actomyosin ring breakdown and DNA content; data not shown).

strikingly, this timing was similar to the time at which Cdc14 is released from the nucleolus in the same protocol.[6] Additionally, mutants in Cdc14 (*cdc14-1*) or in the signaling pathways leading to its release (*cdc15-2*) show Bnr1 localized to the bud neck and Bni1 absent from the bud neck (data not shown), consistent with a functional role for Cdc14 in formin localization. Together with the biochemical evidence that Cdc14 dephosphorylates the formins, these results are consistent with the hypothesis that Cdc14 dephosphorylation is directly responsible for formin localization.

Mutations within the NES of Cdc14 were reported to allow destruction of mitotic cyclins but block cytokinesis (23). Using this conditional, separation-of-function mutant, we investigated the effect of Cdc14 release when it is incapable of reaching cytoplasmic targets. We confirmed the absence of Cdc14-dNES at the bud neck, and Bnr1 localized to the bud neck of cdc14-dNES arrested cells (data not shown). However in our experiments, Clb2 degradation was not as efficient as

[6] Y. Lu, personal communication.

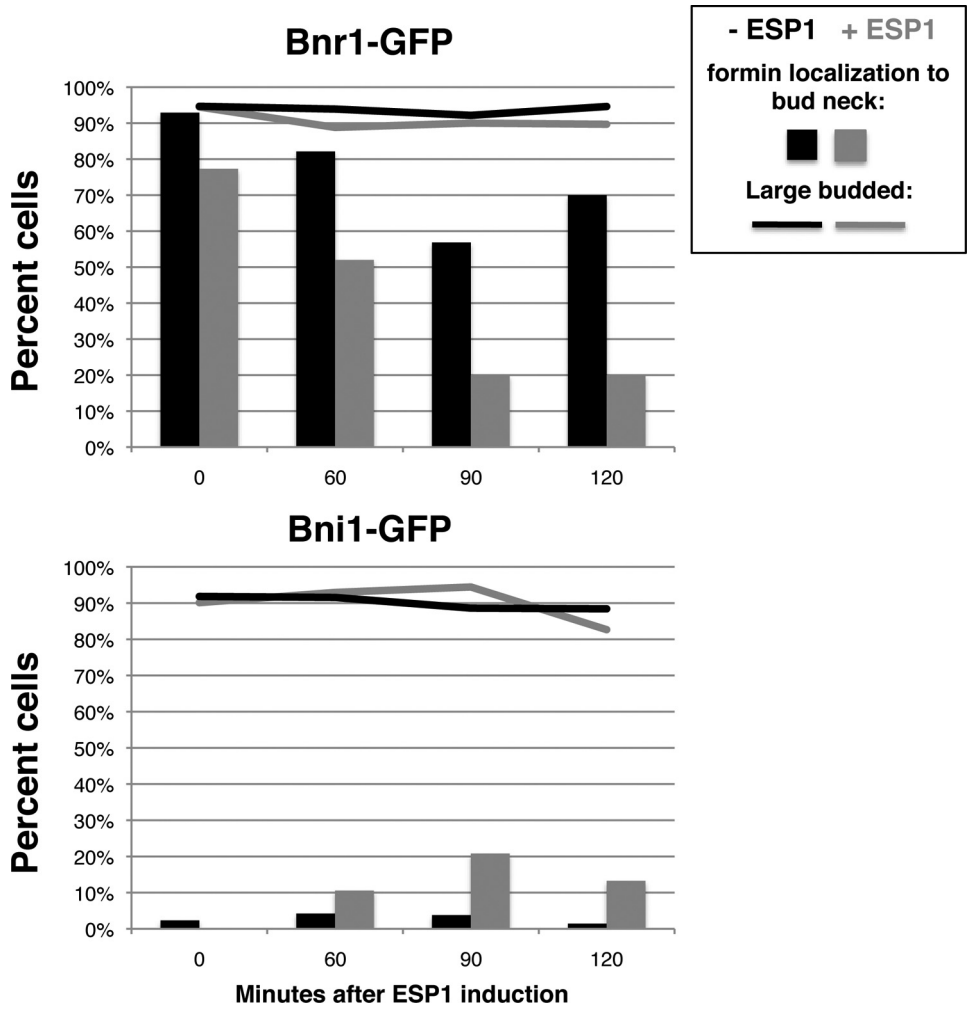



FIGURE 8. **Cells sustained in anaphase with Cdc14 release show a coincident relocalization of the formins.** *MET-CDC20 cdh1 GAL-ESP1* strain was arrested in metaphase and either induced with ESP1 (+, in *gray*) to allow Cdc14 release with delayed cell cycle progression or not (−, *black*). The localization of the formin to the bud neck at timepoints after the induction is shown for Bnr1-GFP (*top panel*) and Bni-GFP (*bottom panel*) by *bar graph*. To assay cell cycle progression, the percent of large budded cells is shown by the line graphs.

previously reported during the cdc14dNES arrest (data not shown), meaning that the separation of overall Cdc14 function from bud-neck-specific function by cdc14dNES may not be very tight under our conditions. This lessens the specificity of results with this mutant when assayed under our conditions.

We next determined the localization of formins in cells maintained in metaphase by depletion of Cdc20 and induced to overexpress Cdc14 (Fig. 7). An empty vector or plasmids expressing wild type or a catalytically inactive mutant of Cdc14 were transformed into strains with a fluorescently tagged formin. A truncated galactose inducible promoter was used to lessen the overexpression (51). When wild type *CDC14* was overexpressed, we observed a change in both of the formin localizations at the bud neck (Fig. 7); however, cells expressing wild-type Cdc14 were no longer arrested in metaphase and instead exited mitosis as assayed by breakdown of their actomyosin rings and DNA replication (data not shown). This experiment was repeated in a strain with *CDH1* deleted to maintain high levels of mitotic cyclin, but the same release from the block was observed (data not shown). Therefore, while this result is consistent with a direct effect of Cdc14 on formin localization, an indirect effect on formin localization of Cdc14-stimulated exit from mitosis cannot be ruled out.

To resolve this issue, we sought a system where an endogenous level of Cdc14 could be released from the nucleolus without cell cycle progression. This was accomplished through the overexpression of separase (*GALS-ESP1*) in *cdh1 cdc20* cells. This manipulation was reported to cause Esp1-dependent Cdc14 release (20) during a prolonged anaphase (59). The complete inability of these *cdh1 cdc20* cells to degrade mitotic cyclins (59) probably results in a stable block before telophase, allowing us to ask if released Cdc14 could affect formin localization without mitotic exit. Consistent with our hypothesis that Cdc14 affects formin localization, *GALS-ESP1* induction caused the formin Bnr1 to leave the bud neck and Bni1 to appear at the bud neck (Fig. 8), at a time known to be coincident with the release of Cdc14 (59). As expected, cells remained large budded during the time course, consistent with a block of mitotic exit. Overall, these results are consistent with Cdc14 dephosphorylation of the formins, demonstrated biochemically, directly regulating their localization.

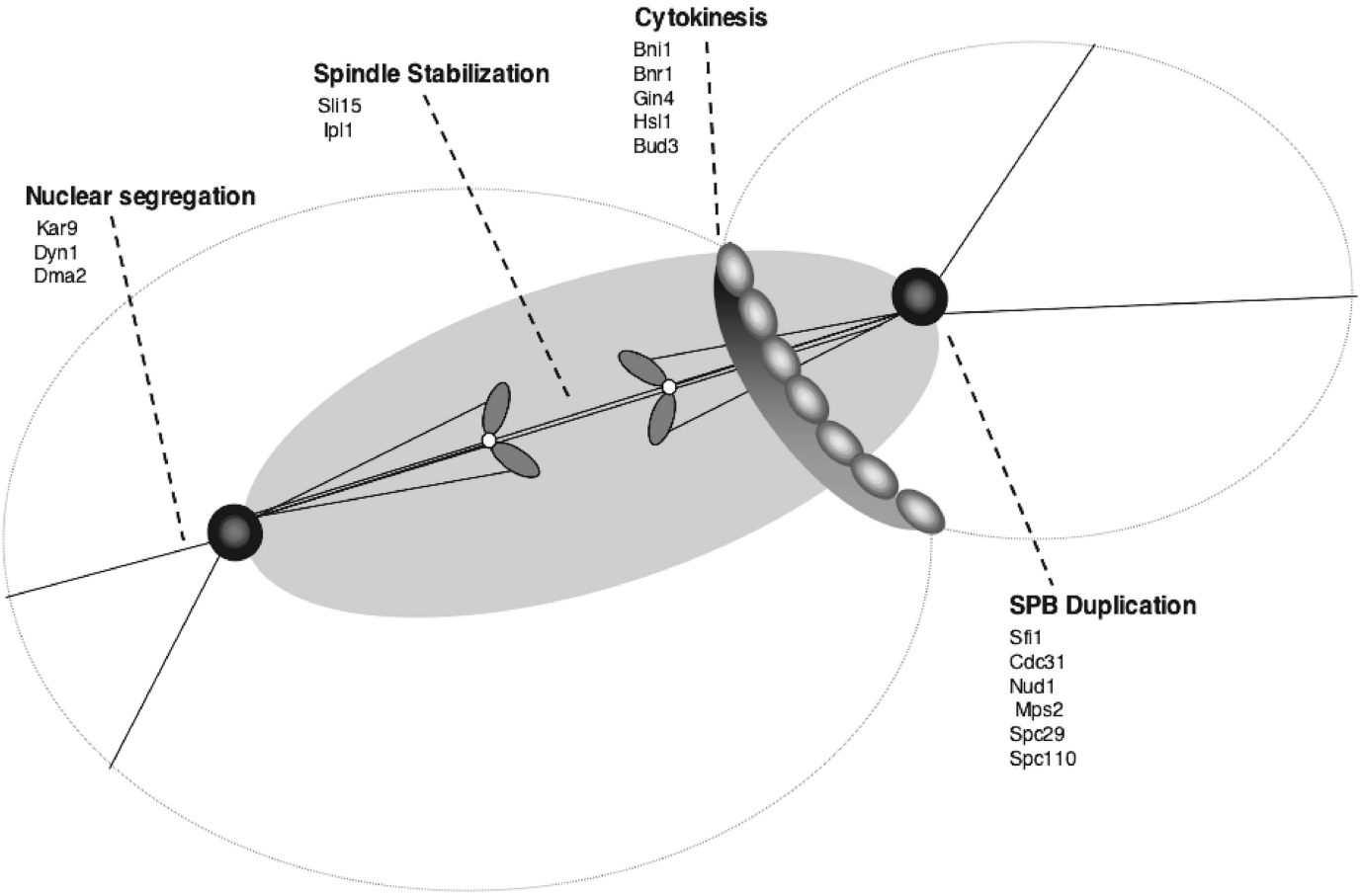



FIGURE 9. **Processes regulated by Cdc14.** Potential substrates of Cdc14, identified in this study, are indicated for each mitotic process. Cdc14 has roles in segregation of nuclei, by generating pulling forces on the mother cell-bound SPB, stabilization of the mitotic spindle, by directing proteins to the spindle midzone, and cytokinesis, by influencing cytokinesis and septin ring organization. Cdc14 also has a potential role in licensing of SPB duplication.

## DISCUSSION

Using mutant yeast strains that affect the localization or the catalytic activity of Cdc14, we identified a set of novel Cdc14-associated proteins. We also confirmed some known Cdc14 interactors including the regulator Net1, components of the SPB, and the established substrates Sli15 and Lte1. Several of these novel interactors may represent interesting new substrates of Cdc14, (discussed below). In addition to their utility as proteomic tools, we found that substrate-trapping mutants are useful for validating Cdc14 substrates. Sli15 and a subset of the Cdc14 interactors showed enhanced binding to the substrate-trapping mutants compared with wild-type Cdc14. Moreover, we were able to confirm several of these interactors as substrates for Cdc14 *in vivo* and *in vitro*.

Given the requirement for release of Cdc14 into the cytoplasm for actomyosin ring contraction and cytokinesis (23), Bni1 and Bnr1 represent intriguing new Cdc14 substrates. Bni1 and Bnr1 are partially functionally redundant formins that nucleate actin cables (45) and appear to be important for formation and contraction of the actomyosin ring (5, 31). Notably, both proteins exhibit cell cycle-regulated localization to the bud neck that is altered during anaphase. Bnr1 localizes to the bud neck for the majority of the cell cycle, but disappears just prior to contraction of the actomyosin ring (60, 61). In contrast, Bni1 localizes to cytoplasmic speckles through most of the cell cycle, but appears at the bud neck following disassembly of the mitotic spindle, and prior to cytokinesis (61, 62). This change in localization of both Bni1 and Bnr1 is temporally coincident with Cdc14 release from the nucleolus. Results from this study demonstrate that Cdc14 interacts with and dephosphorylates both Bni1 and Bnr1 *in vivo* and *in vitro*. Phosphorylation of Bni1 does not affect actin nucleation *in vitro* (63), so it unlikely that Cdc14-dependent dephosphorylation of Bni1 alters its activity. Instead, phosphorylation of Bni1 and Bnr1 may regulate their subcellular localization, and we provide evidence consistent with this role. Deletion of *BNI1* causes a delay of or a failure to complete actomyosin ring contraction (64), and Bni1 may be particularly important for the incorporation of actin filaments into the actomyosin ring during mitosis (31). Overexpression of *BNR1* causes a cytokinesis defect (65). Cdc14-dependent dephosphorylation of Bni1 and/or Bnr1 may alter the localization of these proteins, a step that might be necessary for timely ring contraction and cytokinesis.

Kar9 is an interesting Cdc14 substrate, since there is a role for Cdc14 in nuclear positioning during anaphase (18). Kar9 orients the mitotic spindle by linking the daughter-bound SPB to actin cables (36, 37) and is restricted to the daughter-bound SPB when phosphorylated by Cdk1 (38, 39, 41, 66).

Mutation of the Cdk1 consensus sites in Kar9 or deletion of specific cyclin genes results in redistribution of Kar9 to both SPBs (39–41, 66). Kar9 phosphorylation is cell cycle regulated, being phosphorylated at the G1/S transition and dephosphorylated during mitosis (38). This dephosphorylation of Kar9 occurs concurrent with Cdc14 release, and we have shown here that Cdc14 can dephosphorylate Kar9 *in vivo* and *in vitro*. Cdc14 may dephosphorylate Kar9 during anaphase, allowing it to redistribute to both SPBs in anaphase. This, in turn, could alter the forces acting on replicated nuclei, such that a unidirectional force pulling nuclei into the daughter cell is changed to a bidirectional force pulling the nuclei into both mother and daughter cells for proper DNA segregation. This could partially account for the observed phenotype of nuclei accumulating in daughter cells in cells lacking FEAR network-released Cdc14 (18). Preliminary results from our lab indicate that Kar9 moves from the daughter-bound SPB to both SPBs during anaphase.[6] It will be interesting to see if Kar9 distribution is altered in mutant yeast strains that affect the localization of Cdc14.

Substrate-trapping methodology has proven fruitful for the identification and confirmation of substrates of multiple PTPs (reviewed in Ref. 67), and a proteomic approach has been used for the identification of substrates of the PTP, PTPN22, by MS analysis of proteins associated with a substrate-trapping mutant (68). In this study, we demonstrate that a substrate-trapping form of Cdc14 can be used to identify and validate novel substrates, including Bni1, Bnr1, and Kar9. Additionally, we show that mutants with altered Cdc14 localization may be useful as proteomic probes to identify new targets. This strategy has elucidated new Cdc14 substrates, and perhaps regulators, for future characterization, which are likely to be involved in diverse mitotic processes (Fig. 9). Moreover, this approach is likely to have potential for the identification of substrates of other phosphatases.

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