Mitotic Exit in the Absence of Separase Activity

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Submitted October 17, 2008; Revised December 15, 2008; Accepted January 5, 2009
Monitoring Editor: Daniel J. Lew

In budding yeast, three interdigitated pathways regulate mitotic exit (ME): mitotic cyclin–cyclin-dependent kinase (Cdk) inactivation; the Cdc14 early anaphase release (FEAR) network, including a nonproteolytic function of separase (Esp1); and the mitotic exit network (MEN) driven by interaction between the spindle pole body and the bud cortex. Here, we evaluate the contributions of these pathways to ME kinetics. Reducing Cdk activity is critical for ME, and the MEN contributes strongly to ME efficiency. Esp1 contributes to ME kinetics mainly through cohesin cleavage: the Esp1 requirement can be largely bypassed if cells are provided Esp1-independent means of separating sister chromatids. In the absence of Esp1 activity, we observed only a minor ME delay consistent with a FEAR defect. Esp1 overexpression drives ME in Cdc20-depleted cells arrested in metaphase. We have found that this activity of overexpressed Esp1 depended on spindle integrity and the MEN. We defined the first quantitative measure for Cdc14 release based on colocalization with the Net1 nucleolar anchor. This measure indicates efficient Cdc14 release upon MEN activation; release driven by Esp1 in the absence of microtubules was inefficient and incapable of driving ME. We also found a novel role for the MEN: activating Cdc14 nuclear export, even in the absence of Net1.

INTRODUCTION

Initiation of mitosis requires a combination of B-type cyclin-dependent kinase (Cdk) activity promoting various mitotic events, including spindle morphogenesis and function (Fitch *et al.*, 1992; Rahal and Amon, 2008). The anaphase-promoting complex (APC) with the Cdc20 coactivator removes the anaphase inhibitor Pds1 at the metaphase-to-anaphase transition to allow progression to telophase. A further set of events, collectively referred to as mitotic exit (ME), must occur to reset the cell cycle to G1. ME encompasses spindle disassembly, cyclin/CDK inactivation, cytokinesis, and relicensing of replication origins. Collectively, these events convert a late mitotic cell into two progeny cells that are prepared to reenter the replicative cycle (DNA replication, spindle morphogenesis, and function, and budding to produce the next daughter cell body in the case of budding yeast).

In budding yeast *Saccharomyces cerevisiae*, the phosphatase Cdc14 is required for ME. Through most of the cell cycle, Cdc14 is kept sequestered and inactive in the nucleolus due to binding to a nucleolar protein Net1 (Shou *et al.*, 1999; Visintin *et al.*, 1999), and it is released during mitosis to fulfill various cellular functions. The release is biphasic, with a first wave of Cdc14 release happening in early anaphase, controlled by the Cdc fourteen early anaphase release (FEAR) network, which includes separase (Esp1), Spo12, Slk19, and Cdc5 (Stegmeier *et al.*, 2002). FEAR network activity is thought to be initiated by APC-Cdc20–induced degradation of Pds1, which is a chaperone and inhibitor of the separase

Esp1. After Pds1 proteolysis, free Esp1 cleaves cohesins, allowing sister chromatid separation and anaphase (Uhlmann *et al.*, 2000). Independent of cohesin cleavage and Esp1 proteolytic activity, free Esp1 can promote FEAR network-induced Cdc14 release. FEAR-released Cdc14 modulates nuclear movement, rDNA segregation and spindle stability; these functions may facilitate mitotic progression before ME (Azzam *et al.*, 2004; D'Amours *et al.*, 2004; Ross and Cohen-Fix, 2004; Sullivan *et al.*, 2004; Higuchi and Uhlmann, 2005).

Early anaphase release of Cdc14 by the FEAR network may promote mitotic exit network (MEN) activation through Cdc15 dephosphorylation and activation (Jaspersen and Morgan, 2000; Queralt *et al.*, 2006). MEN activity can promote additional Cdc14 release, thus forming a potential positive feedback loop, which could eventually release enough Cdc14 to drive ME. Esp1 overexpression was shown to be able to drive mitotic cyclin degradation independently of anaphase initiation (Tinker-Kulberg and Morgan, 1999).

The nonproteolytic function of Esp1 has been proposed to be essential for ME (Queralt *et al.*, 2006), whereas removal of other tested FEAR network components causes only a 15- to 20-min delay of ME (Stegmeier *et al.*, 2002). This suggests that Esp1 plays an additional nonproteolytic role in ME beyond its FEAR network functions.

In an alternative (not necessarily mutually exclusive) model, Cdc14 release leads to ME in response to MEN activation that is triggered by migration of a spindle pole body (SPB) into the bud activates the MEN (Yeh *et al.*, 1995; Bardin *et al.*, 2000). This mechanism is termed the spindle-positioning checkpoint. The MEN is considered to be responsible for continued Cdc14 release in late anaphase and telophase. Cdc14-driven Cdc5 degradation by Cdh1 and Bub2–Bfa1 reactivation may contribute to MEN inactivation (Pereira *et al.*, 2002; Visintin *et al.*, 2008).

In addition to Cdc14 release by the FEAR/MEN pathways, it is clear that degradation of mitotic cyclins is essential for ME, because high mitotic Cdk activity blocks ME (Wäsch and Cross, 2002; Thornton and Toczyski, 2003). Mitotic cyclin degradation is initiated by APC-Cdc20 at the

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-10-1042>) on January 14, 2009.

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Abbreviations used: Cdk, cyclin-dependent kinase; FEAR, Cdc fourteen early anaphase release; ME, mitotic exit; MEN, mitotic exit network.

same time that the securin Pds1 is degraded (Yeong *et al.*, 2000), and complete cyclin degradation is carried out later by APC-Cdh1 (Schwab *et al.*, 1997; Visintin *et al.*, 1997). Cdh1 is likely activated by MEN-released Cdc14 (Jaspersen *et al.*, 1998; Visintin *et al.*, 1998), and Cdc14 also promotes ME via accumulation of the Sic1 Cdk inhibitor (Visintin *et al.*, 1998).

The relationship between Cdk inactivation, Esp1 function, and MEN activation is not clear. Cdk activity was proposed to promote the FEAR pathway by phosphorylating Net1 (Azzam *et al.*, 2004). Recently, it was proposed that Cdk activity delays anaphase initiation (and presumably the FEAR network) by phosphorylating Pds1, delaying its degradation; this delay was proposed to cause simultaneous cleavage of all cohesin via a mechanism in which Cdc14 released by the FEAR network dephosphorylated Pds1, leading to more Esp1 activation and a positive feedback loop (Holt *et al.*, 2008). Cdc15 may be phosphorylated by cyclin (Clb)-Cdk, potentially leading to inhibition of the MEN (Jaspersen and Morgan, 2000); despite this, high Clb-Cdk does not block Cdc14 release from the nucleolus (Stegmeier *et al.*, 2002). Overall, the interaction of Cdk activity with the MEN has only been partially characterized (Menssen *et al.*, 2001; Stegmeier *et al.*, 2002).

Thus, Cdk inactivation, cohesin cleavage, Esp1's nonproteolytic function, and the spindle-positioning checkpoint-dependent activation of the MEN all have the potential to regulate ME, but the interregulation of these pathways is not well characterized. In this article, we aim to clarify which of these pathways are most critical for timing and efficiency of ME in the wild-type (WT) cell cycle. Therefore, we attempted to independently control these pathways to achieve a balanced view of the relative contributions of major regulators in ME under approximately physiological conditions. These experiments have led us to a view of ME emphasizing the criticality of lowering Clb-Cdk activity; activation of the MEN is a largely independent mechanism that is very important for driving normal ME kinetics. Our results suggest that the primary (though not the sole) contribution of Esp1 to the kinetics of ME in the wild-type cell cycle is to promote sister chromatid separation by proteolytic cohesin cleavage, leading to spindle elongation-dependent MEN activation.

MATERIALS AND METHODS

Strains and Plasmids

All strains are in the W303 background (Supplemental Table 1). Strain constructions were carried out by standard tetrad analysis and transformation methods. In many experiments, we used strains containing a *CDC14* allele endogenously tagged with yellow fluorescent protein (YFP), to follow Cdc14 trafficking. This *CDC14-YFP* was shown previously to fully complement and to be competent for FEAR- and MEN-induced nucleolar exit (Pereira *et al.*, 2002). We also have confirmed key results in isogenic strains with untagged *CDC14* (Supplemental Figure 4A). A complete strain list is in Supplemental Material.

The pYL8 plasmid (*pRS303-GALS-esp1_{frag}*, *esp1_{frag}*, created by truncating a 2.6-kb region from the *ESP1* open reading frame [ORF] by using SphI) was linearized with BlnI and integrated into the genome to make *esp1::GALS-ESP1*. *ESP1::GALS-ESP1* was made similarly, but using plasmid pYL10, which had a full-length *ESP1* ORF under the *GALS* promoter. Correct number of integration was confirmed by real-time polymerase chain reaction (PCR). The construction of *PDS1-mdl esp1::GALS-EPS* (or *ESP1::GALS-ESP1*) strains was as follows: A *pds1::LEU2* strain was first transformed with pYL7 (*pRS406-PDS1-mdl*, linearized with MluI), and then the *URA3* marker in the resulting strain was counterselected on G+FOA plates to obtain *Leu⁻ Ura⁻* clones. The structure of the pop-out strain was confirmed by PCR and Southern blot.

Time Course Experiments

For α factor block, 100 nM α factor was used. For hydroxyurea (HU) block, 0.16 M HU was used. In both cases, cells were washed three times by centrifugation and resuspension in fresh media for release. Arresting the

MET3-CDC20 cell in metaphase was done by addition of methionine to culture medium, as described previously (Uhlmann *et al.*, 2000), and release was done by centrifugation and resuspension in fresh medium lacking methionine. Nocodazole (15 μ g/ml) and 10 μ g/ml benomyl were added to cultures for spindle depolymerization (note that in figures, this is referred to as NOC; we found that adding benomyl as well was important for obtaining a stable block). Protein extraction, immunoblotting, and Clb2-associated histone H1 kinase assay were performed as described previously (Wäsch and Cross, 2002). DNA flow cytometry was performed as described previously (Epstein and Cross, 1992). Budding was assessed by microscopic observation. Nuclear content was assessed by examining samples with nuclei stained with propidium iodide by fluorescence microscopy. Growth curve including cell density and mean cell volume was obtained using a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA). The final carbon source concentration was 2% glucose, 3% galactose, and 3% raffinose. The *MET-CDC20* block experiment in Figure 1 was performed in YEP medium; otherwise, in SC medium. The hydroxyurea and α factor block and release experiment in Figure 3 were performed in SC medium. The α factor block and release experiment in Figure 6 was performed in YEP medium. The time course experiment in Figure 2 was performed in YEP medium. When making a time-lapse movie, only SC medium was used.

Time-Lapse and Fluorescence Microscopy

For time-lapse microscopy, cells were prepared as described in Bean *et al.*, (2006). We used a DMIRE2 inverted fluorescence microscope (Leica Microsystems, Deerfield, IL) equipped with an environmental chamber and objective heater to observe the growth of the yeast cells at various temperatures. Images were acquired every 3 min with an Orca-ER camera (Hamamatsu, Bridgewater, NJ). We used custom Visual Basic software integrated with Image-Pro 5.0 (Media Cybernetics, Bethesda, MD) for microscope control and image acquisition. For still-image fluorescent microscopy, cells were fixed with 4% paraformaldehyde for 10 min before microscopy. For 4,6-diamidino-2-phenylindole (DAPI) staining, cells were treated briefly with 30% ethanol after the paraformaldehyde fixation step, and then they were stained with 100 μ g/ml DAPI. For imaging these cells, we used an Axioplan2 fluorescent microscope (Carl Zeiss, Jena, Germany) with a Hamamatsu C4742-95 camera. OpenLab 5.0 (Improvision, Lexington, MA) was used for microscope control and image acquisition. Three Z-stacks at 0.6- μ m intervals were taken for each fluorescent channel and projected onto a single image per channel.

Image Analysis

Time-lapse movie segmentation and analysis was done using custom software as described in Charvin *et al.* (2008). For quantitative analysis of fluorescent microscopy, we designed custom software in the Matlab environment. The Z-stack with the highest signal SD chosen for *r* value determination; *r* is calculated by taking the average Cdc14-YFP intensity of the brightest 5% pixels within a cell area, and subtracting the average of the dimmest 5% pixels; this value was then divided by a similar value for the Net1-cyan fluorescent protein (CFP) signal to yield *r*. In all experiments involving *r* value measurement, at least 50 cells were used to generate the distribution for each category at each time point.

Because Cdc14-YFP also localizes to the SPB and bud-neck (Yoshida *et al.*, 2002; Bembek *et al.*, 2005), which could be misinterpreted as a nucleolar signal in *r* value calculation, we exclude the bud-neck region in segmentation, and exclude Cdc14-YFP signal at SPB by removing the YFP pixels whose underlying CFP signal falls out of the top 5% brightest CFP intensity. Thus, this statistic effectively examines the degree of concentration of Cdc14 in the region where Net1 is localized, without regard to concentration of Cdc14 in other locations. Empirically, we found that the correction of removing high YFP pixels that did not coincide with high CFP pixels had essentially no effect on the results of any experiments (data not shown).

RESULTS

Efficient Bypass of the Cdc20 Requirement for Mitotic Exit by Simultaneous Ectopic Cohesin Cleavage and Cdk Inactivation

Cdc20 is an essential activator of the APC, and in its absence cells arrest with high Cdk activity due to mitotic Clb stabilization (Yeong *et al.*, 2000), and with unseparated sister chromatids due to Pds1 stabilization; Pds1 inhibits Esp1 and blocks cohesin cleavage and subsequent anaphase (Shirayama *et al.*, 1999). Remarkably, simultaneous deletion of Pds1 and the B-type cyclin Clb5 bypasses the Cdc20 requirement (Shirayama *et al.*, 1999), suggesting that the major roles of Cdc20 in mitosis are release of Esp1 and down-regulation of B-type cyclins. We addressed this issue by a different experimental protocol, based on an assay

(BD96b-4C). + +, + -, - +, or - - indicate the presence or absence of *GAL1-TEV/SCC1-TEV* and *GAL1-SIC1-4A*. The fraction of large budded cells (excluding rebudded and small budded cells) was calculated from >200 cells at each time point. DNA flow cytometry profiles from the beginning and end of the time course for each sample are shown (complete DNA flow cytometry data in Supplemental Figure 1), as well as sketches of the cell morphologies at the end of the experiment. Note that the elongated buds are a consequence of rebudding (with or without prior cytokinesis) in the presence of high Sic1 levels (Lew and Reed, 1993) and thus allow unambiguous discrimination between the large round buds found at the beginning of the experiment and the new buds formed after complete or partial mitotic exit.

developed by Sullivan and Uhlmann (2003). Cells were depleted of Cdc20 and arrested in metaphase, by using a methionine-suppressible *MET3-CDC20* construct (Sullivan and Uhlmann, 2003). To compensate for the absence of Esp1 activity due to accumulated Pds1, we used the *GAL1-TEV/SCC1-TEV* system, in which inducible Tobacco etch virus (TEV) protease can cleave an Scc1 subunit with an engineered TEV site (Uhlmann *et al.*, 2000). To allow Cdc20-independent down-regulation of Clb-Cdk activity, we used *GAL1-SIC1-4A*, which allows conditional overexpression of a stable version of the Sic1 inhibitor, thus inactivating Clb-Cdk activity without a requirement for Cdc20-driven Clb proteolysis (Verma *et al.*, 1997). These manipulations are indicated in red on the control diagram in Figure 1A. We then examined whether these *cdc20*-blocked cells can carry out ME, as a function of ectopic regulation of anaphase by using *GAL1-TEV/SCC1-TEV* (Uhlmann *et al.*, 2000) and *GAL1-SIC1-4A* (Verma *et al.*, 1997). To monitor ME, we used phase contrast microscopy to detect cytokinesis and rebudding in the next cell cycle. We also used DNA flow cytometry to analyze replication and effective chromosome segregation to daughter cells. Simultaneously providing ectopic sources of cohesin cleavage and Clb-Cdk inactivation allowed quantitative and rapid ME, by the assays of cytokinesis and rebudding in the next cell cycle (Figure 1B), resulting in the efficient accumulation of 1C budded cells. DNA replication in the next cell cycle did not occur, presumably because of stable Sic1 accumulation (Schwob *et al.*, 1994; Verma *et al.*, 1997). Of the two factors driving ME in this remarkably effective synthetically driven ME, Cdk inactivation is essential, whereas TEV-mediated cohesin cleavage and consequent spindle elongation is important for efficiency but not absolutely required (Figure 1B). The *cdc20* bypass is quantitative: nearly all the cells progress synchronously through ME based on these assays. We consider this "synthetic" ME to be kinetically efficient because its timing compares very closely to the timing obtained by release of the Cdc20 depletion block by removal of methionine (Figure 1B, control; note that this strain also contains *GAL1-SIC1-4A*, resulting in cells accumulating after mitosis blocked with elongated buds and 1C DNA; Verma *et al.*, 1997).

Figure 1. Combinatorial control of mitotic exit by Cdk inactivation and cohesin cleavage, in the absence of Cdc20. (A). Major components and interactions in ME system. Arrows, induction; bars, inhibition. Components indicated in red are exterior control points used here to manipulate the system. (B) Cultures of *MET3-CDC20* strains were first arrested in metaphase at 23°C by incubation in raffinose + methionine medium to deplete Cdc20, and then galactose was added to the cultures to induce the expression of *GAL1-TEV* and *GAL1-SIC1-4A* where present. Methionine was kept in the medium throughout to maintain *cdc20* depletion, except for the experiment labeled control, which was released into galactose medium lacking methionine to reinduce Cdc20. Strain genotypes: 1, control, *MET3-CDC20 GAL1-SIC1-4A* (YL1721); 2, + +, *MET3-CDC20 scc1Δ SCC1-TEV GAL1-TEV GAL1-SIC1-4A* (YL353); 3, + -, *MET3-CDC20 scc1Δ SCC1-TEV GAL1-TEV* (353); 4, - +, *MET3-CDC20 GAL1-SIC1-4A* (YL1721); and 5, - -, *MET3-CDC20*

This experiment resembles one published by Sullivan and Uhlmann (2003), except that in that work, *clb5* deletion was used for Clb-Cdk down-regulation, which is very likely to provide only partial decrease in Clb-Cdk activity (as evidenced by viability of *clb5* cells), whereas in contrast, *GAL1-SIC1-4A* quantitatively eliminates all Clb-Cdk activity (Supplemental Figure 1B), and results in complete inviability (Verma *et al.*, 1997). Shirayama *et al.* (1999) showed that Clb down-regulation by *clb5* deletion, combined with liberation of Esp1 by *pds1* deletion, would bypass the Cdc20 requirement for mitotic exit. Our results differ from theirs in one critical feature: liberated active Esp1 can in principle provide both proteolytic and nonproteolytic Esp1 activities to drive ME. Because in our experiment, Esp1 inhibition by endogenous stable Pds1 will continue, we only complement the proteolytic function of Esp1 by using the *GAL1-TEV/SCC1-TEV* system. ME in the *GAL1-SIC1-4A GAL1-TEV/SCC1-TEV cdc20*-blocked cells was only slightly slower than ME in cells released from the *cdc20* block (Figure 1B, + + vs. blue control curve). We assume that persistent Pds1 due to the *cdc20* block effectively inhibited Esp1, because anaphase was completely inhibited without *GAL1-TEV* expression. We confirmed that Pds1 was not degraded despite expression of *GAL-SIC1-4A* (in fact, its level increased [Supplemental Figure 1C], perhaps due to increased transcription after mitosis [Spellman *et al.*, 1998]).

Therefore, these results suggest that Esp1 does not play a major role in setting the kinetics of ME beyond its function in sister chromatid separation. The minor delay of ME in the *GAL1-SIC1-4A GAL1-TEV/SCC1-TEV cdc20*-blocked cells compared with release by Cdc20 reactivation is consistent with the lack of FEAR network activity due to lack of Esp1 activity (Stegmeier *et al.*, 2002).

Endogenous Undegradable Pds1 Blocks Sister Chromatid Separation

We wanted to confirm that endogenous levels of Pds1 could effectively block Esp1 activity in the absence of Cdc20-dependent Pds1 degradation, because this was an important assumption in the experiments in Figure 1. Pds1 degradation by Cdc20-APC is dependent on the Pds1 "destruction

box" (Cohen-Fix *et al.*, 1996). Inviability of cells expressing endogenous levels of undegradable Pds1 was suggested by failure to recover transformants of *PDS1*-mutated destruction box (*mdb*) under control of the *PDS1* promoter in low-copy number plasmids (Cohen-Fix *et al.*, 1996), but the reason for the failure to recover these transformants was not elucidated. We replaced the endogenous *PDS1* coding sequence in the endogenous locus with the *PDS1-mdb* coding sequence, by using exact gene replacement. The potential lethality of this allele was overcome by mildly overexpressing Esp1 under a truncated *GAL1* promoter (Mumberg *et al.*, 1994), *GALS-ESP1*. This strain is fully viable on galactose media but inviable on glucose (Figure 2A), confirming that endogenous levels of undegraded Pds1 were lethal, specifically because of Esp1 sequestration. Pds1-*mdb* was stable through the cell cycle at endogenous levels, whereas Pds1-wt was degraded before anaphase as expected (Figure 2B). Because Esp1 is a stable protein, transcriptional repression of *GALS-ESP1* by glucose in a *PDS1-mdb* background allows two or more near-normal cell cycles. Subsequently, we observe a gradual increase of large budded cells with 2C DNA content (Supplemental Figure 2) and unseparated chromosome dots (Bachant *et al.*, 2005) (Figure 2C). These cells were highly delayed in mitotic exit, although ultimately most cells underwent aberrant mitosis with generation of aneuploid or aploid cells (Supplemental Figure 2). From these results, we conclude that the endogenous level of Pds1 can effectively inhibit Esp1 and block sister chromatid separation, provided its Cdc20-mediated degradation is blocked, even in a background where all other Cdc20-mediated functions are presumably intact. This result confirms and extends previous findings that the Cdc20 requirement for sister chromatid separation can be bypassed by *PDS1* deletion (Yamamoto *et al.*, 1996; Lim *et al.*, 1998; Shirayama *et al.*, 1999).

Inhibition of Esp1 by Overexpression of Undegradable Pds1 Blocks ME via Blockage of Cohesin Cleavage

Overexpression of undegradable Pds1 causes a complete block to anaphase and a many-hour delay in ME (Cohen-Fix *et al.*, 1996; Sullivan and Uhlmann, 2003; Queralt *et al.*, 2006). Similarly, in our experiments in Figure 1, Cdc20 depletion (leading to Pds1 accumulation and Esp1 inhibition), led to a significant delay in ME even when Clb–Cdk inhibition was provided ectopically by *GAL1-SIC1-4A*. However, this delay was efficiently rescued by ectopic cohesin cleavage by using *GAL1-TEV* (Figure 1B), suggesting that most of the delay was due to failure of cohesin cleavage. We were concerned, however, that we had not achieved complete Esp1 inhibition by using endogenous levels of accumulated Pds1. To ensure that we achieved full inactivation of endogenous Esp1, we overexpressed Pds1-*mdb* from the *GAL1* promoter. We expressed *GAL1-PDS1-mdb* expression for an hour to accumulate abundant Pds1-*mdb* in *cdc20*-blocked cells, and then we released the *cdc20* block by methionine removal. In this protocol, Clb–Cdk inactivation is expected to proceed via the Cdc20-APC system, whereas Esp1 is sequestered by Pds1-*mdb* overexpression. We provided an ectopic source of cohesin cleavage by using the *GAL1-TEV/SCC1-TEV* system. As a control, we carried out the same protocol substituting *GAL1-PDS1(wt)*, expressing the degradable form of Pds1, for *GAL1-PDS1-mdb*. Expression of Pds1-*mdb* caused no delay in cytokinesis or Clb2 degradation compared with expression of Pds1-wt, despite persistence of Pds1-*mdb* but not Pds1-wt, suggesting that Pds1 is not an effective ME inhibitor provided the need for the proteolytic activity of Esp1 is bypassed (Figure 3C). Although this experiment

Figure 2. Endogenous undegradable Pds1 blocks sister chromatid separation. (A) *PDS1-mdb ESP1::GALS-ESP1* (YL018) or a wild-type control were plated by $10\times$ serial dilution on either glucose (D) or galactose (G) plates at 30°C to assess viability. (B) Cultures (*PDS1-mdb-myc ESP1::GALS-ESP1* or control *PDS1-wt-myc*) synchronized by α -factor block release were assayed by Western blotting with anti-Myc antibody. Pgk1 Western blot was used as a loading control. (C) Endogenous Pds1 is sufficient to block sister chromatid separation. Glucose was added to galactose-grown mid-log cultures of *PDS1-mdb trp1::LacO LacI-GFP*, containing either *esp1::GALS-ESP1* (YL115, filled bar) or *ESP1::GALS-ESP1* (YL113, hatched bar). Samples were fixed with paraformaldehyde and examined by fluorescence microscopy to score separation of the GFP-labeled chromosome "dots." Fraction of large budded cells with unseparated GFP dots (red), with well-separated GFP dots (green) or closely separated GFP dots (blue) are shown. Cell morphologies at the beginning and end of the experiment in the various conditions are diagrammed in the cartoons.

provides a direct comparison between Pds1-wt and Pds1-*mdb* at equally overexpressed levels, overexpressed Pds1-wt could delay ME compared with endogenous levels. However, in the remaining experiments we compared overexpressed Pds1-*mdb* to endogenously expressed Pds1-wt.

We confirmed and extended this result by using S phase block with hydroxyurea, with the four combinations of pres-

Figure 3. Endogenous Esp1 is not necessary for efficient mitotic exit. (A) Artificial cleavage of Scc1 with TEV protease ensures efficient ME in the absence of active Esp1. *mad2Δ* strains were arrested in S phase at 30°C with 0.16 M hydroxyurea and released into galactose with α factor. Pds1-mdb and TEV protease were overexpressed from the *GAL1* promoter to inactivate Esp1 and to cleave Scc1-TEV (YL057). Strains lacking *GAL1-TEV* (YL045) *GAL1-PDS1-mdb* (YL049), or both (2151-7B) were used as controls. (B) Inactivation of cohesin Scc1 restores the efficiency of ME in the absence of active Esp1. *mad2Δ* *GAL1-PDS1-mdb* strains, either *scc1-73* (YL044) or *SCC1* (YL045) were arrested by α factor at 25°C, then released into the absence of α factor either in galactose media to induce undegradable Pds1-mdb or raffinose as a control. Cultures were released at 37°C to inactivate cohesin (*scc1-73*). The α factor was reintroduced 1.5 h after release to cause accumulation of cells in G1 after ME. DNA flow cytometry was used to assess cell cycle progression. (C) Pds1-mdb does not independently block ME in cells provided with ectopic chromosome separation by TEV protease. *GAL1-TEV/SCC1-TEV* *MET3-CDC20* strains, containing *GAL1-PDS1-wt* (YL042) or *GAL1-PDS1-mdb* (YL043), were arrested at the *cdc20* block by incubation in raffinose plus methionine medium and then pulsed with galactose plus methionine for 1 h. The cultures were then transferred into glucose without methionine to release the *cdc20* block. DNA content was measured by DNA flow cytometry and Clb2 protein level by Western blot. Even loading was shown by amido-black staining (data not shown).

ence or absence of *GAL1-PDS1-mdb* and *GAL1-TEV/SCC1-TEV*, all in the presence of wild-type endogenous *PDS1*. Because premature cohesin cleavage can activate the Mad2-dependent spindle checkpoint (Severin *et al.*, 2001), we carried out these experiments in a *mad2Δ* background. In this protocol, we allow the endogenous Cdc20-dependent Clb degradation system to eliminate Clb-Cdk activity and endogenous Pds1. As reported previously (Cohen-Fix *et al.*, 1996), Pds1-mdb overexpression blocks mitotic progression, but when chromosome separation is allowed by using *GAL1-TEV/SCC1-TEV*, we observed efficient ME, which occurred almost as rapidly as in the *GAL1-TEV/SCC1-TEV* strain lacking *GAL1-PDS1-mdb*, or the wild-type control (Figure 3A). The *GAL1-PDS1-mdb* *GAL1-TEV/SCC1-TEV* strain exhibited a delay of ~20 min judging from DNA flow cytometry and bud-count. This delay was consistent with

the ME delay in FEAR network mutants (Stegmeier *et al.*, 2002), but much shorter than the >3-h delay caused by *GAL1-PDS1-mdb* overexpression (Cohen-Fix *et al.*, 1996) (Figure 3A). Therefore, most of the long delay caused by Esp1 inhibition by Pds1-mdb overexpression is due to failure of cohesin cleavage.

The temperature-sensitive *scc1-73* cohesin allele (Michaelis *et al.*, 1997) allows sister chromatid separation without Esp1 activity at restrictive temperature (Uhlmann *et al.*, 1999). We constructed *mad2Δ* strains that were *scc1-73* or *SCC1*, with or without *GAL1-PDS1-mdb*, synchronized cells in G1 with α factor, and we expressed *GAL1-PDS1-mdb* for 1 h before release at 37°C (restrictive temperature for *scc1-73*). *SCC1* *GAL1-PDS1-mdb* cells show a lengthy delay before ultimately undergoing aberrant ME with accumulation of aneuploid cells. (Eventual accumulation of aneuploid cells is

a consequence of *GAL1-PDS1-mdb* expression at 37°C, where the G2 block is less stable than at 30°C. This may be due to lower expression of *GAL1-PDS1-mdb* at 37°C (Figure 3B). In contrast, *scc1-73 GAL1-PDS1-mdb* cells had indistinguishable ME kinetics compared with *scc1-73* cells lacking *GAL1-PDS1-mdb*, and similar ME kinetics compared with *SCC1* cells lacking *GAL1-PDS1-mdb*. This result confirms that cohesin inactivation can bypass the *GAL1-PDS1-mdb* block to ME. Overall, three independent experiments (Figure 3, A–C) show that the Esp1 requirement for ME can be largely bypassed by complementing its proteolytic function in *Scc1* inactivation. This idea is also suggested by the similar kinetics of ME in *cdc20*-blocked cells with *GAL1-SIC1-4A* and *GAL1-TEV* overexpression to the kinetics of ME upon direct release of the *cdc20* block (Figure 1B).

Stegmeier *et al.* (2002) carried out a similar experiment to the one in Figure 3B. Instead of inhibiting Esp1 with *GAL1-PDS1-mdb*, they used the *esp1-1* temperature-sensitive allele, and instead of *scc1-73*, they used the *mcd1-1* temperature-sensitive allele (*MCD1* is the standard name for the cohesin subunit also named *SCC1*). As in our experiment (Figure 3A), these strains (with the spindle checkpoint disabled by *MAD1* deletion) were released from an α -factor block at nonpermissive temperature. They observed a significant reduction in ME delay by inclusion of *mcd1-1* in the *esp1-1* background, which is qualitatively similar to our findings. Distinct from our findings, they observed that the *esp1-1 mcd1-1* strain exhibited an ~45-min delay in ME based on timing of mitotic cyclin (Clb2) degradation compared with the *ESP1 mcd1-1* strain, whereas we observed little delay in ME based on direct examination of cytokinesis comparing the *GAL1-PDS1-mdb* strains that were *SCC1-ut* or *scc1-73*. We do not know whether the differences in results between our experiment and the results in Stegmeier *et al.* (2002) are due to differences between thermal inactivation of *mcd1-1* versus *scc1-73*, to the use of *esp1-1* versus *GAL1-PDS1-mdb* to inhibit Esp1 activity, or to the difference in assay for ME. Many previous experiments support the efficacy of *GAL1-PDS1-mdb* in full inhibition of Esp1, both for its proteolytic and nonproteolytic functions (Cohen-Fix *et al.*, 1996; Sullivan and Uhlmann, 2003; Queralt *et al.*, 2006), and the results in Figure 3B are consistent with the results in Figure 3A using *GAL1-TEV* rather than *scc1/mcd1* mutations to inactivate cohesin. In a recently published similar experiment (Visintin *et al.*, 2008), a *mad1Δ GAL-PDS1Δdb mcd1-1* strain exhibited a ME delay of 30 min or less. This result is qualitatively consistent with our observations in Figure 3, A and B.

Thus, we conclude that liberation of Esp1 from Pds1 inhibition is not required for ME in multiple experimental conditions, provided the requirement for cohesin cleavage is bypassed, although inhibition of Esp1 may cause a <30-min ME delay due to failure of FEAR network activation. We propose that provided sufficient Clb–Cdk inactivation, the timing of ME is largely regulated by the spindle-positioning checkpoint-regulating MEN activation, as proposed by Bardin *et al.* (2000). Cohesin cleavage may be a requirement for efficient activation of the MEN by this mechanism, because anaphase spindle elongation requires cohesin cleavage, and anaphase efficiently drives one SPB into the bud.

Consistent with the hypothesis that allowing cohesin cleavage bypasses the Pds1–*mdb* block by allowing spindle elongation and consequent MEN activation, the *GAL1-PDS1-mdb* block to cytokinesis can be effectively bypassed by ectopic activation of the MEN by deletion of the MEN inhibitor *BUB2*, in the absence of chromosome separation (Queralt *et al.*, 2006); we have confirmed this result using HU block-release instead of *cdc20* block-release (Supplemental

Figure 3). We explore the connection between cohesin cleavage, spindle elongation, and MEN activation further in the following sections.

Direct Block of *Scc1* Cleavage Delays ME in Cells with Active Esp1

The results mentioned above indicate that Esp1 contributes little to ME kinetics beyond its role in cohesin cleavage, leading to the conclusion that beyond the requirement for cohesin cleavage, Esp1 is not necessary for efficient ME (although as noted, its inhibition does lead to a delay of <30 min in ME). A converse question, so far not addressed in our experiments, concerns the ability of Esp1 to drive ME in cells in which cohesin cleavage and sister chromatid separation fails—is Esp1 sufficient to drive ME without cohesin cleavage? This question has been examined previously with the use of the noncleavable version of *Scc1* expressed from the *GAL1* promoter (*GAL1-SCC1-RRDD*) (Uhlmann *et al.*, 1999). In these experiments, blocking sister chromatid separation does not block Esp1 activation, because endogenous *Scc1* is cleaved on schedule even in the presence of ectopic *Scc1-RRDD* (Uhlmann *et al.*, 1999). Blocking sister separation with *Scc1-RRDD* in the presence of active Esp1 causes a delay in ME estimated between 20 and 60 min, depending on the assays for ME and/or on the exact experimental conditions (Uhlmann *et al.*, 2000; Stegmeier *et al.*, 2002). We examined this question using a different assay, by time-lapse microscopy of *GAL1-SCC1-RRDD* cells pregrown in raffinose (uninduced) and plated on galactose medium to induce *GAL1-SCC1-RRDD* expression. We observed a variable delay averaging ~1 h between the first bud emergence (unaffected by *SCC1-RRDD* expression) and the second bud emergence, which requires prior ME, due to *SCC1-RRDD* expression (Table 1). We included a Myo1–green fluorescent protein (GFP) marker (Bi *et al.*, 1998) to allow measurement of the time between budding and cytokinesis (determined by Myo1 ring disappearance), and observed a delay of ~0.5 h due to *SCC1-RRDD* expression. The difference in delay times between these two assays is interesting and suggests that even in cells that complete cytokinesis, defects due to failure of cohesin cleavage cause an additional ~0.5 h delay in rebudding. Thus, our results generally confirm the previous findings of a significant delay in ME solely due to failure of cohesin cleavage. An advantage of our assay is that it requires no previous synchronization of the cells, which avoids potential artifacts, and in addition allows determination of the variability among individual cells, which can give misleading results in population studies. Furthermore, the method allows us to observe events preceding ME.

Using cells labeled with GFP-tubulin and blocked in metaphase by *Scc1-RRDD*, we observed rapid spindle oscillations (Supplemental Video 3), which pushed one SPB back and forth between daughter and mother cells (Palmer *et al.*, 1989). This oscillatory movement could potentially activate the MEN by allowing one SPB to contact *Lte1* near the bud cortex (Bardin *et al.*, 2000). This makes a complete assessment of Esp1's contribution to ME in this experimental context difficult, because the spindle oscillations might activate the MEN without any Esp1 activity. This difficulty was circumvented in the next section by using nocodazole to depolymerize the spindle.

Regardless, based on our and others' results with *GAL1-SCC1-RRDD*, it is clear that blocking cohesin cleavage while allowing Esp1 activity causes a substantial delay in ME. In turn, this suggests that the kinetics of ME in the wild-type cell cycle are driven by cohesin cleavage, because the time from cohesin cleavage to ME in wild-type cells is only

Table 1. Mitotic exit delay caused by noncleavable cohesin

	Myo1-GFP appearance to disappearance (min)	p value relative to WT	Bud to rebud (min)	p value relative to WT	n
30°C					
WT	75 ± 14		110 ± 29		18
SCC1-RRDD					
Mo.	102 ± 20 (Δ 27 min)	5 × 10 ⁻³	169 ± 60 (Δ 59 min)	1 × 10 ⁻²	11
Da.	112 ± 21 (Δ 37 min)	1 × 10 ⁻⁴	228 ± 59 (Δ 118 min)	1 × 10 ⁻⁵	19
37°C					
WT	N.D.		102 ± 8		12
SCC1-RRDD					
Mo.	N.D.		153 ± 28 (Δ 51 min)	1 × 10 ⁻⁴	9
Da.	N.D.		189 ± 99 (Δ 87 min)	1 × 10 ⁻²	12

Strain *GAL-SCC1-RRDD TUB1-GFP MYO1-GFP* (YL066) was pregrown in raffinose medium, plated on galactose medium, and subjected to time-lapse analysis at 30 or 37°C as described in *Materials and Methods*. Cells that were unbudded at the time of plating were timed for both the interval from first budding to first cytokinesis (Myo1-GFP ring contraction and disappearance) and from first budding to the second budding. For comparison with other published data, we also carried this experiment out at 37°C; at this temperature, high fluorescent background prevented reliable assignment of time of cytokinesis, so only bud-to-bud times were assayed for initially unbudded cells. Cells were classified according to whether the short spindle ended in the mother (Mo.) or the daughter cell (Da.). (At 30°C, almost 100% initially unbudded cells showed defective division, in which the spindle did not elongate but ended up intact in mother or daughter, in the first cell cycle. At 37°C, 35% initially unbudded cells elongated the spindle in spite of galactose addition, suggesting inefficient expression of *GAL1-SCC1-RRDD* at 37°C. Because we are tracking individual cells through time, we can exclude such cells from the analysis.) A *MYO1-GFP TUB1-GFP* strain lacking *GAL1-SCC1-RRDD* (BD78-2C) was treated in parallel as a control, pooling bud-to-bud data for mothers and daughters. All numbers are in minutes ± standard deviation. The average differences (Δ) from wild type are shown along with the p value for these differences (by *t* test). The numbers of cells (n) examined in each condition are shown in the last column. N.D., not determined.

~15–20 min (Stegmeier *et al.*, 2002), shorter than our estimate of the time required for ME in the presence of active Esp1, but without cohesin cleavage.

Efficient Mitotic Exit Promoted by Esp1 Overexpression Depends on Spindle Integrity and MEN Activation

Despite our conclusion that nonproteolytic functions of Esp1 do not make a major contribution to ME kinetics, it is clear that Esp1 does have nonproteolytic biological activity (D'Amours *et al.*, 2004; Ross and Cohen-Fix, 2004). For example, Esp1 overexpression, but not TEV-induced spindle elongation, was shown to drive ME in *cdc20*-depleted cells, without a requirement for Esp1 proteolytic activity (Sullivan and Uhlmann, 2003; Queralt *et al.*, 2006). We have confirmed this finding, even using the attenuated *GALS* promoter driving *ESP1* (Figure 2) instead of six copies of *GAL1-ESP1* (Sullivan and Uhlmann, 2003; Queralt *et al.*, 2006).

Expression of *GALS-ESP1* causes an ~30-fold overexpression of Esp1 based on comparison of accumulation of Myc-tagged Esp1 from the *GALS* versus the endogenous promoter (data not shown). It is also an effective overexpressor based on rescue of *PDS1-mdb* lethality (see above). For most purposes, we prefer the *GALS-ESP1* construct because it allows viability, unlike *6xGAL1-ESP1*. We found efficient induction of ME by *GALS-ESP1*, with all markers of ME (cytokinesis, rebudding, Clb2 degradation, and DNA replication in the next cell cycle) occurring promptly upon *GALS-ESP1* induction in *Cdc20*-depleted cells (Figure 4A). This Esp1-induced ME was much more efficient than that described previously (Sullivan and Uhlmann, 2003). This is likely a consequence of performing the experiment at 30°C rather than at 23°C, because *6xGAL1-ESP1* also drove much more efficient ME at 30°C than at 23°C (Supplemental Figure 4D). *GALS-ESP1* drives essentially complete and efficient ME, including Clb2 degradation, cytokinesis, and a second round of budding and DNA replication in the next cell cycle, within 1–2 h of galactose addition to *cdc20*-blocked cells. In

contrast, at 23°C, *6xGAL1-ESP1* requires at least 3 h to drive only partial ME (Sullivan and Uhlmann, 2003), and inefficient Clb2–Cdk inactivation (Supplemental Figure 1).

Because of the high efficiency of *GAL-ESP1*-induced ME in our assay compared with the results of Sullivan and Uhlmann (2003), we characterized our assay further. First, we tested whether overexpressed Esp1 could drive ME in the presence of nocodazole to depolymerize the spindle microtubules. Inclusion of nocodazole blocked Esp1-induced ME in all aspects we have tested (cytokinesis, rebudding, and Clb2 degradation) for at least 3 h (Figure 4A). Thus, in our conditions, overexpressed Esp1 may not be intrinsically sufficient to drive ME in *cdc20*-depleted cells, or at least, requires an intact spindle to carry out this function. Nocodazole per se does not prevent ME, as the double knockout of spindle-checkpoint component *MAD2* and spindle-positioning checkpoint component *BUB2* restores efficient ME in the presence of nocodazole (Alexandru *et al.*, 1999). The involvement of the spindle integrity checkpoint surveillance system in our results is unlikely, because the experiment was performed in a *cdc20*-depleted background, removing the target of the checkpoint (Hwang *et al.*, 1998; Kim *et al.*, 1998). In addition, we have performed the same experiment in the absence of the essential spindle checkpoint component *Mad2*, with identical results (Supplemental Figure 4B).

Esp1 binds to the spindle (Jensen *et al.*, 2001), and Esp1 might require an intact spindle to drive ME for reasons unrelated to ultimate spindle elongation. However, our conclusions are consistent with the finding that both *Cdc14* release and ME (assessed by spindle disassembly) in the *dyn1Δ* mutant coincide with SPB moving into the bud, rather than spindle elongation per se (implying separase activation), which frequently happens within the mother cell body in this mutant (Bardin *et al.*, 2000). In these cells, the spindle is intact, but Esp1 still seems unable to promote ME on its own. Thus, the simplest interpretation of our finding is that the ability of Esp1 to drive efficient ME in the absence of

Figure 4. Mitotic exit promoted by Esp1 overexpression depends on an intact spindle and MEN activation. (A) A *MET3-CDC20 GALS-ESP1* strain (YL1361) was arrested by Cdc20 depletion, as in Figure 1. Esp1 was expressed from the *GALS* promoter at time zero by adding galactose (G) in the absence or presence of nocodazole + benomyl (NOC) (methionine was kept in the medium throughout to maintain Cdc20 depletion). DNA flow cytometry and protein samples were taken. Microscopic examination allowed quantification of the following phenotypes: black, large-budded mononucleate cell; red, large-budded binucleate cell; green, rebudded cell without cytokinesis; and blue, unbudded or small-budded cell (bottom right). Squares, without nocodazole; circles, with nocodazole. Western blotting was used to assess the level of Clb2 (amido-black staining of the gels showed equal loading of all lanes; data not shown). (B) *MET3-CDC20 GALS-ESP1* strains, either *CDC15* (YL1361) or *cdc15-2* (YL1362) were treated and analyzed as described in A, except that the cultures were maintained at 35.5°C to inactivate *cdc15-2*. Squares, *CDC15-wt*; circles, *cdc15-2*. Nonspecific band (*) was used as a loading control.

Cdc20 may rely on spindle elongation consequent to Esp1-dependent cohesin cleavage.

Spindle elongation could promote ME by driving the daughter spindle pole into proximity to the bud cortex, activating the MEN (Yeh *et al.*, 1995; Bardin *et al.*, 2000; Molk *et al.*, 2004; Stegmeier and Amon, 2004). To test this, we inhibited MEN activation with a temperature-sensitive *cdc15-2* mutation. *cdc15-2* completely inhibits *GALS-ESP1*-induced ME at 35.5°C (Figure 4B).

Thus, promotion of ME by Esp1 overexpression in *cdc20*-blocked cells requires both an intact spindle and a functional MEN. We hypothesize that the requirement for spindle integrity for Esp1 promotion of ME arises because Esp1-mediated cohesin cleavage allows spindle elongation, promoting effective contact between the daughter SPB and the bud cortex and thereby promoting MEN activation (Yeh *et al.*, 1995; Bardin *et al.*, 2000), although we cannot rule out the possibility that Esp1's ability to drive ME requires microtubule or spindle integrity for some other reasons.

Deletion of *CLB5* may lower the threshold of Esp1 activity required for triggering ME, because it rescues viability of *cdc20 pds1* cells and significantly increases ME efficiency in *6xGAL1-ESP1 cdc20* cells at 23°C (Shirayama *et al.*, 1999; Sullivan and Uhlmann, 2003). Thus, *CLB5* deletion might

sensitize the cell to the ME-promoting ability of Esp1. However, the result in Figure 4A is robust to deletion of *CLB5*, implying that spindle function is strongly required for efficient *GALS-ESP1*-induced ME (Supplemental Figure 4C).

As shown in Figure 1, partial ME is obtainable with complete Clb-Cdk inhibition driven by *GAL1-SIC1-4A*, and ME becomes more efficient with concurrent expression of *GAL1-TEV* to promote ectopic cohesin cleavage. We examined the ability of *GALS-ESP1* to substitute for *GAL1-TEV* in this protocol, comparing ME in the presence or absence of *GALS-ESP1* and of nocodazole, all in the presence of *GAL1-SIC1-4A*. *GALS-ESP1* promoted strong ME only in the absence of nocodazole. In the presence of nocodazole, we observed the partial ME attributable to *GAL1-SIC1-4A* (Figure 1B), independently of the presence or absence of *GALS-ESP1* (Supplemental Figure 5). In contrast, the partial ME driven by Clb-Cdk inactivation in *cdc20*-blocked cells is unaffected by nocodazole. Thus, even with complete Clb-Cdk inhibition, Esp1 is ineffective at promoting ME in the presence of nocodazole.

In these experiments, our assays for ME are direct and require a functional mitosis with effective resetting of the divided cells to the next G1. Replication origins are presumably relicensed because DNA replication occurs in the next

cell cycle (Figure 4A), and after quantitative cytokinesis and cell division, budding occurs again in the next cell cycle, suggesting the probable reloading of Swi4 onto promoters of the G1/S regulon (another event of mitotic exit) (Koch and Nasmyth, 1994; Skotheim *et al.*, 2008). Therefore, these results are strong evidence for the sufficiency of overexpressed Esp1 to drive functional ME, but this ability is completely dependent on microtubule integrity and the MEN.

Quantitative Measurement of Esp1-induced Cdc14 Release and Activity

The ability of overexpressed Esp1 to promote ME was attributed to its ability to promote MEN-independent Cdc14 release from the nucleolus (Sullivan and Uhlmann, 2003). It is well established that the activity of Cdc14 is regulated by its localization in the nucleolus, where it is stably bound to its inhibitor Net1 and also sequestered from many potential dephosphorylation targets (Stegmeier and Amon, 2004).

It is important to note that the release status of Cdc14 is not all-or-none. Cdc14 has been qualitatively described as “partially” or “fully” released from the nucleolus (Stegmeier *et al.*, 2002), but a quantitative measure of Cdc14 nucleolar localization has never been developed. Here, we use two-color imaging with Cdc14-YFP and Net1-CFP (Pereira *et al.*, 2002) and define a parameter, r , for any individual cell based on quantitative fluorescence microscopy: $r = (\text{the mean intensity of 5\% brightest YFP pixels} - \text{the mean intensity of 5\% dimmest YFP pixels}) / (\text{the mean intensity of 5\% brightest CFP pixels} - \text{the mean intensity of 5\% dimmest CFP pixels})$.

This value will be high when Cdc14-YFP and Net1-CFP are colocalized, and low when Cdc14-YFP is significantly more dispersed than Net1-CFP (which is thought to remain nucleolar throughout the cell cycle). Thus, a lower r value should indicate higher Cdc14 release from Net1 and consequently increased Cdc14 activity. (This will not be true if Cdc14 is highly concentrated in a location different from where Net1 is concentrated; therefore, we eliminated YFP pixels from the top 5% list that did not overlay one of the 5% brightest CFP pixels, before calculating r . The results were essentially identical with or without this correction; data not shown). An advantage of this approach is that it controls for changes in shape of the nucleolus, because Net1 remains stably nucleolar throughout the cell cycle (Shou *et al.*, 1999). In the absence of a nucleolar marker, nucleolar shape changes (especially nucleolar broadening or enlargement, which we have observed in some conditions) could provide a potentially misleading impression of Cdc14 release, because Cdc14 loses the typical crescent-shaped nucleolar distribution.

To establish the validity of this parameter, we measured r throughout a *cdc20* block-release experiment. At 30 min after release, cells with low r values seem exclusively in the anaphase subpopulation. Ten minutes later, Cdc14-YFP is resequenced into the nucleolus as the low r fraction diminishes (Figure 5A). Thus, the r statistic clearly reflects the known dynamic localization behavior of Cdc14. It is notable in these images that we essentially never observe complete absence of Cdc14 from the nucleolus; corresponding to this, the r value is never below ~ 0.3 in this experiment, where a value of 0 would correspond to uniform spreading of Cdc14 through the cell.

We then examined the ability of overexpressed Esp1 to drive Cdc14 release from the nucleolus in *cdc20*-blocked cells, with or without spindle depolymerization induced by nocodazole. In these experiments, we used *6xGAL1-ESP1* (Sullivan and Uhlmann, 2003) instead of *GALS-ESP1* because the higher overexpression produced a stronger and

more synchronous phenotype. (Qualitatively similar results were also obtained with *GALS-ESP1*; data not shown). We observe efficient Cdc14 release (low r value cells) 1 h after galactose addition to *cdc20*-blocked *6xGAL1-ESP1* cells (Figure 5B), comparable with r values occurring in a normal cell cycle (compare with Figure 5A). After release, the cells re-sequester Cdc14, and undergo a second release cycle ~ 3 h later (Figure 5B), probably corresponding to ME after completion of a second cell cycle (Figure 4A). Addition of nocodazole strongly reduced Cdc14 release measured by this assay (Figure 5B).

Esp1-induced Cdc14 release was reported previously to occur in nocodazole (Sullivan and Uhlmann, 2003; Visintin *et al.*, 2003). We do not understand the basis for the discrepancy in results; however, the experiments were done differently. The previous reports used immunofluorescence to detect Cdc14 and did not report a double-label with Cdc14's inhibitor Net1 or another nucleolar marker, making a full assessment of Cdc14's nucleolar localization difficult. In addition, no independent markers of ME were reported in those experiments beyond Cdc14 release. We used fluorescent protein fusions in lightly fixed cells rather than immunofluorescence (which requires harsher fixation and extraction conditions), and included a Net1-CFP marker, both to mark the nucleolus and to quantitatively measure the amount of Cdc14 released from Net1. Although our assay may be less sensitive than the immunofluorescence assay for detection of Cdc14 release, we find that ME does not occur under these conditions, based on lack of cytokinesis, rebudding and Clb2 degradation (Figure 4A), and our quantitative assay unambiguously shows that Esp1-induced Cdc14 release is significantly less efficient in the presence of nocodazole (Figure 5B). On long incubation in nocodazole, we observed a spreading of Cdc14-YFP signal in *cdc20*-depleted *GAL1-ESP1*-expressing cells, but an essentially identical spreading of Net1-CFP was also observed, which colocalized with Cdc14-YFP, accounting for the maintenance of a high r value (Figure 5B). We do not know whether this spreading is due to the release of Net1 as well as Cdc14 from the nucleolus or to a general disruption of nucleolar structure. The tight colocalization suggests that Net1 has the potential to bind and inactivate Cdc14 in this condition, which could explain the lack of mitotic exit in this context.

We do not know what aspects of experimental design contribute to the discrepancy between our results and those of Sullivan and Uhlmann (2003) and Visintin *et al.* (2003). However, we believe that inclusion of the Net1 marker and scoring of independent ME phenotypes in addition to Cdc14 release makes our experimental design more complete. Direct quantitative comparison shows that Esp1-driven Cdc14 release from Net1 in the presence of nocodazole is significantly lower than release in the absence of nocodazole. According to our results, Esp1-driven Cdc14 released from Net1 in the presence of nocodazole is inadequate to drive ME, either directly, or indirectly through the proposed Cdc14-Cdc15-positive feedback (Queralt *et al.*, 2006).

We have quantified Cdc14 release in *CDC15* or *cdc15-2* cells, released from a *cdc20* block at 37°C to inactivate *cdc15-2*. On release, r shifted strongly and transiently to a low value 20 min after release in the *CDC15* control. We observed a modest decrease in r in the *cdc15-2* cells; this decrease was maximal at 20 min after release. Some punctate Cdc14-YFP signal outside the nucleolus are observed in *cdc15-2* cells but are largely absent in the *CDC15*-wt control (Supplemental Figure 6); we do not know what these signify, but similar punctate signal from Cdc14-YFP in cells undergoing Cdc15-independent Cdc14 release was apparent

Figure 5. Quantitative measurement of Cdc14 release. (A) A *MET3-CDC20 CDC14-YFP NET1-CFP* strain (YL1452) was arrested in metaphase by incubation in +Met medium and released at time zero by removal of Met. The r value (characterizing the degree of cellular dispersion of Cdc14 relative to Net1) for cells at various time points was determined as described in *Materials and Methods*. Yellow curve, r value distribution in anaphase subpopulation. Green curve, r value distribution in unbudded/small-budded/rebudded cells. Red curve, r distribution in metaphase subpopulation. x -axis, r value; y -axis, frequency. The red, green, and yellow distributions sum to the total histogram of r values (bars) and represent at least 50 cells per category, per time point. Arrowhead on the picture highlights the bud-neck localization of Cdc14-YFP at ME. White numbers indicate r values for specific representative cells. Bar, 10 μ m. (B) A *MET3-CDC20 6xGAL1-ESP1* strain (YL1451) was arrested in metaphase by incubation in +Met medium. Galactose (G) or raffinose (R) were added at time zero in the presence (+NOC) or absence (-NOC) of nocodazole + benomyl (methionine was kept in the medium throughout to maintain Cdc20 depletion). At the indicated times, r values were determined as described in A.

Figure 6. Cdc14 release occurs despite persistent endogenous Clb-Cdk activity. (A) Strain *CLB2-kd GAL1-SIC1 CDC14-YFP NET1-CFP MYO1-mCherry* (ALG611) was arrested in α factor in galactose media and then released into either glucose to turn off *GAL1-SIC1* (Glu) or galactose media (Gal) to keep *GAL1-SIC1* on. Cdc14 localization was quantified as shown in Figure 5. DNA flow cytometry, Clb2 Western blot, and r value measurements were performed as described in *Materials and Methods*. The inset cartoon shows the cell morphology of each category. (B) *MET3-CDC20 cdh1Δ GAL5-ESP1* (YL165) cells were first arrested in metaphase by incubation in raffinose plus methionine medium, and then galactose plus methionine was added to induce Esp1 overexpression at time zero. DNA flow cytometry, Clb2 Western blot and r value test were performed as described in *Materials and Methods*; r value graphs were constructed as shown in Figure 6.

in previously published images (Pereira *et al.*, 2002). Thus, our quantitative assay provides only a weak (though reproducible) signal from FEAR pathway-induced Cdc14 release; however, the assay readily detects release levels of Cdc14 that correlate with efficient ME by direct cell biological measures.

We conclude that separase Esp1 promotes efficient ME in *cdc20*-blocked cells in a spindle-dependent manner, which we hypothesize is due to the requirement for spindle elongation consequent to cohesin cleavage. The requirement for active Cdc15 in this process implicates MEN activation as the proximal target of spindle elongation, resulting in effective Cdc14 release and consequent ME.

A high level of Cdk activity was shown to induce Cdc15 phosphorylation and lower Dbf2 kinase activity, and these reactions could have the potential to impair Cdc14 release (Jaspersen and Morgan, 2000; Menssen *et al.*, 2001; Stegmeier *et al.*, 2002); conversely, Clb-Cdk was proposed to phosphorylate Net1, facilitating FEAR network activity (Azzam *et al.*, 2004). We were concerned, therefore, that our experimental manipulation of ectopically regulating Cdk inactivation was not truly independent of promotion or prevention of Cdc14 release. To investigate this, we quantified the release kinetics of Cdc14 in the presence of undegradable Clb2 (*Clb2-kd*, lacking the KEN boxes and destruction box. *CLB2-kd* at its endogenous locus is lethal, but it can be rescued by Sic1 overexpression from the *GAL1* promoter; *GAL1-SIC1* turnoff in this strain results in a block to ME; Wäsch and Cross, 2002). We synchronized a *GAL1-SIC1 CLB2-kd CDC14-YFP NET1-CFP MYO1-mCherry* strain in G1 with α factor, and released into glucose to shut off Sic1 expression, or into galac-

tose as a control. Myo1-mCherry forms a ring at the bud neck which disappears at cytokinesis (Bi *et al.*, 1998). Cell cycle progression was monitored by budding, Myo1-mCherry localization, and the separation of the Net1-CFP signal across the bud neck to assay anaphase. Clb2-kd cells arrest in telophase as described previously (Wäsch and Cross, 2002), but Cdc14 release was very efficient, essentially coincident with anaphase and then persisting for ~30 min (Figure 6A).

Cdc14 release in the presence of undegradable Clb2 was described previously (Stegmeier *et al.*, 2002), by using *CLB2-db* overexpression from the *GAL1* promoter; however, in that experiment, Cdc14 release was delayed relative to anaphase, compared with wild-type controls, and some defects in MEN activation were also noted. We have confirmed delays in Cdc14 release in cells strongly overexpressing Clb2-kd (data not shown); but in Figure 6A, undegradable Clb2 is expressed from the endogenous promoter, providing more physiological Clb2 levels and no delay in Cdc14 release. (Western blotting indicates about a 5- to 10-fold higher level of Clb2-kd when expressed from the *GAL1* promoter than from the endogenous promoter; data not shown.)

In a different experimental approach to the same question, we assayed *GAL5-ESP1* induction of ME in *cdc20*-blocked cells (as in Figure 4A), in the absence of *CDH1*. These cells lack any factor to activate the APC for Clb degradation, because at least one of either Cdc20 or Cdh1 is required for Clb degradation (Schwab *et al.*, 1997; Wäsch and Cross, 2002). Unlike *CDH1* controls (Figure 4; data not shown), the *cdh1Δ* cells maintained a stable telophase block without any evidence of ME in this protocol, for up to 4 h (when some

degree of rebudding occurs). Despite this stable block, very efficient Cdc14 release was observed throughout this period, persisting for ~3 h (Figure 6B). These results show that stabilized Clb cyclins cannot block Cdc14 release when expressed at endogenous levels. The long period of Cdc14 release is unlikely to be due simply to overactivation of the nonproteolytic function of Esp1, because in *CDH1+* cells, 6xGAL-*ESP1* only causes a transient Cdc14 release (Figure 5B). APC-Cdh1-dependent inactivation of Cdc5 has been proposed to contribute to Cdc14 resequestration in the nucleolus (Visintin *et al.*, 2008); therefore, the long Cdc14 release in *cdh1Δ* cells could be due to persistent Cdc5 activity in addition to persistent Clb cyclins.

The experiments in Figures 4 and 6 allow us to propose a model for efficient induction of ME by *ESP1* overexpression in *cdc20*-blocked cells: the overexpressed Esp1 cleaves cohesin and allows spindle elongation, prompting MEN activation when the daughter spindle pole approaches the bud cortex (Yeh *et al.*, 1995; Bardin *et al.*, 2000; Molk *et al.*, 2004). MEN activation promotes highly efficient Cdc14 release, which can activate Cdh1 by dephosphorylation (Visintin *et al.*, 1998; Jaspersen *et al.*, 1999), leading to Clb degradation. Our results with nocodazole, *cdc15-2*, *CLB2-*kd**, and *cdh1Δ* suggest that all of these steps are required for overexpressed Esp1 to induce effective ME.

These results also demonstrate that endogenous expression of Clb mitotic cyclins is sufficient to counteract the ability of fully released Cdc14 to drive ME, provided cyclin degradation is prevented. Combined with the findings that mitotic Clb degradation is essential for ME (Wäsch and Cross, 2002) and that Clb-Cdk inactivation can cause partial ME even without cohesin cleavage (Figure 1B), these results emphasize the criticality of mitotic cyclin degradation for ME.

The Mitotic Exit Network Controls Cdc14 Nuclear Export

Net1 sequestration in the nucleolus is the only characterized mechanism for regulation of Cdc14 activity; hence, *net1* deletion might be expected to completely relieve any MEN-dependent regulation of Cdc14. However, we found that in *net1Δ* cells, Cdc14-YFP localization is still cell-cycle regulated, being concentrated in the nucleus for most of the cell cycle but spread throughout the cell transiently at the time of cell division (Figure 7B).

To examine the possibility that this result is due to residual binding of Cdc14 to other nucleolar components, we examined colocalization of Cdc14-YFP with DNA, with the nucleolar marker Nop1-dsRed (Gadal *et al.*, 2001), and with a general marker of nuclear volume, dsRed-NLS (Rodrigues *et al.*, 2001). In *NET1* cells, Cdc14 is in a typical crescent-shaped nucleolar distribution flanking the bulk of nuclear DNA, whereas in *net1* cells, Cdc14-YFP staining is enlarged to contain the DNA signal. In *net1* cells, Cdc14-YFP is localized much more broadly than the Nop1-DsRed nucleolar marker, but it is coincident with the dsRed-NLS marker for the nuclear interior. Thus, in *net1* cells, Cdc14 is not retained in the nucleolus but is nevertheless restricted to the nucleus (Figure 7A), through most of the cell cycle.

We used time-lapse fluorescence microscopy to study whether the MEN is responsible for Cdc14 nuclear export in the absence of Net1. When the MEN is inactivated at restrictive temperature in a *cdc15-2 net1Δ* strain, Cdc14-YFP remains concentrated in the nucleus throughout the cell cycle, in contrast to *CDC15 net1Δ* cells with an intact MEN (Figure 7, B and C, and Supplemental Videos 1 and 2). The Cdc14 nuclear export phenotype can be quantified using time-lapse microscopy to calculate the dispersion of Cdc14-YFP signal inside individual cells. A decrease of coefficient of variation

(CV) corresponds to Cdc14 nuclear export, because Cdc14 is no longer concentrated; therefore, the signal across the cell is less variable (we cannot apply the *r* value test in these experiments because *NET1* has been deleted, but the CV measurement correlates well with the *r* value test at least for cells with normal nucleoli, as in this experiment; data not shown). A drop in the CV for Cdc14-YFP at around the time of mitosis (as judged by the time of nuclear division) is clearly detected in *CDC15* cells at low and high temperatures, but absent in *cdc15-2* cells specifically at 37°C (Figure 7D). Cdk activity by itself is unlikely to control Cdc14 export, because the timing of Clb2 degradation in *cdc15-2 net1Δ* is almost identical to *CDC15 net1Δ* (Supplemental Figure 7), as expected (Shou *et al.*, 1999). Cdc14 nuclear export is probably not directly driven by Esp1 activity, because Cdc14 nuclear export is impaired in *net1Δ cdc15-2* cells where Esp1 activity is presumably normal based on occurrence of cohesin cleavage and chromosome segregation. A nuclear export sequence in yeast Cdc14 was recently reported. Mutations of that sequence cause Cdc14 to fail to localize to the bud neck during mitotic exit and also cause defects in cytokinesis (Bembenek *et al.*, 2005). Function of the Cdc14 nuclear export signal may be tied to MEN activation.

The *net1* deletion bypasses the *cdc15* block to telophase exit and rebudding, as expected because these double mutants are viable (Shou *et al.*, 1999; Visintin *et al.*, 1999). Interestingly, we observed a significant delay in rebudding after ME in the *cdc15 net1* mutants compared with the *CDC15 net1* mutants; this could be consistent with a cytoplasmic function of Cdc14 in promoting budding, perhaps via earlier Cdc14 localization to the bud neck (Figure 7D, red vs. blue bars).

Mutations in the nuclear transport factors *SRP1* and *KAP104* alleviate the telophase arrest of MEN mutants (Shou and Deshaies, 2002); it is possible that our finding of MEN-dependent Cdc14 nuclear export is related to these observations, although the critical cargo responsible for this genetic effect was not identified. A *kap104* mutation was shown to cause partial delocalization of Cdc14 (12%) from the nucleolus in interphase cells; however, this reaction was independent of the MEN, unlike the Cdc14 nuclear export we observe (Asakawa and Toh-e, 2002).

Our observations implicate the MEN in release of Cdc14 from the nucleus, leading to its dispersal throughout the cell. This activity may contribute to the functional dichotomy of the FEAR and MEN network. Quantitatively, the FEAR network releases less Cdc14, and in addition, FEAR network-released Cdc14 may not disperse through the cytoplasm, without subsequent MEN activation.

In the fission yeast *Schizosaccharomyces pombe*, the Cdc14 orthologue Clp1/Flp1 is also regulated through nuclear export by the SIN network (the MEN orthologue in *S. pombe*), although Clp1/Flp1 plays a different role in regulating mitosis in *S. pombe* than in *S. cerevisiae* (Trautmann *et al.*, 2001; Chen *et al.*, 2008). Thus, nuclear import/export could be a conserved mechanism for regulating Cdc14 (Clp1/Flp1) activity.

DISCUSSION

The Global Structure of Control of Mitotic Exit: Clb Degradation and MEN Activation Are the Main Drivers of ME Kinetics

In this article, we aim to clarify the global structure of the ME control system in *S. cerevisiae* involving three pathways: Clb degradation and other mechanisms of Clb-Cdk inactivation, the nonproteolytic function of Esp1, and the spindle-positioning checkpoint-dependent activation of the MEN. From the

Figure 7. Mitotic exit network controls Cdc14 nuclear export. (A) WT and *net1Δ* cells containing *CDC14-YFP* (YL1701) were briefly fixed and stained with DAPI to label DNA (top); *net1Δ CDC14-YFP* strains containing *NOP1-dsRed* (YL1702) or *dsRed-NLS* (YL174) were examined separately. (B) Selected frames of a time-lapse movie (Bean *et al.*, 2006) with indicated strain genotypes. In *net1Δ CDC14-YFP* (YL1701) cells, Cdc14-YFP was transiently excluded from the nucleus approximately at the time of ME. This transient nuclear exclusion was not observed in *net1Δ CDC14-YFP cdc15-2* (YL161) cells at 25° and 37°C. Time-lapse movies showing Cdc14 release kinetics of YL161 strain at 25° and 37°C are included in Supplemental Material. (C) The percentage of cell cycles, tracked using fluorescent time-lapse microscopy, in the course of which Cdc14-YFP release from nucleus was observed. Fifty cell cycles were examined in each condition. (D) Quantification of Cdc14 release from nucleus in a *net1Δ* background. The CV of Cdc14-YFP signal inside a single cell, computed from fluorescent time-lapse microscopy data, is the SD of YFP pixel intensity across the cell, divided by the mean intensity; this number will be high in cells with Cdc14-YFP concentrated in specific regions and low when Cdc14-YFP is dispersed through the cell. Four examples of *CDC15-wt* (blue) and *cdc15-2* (red) cells, both at 37°C, are shown. Curves are aligned by nuclear division as judged by initial stretching of the Cdc14-YFP signal across the bud neck, at $t = 0$. Color bars indicate rebudding in the next G1.

results of experiments independently controlling these activities, we concluded that Clb degradation was an essential requirement for ME, independently of the other pathways. The activation of the MEN, dependent on the spindle-position checkpoint, contributes strongly to ME efficiency (Figures 1, 3). The proteolysis-independent function of Esp1 made a minor contribution to the timing of ME. By modifying a published assay (Sullivan and Uhlmann, 2003), we found conditions in which overexpressed Esp1 promoted highly efficient ME and

indeed at least one additional complete cell cycle in the absence of Cdc20. This activity of overexpressed Esp1 required an intact spindle and a functional MEN (Figure 4).

Esp1 Is Not Essential for Mitotic Exit, and Contributes to Timing of ME Mainly through Promoting Cohesin Cleavage

Recently, it was proposed that Esp1 was essential for ME (Queralt *et al.*, 2006). The *esp1-2td* allele is thought to com-

pletely remove separase activity and was reported to completely block ME (Queralt *et al.*, 2006). In our hands, this allele only causes a 2-h delay in ME (data not shown), confirming a recent result of Visintin *et al.*, (2008). These results support our conclusions derived using Pds1 as an Esp1 inhibitor (see above) in suggesting that Esp1 is not essential for ME. Its absence does clearly delay ME very significantly, but our results suggest that this delay is mainly due to failure of cohesin cleavage, which prevents spindle elongation, greatly delaying MEN activation. Interestingly, separase is also not required for ME in mammalian cells or fission yeast (Hirano *et al.*, 1986; Wirth *et al.*, 2006). The conclusion that Esp1 is not required for ME is consistent with the fact that the FEAR network, although accelerating progression through mitosis, is dispensable for the cell cycle, whereas the MEN is essential (Shou *et al.*, 1999; Visintin *et al.*, 1999; Hofken and Schiebel, 2002; Stegmeier *et al.*, 2002).

Cdc20-APC Is Central in Control of Mitotic Exit

The requirement for degradation of both mitotic B-type cyclins and Pds1 for efficient ME places APC-Cdc20 in a uniquely important position in the ME control system. Mitotic cyclin degradation in budding yeast is biphasic (Yeong *et al.*, 2000). APC-Cdc20 degrades Clb5,6 and partially degrades Clb1,2 at the metaphase to anaphase transition, which may lower the overall Cdk activity level sufficiently to allow ME once the MEN is triggered by spindle elongation. Consistently, deleting *CLB5* to lower Clb-Cdk activity can restore viability to *cdc20Δ pds1Δ* strains (Shirayama *et al.*, 1999), and the APC can be bypassed altogether by deletion of *PDS1* combined with overexpression of Sic1 to inhibit Clb-Cdk activity (Thornton and Toczyski, 2003). Thus, the tight block to ME in *cdc20* cells cannot be overcome by simple bypass of the cohesin cleavage requirement (Sullivan and Uhlmann, 2003). Cdk inactivation is independently required. In wild-type cells, sufficient inactivation may be provided in the first Cdc20-dependent phase of Clb degradation (Yeong *et al.*, 2000); we could bypass this requirement with overexpressed stable Sic1 (Figure 1). We also found efficient mitotic cyclin degradation driven by overexpressed Esp1 in the absence of Cdc20 (Figure 4). Clb2 degradation, and all other aspects of ME scored, were dependent on an intact spindle, the MEN, and Cdh1, in Esp1 overexpressors, suggesting that full *cdc20* bypass by overexpressed Esp1 requires Clb-Cdk inactivation via Cdh1 activation through the MEN (Visintin *et al.*, 1998; Jaspersen *et al.*, 1999). It is interesting that this mechanism is not executed during *GAL1-TEV*-induced spindle elongation; we speculate that this may be due to rapid spindle breakage in *GAL1-TEV*-induced anaphase (Higuchi and Uhlmann, 2005), resulting in inefficient MEN activation. According to this speculation, spindle stabilization by the FEAR pathway including Esp1 (Jensen *et al.*, 2001; Higuchi and Uhlmann, 2005) may largely account for the functional difference in promoting ME after anaphase induction by *GAL1-ESP1* compared with *GAL1-TEV*, in the absence of *CDC20* (Sullivan and Uhlmann, 2003).

The mechanism by which Clb-Cdk inhibits ME is unknown. High Clb-Cdk may block cytokinesis and rebudding directly (Padmashree and Surana, 2001; Eluere *et al.*, 2007).

Cdc14 Release and Clb-Cdk Inactivation Are Interregulated

Cdc14 is essential for ME, but it cannot promote ME until it is released from its nucleolar anchor Net1 (Stegmeier and Amon, 2004). Our results showing strong dependence of ME kinetics on cohesin cleavage can be explained by the idea that cohesin cleavage is needed for rapid and efficient MEN

activation to drive Cdc14 release. Our quantitative assay for Cdc14 release showed that this release did not require Cdk inactivation. However, Clb-Cdk may phosphorylate Net1, contributing to early release of Cdc14 (Azzam *et al.*, 2004); in addition, the MEN may be inhibited by Clb-Cdk, at least at very high levels (Jaspersen and Morgan, 2000; Stegmeier *et al.*, 2002), and Cdc14 released by the MEN promotes Clb-Cdk inactivation (Stegmeier and Amon, 2004). Finally, Cdc14 probably antagonizes Clb-Cdk indirectly by dephosphorylating Clb-Cdk substrates. Thus, pathways for Clb-Cdk inactivation and for Cdc14 release are only partially independent.

Esp1-induced Cdc14 Release May Function Primarily in Mitotic Progression

Esp1 in combination with the FEAR pathway has the ability to promote Cdc14 release independent of cohesin cleavage (Stegmeier *et al.*, 2002; Sullivan and Uhlmann, 2003; D'Amours *et al.*, 2004; Ross and Cohen-Fix, 2004). FEAR-released Cdc14 modulates nuclear movement, rDNA segregation and spindle stability to facilitate mitotic progression before ME (Jensen *et al.*, 2001; Azzam *et al.*, 2004; Sullivan *et al.*, 2004; Higuchi and Uhlmann, 2005; Khmelinskii *et al.*, 2007). It is plausible that such events occurring before ME would delay ultimate exit timing, for example by delaying effective spindle elongation.

The FEAR pathway synergizes with the MEN; for example *LTE1* is an important but nonessential MEN component, but *lte1* mutants arrest in anaphase in the absence of the FEAR pathway (Stegmeier *et al.*, 2002). The mechanism of this synergy remains to be determined; one plausible mechanism that has been proposed is that early Cdc14 release promotes later MEN-dependent release in a positive feedback (Queralt *et al.*, 2006). As another possible mechanism of synergy, we speculate that contribution of FEAR pathway components to effective spindle function (Zeng *et al.*, 1999; Pereira and Schiebel, 2003; Higuchi and Uhlmann, 2005) might result in less efficient MEN activation via spindle defects in FEAR-defective strains. Recently, an interesting model was proposed in which early release of Cdc14 driven by Esp1 could play a role in coordinated and simultaneous segregation of all sister chromatids (Holt *et al.*, 2008). Independently of the well-documented effects of FEAR/Esp1 on mitotic progression, our results suggest that Clb degradation and MEN activation are major determinants of timing of mitotic exit.

ACKNOWLEDGMENTS

We thank A. Amon (Whitehead Institute), O. Cohen-Fix (NIH), M. Corte-Real (Centro de Biologia Molecular e Ambiental), J. Bloom, B. Drapkin, A. Geoghegan, and J. Robbins (Rockefeller University), D. Morgan (University of California, San Francisco), K. Nasmyth (Oxford University), E. Schiebel (Zentrum für Molekulare Biologie der Universität Heidelberg), and F. Uhlmann (Cancer Research UK) for providing strains and plasmids. We thank A. Amon, N. Buchler, B. Drapkin, H. Unalike, D. Morgan, J. Robbins, and W. Shou for comments on the manuscript. This work was supported by a grant from the National Institutes of Health to F. C.

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