Mutual regulation of cyclin-dependent kinase and the mitotic exit network

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The mitotic exit network (MEN) is a spindle pole body (SPB)–associated, GTPase-driven signaling cascade that controls mitotic exit. The inhibitory Bfa1–Bub2 GTPase-activating protein (GAP) only associates with the daughter SPB (dSPB), raising the question as to how the MEN is regulated on the mother SPB (mSPB). Here, we show mutual regulation of cyclin-dependent kinase 1 (Cdk1) and the MEN. In early anaphase Cdk1 becomes recruited to the mSPB depending on the activity of the MEN kinase Cdc15. Conversely, Cdk1 negatively regulates binding of Cdc15 to the mSPB. In addition, Cdk1 phosphorylates the Mob1 protein to inhibit the activity of Dbf2–Mob1 kinase that regulates Cdc14 phosphatase. Our data revise the understanding of the spatial regulation of the MEN. Although MEN activity in the daughter cells is controlled by Bfa1–Bub2, Cdk1 inhibits MEN activity at the mSPB. Consistent with this model, only triple mutants that lack *BUB2* and the Cdk1 phosphorylation sites in Mob1 and Cdc15 show mitotic exit defects.

Introduction

Budding yeast Cdc14 belongs to a conserved class of dual-specificity protein phosphatases. During interphase and early mitosis yeast Cdc14 is kept inactive by entrapment in the nucleolus as a consequence of its association with Net1/Cfi1 (Shou et al., 1999; Visintin et al., 1999). However, upon anaphase onset Cdc14 is released from the nucleolus. The active Cdc14 then resides in the nucleoplasm, the cytoplasm, at the budding yeast centrosome (the spindle pole body [SPB]), and at the site of cytokinesis (Pereira et al., 2002; Yoshida et al., 2002; Stegmeier and Amon, 2004) where it dephosphorylates proteins that have previously been phosphorylated by cyclin-dependent kinase 1 (Cdk1). In addition, Cdc14 promotes inactivation of the Cdk1–Clb2 complex at the end of mitosis, thereby promoting the transition from mitosis to G1 phase of the cell cycle (Visintin et al., 1998).

Two pathways regulate Cdc14 localization. The Cdc14 early release pathway (FEAR) triggers a transient and partial release of Cdc14 at the beginning of anaphase (Pereira et al., 2002; Stegmeier et al., 2002). This short burst of Cdc14 activity is sufficient to promote the segregation of the rDNA locus, the targeting of the INCENP homologue Sli15 to the mitotic spindle, changes in microtubule (MT) dynamics, and spindle midzone assembly (Pereira and Schiebel, 2003; D'Amours et al., 2004; Lavoie et al., 2004; Sullivan et al., 2004; Higuchi and Uhlmann, 2005; Khmelinskii et al., 2007; Woodbury and Morgan, 2007). However, because the FEAR-activated Cdc14 is only transiently active and Cdk1–Clb2 activity is still high in early anaphase, the FEAR pathway does not induce mitotic exit.

Mitotic exit and cytokinesis require full activation of Cdc14 by the mitotic exit network (MEN), a GTPase-driven signaling cascade that is associated with the SPB (Shirayama et al., 1994; Luca and Winey, 1998; Cenamor et al., 1999; Gruneberg et al., 2000; Xu et al., 2000; Menssen et al., 2001; Pereira and Schiebel, 2001; Stegmeier and Amon, 2004). One of the most upstream MEN components is the Ras-like GTPase Tem1 that is controlled by the putative guanine nucleotide exchange factor Lte1 and the GTPase-activating protein (GAP) complex Bfa1–Bub2 (Shirayama et al., 1994; Bardin et al., 2000; Pereira et al., 2000; Geymonat et al., 2002). Tem1 interacts with the Pak-like kinase Cdc15 (Asakawa et al., 2001), which in turn activates the Dbf2–Mob1 kinase complex via phosphorylation of the kinase subunit Dbf2 (Mah et al., 2001). One function of the

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Abbreviations used in this paper: FEAR, Cdc14 early release pathway; GAP, GTPase-activating protein; MEN, mitotic exit network; MT, microtubule; SPB, spindle pole body; YPAD, yeast extract adenine dextrose.

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Dbf2–Mob1 complex is to phosphorylate Cdc14 at sites adjacent to its nuclear localization sequence, thereby retaining Cdc14 in the cytoplasm (Mohl et al., 2009).

The MEN activation scheme closely follows binding of MEN components to SPBs; SPB binding of Cdc15 requires Tem1 and Dbf2–Mob1 only associates with SPBs when Tem1 and Cdc15 are functional (Visintin and Amon, 2001). The close correlation between MEN activation and SPB localization indicates that MEN regulation occurs at SPBs. Consistently, mutants of the SPB component *NUD1* are defective for MEN signaling (Gruneberg et al., 2000).

Growth by budding generates an inherent polarity in the yeast cell. This polarity is reflected in the two SPBs as they are functionally and biochemically distinct. The preexisting, older SPB is inherited by the daughter cell, the bud (Pereira et al., 2001). In addition, MEN proteins bind differently to the mSPB and dSPB. During an unperturbed cell cycle the inhibitory Bfa1–Bub2 GAP complex localizes preferentially at the dSPB, where it inhibits the MEN, until Cdc5 polo-like kinase inactivates the Bfa1–Bub2 complex in late anaphase (Bardin et al., 2000; Pereira et al., 2000; Hu et al., 2001; Caydasi and Pereira, 2009). Throughout the majority of anaphase Tem1 at the dSPB resides in a complex with the Bfa1–Bub2 GAP and therefore is probably in its inactive GDP-bound form (Pereira et al., 2000). The mSPB recruits only a small fraction of Tem1 (Molk et al., 2004). Cdc15 was reported to bind either first to the mSPB, the dSPB, or with equal timing to both SPBs (Cenamor et al., 1999; Xu et al., 2000; Menssen et al., 2001; Molk et al., 2004). These conflicting data are most easily explained by varying levels of the overexpressed Cdc15 protein (Cenamor et al., 1999). Finally, the Mob1 protein associates in early anaphase with the mSPB, well before the protein is recruited to the dSPB (Luca et al., 2001).

The FEAR pathway, the MEN, and Cdk1 are all linked by complex interdependencies. FEAR activation promotes phosphorylation of Net1 by Cdk1–Clb2 in early anaphase, which is a prerequisite for the FEAR-dependent release of Cdc14 (Azzam et al., 2004; Queralt et al., 2006). In addition, Cdk1 phosphorylation activates the FEAR pathway component Spo12 (Tomson et al., 2009). In contrast, Cdk1 negatively regulates the function of the MEN component Cdc15 (Jaspersen and Morgan, 2000). FEAR-released Cdc14 eventually dephosphorylates Cdc15 in early anaphase, which, in a positive feedback loop, further activates Cdc14 (Jaspersen and Morgan, 2000; Menssen et al., 2001; Pereira et al., 2002; Stegmeier et al., 2002). It is therefore puzzling that a mutant version of Cdc15, which is no longer regulated by Cdk1, does not show mitotic exit defects (Jaspersen and Morgan, 2000).

Here, we describe mutual regulation between Cdk1 and MEN proteins. Activation of the MEN component Cdc15 in early anaphase recruits Cdk1 kinase to the mSPB, whereas Cdk1 reduces mSPB binding of Cdc15. In addition, Cdk1 inhibits the activity of the Mob1–Dbf2 kinase complex through Mob1 phosphorylation. We suggest that the Cdk1–Cdc15/Mob1 regulation loop restrains full activation of the MEN at the mSPB in early anaphase. The MEN at the dSPB is inhibited by the Bfa1–Bub2 GAP complex until Cdc5 polo kinase phosphorylates Bfa1 (Hu et al., 2001).

Results

Cdk1 binds to the mSPB in anaphase

Previously, we have shown that Cdk1 binds to the dSPB in G1/S phase of the cell cycle from where it translocates to the plus end of cytoplasmic MTs (Maekawa et al., 2003; Maekawa and Schiebel, 2004; Fig. 1 A, −8 and −6, yellow arrows). We now describe that Cdk1 also changes SPB localization in anaphase. In time-lapse experiments of early anaphase cells, Cdk1-GFP was detected at the SPB in the mother cell but not at the SPB in the bud (Fig. 1 A, 4–9, white arrows). In some cells, a second, dot-like, Cdk1-GFP signal appeared later at the SPB in the daughter cell (Fig. 1 A, 11–13, white arrows). With mitotic exit Cdk1 was no longer present at either of the two SPBs (Fig. 1 A, 14–24). This result was confirmed using α-factor–synchronized *CDK1-3GFP SPC42-eqFP611* cells in which the length of the mitotic spindle was used to monitor progression through anaphase (Fig. 1 B). In cells with a short metaphase spindle Cdk1 was not enriched at SPBs. Cdk1-GFP was found in the majority of early-mid anaphase cells (Fig. 1 B, >70%; 3–8-µm spindle length) in a polar manner at the mSPB. In late anaphase cells (8–10 µm) Cdk1-3GFP dissociated from SPBs. Thus, in early anaphase Cdk1 binds preferentially to the mother spindle pole. This recruitment suggests that Cdk1 is likely to be performing a novel, uncharacterized function at this location.

As yeast kinetochores cluster close to SPBs for the majority of the cell cycle (Jin et al., 1998), the pole signal may arise from the binding of Cdk1 to SPBs, kinetochores, or both. Conditional lethal *ndc10-1* cells lack functional kinetochores (Goh and Kilmartin, 1993). A persistence of the pole signal in *ndc10-1* cells therefore suggests that the Cdk1 signal arises from its recruitment to SPBs. In *ndc10-1* cells Cdk1-GFP associated with the pole marker Spc42-RFP (Fig. 1 C), indicating that it is at SPBs in anaphase.

We next asked whether Cdk1 binding to SPBs requires one of the four mitotic cyclins, Clb1–Clb4. Cdk1 localization at SPBs was not grossly perturbed in *clb1Δ*, *clb2Δ*, *clb3Δ*, or *clb4Δ* cells. Neither was it altered in the *clb3Δ clb4Δ* double mutant cells (unpublished data). However, in *clb1Δ* Gal1-*CLB2* cells, from which Clb2 had been depleted (resulting in cell cycle arrest in anaphase; Azzam et al., 2004), Cdk1-GFP failed to bind to the mSPB and dSPB (Fig. 1 D). It is therefore likely that it is Cdk1 in a complex with either Clb1 or Clb2 that associates with anaphase SPBs. This result is consistent with the reported SPB localization of Clb2 in anaphase cells (Hood et al., 2001; Bailly et al., 2003) and supports the notion that the functional Cdk1–Clb2 kinase complex binds to the mSPB in anaphase.

The MEN regulates binding of Cdk1 to SPBs

The MEN is an SPB-associated signaling cascade that becomes active in anaphase (Stegmeier and Amon, 2004). MEN activation could promote the recruitment of Cdk1 to SPBs. To test this hypothesis, we analyzed the localization of Cdk1 in cells lacking either the *KAR9* or *DYN1* genes. In *kar9Δ* or *dyn1Δ* cells, ∼10–20% of anaphase spindles become misaligned in the mother cell body (Li et al., 1993; Miller and Rose, 1998). The

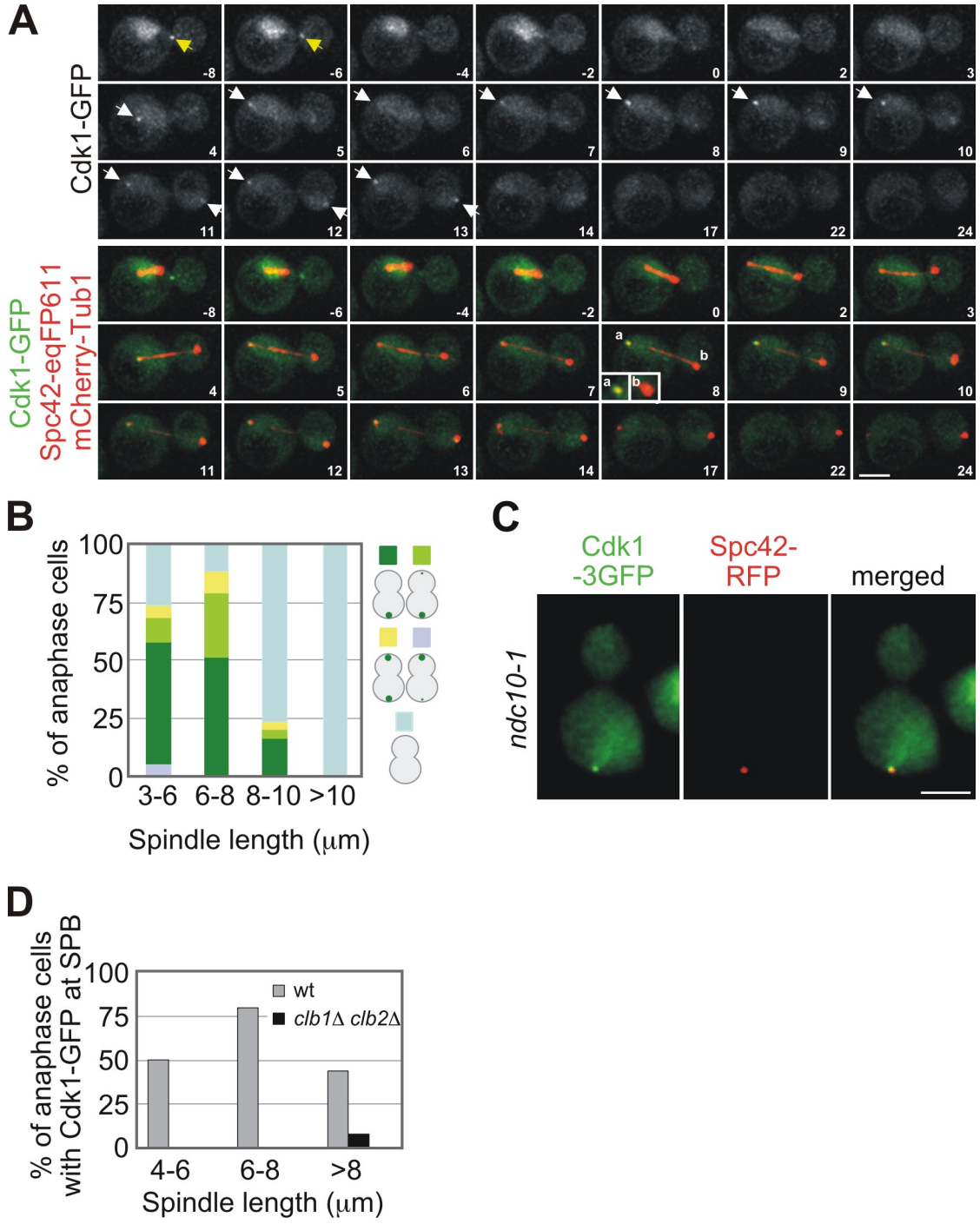

Figure 1. **Cdk1 binds to the mSPB in early anaphase.** (A) Time-lapse analysis of *CDK1-GFP SPC42-eqFP611 mCherry-TUB1* cells grown in low fluorescence medium at 30°C. Consecutive sections were taken every 60 s. Shown are deconvolved and projected images. The yellow arrows show Cdk1-GFP at the plus end of cytoplasmic MTs (Maekawa and Schiebel, 2004). The white arrows highlight Cdk1 at the SPBs. The insets (a and b) are enlargements of the two spindle poles. Bar, 5 μm. (B) Cdk1 associates with mSPB in anaphase. *CDK1-3GFP SPC42-eqFP611* cells were synchronized with α-factor at 30°C. $n > 130$ anaphase cells were analyzed and classified for colocalization of the SPB marker Spc42-eqFP611 and Cdk1-3GFP as indicated. Shown is one representative experiment out of five independent experiments. (C) Cdk1-3GFP associates with SPBs independently of the kinetochore structure. *CDK1-3GFP SPC42-RFP ndc10-1* cells were synchronized with α-factor and released into medium at 37°C. After 150 min at 37°C, fixed cells were stained with DAPI. Bar, 5 μm. (D) Cdk1-GFP does not localize to SPBs in *clb1Δ clb2Δ* cells. α-Factor–synchronized wild-type and *clb1Δ* Gal1-*CLB2* cells were arrested in G1 phase and released in YPAD medium at 30°C to induce Clb2 depletion. SPB localization of Cdk1-GFP of cells in anaphase was quantified for wild-type (wt; $n = 119$) and *clb1Δ* Gal1-*CLB2* (*clb1Δ clb2Δ*; $n = 70$) cells.

MEN of *kar9Δ* and *dyn1Δ* cells is inactive when the anaphase spindle is mispositioned, but active when the spindle is correctly aligned along the mother–bud axis (Bardin et al., 2000; Pereira et al., 2000). In *kar9Δ* cells with a correctly aligned spindle, Cdk1-3GFP bound in early anaphase to the mSPB (Fig. 2 A, *kar9Δ*, enlargement 4; and Fig. 2 B) in the same way as it did in

wild-type cells (Fig. 2 A, *KAR9*, enlargement 1; and Fig. 2 B). In contrast, Cdk1 was not detectable at either of the two SPBs in *kar9Δ* cells with a misaligned spindle (Fig. 2 A, *kar9Δ*, asterisks and enlargements 2 and 3; and Fig. 2 B). In these cells Cdk1 was distributed throughout the nucleus. Similar data were obtained for *dyn1Δ* cells (Fig. S1, A and B). Thus, MEN activity may regulate the SPB recruitment of Cdk1.

To directly confirm a role of the MEN in the localization of Cdk1 to the anaphase SPB, we inactivated the polo-like kinase Cdc5 and Tem1 that are both essential for MEN activity (Shirayama et al., 1998; Shou et al., 1999; Hu et al., 2001). We used Gal1-*CDC5* and Gal1-*UPL-TEM1* cells (*TEM1* fused to a degron element) for this experiment. Gal1-expressed Cdc5 and UPL-Tem1 are both rapidly depleted from cells after their expression is repressed by the addition of glucose to the growth medium. Cells then arrested in late anaphase as a consequence of the lack of MEN activity (Shirayama et al., 1998; Shou et al., 1999). In such repressed Gal1-*CDC5* and Gal1-*UPL-TEM1* cells, Cdk1 was no longer detectable at SPBs at any stage of anaphase (Fig. 2 C), indicating that an active MEN is required for the binding of Cdk1 to SPBs.

We next addressed how inactivation of the MEN inhibitor Bfa1 affects the recruitment of Cdk1 to SPBs. In contrast to wild-type cells, which showed a polar SPB binding of Cdk1 (Fig. 2 D, enlargements 1 and 2), Cdk1 of *bfa1Δ* cells bound with equal intensity and timing to both SPBs in early-mid anaphase (Fig. 2 D, enlargements 3 and 4; and Fig. 2 E). Thus, in wild-type cells the Bfa1–Bub2 GAP complex inhibits the binding of Cdk1 to the dSPB.

The FEAR pathway regulates release of Cdc14 in early anaphase and could be essential for the binding of Cdk1 to the mSPB (Stegmeier et al., 2002). To test this possibility, we analyzed binding of Cdk1-GFP to the mSPB in FEAR-defective *spo12Δ* or *slk19Δ* cells. In addition, we compared timing of binding of Cdk1 to the mSPB of *spo12Δ* cells with the release of Cdc14-3mCherry from the nucleolus as an indication for full activation of the MEN. Time-lapse data showed that Cdk1 bound in mid-anaphase to the mSPB of FEAR-defective *spo12Δ* cells (Fig. S1 C) and *slk19Δ* cells (not depicted). In *spo12Δ* cells, Cdk1-GFP bound to the mSPB 6–8 min ($n = 5$) before Cdc14-3mCherry was released from the nucleolus (Fig. S1 C). These data suggest that the FEAR network is not important for Cdk1 binding to the mSPB.

SPB localization of Cdk1 requires Cdc15 kinase activity but persists in *DBF2* and *MOB1* mutants

Our analysis already demonstrated the essential role of the GTPase Tem1 in recruiting Cdk1 to SPBs (Fig. 2 C). We now asked whether the MEN kinase Cdc15 was also required to promote the binding of Cdk1 to the dSPB. The role of *CDC15* was first analyzed in cells expressing the *cdc15-as1* allele that can be inhibited by the ATP analogue "PP1 analog 8" (D'Aquino et al., 2005). Inhibition of cdc15-as1 kinase activity strongly reduced the efficiency of Cdk1-GFP binding to the mSPB in any stage of anaphase (Fig. 3, A and B). This failure in Cdk1 recruitment was not caused by a deficiency in the binding of the cdc15-as1 protein to SPBs. On the contrary, cdc15-as1-GFP accumulated to a much higher level at SPBs than Cdc15-GFP (Fig. 3 C), even though the cellular levels of Cdc15 and cdc15-as1 were identical as shown by immunoblot (Fig. 3 D). The dependency of SPB localization upon Cdc15 activity was confirmed in *cdc15-1* cells (Fig. S2, A and B).

In contrast to *cdc15* cells, Cdk1-GFP still associated with the mSPB of *dbf2-2* and *mob1-67* cells (Fig. 3, E and F). This could mean that it is only the most upstream branch of the MEN, consisting of Tem1 and Cdc15, that directs Cdk1 to SPBs. Alternatively, Cdk1 could bind to the inactive dbf2-2 and mob1-67 proteins at SPBs. Analysis of the localization of GFP-tagged mob1-67 and dbf2-2 showed that the proteins were no longer at SPBs when cells were incubated at the restrictive temperature (Fig. S2, C and D). In addition, Cdk1 was still targeted to the mSPB in *dbf2-2 dbf20Δ* cells (not depicted), indicating that the Dbf2 paralogue Dbf20 could not substitute for this function of Dbf2. In conclusion, the Dbf2–Mob1 complex is not essential for the mSPB binding of Cdk1.

Mutual regulation of Cdc15 and Cdk1 at the mSPB

What function does Cdk1 execute at anaphase SPBs? Cdk1 has been shown to negatively regulate the MEN by phosphorylating the MEN component Cdc15 at seven sites (Jaspersen and Morgan, 2000; Stegmeier et al., 2002). The function of this modification has remained obscure because *CDC15-7A* cells do not show growth or mitotic exit defects and the specific activity of the Cdc15 kinase is not altered by Cdk1 phosphorylation (Jaspersen and Morgan, 2000; Xu et al., 2000). We wondered whether phosphorylation by Cdk1 may be regulating the binding of Cdc15 to the mSPB. Conflicting data on the cell cycle–dependent binding of overexpressed Cdc15 to SPBs have been reported (Cenamor et al., 1999; Xu et al., 2000; Menssen et al., 2001), and the analysis of the localization of nonoverexpressed Cdc15-GFP was only qualitative (Molk et al., 2004). We therefore quantified SPB binding of the functional *CDC15-GFP* gene product that was expressed as the sole *CDC15* gene copy at the native *CDC15* locus from its native promoter in wild-type cells and in *td-cdc14* degron cells. *td-cdc14* degron cells lack Cdc14, the phosphatase that dephosphorylates Cdc15 in early anaphase, and have high Cdk1–Clb2 activity. Cdc15 therefore remains hyperphosphorylated in anaphase of *td-cdc14* cells (Jaspersen and Morgan, 2000; Pereira and Schiebel, 2003).

In synchronized *CDC15-GFP* cells, Cdc15 showed a weak association with both the mSPB and dSPB in early anaphase (Fig. 4 B, yellow bar, 4–6 µm) and, as reported (Visintin and Amon, 2001), this association was enhanced in mid- to late anaphase (Fig. 4, A and B, blue bar, >6 µm). Importantly, Cdk1 regulated SPB binding of Cdc15 as shown in *td-cdc14* degron cells. In *td-cdc14* cells the hyperphosphorylated Cdc15-GFP did not bind strongly to SPBs, even after the spindle had extended to lengths exceeding 6 µm (Fig. 4, A and B, red bars). Cdk1 phosphorylation of Cdc15 may therefore inhibit SPB

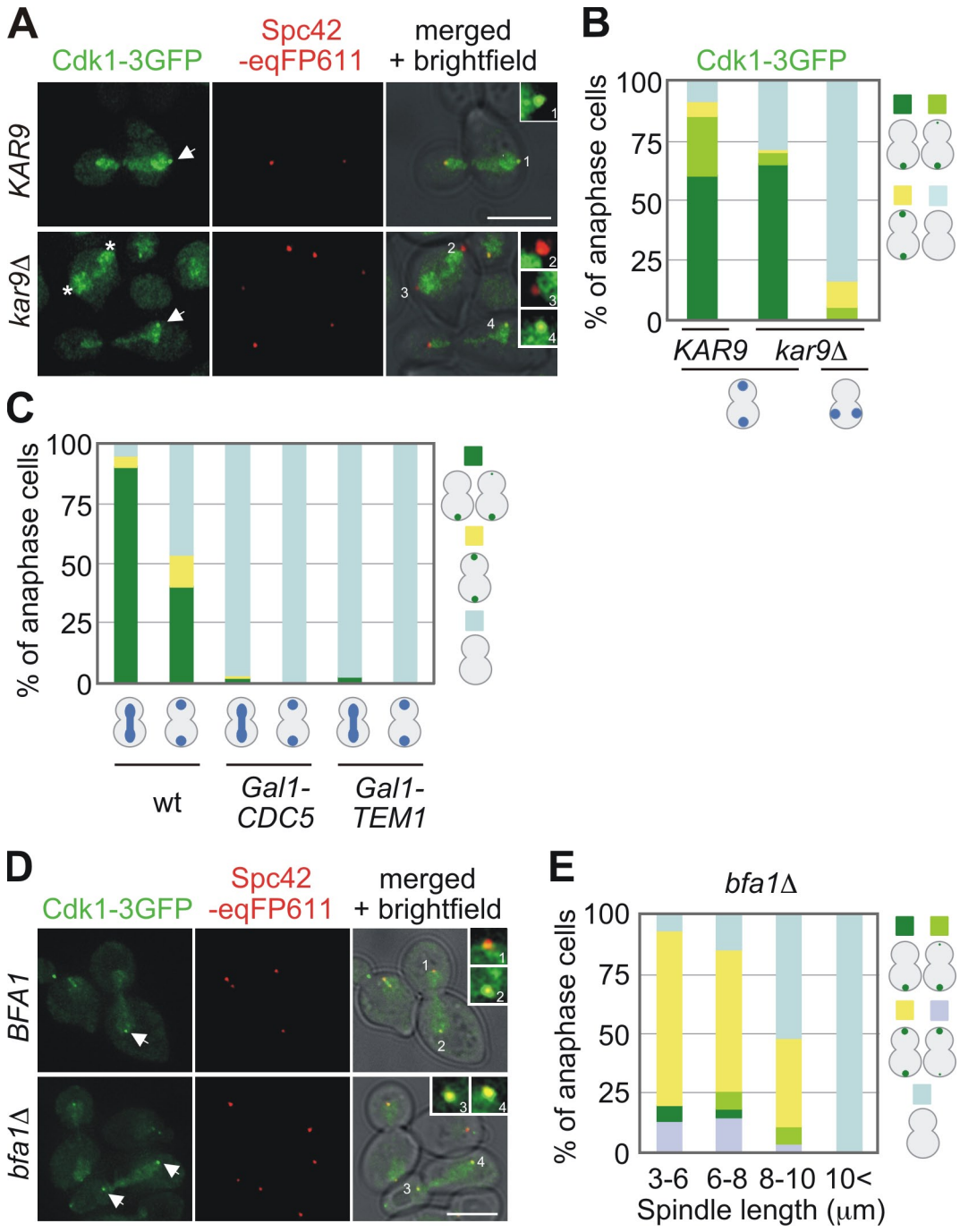

Figure 2. **Activity of the MEN regulates binding of Cdk1 to SPBs.** (A) Cdk1 does not associate with SPBs in cells with a misaligned anaphase spindle. *KAR9 CDK1-3GFP SPC42-eqFP611* and *kar9Δ CDK1-3GFP SPC42-eqFP611* cells grown in YPAD were synchronized with α-factor at 30°C and released at 37°C. The pictures in the right corner show enlargements of SPB signals. (B) Quantification of anaphase cells of experiment A with correctly or misaligned spindles. *n* > 200 cells per strain. (C) Cdc5 and Tem1 are required for Cdk1 localization to SPBs. α-Factor–synchronized wild-type, Gal1-*CDC5*, and Gal1-*UPL-TEM1* cells were grown in YPD medium at 30°C to deplete Cdc5 and Upl-Tem1. Anaphase cells were analyzed for SPB localization of Cdk1-3GFP. *n* > 75 cells for each strain. (D) α-Factor–synchronized *BFA1 CDK1-3GFP SPC42-eqFP611* and *bfa1Δ CDK1-3GFP SPC42-eqFP611* cells were grown in YPAD medium at 30°C and examined in anaphase for colocalization of Cdk1-3GFP and the SPB marker Spc42-eqFP611. The pictures in the right corner show enlargements of SPB signals. (E) Quantification of D as illustrated in the figure. *n* > 80 anaphase cells were analyzed. Bars, 5 μm.

association. To confirm this, we quantified SPB binding of the nonphosphorylated Cdc15-7A-GFP in *td-cdc14* cells. Cdc15-7A-GFP bound strongly to SPBs nearly independently of anaphase progression (Fig. 4 C, red bars).

The data above suggest that phosphorylation by Cdk1 reduces the binding of Cdc15 to the mSPB. However, Cdc15 activity is required to recruit Cdk1 to SPBs (Fig. 3 A). This mutual regulation between Cdc15 and Cdk1 should limit accumulation of both cell cycle regulators at the mSPB. Disrupting the negative regulation between Cdk1 and Cdc15 should result in the hyperaccumulation of proteins at the mSPB. This is the case for Cdc15, as cdc15-as1-GFP accumulates to higher levels at SPBs

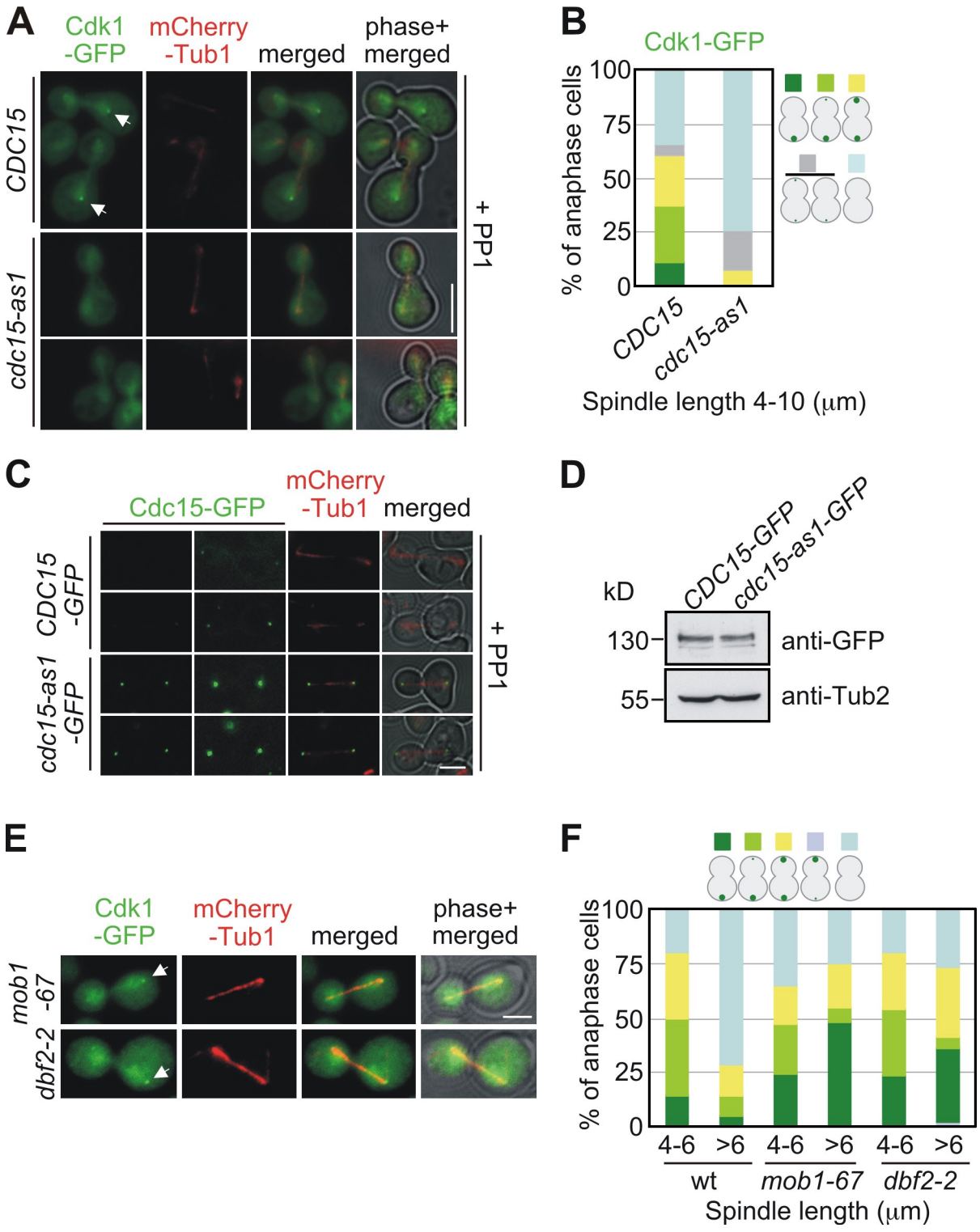

Figure 3. **SPB localization of Cdk1 depends on Cdc15 kinase activity.** (A and B) *CDC15* and *cdc15-as1* cells were synchronized with α-factor and released at 30°C into YPAD medium with the PP1 analogue 8 to inhibit activity of cdc15-as1 kinase (D'Aquino et al., 2005). Anaphase cells with a 4–10-μm spindle were examined for the Cdk1-GFP localization. $n = 38$ for *CDC15*, $n = 43$ for *cdc15-as1*. The arrows in A point toward the Cdk1-GFP signal at mSPBs. (C) Localization of Cdc15-GFP and cdc15-as1-GFP in anaphase. Two representative cells are shown for each strain grown in YPAD at 30°C in the presence of PP1 analogue 8. The first Cdc15-GFP and cdc15-as1-GFP pictures were taken under identical conditions. The second pictures in this row are linear enhancements of the first pictures. Bar, 5 μm. (D) Protein levels of Cdc15-GFP and cdc15-as1-GFP detected with anti-GFP antibody. Anti-Tub2 was used as loading control. (E) The Dbf2–Mob1 kinase complex is not required for SPB association of Cdk1 in anaphase. Synchronized wild-type, *mob1-67*, and *dbf2-2* cells were grown in YPAD at 37°C after the release of the α-factor block. Anaphase cells were examined for SPB localization of Cdk1-GFP. (F) Quantification of E. $n > 75$ anaphase cells per strain. Bars, 5 μm.

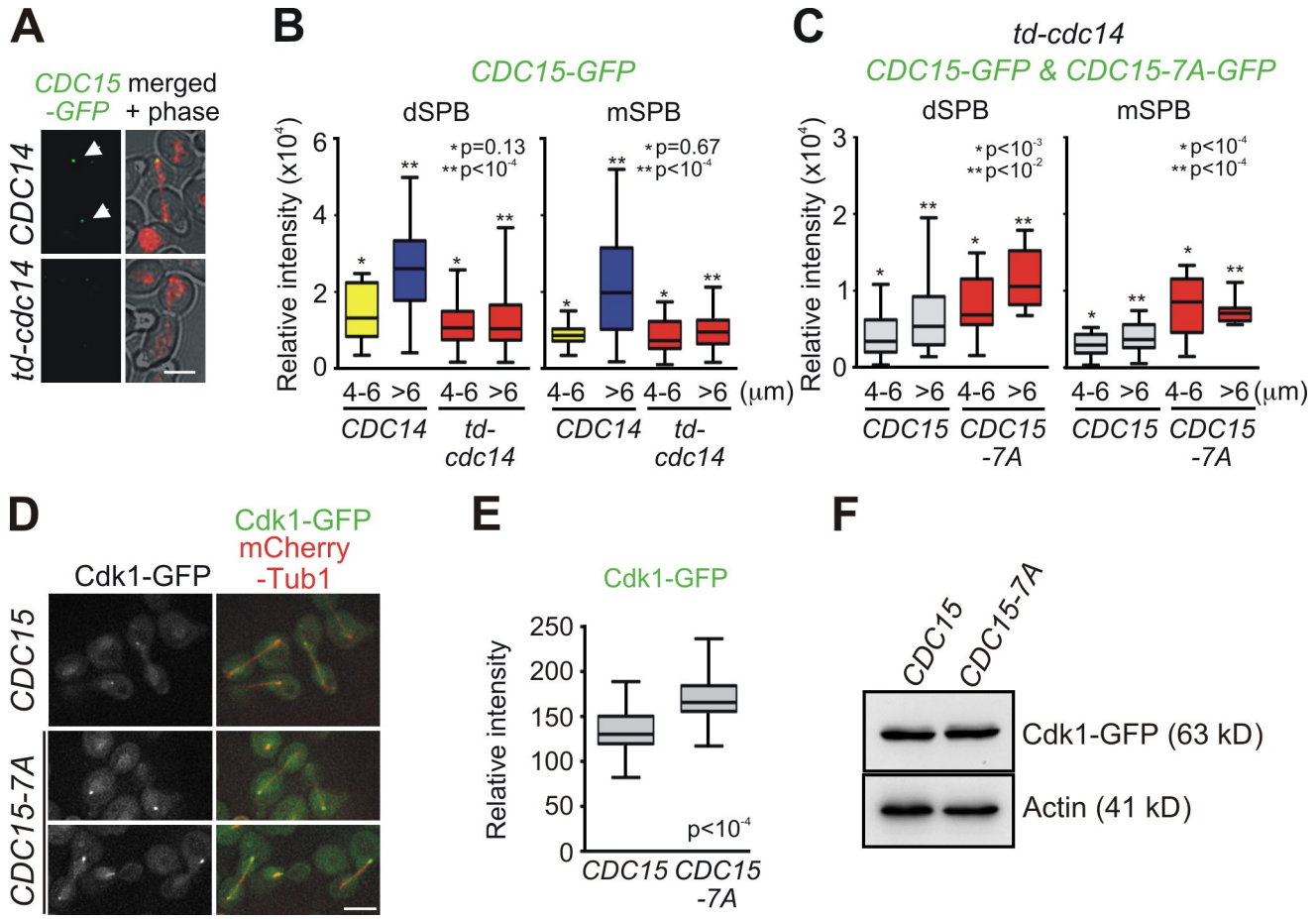

Figure 4. **Cdc15 and Cdk1 show mutual regulation at the mSPB.** (A) *CDC14* and *td-cdc14* cells harboring *CDC15-GFP mCherry-TUB1* were examined for Cdc15-GFP localization in anaphase. The arrows highlight Cdc15-GFP at SPBs. Bar, 5 µm. (B and C) Anaphase cells of *CDC14 CDC15-GFP*, *td-cdc14 CDC15-GFP*, and *td-cdc14 CDC15-7A-GFP* were grown in YPAD and analyzed for GFP signal at SPBs. Quantified relative fluorescent intensities are summarized in box-and-whisker plots: boxes span between the 25th and 75th percentile with a line at the median; whiskers extend from the 10th to 90th percentile. P-values were calculated using unpaired *t* tests and indicate significant differences between * or ** marked bars. (B) $n > 50$ anaphase cells per strain. (C) $n > 50$ for *CDC15-GFP* cells and $n = 24$ for *CDC15-7A-GFP* cells. (D) *CDC15* and *CDC15-7A* cells were grown in SC medium. Cells in anaphase were examined for Cdk1-GFP localization to SPBs. Bar, 5 µm. (E) Quantification of Cdk1-GFP signal at the mSPB. Relative fluorescent intensities in box-and-whisker plots as in B and C. $n > 50$ cells were analyzed per strain. (F) Cdk1-GFP protein levels measured with anti-GFP antibody and actin as loading control.

than Cdc15 (Fig. 3 C). To test this further, we analyzed SPB localization of Cdk1-GFP in *CDC15-7A* cells. Cdk1-GFP at the mSPB was clearly increased in *CDC15-7A* cells compared with *CDC15* wild-type cells (Fig. 4, D and E). Cdk1-GFP protein levels were the same in both cell types (Fig. 4 F). Taken together, our data suggest that Cdk1 negatively regulates binding of Cdc15 to the mSPB, whereas Cdk1 requires the activity of Cdc15 to bind to SPBs. This interdependency restricts binding of both Cdk1 and Cdc15 to the mSPB in early anaphase.

Phosphorylation of Mob1 by Cdk1

CDC15-7A cells do not have a mitotic exit defect (Jaspersen and Morgan, 2000). Cdk1 may regulate additional MEN proteins. Mob1 is a good candidate because it is phosphorylated by Cdk1 in vitro (Holt et al., 2007). Analysis of the amino acid sequence of Mob1 identified two full Cdk1 consensus sites (Fig. 5 A; S/T-P-x-K/R). Mutagenesis of these two sites, S36 and T85, to alanine reduced the phosphorylation of Mob1-2A by Cdk1–Clb2 to 11% of wild-type Mob1 (Fig. 5 B). Mutagenesis of the five remaining S/T-P sites (Mob1-7A) further diminished the ability of Cdk1–Clb2 to phosphorylate Mob1 (Fig. 5 B). Thus, Cdk1–Clb2 phosphorylates Mob1 in vitro predominantly at two sites, S36 and T85.

We next asked whether Mob1 is phosphorylated in vivo. Gal1-*CDC20 MOB1-6HA* cells were arrested in metaphase by depletion of Cdc20. Mob1 appeared as multiple bands in SDS-PAGE (Fig. 5 C). Treatment of Mob1 with lambda phosphatase caused the collapse of the Mob1 bands to a single faster migrating species. Adding the inhibitor EDTA prevented this collapse, suggesting that Mob1 is a phosphoprotein in vivo.

α-Factor–synchronized cells were analyzed in order to monitor changes in Mob1 phosphorylation that accompany cell cycle progression. In G1 phase, Mob1 migrated as a single band corresponding in mobility to dephosphorylated Mob1 protein (Fig. 5 D). 20–30 min after release of the G1 cell cycle block, as soon as cells started to develop a bud, and well before the start of anaphase (Fig. 5 D, 50 min), the upshift of the protein band indicated that a fraction of Mob1 became phosphorylated

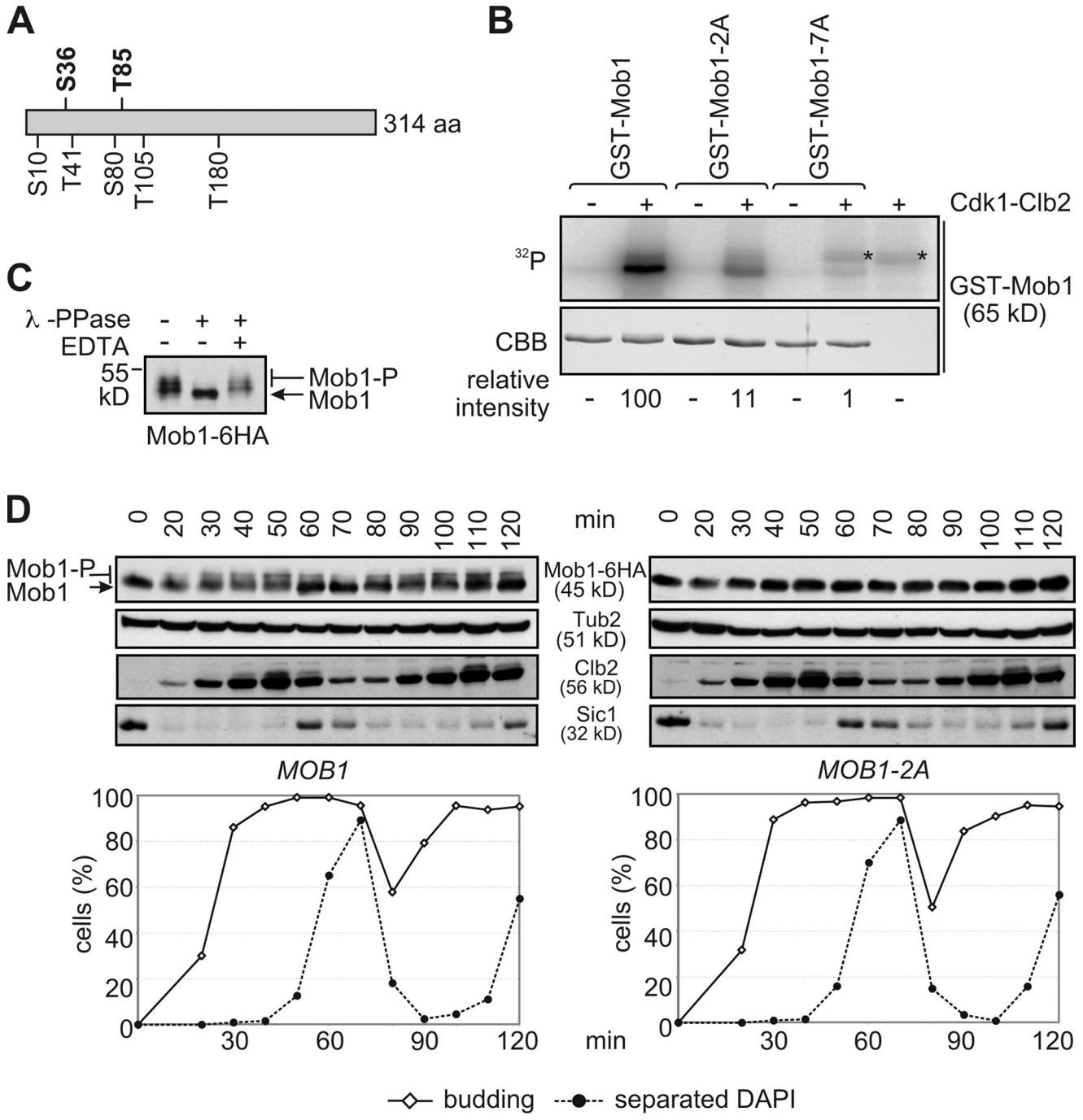

Figure 5. **Phosphorylation of Mob1 by Cdk1 kinase inhibits the MEN.** (A) Distribution of the two full consensus sites [S/T-P-x-K/R] (bold) and the five minimal consensus sites [S/T-P] in the Mob1 protein. (B) Phosphorylation of Mob1-2A and Mob1-7A by Cdk1–Clb2 is strongly reduced. Purified GST-Mob1, GST-Mob1-2A, and GST-Mob1-7A were incubated with Cdk1–Clb2 in the presence of γ-[^{32}P]ATP. ^{32}P-Mob1 was determined by autoradiography. The two asterisks indicate a protein band in the Cdk1–Clb2 preparation that is phosphorylated by Cdk1–Clb2. CBB: Coomassie brilliant blue–stained gel. (C) Immunoprecipitated Mob1-6HA was incubated as indicated. Immunoblot with anti-HA antibodies. (D) *MOB1-6HA* and *MOB1-2A-6HA* cells were arrested with α-factor in G1 phase (t = 0) and released into a synchronized cell cycle at 30°C. Cells were analyzed for Mob1 phosphorylation and Clb2 and Sic1 protein levels by immunoblotting. Budding index and timing of anaphase are shown below the graphs.

(Fig. 5 D, 20 min). Dephosphorylation of Mob1 coincided with mitotic exit when the levels of Clb2 decreased and the Sic1 protein accumulated (Fig. 5 D, 60–70 min). In contrast, Mob1-2A showed strongly reduced phosphorylation at all stages of cell cycle progression (Fig. 5 D, Mob1-2A), indicating that S36 and T85 are the two major in vivo Cdk1 phosphorylation sites.

Cdk1 inhibits activity of the Dbf2–Mob1 kinase complex

A genetic approach was used to address whether Cdk1 phosphorylation activates or inhibits Mob1 function. Conditional lethal *dbf2-2* cells were combined with *MOB1*, *MOB1-S36A*, *MOB1-T85A*, *MOB1-2A*, or *MOB1-7A* alleles. All strains grew equally well at 23°C (Fig. 6 A). However, at 37°C *MOB1-T85A*,

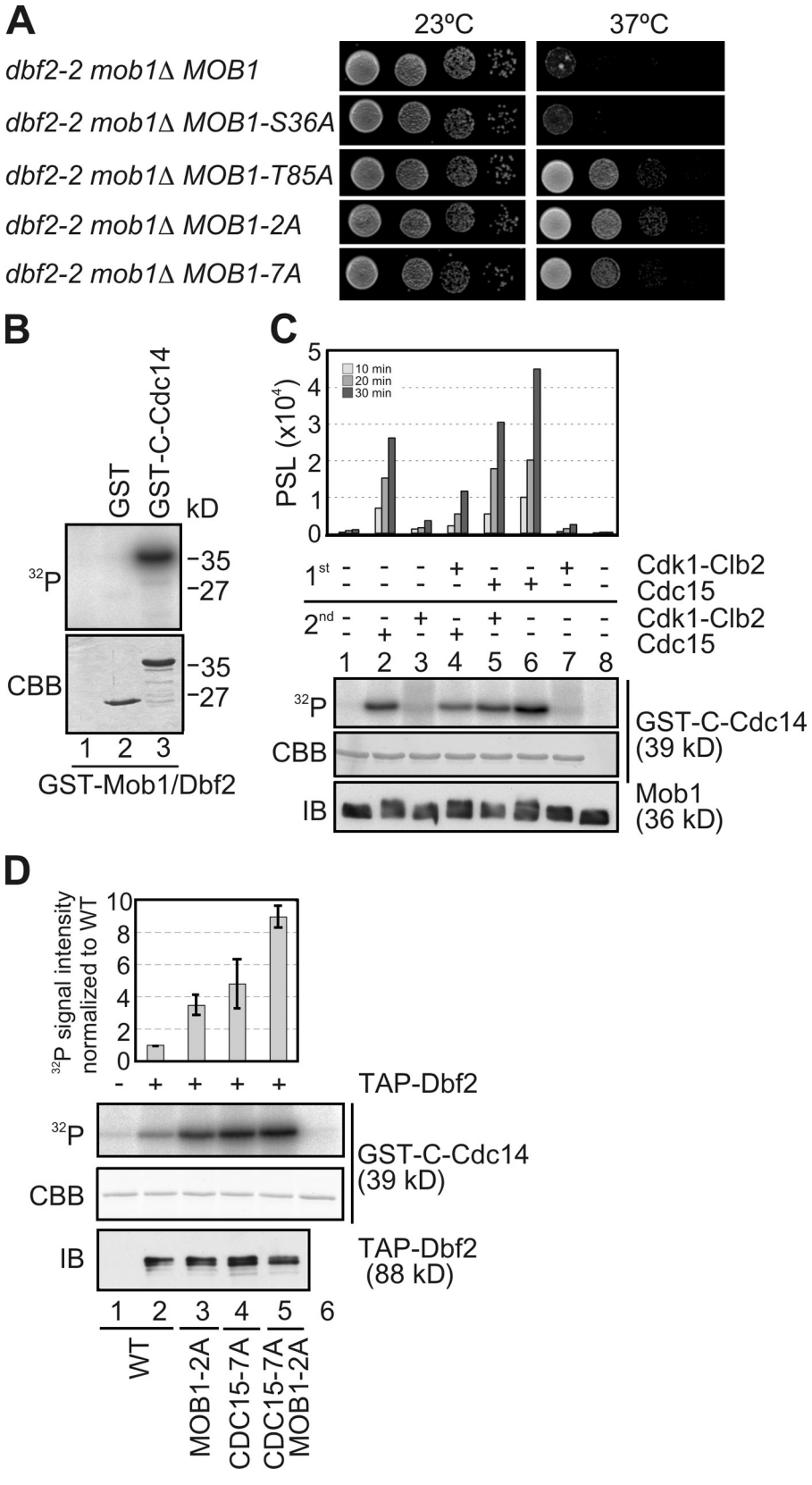

Figure 6. **Cdk1 regulates kinase activity of Dbf2–Mob1.** (A) Log-phase cells with the indicated phenotypes were serially diluted 10-fold and spotted onto YPD plates. Plates were incubated for 2 d at 23 or 37°C. (B) Active Dbf2–Mob1 complex was incubated without substrate (lane 1), GST (lane 2), and GST-C-Cdc14 (lane 3) in the presence of γ-[^{32}P]ATP. Shown is an autoradiography. (C) Cdk1–Clb2 kinase inhibits the activation of Dbf2–Mob1 kinase by Cdc15 in vitro. GST-Mob1 in a complex with Dbf2 was incubated with Cdk1–Clb2 or Cdc15 in the first and second kinase reaction as indicated in the figure. After the second reaction, Mob1-Dbf2 kinase assays with GST-C-Cdc14 as substrate and anti-Mob1 immunoblots were performed. The top graph shows the specific Dbf2–Mob1 kinase activity. Shown is the outcome of one out of two independent experiments. Both results were identical. (D) Gal1-*CLB2-ΔDB* cells were arrested with α-factor in G1 phase in YPAR and released into a synchronized cell cycle at 30°C in YPAR. After ∼60 min, galactose (2%) was added. Cells in anaphase were used for immunoprecipitation of TAP-Dbf2 followed by kinase assays using GST-C-Cdc14 as substrate. Phosphorylation was determined by autoradiography and normalized to immunoprecipitated TAP-Dbf2 (immunoblot anti-TAP). Dbf2–Mob1 kinase activity is shown as mean ± SD of three experiments with the activity of wild-type cells set to 1.

MOB1-2A, and *MOB1-7A* were clearly fitter than wild-type *MOB1* cells, indicating that nonphosphorylated mutant Mob1 proteins possessed MEN-activating function. The overall impact of the mutations was insensitive to the additional deletion of *DBF20* with the exception that *dbf2-2 dbf20Δ* cells were more temperature sensitive than *dbf2-2* cells (Fig. S3 A). This indicates that the suppression of the *dbf2-2* growth defect by the *MOB1-2A* allele does not require *DBF20* function. Moreover,

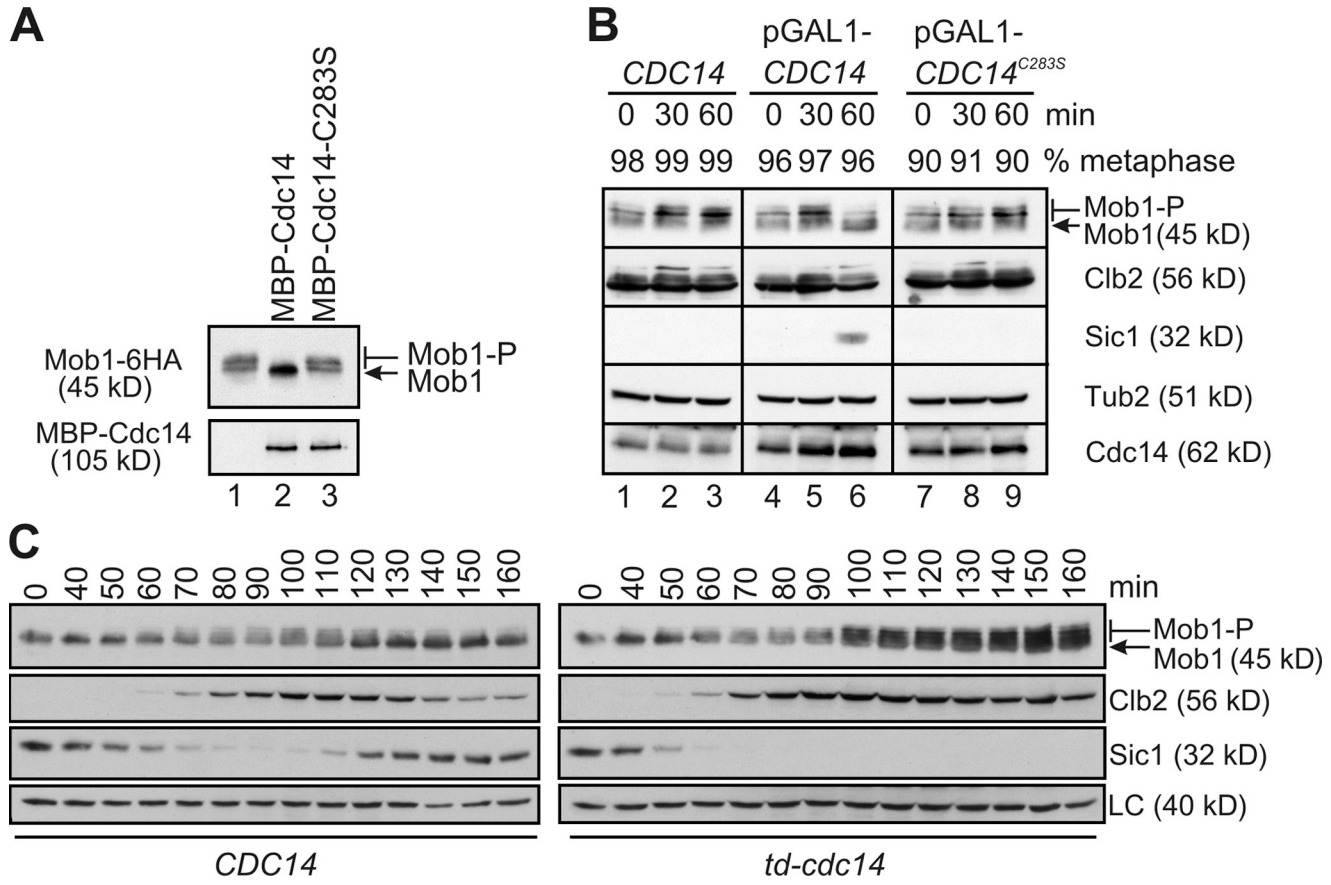

Figure 7. **Cdc14 dephosphorylates Mob1.** (A) Cdc14 dephosphorylates Mob1 in vitro. Gal1-*CDC20 MOB1-6HA* cells were arrested in metaphase through depletion of Cdc20. Immunoprecipitated Mob1-6HA was incubated without any additional protein (lane 1), with GST-Cdc14 (lane 2), or GST-Cdc14-C283S (lane 3) for 1 h at 30°C. Shown is an immunoblot with anti-HA and anti-Cdc14 antibodies. (B) Overexpressed Cdc14 dephosphorylates Mob1 in metaphase. α-Factor–synchronized *MOB1-6HA cdc26Δ* pMET3-*CDC20* (lane 1–3), *MOB1-6HA cdc26Δ* pMET3-*CDC20* Gal1-*CDC14* (lane 4–6), and *MOB1-6HA cdc26Δ* pMET3-*CDC20* Gal1-*CDC14*^{C283S} cells (lane 7–9) were arrested in metaphase through Cdc20 depletion. Cells were shifted to 37°C for 1 h to inactivate the APC. Galactose was then added to induce the Gal1 promoter (t = 0). The percentage of metaphase cells was determined by DAPI staining for each time point. Samples were analyzed with the indicated antibodies. (C) Dephosphorylation of Mob1 is dependent on *CDC14*. *CDC14 MOB1-6HA* and *td-cdc14 MOB1-6HA* cells were synchronized with α-factor at 23°C and released from the G1 block at 37°C. Samples were withdrawn as indicated and analyzed for Mob1 phosphorylation, Clb2, and Sic1 protein levels. A crossreacting band of the anti-Clb2 antibody was used as loading control (LC).

MOB1-2A did not suppress the growth defect of *tem1-3*, *cdc15-1*, and *cdc14-1* cells (unpublished data). Thus, phosphorylation of Mob1 by Cdk1 inhibits the MEN at the level of the Dbf2–Mob1 complex.

We performed coimmunoprecipitation experiments to show that Mob1 phosphorylation by Cdk1 does not regulate complex formation between Mob1 and Dbf2 (Fig. S3, B and C). Instead, Cdk1 controls the kinase activity of the Dbf2–Mob1 complex as shown by two approaches. First, we reconstituted the MEN pathway using the purified Cdc15 and Dbf2–Mob1 proteins (Mah et al., 2001; Geymonat et al., 2007). Cdk1–Clb2 was purified from yeast cells (Loog and Morgan, 2005). As a substrate for Dbf2–Mob1 kinase, we used a C-terminal fragment of Cdc14 (C-Cdc14) that was readily phosphorylated by Dbf2–Mob1 but not by Cdk1–Clb2 kinase (Fig. 6 B; unpublished data; Mohl et al., 2009). The purified Dbf2–Mob1 complex bound to glutathione beads was preincubated with Cdk1–Clb2 and Cdc15 as outlined in Fig. 6 C. The glutathione beads were than washed and incubated with γ-[^{32}P]ATP and C-Cdc14 to determine the Dbf2–Mob1 kinase activity. This activity was corrected for the amount of Dbf2–Mob1 in the kinase reaction to generate the read-out of specific activity (Fig. 6 C, top graph). The Dbf2–Mob1 kinase complex was only active when preincubated with Cdc15 (Fig. 6 C, compare lanes 1, 2, and 6; Mah et al., 2001). Importantly, incubation of Dbf2–Mob1 with Cdk1–Clb2 led to a roughly twofold reduction in the specific activity of the Dbf2–Mob1 kinase complex (Fig. 6 C, compare lanes 2 with 4, and 5 with 6). The inhibition of Dbf2–Mob1 kinase activity by Cdk1–Clb2 was seen irrespective of whether Cdc15 was first incubated with Dbf2–Mob1 followed by Cdk1–Clb2 or vice versa (Fig. 6 C, lanes 2 and 4 vs. 5 and 6). Thus, Cdk1–Clb2 inhibits the activity of the Dbf2–Mob1 kinase complex.

Second, to verify that Cdk1–Clb2 inhibits the MEN at the level of Cdc15 and Mob1, we overexpressed a stable version of *CLB2* (*CLB2-ΔDB*) in synchronized yeast cells and allowed cell cycle progression into anaphase. Immunoprecipitation of Dbf2 showed that the specific kinase activity of the Dbf2–Mob1 complex was low in the presence of high Cdk1–Clb2 activity (Fig. 6 D; Stegmeier et al., 2002). Importantly, the specific kinase activity of

Dbf2–Mob1 was elevated between 4- and 10-fold in *MOB1-2A*, *CDC15-7A*, and *MOB1-2A CDC15-7A* cells. These data indicate that Cdk1 kinase inhibits cellular Dbf2–Mob1 kinase activity via phosphorylation of both Cdc15 and Mob1.

Cdc14 dephosphorylates Mob1

Abrupt dephosphorylation of Mob1 coincided with mitotic exit (Fig. 5 D). The abruptness of this transition suggests that the phosphatase Cdc14 may be driving this dephosphorylation. Three lines of evidence support this possibility. Mob1 was dephosphorylated by recombinant wild-type Cdc14 but not by the catalytically inactive Cdc14-C283S mutant protein (Fig. 7 A). Premature activation of *CDC14* by overexpression from the Gal1 promoter led to Mob1 dephosphorylation in metaphase-arrested cells (Fig. 7 B). Finally, synchronized *td-cdc14* cells showed that Mob1 dephosphorylation depended on Cdc14 activity (Fig. 7 C). Together, these data suggest that Cdc14 directly dephosphorylates Mob1 during mitotic exit.

Cdk1 together with the Bfa1–Bub2 complex inhibits mitotic exit of cells

Deletion of *BUB2* or the introduction of the *CDC15-7A* mutation hardly influenced cell cycle progression (Fraschini et al., 1999; Jaspersen and Morgan, 2000). However, the function of Bfa1–Bub2 GAP complex in inhibiting mitotic exit becomes apparent when combined with mutants like *kar9Δ* that cause spindle misalignment (Pereira et al., 2000). Similarly, *CDC15-7A* and *MOB1-2A* showed genetic interactions with *kar9Δ*. The *CDC15-7A MOB1-2A kar9Δ* triple mutant was no longer able to inhibit mitotic exit when the spindle was misaligned (Fig. S4, A and B). These data suggest that dual inhibition of the MEN by Bfa1–Bub2 and Cdk1 is essential when the spindle is misaligned. However, this MEN inhibition by Cdk1 probably does not take place at the SPB because *kar9Δ* cells with misaligned spindles do not accumulate Cdk1 at SPBs (Fig. 2, A and B).

During normal cell cycle progression, inhibition of the MEN by either Cdk1 or the Bfa1–Bub2 GAP complex may be sufficient to prevent premature mitotic exit. This possibility was tested using combinations of single, double, and triple mutants of *bub2Δ*, *CDC15-7A*, and *MOB1-2A*. Analysis of the growth of cells at different temperatures revealed that only the triple mutant *bub2Δ CDC15-7A MOB1-2A* cells grew slower at 23°C and showed an elevated growth defect at 37°C (Fig. 8 A).

We analyzed the MT cytoskeleton and the Cdc14-GFP distribution of cells grown at 23°C. Wild-type, single, and *CDC15-7A MOB1-2A* and *bub2Δ MOB1-2A* double mutants had normal proportions of cells in G1, S, and mitotic phases of the cell cycle (Fig. 8, B and C; unpublished data). Synchronization of these cells by α-factor block and release did not show obvious mitotic exit defects (not depicted). In contrast, ∼8% of log phase *bub2Δ CDC15-7A* cells exhibited the abnormal phenotypes of premature Cdc14 release (i.e., released Cdc14 in cells with a short spindle), cytokinesis failure (cells with multiple buds), and defects in nucleolar division (cells with multiple Cdc14 nucleoli foci) (Fig. 8 C). These defects were strongly enhanced in *bub2Δ CDC15-7A MOB1-2A* triple mutant cells (Fig. 8, B and C). Moreover, analysis of α-factor–synchronized *bub2Δ CDC15-7A MOB1-2A* cells confirmed the premature release of Cdc14 from the nucleolus in about half of cells with a short metaphase spindle (Fig. 8 B, top *bub2Δ CDC15-7A MOB1-2A* cell). In contrast, deletion of the spindle orientation checkpoint gene *KIN4* in *CDC15-7A MOB1-2A* cells did not cause mitotic exit defects (not depicted). Kin4 blocks mitotic exit of cells with a misaligned anaphase spindle by keeping the Bfa1–Bub2 complex active (D'Aquino et al., 2005; Pereira and Schiebel, 2005; Caydasi and Pereira, 2009; Monje-Casas and Amon, 2009). Thus, the Bfa1–Bub2 GAP complex together with Cdk1 phosphorylation of Cdc15 and Mob1 inhibit mitotic exit during an unperturbed cell cycle.

Discussion

The MEN is depicted as a linear SPB-associated pathway that is regulated by the inhibitory Bfa1–Bub2 GAP complex and, in an undefined way, by Cdk1 (Stegmeier and Amon, 2004). In one report it was suggested that Cdk1 inhibits the relocalization of Cdc15 from the mSPB to the dSPB (Menssen et al., 2001). However, the overexpressed Cdc15 construct used in this previous study already bound to the SPB of G1 cells, which is in clear contrast to all other reports on the localization of Cdc15 (Cenamor et al., 1999; Xu et al., 2000; Visintin and Amon, 2001; Molk et al., 2004) including the data presented here using a fully functional, nonoverexpressed *CDC15-GFP* gene fusion. In this study, we revise the view of how the MEN is regulated and provide evidence that the MEN is inhibited concretely by the Bfa1–Bub2 GAP and the phosphorylation of Cdc15 and Mob1 by Cdk1.

Regulation of Cdc14 and Mob1 by Cdk1

Recent models on MEN regulation suggest that inhibition of the MEN by the Bfa1–Bub2 GAP complex at the dSPB and the binding of Lte1 to the cortex of the daughter cell could be sufficient to restrain MEN activation (Bardin et al., 2000; Pereira et al., 2000). However, we consider this relatively simple model as unlikely because inactivation of the Bfa1–Bub2 GAP does not cause premature mitotic exit (Fraschini et al., 1999; Pereira et al., 2000).

How is the MEN regulated throughout the cell cycle? In S phase and metaphase the MEN is kept inactive by the inhibitory Bfa1–Bub2 GAP complex residing at SPBs (Fraschini et al., 1999; Pereira et al., 2000). At this phase of the cell cycle Cdc15 and Mob1 are not enriched at SPBs (Visintin and Amon, 2001); however, Cdk1 phosphorylates both proteins (Fig. 5; Jaspersen and Morgan, 2000). This suggests that Cdk1 in the cytoplasm is able to phosphorylate Cdc15 and Mob1 to reduce MEN activity (Jaspersen and Morgan, 2000; Pereira et al., 2000; Menssen et al., 2001; Figs. 5, 6, and 8).

At the metaphase–anaphase transition APC^{Cdc20} induces a decline of mitotic Cdk1 activity by degrading about half of the mitotic cyclin Clb2 (Shirayama et al., 1999; Yeong et al., 2000). In addition, activation of separase leads to a drop of PP2A activity, allowing the remaining Cdk1–Clb2 together with FEAR network components to trigger the transient release of Cdc14 from the nucleolus (Stegmeier et al., 2002; Azzam et al., 2004; Queralt et al., 2006). These changes in Cdc14 phosphatase

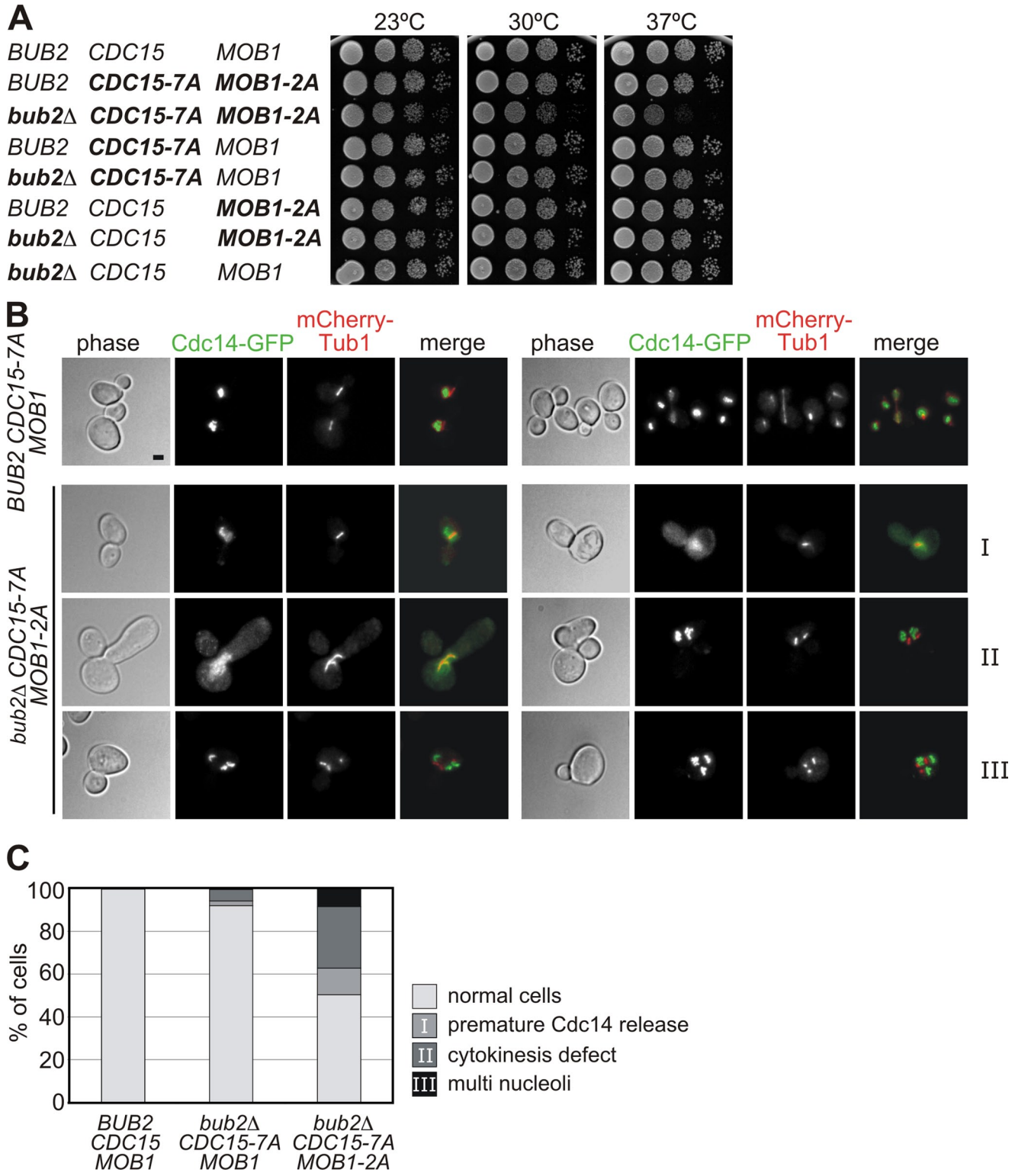

Figure 8. ***bub2Δ CDC15-7A MOB1-2A* cells accumulate phenotypes that are consistent with premature mitotic exit and cytokinesis defects.** (A) *bub2Δ CDC15-7A MOB1-2A* cells show enhanced growth defects at elevated temperatures. Serial dilutions of cells with the indicated phenotypes were spotted onto YPD plates. Plates were incubated for 2–3 d at the indicated temperature. (B and C) Defects of *bub2Δ CDC15-7A* and *bub2Δ CDC15-7A MOB1-2A* cells. Cells were cultured in YPAD medium at 23°C and analyzed by fluorescence microscopy for Cdc14-GFP and mCherry-Tub1 localization. Bar, 2 µm. (C) Quantification of B. Cells were classified as indicated in the figure (n > 100 cells per strain).

and Cdk1 kinase activities and the movement of the spindle toward the MEN activator Lte1 that is associated with the cortex of the bud promote activation of Tem1 and at least partial dephosphorylation of Cdc15 and Mob1 (Bardin et al., 2000; Jaspersen and Morgan, 2000; Pereira et al., 2000; Stegmeier et al., 2002; Fig. 7).

In early anaphase the Bfa1–Bub2 GAP complex probably still inhibits the binding of active Tem1-GTP to the dSPB (Pereira et al., 2002). Active Tem1 may bind to the mSPB devoid of the Bfa1–Bub2 GAP (Molk et al., 2004). In addition, FEAR-released Cdc14 is already sufficiently high to dephosphorylate to some degree cytoplasmic Cdc15 and Mob1, which occurs simultaneously to the degradation of the first wave of Clb2 (Fig. 5, and Fig. 9 for model; Jaspersen and Morgan, 2000; Asakawa et al., 2001; Visintin and Amon, 2001; Stegmeier et al., 2002; Molk et al., 2004). Tem1 then recruits nonphosphorylated Cdc15 to the mSPB (Fig. 4 C). Cdc15 in turn facilitates binding of the Dbf2–Mob1 kinase complex and Cdk1 to the mSPB (Fig. 3 and Fig. S4 C). How Cdk1 binds to the mSPB is presently unclear. However, it is interesting to note that the fission yeast Cdc15 homologue Cdc7 can phosphorylate the SPB scaffold protein Nud1, named Cut11 in fission yeast (Krapp et al., 2003).

Binding of active Cdc15 to the SPB should be sufficient to activate the MEN (Visintin and Amon, 2001). However, measurement of the timing of Cdk1 binding to the mSPB as an indication of Cdc15 activity and of Cdc14 release from the nucleolus as an indicator of full MEN activity in FEAR-defective *spo12Δ* cells suggests a delay of 6–8 min (anaphase in yeast is only 15 min) between Cdc15 activation at the mSPB and release of Cdc14 from the nucleolus (Fig. S1 C). This delay indicates a transient inhibition of full MEN activation (as measured by Cdc14 release) in early anaphase.

The finding that increased levels of active Cdc15 at the mSPB (Cdc15-7A) also increases Cdk1 at the mSPB (Fig. 4 D) is consistent with mutual regulation of both proteins at this pole. In our model (Fig. 9), the proximity of Cdk1, Cdc15, and Mob1 at the mSPB in early anaphase allows efficient phosphorylation of Cdc15 and Mob1 despite decreasing Cdk1 activity in the cytoplasm and nucleoplasm. Phosphorylated Cdc15 then binds with reduced affinity to the SPB, thus diminishing its binding and subsequently also that of Cdk1. In addition, phosphorylation of Mob1 reduces Dbf2–Mob1 kinase activity (Fig. 6). The consequence of the Cdk1–Cdc15/Mob1 regulation loop is the self-inhibition of the MEN in early anaphase. This model is supported by the observations that interfering with the feedback loop by inhibition of Cdc15 kinase activity (Fig. 3 C) or blocking the ability of Cdk1 to phosphorylate Cdc15 (Fig. 4 C; Cdc15-7A) leads to hyperaccumulation of Cdc15 at the mSPB. Moreover, Cdk1 accumulated more strongly at the mSPB in *CDC15-7A* cells than in *CDC15* cells (Fig. 4, D and E).

Down-regulation of the MEN by Cdk1 continues until, in mid/late anaphase, the Cdc14/Cdk1 ratio is shifted in favor of the phosphatase. Cdc15 and Mob1 are then completely dephosphorylated by Cdc14 (Fig. 7; Jaspersen and Morgan, 2000), causing the collapse of the regulation loop at the mSPB concomitant with the full activation of the MEN at the mSPB. Eventually, Cdc5 phosphorylates Bfa1 in late anaphase/telophase to inactivate the Bfa1–Bub2 GAP complex (Hu et al., 2001; Pereira et al., 2002).

In cells with a misaligned anaphase spindle Cdk1 does not bind to SPBs (Fig. 2 and Fig. S1). However, Cdk1 contributes to the inhibition of mitotic exit through phosphorylation of cytoplasmic Cdc15 and Mob1 (Fig. S4). In this respect, it is interesting that in case of spindle alignment defects Bfa1–Bub2 activity becomes transferred from the SPB into the cytoplasm (Caydasi and Pereira, 2009).

The checkpoint kinase Kin4 (D'Aquino et al., 2005; Pereira and Schiebel, 2005; Caydasi and Pereira, 2009; Monje-Casas and Amon, 2009) does not have a function in an unperturbed cell cycle because *kin4Δ CDC15-7A MOB1-2A* cells have the same mitotic exit phenotype as *CDC15-7A MOB1-2A* cells (unpublished data). Thus, Kin4 is only important when the anaphase spindle is misaligned to prevent Cdc5 polo kinase to phosphorylate Bfa1 (Hu et al., 2001; D'Aquino et al., 2005; Pereira and Schiebel, 2005; Maekawa et al., 2007; Caydasi and Pereira, 2009).

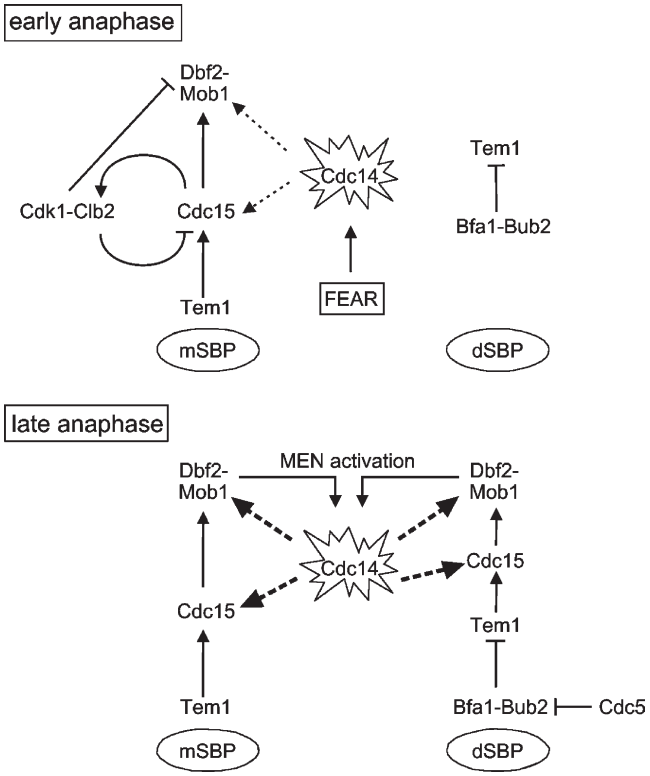

Figure 9. **Model for the function of Cdk1 at the mSPB.** In early anaphase Cdc14 becomes released from the nucleolus by the FEAR pathway (Stegmeier et al., 2002). Cdc15 and Mob1 become partially dephosphorylated (dashed lines). In addition, Tem1 binds to the mSPB (Molk et al., 2004) and recruits nonphosphorylated Cdc15 to this SPB. Cdc15 directs Cdk1 and Dbf2–Mob1 kinases to the mSPB. We propose that the close vicinity of the proteins at the mSPB leads to phosphorylation of Mob1 and Cdc15 by Cdk1. Cdk1-phosphorylated Cdc15 dissociates from the mSPB restricting Cdk1 at the mSPB (symbolized as inhibition of Cdc15). Phosphorylation of Mob1 by Cdk1 leads to a decrease in Dbf2–Mob1 kinase activity. These events restrict full activation of the MEN at the mSPB. At the dSPB, the Bfa1–Bub2 complex inhibits activation of Tem1 (Bardin et al., 2000; Pereira et al., 2000). In late anaphase the increase in Cdc14 activity and the decrease in Cdk1 activity disrupt the regulation loop between Cdk1 and Cdc15 at the mSPB (thick dashed lines symbolize complete dephosphorylation by Cdc14). This together with the phosphorylation of Bfa1 by Cdc5 polo-like kinase at the dSPB (Hu et al., 2001; Geymonat et al., 2003; Maekawa et al., 2007) allows full activation of the MEN. Dbf2–Mob1 kinase then phosphorylates Cdc14 (Mohl et al., 2009).

Opposing functions of Cdk1 kinase in anaphase

Cdk1 kinase has a dual but antagonistic role in the regulation of Cdc14 phosphatase. As part of the FEAR pathway Cdk1 activates the release of Cdc14 in early anaphase (Azzam et al., 2004). Simultaneously, Cdk1 inhibits activation of the MEN (Fig. 6). This dual action of Cdk1 may help to regulate the timing of events in anaphase. Recruitment of Cdk1–Clb2 to the mSPB is an inhibitory mechanism that ensures that the MEN does not become active until the FEAR-released Cdc14 has accomplished its functions in spindle midzone formation and rDNA segregation (Pereira and Schiebel, 2003; D'Amours et al., 2004; Lavoie et al., 2004; Sullivan et al., 2004; Higuchi and Uhlmann, 2005; Khmelinskii et al., 2007). Genetic data impressively demonstrate the importance of the concerted action of MEN inhibition by the Bfa1–Bub2 complex and by Cdk1 for the coordination of anaphase events. *bub2Δ CDC15-7A MOB2-2A* cells are compromised for growth, and even at the permissive temperature, prematurely release Cdc14 from the nucleolus and exhibit defects in both cytokinesis and the segregation of their nucleoli (Fig. 8).

The MEN, the septation initiation network (SIN) of *Schizosaccharomyces pombe*, and the Hippo pathway of *Drosophila* and vertebrates have, in Cdc15- and Dbf2–Mob1-like kinases, common signaling elements. Like the MEN, the SIN pathway is inhibited by Cdk1 activity (McCollum and Gould, 2001). However, a recent chemical genetic analysis concluded that the relevant Cdk1 targets whose phosphorylation prevents activation of the SIN remain to be identified (Dischinger et al., 2008). Little is known about cell cycle control of the Hippo pathway (Pan, 2007). It is, however, interesting that the Dbf2-related kinase Lats1 was originally described as a partner of Cdk1 (Tao et al., 1999). Thus, our analysis on the interplay between Cdk1 and MEN components will help to understand the regulation of MEN-related pathways in other organisms.

Materials and methods

Yeast strains and plasmids

Yeast strains and plasmids are listed in Table I. Yeast strains were derivatives of S288c unless stated otherwise. Yeast strains were constructed by PCR-based methods (Janke et al., 2004). The red fluorescent eqFP611 (Wiedenmann et al., 2002) was used to mark SPBs through a fusion with *SPC42*. The *Cherry-TUB1* construct was described previously (Khmelinskii et al., 2007).

Construction of MOB1 mutants

The *MOB1* gene was cloned into yeast integration vector pRS305H (Taxis and Knop, 2006). Mutations in *MOB1* were introduced by PCR-directed mutagenesis and confirmed by DNA sequencing. Serine or threonine residues of two full Cdk1 consensus sites and five minimal Cdk1 consensus sites were mutated to alanine. The *MOB1-7A* mutant resulted in the exchange of S10, S36, T41, S80, T85, T105, and T180 to alanine. All *MOB1* constructs were functional because they rescued the lethality of a *MOB1* deletion.

Cell cycle analysis and growth conditions

For synchronization, yeast cells were grown in yeast extract adenine dextrose (YPAD) medium and arrested in G1 by treatment with α-factor (10 µg/ml) for 2.5 or 2 h at 23 or 30°C, respectively, until >95% of cells showed a mating projection. Cells were then washed with prewarmed growth medium to remove α-factor and resuspended in YPAD medium at the indicated temperatures. Cells with *CDC5* or *UPL-TEM1* under the control of the Gal1 promoter were grown in YPA with 3% raffinose and 2% galactose (YPAG/R). For the depletion of Cdc5 and Tem1, Gal1-*CDC5* and Gal1-*UPL-TEM1* cells in YPAG/R were synchronized with α-factor. G1-arrested cells were then released into YPAD to repress the Gal1 promoter.

Fluorescence microscopy

Yeast cells with *CDK1-GFP*, *CDK1-3GFP*, *CDC15-GFP*, *cdc15-as1-GFP*, *MOB1-GFP*, *mob1-67-GFP*, *DBF2-GFP*, *dbf2-2-GFP*, and *CDC14-3mCherry* were analyzed by fluorescence microscopy without washing or fixation with the exception of *CDK1-3GFP*, *MOB1-GFP*, and *CDC14-GFP* cells in Figs. 1 (B and C), 2, 3 E, 8 B, S1 A, S2 A, and S4 C. These cells were analyzed after fixation with 4% paraformaldehyde in 150 mM K-phosphate buffer, pH 6.5, for 10 min at 20°C. Cells were incubated in PBS containing DAPI to visualize DNA. Z series of images of 0.35-µm steps were captured with a microscope (Axiophot; Carl Zeiss, Inc.) that was equipped with a 100x NA 1.45 oil immersion objective (Plan-Fluar; Carl Zeiss, Inc.) and a camera (Cascade:1K; Photometrics). Pictures were quantified or processed with MetaMorph software (Universal Imaging Corp.; Figs. 1 [C and D], 2 [B and C], 3 [A and C], 4 [A–C], 8 B, S1 A, S2 [C and D], and S4 C). A microscope (Deltavision; Applied Precision) equipped with GFP and TRITC filters (Chroma Technology Corp.), a 100x NA 1.4 oil immersion objective (PlanApo, IX70; Olympus), and a camera (CoolSNAP HQ; Photometrics) was used for pictures in Figs. 1 (A and B), 2 (A and D), 3 E, 4 D, S1 C, and S2 A. Pictures were processed with SoftWoRx 3.5.0 software (Applied Precision). Fluorescence intensity was measured in one plane with the SPB in focus.

Time-lapse experiments were performed (Fig. 1 A and Fig. S1 C) on Con A–coated glass-bottom dishes (MatTek) using the Deltavision microscope at 30°C. Z series at 0.35-µm steps (2 × 2 binning) were acquired every 1 min (SHM1757) or 2 min (CKY949). Deconvolution was performed using SoftWoRx 3.5.0 software (Applied Precision) with default settings.

Adobe Photoshop and ImageJ were used to mount the images and to produce merged color images. No manipulations other than contrast and brightness adjustments were used.

In vitro kinase assays

Clb2 was expressed from a 2-µm Gal-*CLB2-TAP* plasmid (Übersax et al., 2003). Kinase complex was purified via the TAP tag. Cdk1 kinase assays with GST-Mob1 as substrate were performed as described previously (Loog and Morgan, 2005), except that the reaction buffer was 25 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol, 10 mM $MgCl_2$, 10 µM ATP, 5 mM β-glycerophosphate, and 5 µCi γ-[^{32}P]ATP in a 20-µl reaction volume.

The GST–Mob1–Dbf2 complex of Fig. 6 was affinity purified from yeast strains expressing *GST-MOB1* and *DBF2* under the control of the pGal1-10 promoter without eluting the complex from the glutathione affinity beads (Geymonat et al., 2002). Co-purification of Dbf2 was confirmed by MALDI-TOF/TOF. In Fig. 6 C, the beads with ∼80 ng Dbf2–Mob1 were incubated in 40 µl Cdc15/Cdk1 kinase buffer (50 mM Hepes-KOH, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 5 mM β-glycerophosphate, and 1 mM DTT) containing 5 mM ATP and complete EDTA-free protease inhibitor cocktail (Roche) for 1 h at 30°C with 3 ng of Cdc15 or Cdk1–Clb2 or without kinase (first reaction). The beads were washed three times with Cdc15/Cdk1 kinase buffer. The second reaction was performed as the first reaction. One eighth of the beads were incubated in Dbf2 kinase buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 5 mM β-glycerophosphate, 1 mM DTT, and 5 µCi γ-[^{32}P]ATP) with 1 µg GST-C-Cdc14 at 20°C for 10, 20, and 30 min. The kinase reaction was stopped by the addition of 5 µl 6x sample buffer and heating the samples at 95°C for 5 min. Samples were loaded onto a 10% SDS-PAGE gel. Gels were stained with SimplyBlue Safe Stain (Invitrogen) and radioactivity was detected by a PhosphorImager (FLA-300; Fujifilm) and quantified with Image Gauge v3.45 (Fujifilm).

TAP-Dbf2 immunoprecipitations were performed in 25 mM K-phosphate, pH 7.6, 150 mM KCl, 1% NP-40, 10% glycerol, 5 mM $MgCl_2$, 1 mM EDTA, complete EDTA-free protease inhibitor cocktail (Roche), and 80 mM β-glycerophosphate using rabbit IgG coupled to M-270 Epoxy Dynabeads. Half of each reaction was used for immunoblot analysis and the other half for kinase assays in Dbf2 kinase buffer using GST-C-Cdc14 as substrate as in Fig. 6 C with incubation for 15 min at 30°C.

Online supplemental material

Fig. S1: Cdk1 localization in *dyn1Δ* cells and time-lapse analysis of *CDK1-GFP CDC14-3mCherry* cells. Fig. S2: Cdk1 localization in *cdc15-1* cells and localization of mob1-67 and dbf2-2 proteins. Fig. S3: suppression analysis in *dbf2-2 dbf20Δ* cells and binding of Mob1-2A to Dbf2.

Table I. **Yeast strains and plasmids**

Name	Genotype/construction	Source or reference
Yeast strains		
CKY310	*pGal1-CDC20-kanMX6 MOB1-6HA-klTRP1*	This study
CKY445	*Δmob1::klTRP1 leu2Δ1::MOB1-hphNT1*	This study
CKY448	*Δmob1::klTRP1 leu2Δ1::MOB1-2A-hphNT1*	This study
CKY457	*Δmob1::klTRP1 leu2Δ1::MOB1-hphNT1 MOB1-6HA-KanMX*	This study
CKY463	*Δmob1::klTRP1 leu2Δ1::MOB1-2A-hphNT1 MOB1-2A-6HA-KanMX*	This study
CKY489	*Δmob1::klTRP1 leu2Δ1::MOB1-2A-hphNT1 MOB1-2A-6HA-KanMX pGal1-CDC20::LEU2*	This study
CKY548	*Δmob1::klTRP1 leu2Δ1::MOB1-hphNT1 MOB1-6HA-NatNT2 TAP-DBF2-KanMX4*	This study
CKY549	*Δmob1::klTRP1 leu2Δ1::MOB1-2A-hphNT1 MOB1-6HA-NatNT2 TAP-DBF2-KanMX4*	This study
CKY592	*Δmob1::klTRP1 leu2Δ1::MOB1-hphNT1 MOB1-6HA-KanMX pGal1-CDC20::LEU2*	This study
CKY593	*Δmob1::klTRP1 leu2Δ1::MOB1-hphNT1 MOB1-6HA-NatNT2 TAP-DBF2-KanMX4 pGal1-CDC20::LEU2*	This study
CKY594	*Δmob1::klTRP1 leu2Δ1::MOB1-2A-hphNT1 MOB1-6HA-NatNT2 TAP-DBF2-KanMX4 pGal1-CDC20::LEU2*	This study
CKY604	*CDC15-7A mCherry-TUB1-TRP1 Δmob1::NatNT2 leu2Δ1::MOB1-hphNT1*	This study
CKY606	*CDC15-7A mCherry-TUB1-TRP1 Δmob1::NatNT2 leu2Δ1::MOB1-2A-hphNT1*	This study
CKY623	*HIS3-pGal1-HA-UBR1 MOB1-6HA-hphNT1*	This study
CKY624	*HIS3-pGal1-HA-UBR1 KanMX6-td-CDC14 MOB1-6HA-hphNT1*	This study
CKY629	*MOB1-6HA-klTRP1 cdc26Δ::NatNT2 CDC20::pKN109 (pMet3-CDC20)*	This study
CKY630	*MOB1-6HA-klTRP1 cdc26Δ::NatNT2 pGal-CDC14::LEU2 CDC20::pKN109*	This study
CKY631	*MOB1-6HA-klTRP1 cdc26Δ::NatNT2 pGal-CDC14-C283S::LEU2 CDC20::pKN109*	This study
CKY663	*mCherry-TUB1-TRP1 bub2Δ::HIS3MX6 CDC14-GFP-URA3*	This study
CKY664	*CDC15-7A mCherry-TUB1-TRP1 bub2Δ::HIS3MX6 CDC14-GFP-URA3*	This study
CKY666	*CDC15-7A mCherry-TUB1-TRP1 Δmob1::NatNT2 leu2Δ1::MOB1-2A-hphNT1 bub2Δ::HIS3MX6 CDC14-GFP-URA3*	This study
CKY669	*Δmob1::klTRP1 leu2Δ1::MOB1-2A-hphNT1 bub2Δ::HIS3MX6 CDC14-GFP-URA3*	This study
CKY682	*mCherry-TUB1-TRP1 CDC14-GFP-URA3*	This study
CKY683	*CDC15-7A mCherry-TUB1-TRP1 CDC14-GFP-URA3*	This study
CKY685	*Δmob1::klTRP1 leu2Δ1::MOB1-2A-hphNT1 CDC14-GFP-URA3*	This study
CKY687	*CDC15-7A mCherry-TUB1-TRP1 Δmob1::NatNT2 leu2Δ1::MOB1-2A-hphNT1 CDC14-GFP-URA3*	This study
CKY708	*cdc15-as SPC42-eqFP611-HIS3MX6 MOB1-GFP-KanMX6*	This study
CKY769	*Δmob1::klTRP1 leu2Δ1::MOB1-hphNT1 TAP-DBF2-KanMX4 pGal-CLBΔDB::URA3*	This study
CKY770	*Δmob1::klTRP1 leu2Δ1::MOB1-2A-hphNT1 TAP-DBF2-KanMX4 pGal-CLBΔDB::URA3*	This study
CKY771	*CDC15-7A mCherry-TUB1-TRP1 Δmob1::NatNT2 leu2Δ1::MOB1-hphNT1 TAP-DBF2-KanMX4 pGal-CLBΔDB::URA3*	This study
CKY772	*CDC15-7A mCherry-TUB1-TRP1 Δmob1::NatNT2 leu2Δ1::MOB1-2A-hphNT1 TAP-DBF2-KanMX4 pGal-CLBΔDB::URA3*	This study
CKY870	*Δmob1::klTRP1 leu2Δ1::MOB1-hphNT1 kar9Δ::HIS3MX6*	This study
CKY871	*Δmob1::klTRP1 leu2Δ1::MOB1-2A-hphNT1 kar9Δ::HIS3MX6*	This study
CKY872	*CDC15-7A mCherry-TUB1-TRP1 Δmob1::NatNT2 leu2Δ1::MOB1-hphNT1 kar9Δ::HIS3MX6*	This study
CKY873	*CDC15-7A mCherry-TUB1-TRP1 Δmob1::NatNT2 leu2Δ1::MOB1-2A-hphNT1 kar9Δ::HIS3MX6*	This study
CKY935	*CDK1-3xGFP-hphNT1 SPC42-eqFP611-KanMX6 dyn1Δ:: NatNT2*	This study
CKY949	*CDK1-GFP-klTRP1 CDC14-3mCherry-hphNT1 spo12Δ:: NatNT2*	This study
ESM356-1		Pereira et al., 2001
SHM233	*CDK1-3xGFP-kanMX6 pGal1-TEM1-TRP1*	This study
SHM234	*CDK1-3xGFP-kanMX6 pGal1-3HA-CDC5-TRP1*	This study
SHM982	*CDK1-3xGFP-hphNT1 SPC42-eqFP611-KanMX6*	This study
SHM989	*CDK1-3xGFP-hphNT1 SPC42-eqFP611-KanMX6 kar9Δ::klTRP1*	This study
SHM990	*CDK1-3xGFP-hphNT1 SPC42-eqFP611-KanMX6 bfa1Δ::klTRP1*	This study
SHM187	*CDK1-3xGFP-kanMX6*	This study
SHM1757	*CDK1-GFP-klTRP1 mCherry-TUB1-URA3*	This study
SHM1764	*dbf2-2 mCherry-TUB1-URA3 CDK1-GFP-klTRP1*	This study
SHM1765	*mob1-67 mCherry-TUB1-URA3 CDK1-GFP-klTRP1*	This study
SHM1781	*HIS3-pGAL-HA-UBR1 mCherry-TUB1-URA3 CDC15-GFP-klTRP1*	This study
SHM1784	*HIS3-pGal1-HA-UBR1 KanMX6-td-CDC14 mCherry-TUB1-URA3 CDC15-GFP-klTRP1*	This study
SHM1801	*cdc15-1 CDK1-GFP-klTRP1 mCherry-TUB1-URA3*	This study
SHM1802	*mob1-67-GFP-klTRP1 mCherry-TUB1-URA3*	This study
SHM1806	*cdc15-as mCherry-TUB1-URA3 CDK1-GFP-klTRP1*	This study

Table I. **Yeast strains and plasmids** (*Continued*)

Name	Genotype/construction	Source or reference
Yeast strains		
SHM1817	*cdc14-1 CDK1-GFP-klTRP1 mCherry-TUB1-URA3*	This study
SHM1857	*cdc15-as-GFP-klTRP1 mCherry-TUB1-URA3*	This study
SHM1858	*CDC15-GFP-klTRP1 mCherry-TUB1-URA3*	This study
SHM1860	*MOB1-GFP-klTRP1 mCherry-TUB1*	This study
SHM1862	*mCherry-TUB1-URA3 DBF2-GFP-klTRP1*	This study
SHM1871	*dbf2-2-GFP-hphNT1 mCherry-TUB1-URA3*	This study
SHM1910	*CDK1-GFP-klTRP1 mCherry-TUB1-URA3 natNT2-pGal1-CLB2 clb1Δ::hphNT1*	This study
SHM1920	*cdc15Δ::HIS3MX6 CDC15-3HA::LEU2 mCherry-TUB1-klTRP1 CDK1-GFP-hphNT1*	This study
SHM1922	*cdc15Δ::HIS3MX6 CDC15-7A-3HA::LEU2 mCherry-TUB1-klTRP1 CDK1-GFP-hphNT1*	This study
SHM2075	*HIS3-pGal1-HA-UBR1 KanMX6-td-CDC14 mCherry-TUB1-URA3 leu2::CDC15-GFP-hphNT1 cdc15Δ::klTRP1*	This study
SHM2076	*HIS3-pGal1-HA-UBR1 KanMX6-td-CDC14 mCherry-TUB1-URA3 leu2::CDC15-7A-GFP-hphNT1 cdc15Δ::klTRP1*	This study
All strains above carry *MATa ura3-52 his3Δ200 trp1Δ63 leu2Δ1 and are based on ESM356-1.*		
K699	*MATa ade2-1 trp1-1 can1-100 leu2-3, -112 his3-11, -15 ura3.*	K. Nasmyth
CKY531	*MATa ura3-1 trp1-28 leu2Δ0 lys2Δ0 his7 mob1Δ::kanMX4 pep4Δ::LEU2 pESC-424-GST-MOB1/DBF2*	This study
CKY560	*dbf2-2 mob1Δ::klTRP1 leu2Δ1::MOB1-hphNT1*	This study
CKY561	*dbf2-2 mob1Δ::klTRP1 leu2Δ1::MOB1-2A-hphNT1*	This study
CKY562	*dbf2-2 mob1Δ::klTRP1 leu2Δ1::MOB1-7A-hphNT1*	This study
CKY583	*dbf2-2 mob1Δ::klTRP1 leu2Δ1::MOB1-S36A-hphNT1*	This study
CKY584	*dbf2-2 mob1Δ::klTRP1 leu2Δ1::MOB1-T85A-hphNT1*	This study
CKY585	*dbf2-2 dbf20Δ::NatNT2*	This study
CKY586	*dbf2-2 mob1Δ::klTRP1 leu2Δ1::MOB1-hphNT1 dbf20Δ::NatNT2*	This study
CKY587	*dbf2-2 mob1Δ::klTRP1 leu2Δ1::MOB1-2A-hphNT1 dbf20Δ::NatNT2*	This study
CKY588	*dbf2-2 mob1Δ::klTRP1 leu2Δ1::MOB1-7A-hphNT1 dbf20Δ::NatNT2*	This study
CKY589	*dbf2-2 mob1Δ::klTRP1 leu2Δ1::MOB1-S36A-hphNT1 dbf20Δ::NatNT2*	This study
CKY590	*dbf2-2 mob1Δ::klTRP1 leu2Δ1::MOB1-T85A-hphNT1 dbf20Δ::NatNT2*	this study
DOM0073	2 μm-Gal-*CLB2-TAP-URA3*	Übersax et al., 2003
MGY150	*pESC-424-GST-MOB1/DBF2 p28-6xHis-CDC15-HA*	Geymonat et al., 2007
SHM439	*ndc10-1 CDK1-3GFP-kanMX6 SPC42-sRFP-NatNT2*	This study
Strains above carry *MATa ade2-1 trp1-1 can1-100 leu2-3, -112 his3-11, -15 ura3 and are based on K699.*		
Plasmids		
pRS306		Sikorski and Hieter, 1989
pRS305H		Taxis and Knop, 2006
pGEX-5X-1		GE Healthcare
pAK010	pRS304 carrying *mCherry-Tub1*	Khmelinskii et al., 2007
pAK011	pRS306 carrying *mCherry-Tub1*	Khmelinskii et al., 2007
pCK117	pGEX-5X-1-*MOB1*	This study
pCK118	pGEX-5X-1-*MOB1-2A*	This study
pCK116	pGEX-5X-1-*MOB1-7A*	This study
pCK071	*MBP-CDC14*	This study
pCK121	*MBP-CDC14-C283S*	This study
pCK094	pGEX-5X-1-*CDC14-451-551 = GST-C-CDC14*	This study
pCK074	pRS305H-*MOB1*	This study
pCK077	pRS305H-*MOB1-S36A*	This study
pCK078	pRS305H-*MOB1-T85A*	This study
pCK079	pRS305H-*MOB1-2A*	This study
pCK089	pRS305H-*MOB1-7A*	This study
pSM970	pRS306-*CDC14-GFP-HisMX6*	This study
pHM267	pRS305H-*CDC15*	This study
pHM268	pRS305H-*CDC15-7A*	This study

NatNT2 encodes the *Streptomyces noursei nat1* gene. *klTRP1* encodes the *Kluyveromyces lactis TRP1* gene. hphNT1 encodes the *Escherichia coli hph* gene.

Fig. S4: genetic interaction of *CDC15-7A MOB1-2A* with *kar9Δ* and localization of Mob1 in *cdc15-as1* cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200911128/DC1.

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