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Mitotic Exit Network Controls the Localization of Cdc14 to the Spindle Pole Body in *Saccharomyces cerevisiae*

Satoshi Yoshida, Kazuhide Asakawa, and Akio Toh-e[1]
Department of Biological Sciences
Graduate School of Science
The University of Tokyo
7-3-1 Hongo
Tokyo 113-0033
Japan

Summary

Budding yeast Cdc14 phosphatase plays essential roles in mitotic exit. Cdc14 is sequestered in the nucleolus by its inhibitor Net1/Cfi1 and is only released from the nucleolus during anaphase to inactivate mitotic CDK. It is believed that the mitotic exit network (MEN) is required for the release of Cdc14 from the nucleolus because liberation of Cdc14 by *net1/cfi1* mutations bypasses the essential role of the MEN. But how the MEN residing at the spindle pole body (SPB) controls the association of Cdc14 with Net1/Cfi1 in the nucleolus is not yet understood [1, 2]. We found that Cdc14-5GFP was released from the nucleolus in the MEN mutants (*tem1*, *cdc15*, *dbf2*, and *nud1*), but not in the *cdc5* cells during early anaphase. The Cdc14 liberation from the nucleolus was inhibited by the Mad2 checkpoint and by the Bub2 checkpoint in a different manner when microtubule organization was disrupted. We observed Cdc14-5GFP at the SPB in addition to the nucleolus. The SPB localization of Cdc14 was significantly affected by the MEN mutations and the *bub2* mutation. We conclude that Cdc14 is released from the nucleolus at the onset of anaphase in a *CDC5*-dependent manner and that MEN factors possibly regulate Cdc14 release from the SPB.

Results and Discussion

Cdc14 Is Released from the Nucleolus in the *tem1*, *cdc15*, *dbf2*, and *nud1* Cells, but Not in the *cdc5* Cells during Early Anaphase

The MEN is activated only after the divided nuclei are distributed in both mother and daughter cells. This mechanism is ensured by the Bub2-Bfa1 complex and is called the spindle position checkpoint [3–6]. In the *bub2* cells in which the MEN is prematurely activated [7, 8], we found that the Cdc14 was released from the nucleolus only during anaphase, as in the wild-type cells, suggesting that the MEN activation is not sufficient for triggering Cdc14 release from the nucleolus.

To earn a clearer view of Cdc14 regulation, we examined Cdc14 localization in detail using a Cdc14-5GFP construct. *CDC14-5GFP* is fully functional as *CDC14*, judging from the facts that the *CDC14-5GFP* cells (SAY801) grew at the wild-type rate at all the temperatures tested (data not shown) and that the kinetics of cell cycle progression in SAY801 cells was almost identical to that of wild-type cells (Figure S1A). The nucleolar localization of Cdc14-5GFP was confirmed by colocalization with nucleolar protein Kri1-myc (Figure S1C). Synchronized G1 cell cultures were obtained by α-factor treatment, and the cells were released into fresh medium at 37°C. To our surprise, the Cdc14-5GFP signal was delocalized in *cdc15-2* cells in early anaphase like in the wild-type cells. The difference between the wild-type and *cdc15* cells was that the Cdc14-5GFP returned to the nucleolus before mitotic exit and cytokinesis in the *cdc15-2* cells (Figure 1A). Similar results were observed with *tem1-3*, *dbf2-2*, and *nud1-44* mutants (Figure S1A), indicating that the Cdc14 release from the nucleolus was not an allele-specific phenomenon of *cdc15-2* but was the common feature of the mutants of the MEN genes. We also performed the same experiments using myc epitope-tagged Cdc14 (Cdc14-myc) to confirm the Cdc14 release from the nucleolus in the *cdc15-2* mutant (Figure S1B). These observations indicate that *TEM1*, *CDC15*, *DBF2*, and *NUD1* are not required for the Cdc14 release from the nucleolus during anaphase but may be required for maintaining Cdc14 in a released state until mitotic exit. The MEN-independent Cdc14 release from the nucleolus was overlooked in the previous experiments in that Cdc14 of the MEN mutants was examined after a longer (~3 hr) incubation at the restrictive temperature [9, 10]. Among the MEN mutants, we found that the *cdc5 (msd2-1)* mutant showed a defect in Cdc14-5GFP release from the nucleolus (Figure 1A); therefore, we believe that Cdc5 may have a different role in Cdc14 regulation than other MEN factors.

The Cdc14 Liberation from the Nucleolus during Anaphase Was Inhibited by the Mad2-Dependent Spindle Checkpoint

The facts that the destruction of the anaphase inhibitor Pds1 is necessary for Cdc14 release [11] and that mitotic exit always occurs within 20 min after anaphase onset in wild-type and *bub2* cells [12] suggest a link between Cdc14 activation and the onset of anaphase. This consideration prompted us to examine the role of the spindle checkpoint in Cdc14 localization.

The spindle checkpoint of budding yeast consists of two branches [13–16]: one is the Mad2 pathway that stabilizes the anaphase inhibitor Pds1 in response to a kinetochore malfunction, and the other is the Bub2 pathway that monitors spindle position. To explore how the spindle checkpoint is involved in the Cdc14 release from the nucleolus, we examined the Cdc14-5GFP localization in the synchronized wild-type, *mad2*, *bub2*, and *mad2 bub2* cell cultures in the presence of the microtubule-destabilizing drug nocodazole.

The cells were synchronized in G1 phase with mating pheromone and were released into medium containing nocodazole at 25°C. In the wild-type cells, nuclear division was inhibited and the Cdc14-5GFP was kept inside

[1]Correspondence: toh-e@biol.s.u-tokyo.ac.jp

the nucleolus throughout the experiment, indicating that active spindle checkpoint prevented Cdc14-5GFP from liberation. Cdc14-5GFP was gradually released from the nucleolus in the *bub2* cells. In the *mad2* cells, Cdc14-5GFP was released for a short period and then reaccumulated in the nucleolus (Figure 1B). We also found that the *pds1* mutant showed similar kinetics of Cdc14-5GFP release as that seen in the *mad2* mutant. These observations suggest that the Cdc14 release from the nucleolus is not only inhibited by the Bub2 control but also by the Mad2 control in a different manner. This notion was further confirmed by the observation that there was no delay in Cdc14 release and in the cell cycle progression (judged by the appearance of rebudded cells) in the *mad2 bub2* double mutant (Figure 1B and [13–15]). These findings indicate that Mad2 and Bub2 prevent the Cdc14 from being released from the nucleolus in a different manner, and both branches of the spindle checkpoint should be inactivated to exit from mitosis in normal kinetics.

Recently, it has shown that Cdc5 phosphorylates Bfa1, a partner of Bub2, and may inactivate the Bub2 checkpoint in anaphase [8, 17]. This result suggests that the defect of Cdc14 release in the *cdc5* (*msd2-1*) strain (Figure 1A) can be brought about via the spindle checkpoint. But, the deletion of the *BUB2* gene only partially rescued the *cdc5* defect in the Cdc14 release, and deletion of the *MAD2* did not (Figure 1A). We also found that overexpression of *CDC5* can promote Cdc14-5GFP release from the nucleolus in nocodazole-arrested cells (Figure 1Cii), indicating that overexpression of *CDC5* overrode spindle checkpoints. We also noticed that *CDC15* is dispensable for the Cdc14 release from the nucleolus when *CDC5* was overexpressed (Figure 1Ciii). Thus, Cdc5 executes its function to promote Cdc14 release from the nucleolus downstream of the spindle checkpoint.

Cdc14-5GFP Is Localized at the SPB

By exploiting Cdc14-5GFP, we observed in vivo Cdc14-5GFP localization in detail. In addition to the nucleolus, a Cdc14-5GFP signal was often found as a spot around the nucleus (Figure 2A). We found that these spots correspond to the SPB by colocalization with SPB component Spc72 (Figure 2C). The SPB localization of Cdc14-5GFP was faint or undetectable during interphase, but it was prominent during anaphase when nucleolar Cdc14-5GFP was released. Cdc14-5GFP was preferentially found on only one SPB that migrates into the bud and on both SPBs in late telophase cells when Cdc14-5GFP began to concentrate in the nucleolus (Figures 2B–2D, not clear in 2A).

MEN Regulates Cdc14 Localization at the SPB

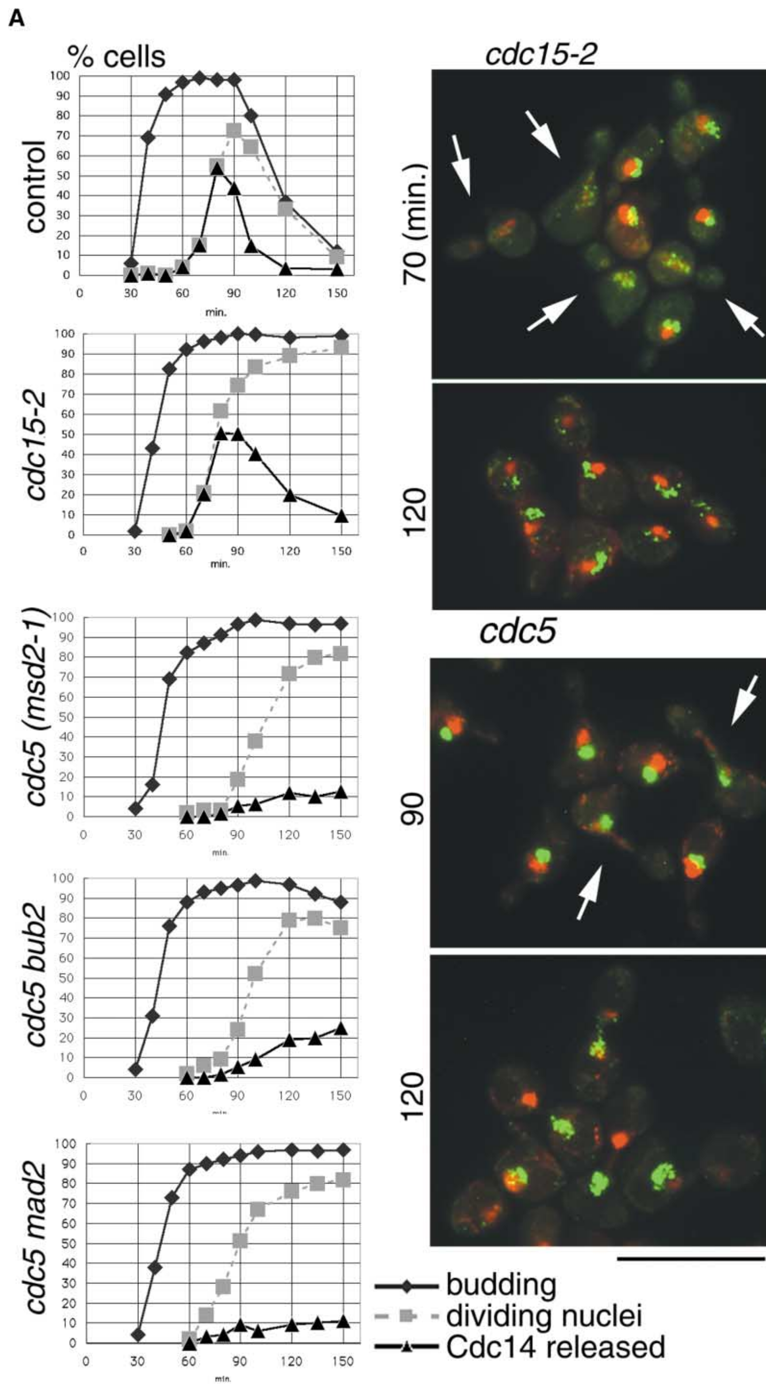
The finding that *TEM1*, *DBF2*, *CDC15*, and *NUD1* were nonessential for triggering Cdc14 raised the possibility that these factors exert some functions after Cdc14 has been released from the nucleolus. To test this possibility, we examined the SPB localization of Cdc14-5GFP in MEN mutants at 34.5°C because the SPB signal of Cdc14-5GFP was weakened at a higher temperature. The nucleolar localization of Cdc14-5GFP at 34.5°C was comparable to that at 37°C. Although SPB localization of Cdc14-5GFP was weakened after fixation and immunostaining of tubulin, a GFP spot was observed at the leading edge of the anaphase spindle in wild-type cells when Cdc14 was released from the nucleolus. The GFP spot was hardly detectable after Cdc14 accumulation in the nucleolus at the end of the mitosis, indicating that the amount of Cdc14-5GFP at the SPB fluctuates during the cell cycle. The Cdc14-5GFP localization at SPB was observed in *cdc15-2* (Figures 2C–2E), *tem1-3*, and *cdc5* (data not shown) cells but was found in only about one third of the anaphase spindles. This observation suggests that MEN is required for the proper Cdc14 localization at the SPB, but it does not essentially mean that the MEN functions to tether Cdc14 at the SPB during anaphase, because we also found that the SPB localization of Cdc14-5GFP was lost in *bub2* mutation and often mislocalized in the cytoplasm (Figures 2C–2E).

To study the SPB localization of Cdc14-5GFP in detail, we used cells depleted of Net1 by replacing the genomic *NET1* promoter with the glucose-repressible *GAL1* promoter (called *net1-d* hereafter) to ignore the nucleolar regulation of Cdc14. In the *net1-d* cells in glucose medium (YPD), the Cdc14-5GFP signal was not nucleolar any more, and only one GFP spot that corresponds to the SPB was prominent throughout the cell cycle (Figure 3A), suggesting that the affinity of Cdc14 to the SPB does not vary much during the cell cycle and that the prominent SPB localization during anaphase observed in wild-type cells might be due to the increased pool of Cdc14-5GFP released from the nucleolus. If so, the faint SPB localization of Cdc14 in MEN mutants may be due to the insufficient release of Cdc14 from the nucleolus during anaphase, and MEN could be involved in full release of Cdc14 from the nucleolus.

In *tem1 net1-d* cells, the SPB signal of Cdc14-5GFP was significantly brighter than in *net1-d* (Figure 3B). In the *bub2 net1-d* cells, in contrast, the Cdc14-5GFP signal was rarely observed (Figure 3B). The failure of Cdc14-5GFP's SPB localization in the *bub2* mutant may not be due to the reduced accessibility but may be due to Tem1-dependent rapid release of Cdc14-GFP from SPB because Cdc14-5GFP accumulated at the SPB in the *tem1 bub2 net1-d* cells and in the *cdc15 net1-d* cells (data not shown). Thus, we suggest that the active Tem1 pathway promotes Cdc14 release from the SPB when nucleolar Cdc14 is constitutively released.

Dual Regulation of Cdc14

In conclusion, Cdc14 is localized to two different compartments, the nucleolus and the SPB. Cdc14 localization in each compartment is regulated by two different pathways: one is the Cdc5/Mad2 pathway controlling Cdc14 release from the nucleolus in early anaphase, and the other is the Tem1/Bub2 pathway controlling Cdc14 at later steps. Recently, Stegmeier and coworkers reported the similar observation that a MEN-independent mechanism triggers Cdc14 release from the nucleolus [18]. They showed that the early Cdc14 release from the nucleolus activates MEN by dephosphorylation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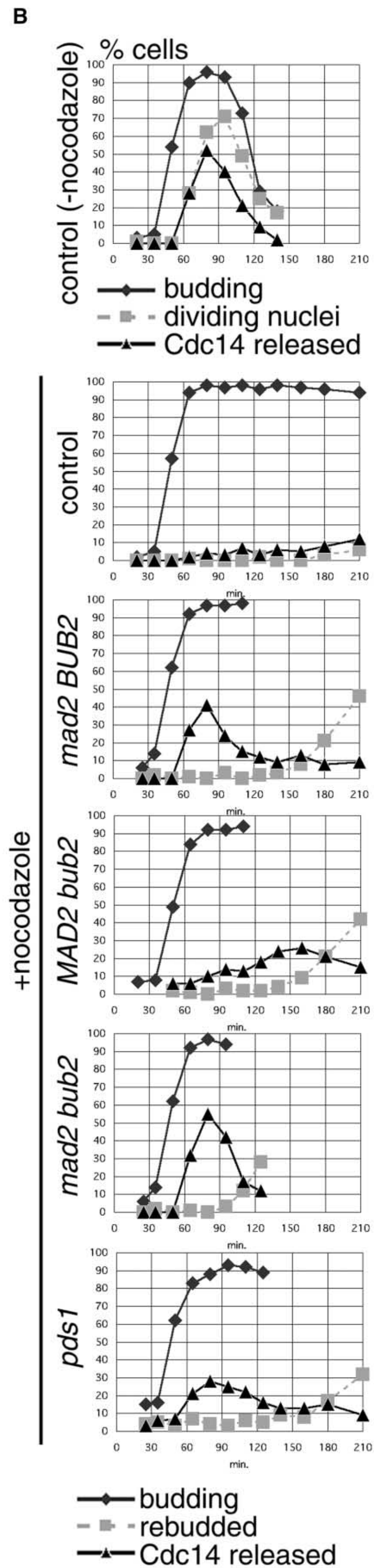
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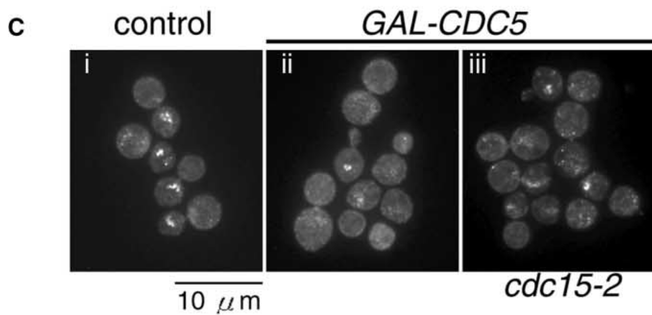
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of Cdc15 and that MEN-independent Cdc14 release is predominantly in the nucleus. We noticed that Cdc14-5GFP localization at the SPB was inefficient during anaphase in MEN mutants (Figure 2D), whereas Cdc14-5GFP highly accumulated at the SPB in *tem1 net1-d* cells in which Cdc14-5GFP is constitutively released from the nucleolus. These observations indicate that the full Cdc14 release from the nucleolus is required for the SPB accumulation of Cdc14. The regulation of Cdc14 at the SPB seems to be important for mitotic exit because mitotic exit was delayed in *tem1 net1-d* cells, in which Cdc14-5GFP accumulates at the SPB, in comparison with *net1-d* cells (Figure S1D). It is possible that Cdc14 released from the nucleolus, a reservoir of inactive Cdc14, is activated at the SPB in which there is no detectable Net1 present and that SPB may act as a reservoir of active Cdc14. It is also possible that the MEN regulates a cytoplasmic pool of Cdc14 by releasing it from the SPB, given that the MEN-independent Cdc14 release from the nucleolus is limited in the nucleus.

Since the human homolog of Cdc14, hCdc14A, has a similar role to its budding yeast counterpart and is localized at the centrosome during mitosis [19], the SPB regulation of Cdc14 seems to have a general importance in mitotic exit. It should be elucidated what components of the SPB interact with Cdc14 and how MEN regulates Cdc14 at SPB at a molecular level; the study on yeast Cdc14 will shed light on our general understanding of mitosis.

Experimental Procedures

All the yeast strains are isogenic or congenic to w303-1A (*Mat*a *ade2-1 trp1-1 leu2-3, 112 his3-11, 15 ura3-1 ssd1-d*) and are listed in Table S1. Detailed construction of the strain is described in the Supplementary Experimental Procedures. Standard yeast techniques for the cell culture, cell synchronization, and indirect immunofluorecence methods were carried out as described in [20, 21] and in the figure legends. α-factor and nocodazole were used at 15 mg/l. Microscopic photos were taken by an Olympus IX70, UplanApo 100×/1.35 objective with a SENSYS III (Nippon roper) cooled CCD camera using IP lab software. Images were analyzed with IP lab software, and the figures were created with Adobe PhotoShop 5.0. Cell lysates for Western blotting were prepared as described in [22]. Antibodies used in this study are described in the figure legends.

Supplementary Material

Supplementary Material including the strain list, Supplementary Experimental Procedures, and additional figures is available at http://images.cellpress.com/supmat/supmatin.htm.

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Figure 1. Nucleolar Release of Cdc14 Is Regulated by Two Different Mechanisms

(A) Kinetics of Cdc14 release in *cdc15* and *cdc5* mutants. The cells of SAY801 (*CDC14-5GFP*), SAY809 (*cdc15-2 CDC14-5GFP*), SAY815 (*cdc5 CDC14-5GFP*), SAY823 (*cdc5 mad2 CDC14-5GFP*), and SAY825 (*cdc5 bub2 CDC14-5GFP*) were synchronized by an α-factor block and were released into fresh medium at 37°C. Cells were fixed with 3.7% formaldehyde, and DNA was stained with DAPI. Elongated and divided nuclei were counted for the "dividing nuclei". The characteristic populations at the indicated time are presented in the right row, and the cells initiating anaphase are indicated by arrows. Also see Figure S1.

(B) Kinetics of Cdc14 release in nocodazole-treated cells. Each cell line of SAY801 (*CDC14-5GFP*), SAY819 (*mad2 CDC14-5GFP*), SAY817 (*bub2 CDC14-5GFP*), SAY821 (*mad2 bub2 CDC14-5GFP*), and SAY827 (*pds1 CDC14-5GFP*) was synchronized with α-factor and was released into fresh medium with or without 15 μg/ml nocodazole at 25°C. α-factor was added to the wild-type cell culture without nocodazole 60 min after the release to prevent the cells from entering the next cell cycle. Budded cells without discrete Cdc14-5GFP foci were counted as "delocalized", and cells with two buds (three-cell body) or unbudded cells without nuclear DNA were counted as "rebudded". At least 300 cells were counted for each point.

(C) Ectopic expression of *CDC5* induces Cdc14 release and *CDC14*-dependent mitotic exit: (i) SAY801 (*CDC14-5GFP*) cells with [YEplac195], (ii) SAY801 (*CDC14-5GFP*) cells with [pGAL1CDC5dbEU], and (iii) SAY809 (*CDC14-5GFP cdc15-2*) cells with [pGAL1CDC5dbEU] were arrested in metaphase in YEPraffinose-containing nocodazole for 3 hr at 25°C. Then, cultures were incubated at 35°C for 30 min to inactivate Cdc15-2, and then 1/4 volume of YEPgalactose (20%)-containing nocodazole was added to the culture. Samples taken 2.5 hr after galactose addition, were fixed with 3.7% formaldehyde, and were processed for microscopic analysis. The population of the cells with GFP signal in the nucleolus is (i) 87.3%, (ii) 53.1%, and (iii) 62.1% (n $>$ 200).

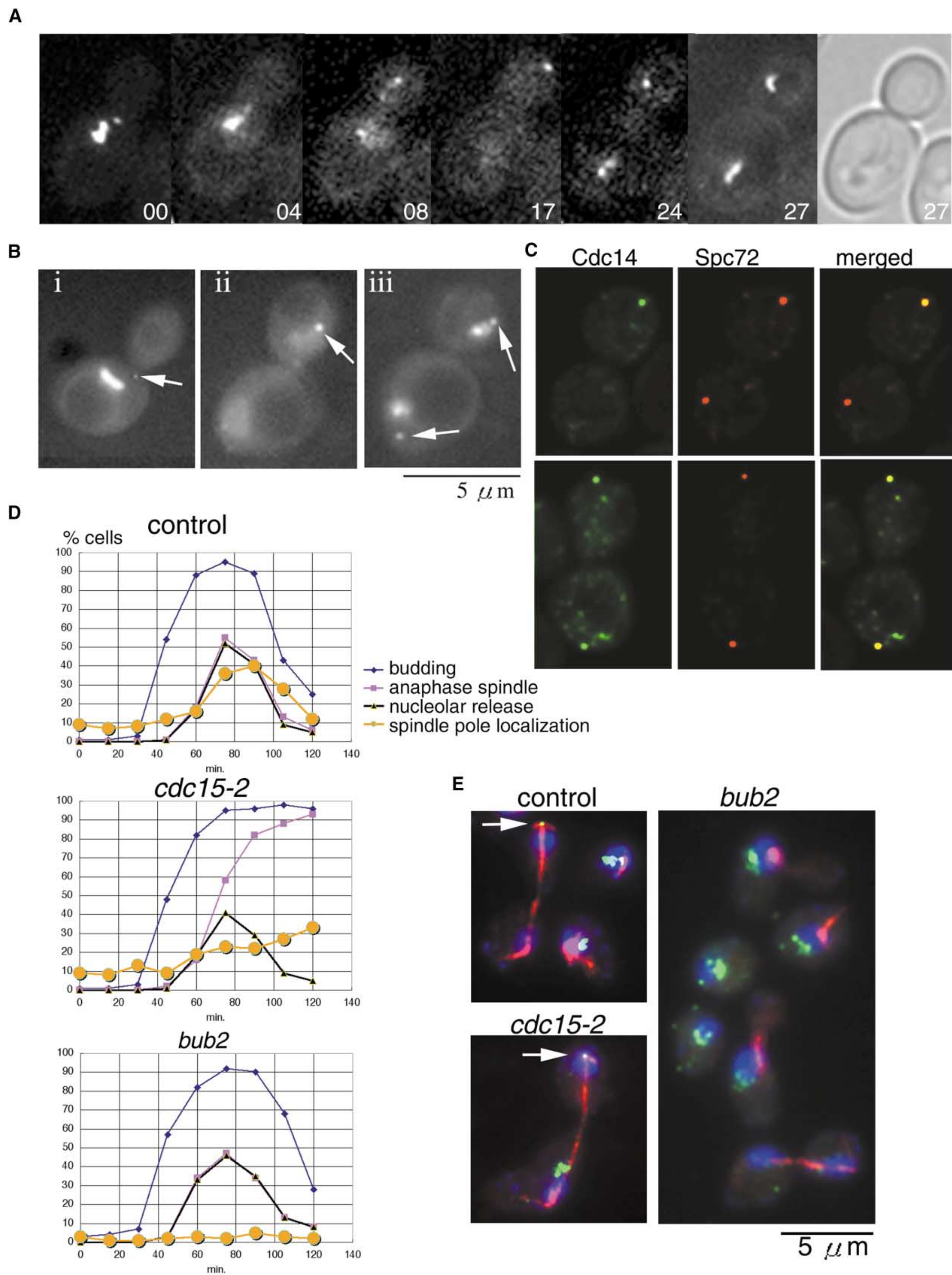

Figure 2. Cdc14-5GFP Is Localized at the SPB

(A) Time-lapsed images of Cdc14-5GFP. Living SAY801 (*CDC14-5GFP*; upper panels) cells were observed at a room temperature. Photos were taken at the indicated time points.

(B) SAY801 (*CDC14-5GFP*) cells were grown at a room temperature. A living cell in the representative stages of M phase was picked up, and

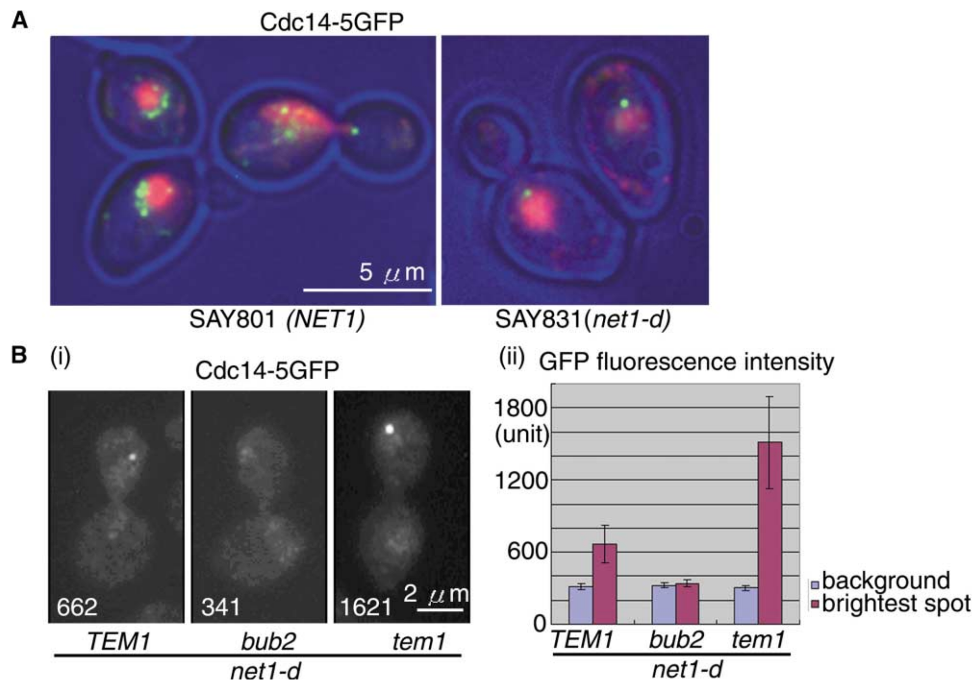

Figure 3. Tem1/Bub2 Affects SPB Localization of Cdc14

(A) Cdc14-5GFP nucleolar localization is lost in *net1-d* cells. SAY801 (*NET1*) and SAY831 (*net1-d*) cells grown in YPD medium were fixed, and images were taken. Cdc14-5GFP (green) and DAPI staining of DNA (red) were overlaid with phase contrast images (blue).

(B) Quantification of the fluorescence signal. Mid-log phase cultures of SAY831 (*TEM1 net1-d*) cells, SAY839 (*bub2 net1-d*) cells, and SAY 833 (*tem1 net1-d*) cells in YPD medium were fixed with 3.7% formaldehyde for 15 min. (i) The photographs were taken for an 800-ms exposure, then the contrast and the brightness of images were enhanced to the same extent. The numbers written in the photographs are the fluorescent intensity (in arbitrary units) of the brightest spot of the cell. (ii) The GFP signal intensity was measured after an 800-ms exposure. The fluorescence intensity of the brightest spot (that always corresponds to an SPB in SAY831 and SAY833) in anaphase cells was measured using IP lab software ($n = 30$ for each strain). The background is the average of the fluorescence intensity of the second brightest point inside the same cell.

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Cdc14 localized to the nucleolus and the SPB was observed. (i) G2/M metaphase, (ii) anaphase, and (iii) telophase. The nonnucleolar spots of Cdc14-5GFP are indicated by arrows.

(C) Cdc14-5GFP is localized at SPB. SAY801 (*CDC14-5GFP*) with *SPC72-myc* plasmid (p72-905CL) was fixed and stained with mouse monoclonal anti-c-*myc* antibody 9E10 (Calbiochem) and Cy3-conjugated goat anti-mouse IgG antibody (Chemicon) as a secondary antibody.

(D) SPB localization of Cdc14-5GFP was prominent during anaphase and was affected by MEN mutations. Cells of SAY801 (control), SAY809 (*cdc15-2*), and SAY817 (*bub2*) were synchronized by an α-factor block and were released at 34.5°C. α-tubulin was immunostained with mouse monoclonal anti-α-tubulin antibody (Sigma) and Rhodamine-conjugated goat anti-mouse IgG antibody (ICN pharmaceuticals). The colocalization of the Cdc14-5GFP spot with the spindle pole was determined and indicated as "spindle pole localization". At least 200 cells were counted for each sample.

(E) Typical samples of the experiment above are shown. Control cells and *cdc15-2* cells were taken 90 min after release. Cdc14-5GFP (green), tubulin (red), and DAPI (blue) are overlaid, and spindle pole localization of Cdc14-5GFP is indicated by an arrow. *bub2* cells were taken 60 min after release.

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