The Mitotic Exit Network and Cdc14 phosphatase initiate cytokinesis by counteracting CDK phosphorylations and blocking polarised growth

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Polarisation of the actin cytoskeleton must cease during cytokinesis, to support efficient assembly and contraction of the actomyosin ring at the site of cell division, but the underlying mechanisms are still understood poorly in most species. In budding yeast, the Mitotic Exit Network (MEN) releases Cdc14 phosphatase from the nucleolus during anaphase, leading to the inactivation of mitotic forms of cyclin-dependent kinase (CDK) and the onset of septation, before G1-CDK can be reactivated and drive repolarisation of the actin cytoskeleton to a new bud. Here, we show that premature inactivation of mitotic CDK, before release of Cdc14, allows G1-CDK to divert the actin cytoskeleton away from the actomyosin ring to a new site of polarised growth, thereby delaying progression through cytokinesis. Our data indicate that cells normally avoid this problem via the MEN-dependent release of Cdc14, which counteracts all classes of CDK-mediated phosphorylations during cytokinesis and blocks polarised growth. The dephosphorylation of CDK targets is therefore central to the mechanism by which the MEN and Cdc14 initiate cytokinesis and block polarised growth during late mitosis.

The EMBO Journal (2012) **31**, 3620–3634. doi:10.1038/emboj.2012.224; Published online 7 August 2012

Subject Categories: cell & tissue architecture; cell cycle

Keywords: Cdc14 phosphatase; CDK; cytokinesis; MEN; yeast

Introduction

Cytokinesis is the process by which the cytoplasm is divided in two at the end of mitosis, and involves a drastic remodeling of the cytoskeleton that must be carefully co-ordinated in time and space, to safeguard inheritance of the genome and of organelles by the two daughter cells (Barr and Gruneberg, 2007; Oliferenko *et al*, 2009; Pollard, 2010). Cells must inhibit

polarised growth before cytokinesis, but this regulation is still poorly understood in most species. In animal cells and yeasts, the cessation of polarised growth during mitosis is associated with a rearrangement of the actin cytoskeleton at the cleavage site to form a contractile ring containing type II myosin and many other factors, which then drives ingression of the plasma membrane. In yeasts, the contractile ring also guides the centripetal deposition of a primary septum, which is flanked by secondary septa and then digested subsequently to allow separation of the two daughter cells (Roncero and Sanchez, 2010). Cell division in yeasts is thus a combination of cytokinesis, septation, and cell separation.

Mitotic forms of cyclin-dependent kinase (CDK) are thought to inhibit cytokinesis until the end of mitosis in most if not all eukaryotes, but at present it is not clear how much the underlying mechanisms have diverged during evolution. In vertebrate cells, CDK blocks the activation of RhoA GTPase at the cleavage site by the GTP exchange factor Ect2, and so inhibits assembly of the contractile actomyosin ring until late anaphase (Yuce *et al*, 2005). Mitotic CDK also inhibits cytokinesis until the end of mitosis in the budding yeast *Saccharomyces cerevisiae* (Ghiara *et al*, 1991; Lew and Reed, 1993), but attempts to understand this regulation have been complicated by the intimate and mutually antagonistic relationship between mitotic CDK and the Mitotic Exit Network (or MEN). Mitotic CDK impairs activation of the MEN before anaphase (Jaspersen and Morgan, 2000; Hwa Lim *et al*, 2003; Konig *et al*, 2010), whereas activation of the MEN during anaphase leads ultimately to efficient inactivation of CDK, but is also thought to have direct roles in promoting cytokinesis and septation, via activation of the actomyosin ring (Bardin and Amon, 2001; Yeong *et al*, 2002; Simanis, 2003; Wolfe and Gould, 2005). The mechanism by which the MEN activates cytokinesis is very poorly characterised at present. In addition, it has been unclear until now whether mitotic CDK in budding yeast inhibits cytokinesis only via its role in blocking the MEN, or also blocks assembly of the actomyosin ring in other ways that might be more analogous to the regulation of cytokinesis in animal cells.

Anaphase is initiated in budding yeast when Cdc20 activates the anaphase promoting complex (APC) that degrades ‘Securin’ and thus allows ‘Separase’ to trigger the segregation of sister chromatids. Separase also has a second role during anaphase as part of the ‘FEAR’ pathway (Fourteen Early Release), leading to partial release of the Cdc14 phosphatase from the nucleolus where it is tethered by the Net1 protein (Stegmeier and Amon, 2004). Cdc14 plays a central role in dephosphorylating targets of CDK (Queralt and Uhlmann, 2008), including components of the MEN, and its partial release by the FEAR pathway coincides with partial degradation of B-type cyclins by APC-Cdc20 (Mocciaro and Schiebel, 2010). The initial accumulation of free Cdc14 is quickly followed by activation of the MEN at Spindle Pole

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Received: 7 June 2012; accepted: 17 July 2012; published online: 7 August 2012

Bodies, driven by entry of one spindle pole into the bud during anaphase and aided by decreasing CDK activity (Stegmeier and Amon, 2004). This leads to release of much more Cdc14 from the nucleolus and the completion of mitotic exit.

The MEN involves the sequential activation of the Tem1 GTPase, the Cdc15 protein kinase, and the Dbf2/Dbf20 protein kinases that work in association with the Mob1 regulatory subunit. Active Dbf2/Dbf20 promote efficient release of Cdc14 from Net1 in the nucleolus, and both Cdc15 and Dbf2 are then required to export Cdc14 out of the nucleus (Lu and Cross, 2009; Mohl *et al*, 2009). Once in the cytoplasm, Cdc14 dephosphorylates key CDK substrates such as the Cdh1 activator of the APC that helps to complete the degradation of B-type cyclins, and the Swi5 transcription factor that governs expression of the Clb-CDK inhibitor Sic1. Cdc14 also prevents degradation of Sic1 by antagonising its phosphorylation by CDK. In all these ways, Cdc14 drives the efficient inactivation of mitotic CDK at the end of mitosis (Stegmeier and Amon, 2004).

Multiple studies have indicated that the MEN is also important for the onset of cytokinesis, independently of its role in CDK inactivation, and apparently analogous to the action of the related Septation Initiation Network (SIN) in fission yeast. The *cdc15-lyt1* allele produces chains of multinucleate cells via delayed mitotic exit followed by a failure to form septa (Jimenez *et al*, 1998), as does partial inactivation of other MEN proteins (Hwa Lim *et al*, 2003). Truncation of the carboxyl terminus of Cdc15 produces a similar phenotype, and this is associated with an apparent delay in contraction of the actomyosin ring (Menssen *et al*, 2001). Defective ring contraction also occurs when MEN proteins are inactivated and mitotic CDK activity is blocked in some other way, for example by use of a *tem1 net1* double mutant that cannot trap Cdc14 in the nucleolus (Lippincott *et al*, 2001), or by over-expressing Sic1 in cells with mutated versions of Dbf2-Mob1 (Luca *et al*, 2001; Meitinger *et al*, 2010). It appears that Cdc14 phosphatase is also important for cytokinesis and not just for inactivation of mitotic CDK, as mutation of a predicted Nuclear Export Sequence in Cdc14 still allows degradation of the mitotic B-type cyclin Clb2 (and does not actually prevent Cdc14 from accumulating in the cytoplasm at the end of mitosis), but delays contraction of the actomyosin ring at high growth temperatures (Bembenek *et al*, 2005). Consistent with all these data, the MEN proteins and Cdc14 relocate to the cleavage site at the end of mitosis, further suggesting that they have independent roles in some aspects of cell division (Frenz *et al*, 2000; Xu *et al*, 2000; Luca *et al*, 2001; Yoshida and Toh-e, 2001; Hwa Lim *et al*, 2003; Bembenek *et al*, 2005).

Currently, the relevant targets of the MEN and Cdc14 for cytokinesis are still poorly understood. Dbf2-Mob1 phosphorylates and inactivates a Nuclear Localisation Sequence of Cdc14, causing Cdc14 to accumulate in the cytoplasm at the end of mitosis (Mohl *et al*, 2009). Mutation of these phosphorylation sites blocks cytoplasmic accumulation of Cdc14 and causes defects in cell division when a temperature-sensitive *cdc15-2* strain is returned to 25°C from 37°C. But this is associated with defective degradation of Clb2, so that the relative contribution to cell division of CDK inactivation and Cdc14 export by Dbf2-Mob1 remains unclear (Mohl *et al*, 2009). The MEN also contributes during

late mitosis to phosphorylation of Hof1, which regulates cytokinesis and septation (Vallen *et al*, 2000; Blondel *et al*, 2005). A recent study showed that Dbf2-Mob1 phosphorylates Hof1 to control its association with the actomyosin ring, and proposed that this regulation is required for actomyosin-ring constriction (Meitinger *et al*, 2011). Moreover, degradation of Hof1 at the end of the cell cycle also seems to be important for timely constriction of the actomyosin ring, and probably depends upon phosphorylation of Hof1 by the MEN proteins and other kinases (Blondel *et al*, 2005). Activation of the contractile ring is still understood very poorly, however, and it remains unclear whether the MEN controls this process directly, or else influences ring function indirectly by controlling aspects of septum formation (Chin *et al*, 2012; Oh *et al*, 2012).

We have exploited an experimental system that allows us to distinguish the contributions of mitotic CDK and the MEN proteins to the regulation of cytokinesis in budding yeast. Our data indicate that the dephosphorylation of CDK targets is the major trigger for cytokinesis and septation at the end of mitosis in budding yeast, analogous to the regulation of cytokinesis by mitotic CDK in animal cells. Cytokinesis is not just regulated by CDK inactivation *per se*, however, but also by the MEN-dependent release of Cdc14 into the cytoplasm, where it presumably dephosphorylates key targets of CDK (and perhaps other kinases too) that govern cytokinesis. As part of this regulation, cytoplasmic Cdc14 also helps to prevent the untimely action of G1 forms of CDK during late mitosis, thereby allowing the efficient action of the actomyosin ring without diversion of the actin cytoskeleton to polarise growth at a new bud site.

Results

Inactivation of mitotic CDK before anaphase induces cytokinesis and septation without cell separation

In addition to inhibition of the MEN and cell division in budding yeast, mitotic CDK prevents a second round of S-phase by stopping the MCM2–7 DNA helicase from loading at origins of DNA replication (Dahmann *et al*, 1995), blocks the selection of a new bud site (Padmashree and Surana, 2001), and also inhibits the resumption of polarised growth by preventing expression of the G1 cyclins Cln1–2 (Fitch *et al*, 1992; Richardson *et al*, 1992; Amon *et al*, 1993; Lew and Reed, 1993; Dahmann *et al*, 1995). Budding yeast cells can be synchronised in G2–M-phase with high mitotic CDK by addition of nocodazole to the culture medium, and this blocks both FEAR and MEN pathways via activation of the spindle checkpoint and the absence of anaphase, respectively. Expressing a stable form of Sic1 (Sic1^{ΔNT}) in such cells inactivates all forms of Clb-CDK and leads to reloading of the MCM2–7 DNA helicase at origins, the establishment of a new bud site, and the resumption of polarised growth following renewed expression of G1 cyclins (Dahmann *et al*, 1995). Accumulation of Sic1^{ΔNT} protein correlates precisely with inhibition of Clb-CDK (Zhai *et al*, 2010), and leads to dephosphorylation of CDK targets such as the Pol12 subunit of DNA polymerase α (Figure 1Ai), and the re-licensing of DNA replication origins (Supplementary Figure 1), as observed previously (Desdouets *et al*, 1998; Noton and Diffley, 2000). The bulk population of Cdc14 phosphatase remains in the nucleolus under such conditions (compare

Figure 1 Inactivation of Clb-CDK before anaphase drives cytokinesis and septation without cell separation. **(A)** (i) Control (W303-1a) and *GAL-SIC1ΔNT* (YLD12) strains were grown in YPRaff medium at 30°C and synchronised in G2-M-phase with nocodazole. Cells were switched to YPGal medium containing nocodazole for the indicated times, and cell extracts were used for immunoblots showing induction of Sic1ΔNT and dephosphorylation of the Clb-CDK target Pol12 (the loading control was the Mcm3 protein). (ii) An identical experiment was performed with *3GFP-RAS2* (YAD204) and *GAL-SIC1ΔNT 3GFP-RAS2* (YASD1759). Live cells were examined by fluorescence microscopy to monitor ingress of the plasma membrane at the budneck. A white arrow denotes undivided cytoplasm at the budneck, and red arrows correspond to cells where examination of each z-level at the budneck showed divided cytoplasm. The scale bars correspond to 2 μm. **(B)** *GAL-SIC1ΔNT ADH1-OsTIR1* 3GFP-RAS2 (YASD1952) and *myo1-aid GAL-SIC1ΔNT ADH1-OsTIR1* 3GFP-RAS2 (YASD1950) were arrested with mating pheromone at 30°C in YPRaff medium, before addition of auxin and incubation for a further 60' to deplete Myo1-aid. Cells were then released from G1-arrest and either used to monitor DNA content by flow cytometry (i), or else were synchronised in G2-M-phase with nocodazole before transfer to YPGal medium containing nocodazole and auxin (ii). Images of representative cells are shown from the 120' time point; scale bars = 2 μm (iii). **(C)** Control and *GAL-SIC1ΔNT* strains were grown as in **(A)**, and samples were processed for electron microscopy following 120' incubation in YPGal medium + nocodazole. The figure shows 40 nm serial sections that start just above the budneck of one control cell (nine consecutive sections) and one cell expressing *GAL-SIC1ΔNT* (15 consecutive sections). Additional examples are shown in Supplementary Figure 4. The scale bars correspond to 1 μm, and 'n' indicates the position of the nucleus in each cell.

Supplementary Figure 2A and B), reflecting a failure to activate the FEAR and MEN pathways in the absence of anaphase.

Although inactivation of mitotic CDK in G2-M-phase does not appear to cause cell division (Hwa Lim *et al*, 2003; Lu and Cross, 2009), it drives Dbf2/Dbf20 (Hwa Lim *et al*, 2003) and the chitin synthase Chs2 (Zhang *et al*, 2006) to the budneck, and leads to the deposition of septal material (Zhang *et al*, 2006). It seemed possible, therefore, that inactivation of mitotic CDK might actually induce cytokinesis and

septation without causing cell separation, even in the absence of anaphase or canonical activation of the MEN. To monitor division of the cytoplasm directly, we utilised a fusion of three copies of GFP to the plasma membrane protein Ras2, expressed from the endogenous *RAS2* locus. Asynchronous cultures of *3GFP-RAS2* and *GAL-SIC1ΔNT 3GFP-RAS2* were grown at 30°C in medium lacking galactose, before synchronisation in G2-M-phase by addition of nocodazole for one generation time. Cells were then transferred to fresh medium containing galactose as well as

nocodazole, and live cells were examined over the course of 2 h. Whereas control cells retained a single cytoplasm throughout the experiment, division of the cytoplasm occurred in almost all cells following inactivation of mitotic CDK by expression of *Sic1ΔNT* (Figure 1Aii). Similarly, cytokinesis was also induced very efficiently following degradation of CDK in G2-M-phase cells (Supplementary Figure 3).

Some yeast strains can survive without type II myosin and manage to divide in the absence of a contractile actomyosin ring, although septum formation is disorganised and cytokinesis is usually less efficient under such circumstances (Bi *et al*, 1998). In the W303 strain with which we work, however, the type II myosin Myo1 is essential for cytokinesis (Tolliday *et al*, 2003). We used the recently described 'auxin degron' approach (Nishimura *et al*, 2009) to deplete Myo1 rapidly in G1 cells and confirmed that this was sufficient to block the subsequent cytokinesis (Figure 1Bi). We then repeated the experiment and used nocodazole to synchronise cells in G2-M-phase after depletion of Myo1-aid, before induction of *GAL-SIC1ΔNT* to inactivate Clb-CDK. Whereas control cells divided their cytoplasm, cytokinesis was blocked in the *myo1-aid* strain, showing that Myo1 is required for cytokinesis following inactivation of Clb-CDK (Figure 1Bii and iii). Taken together, these experiments indicate that inactivation of mitotic CDK before anaphase is sufficient to induce actomyosin-ring-dependent cytokinesis in almost all cells, despite the apparent failure of cell division.

To investigate whether septum formation was complete under such circumstances, we used transmission electron microscopy to analyse 40 nm serial sections across the entire budneck region of 30 control cells and 30 *GAL-SIC1ΔNT* cells, 120' after transferring nocodazole-arrested cultures to medium containing both galactose and nocodazole (all cells were seen to have a single nucleus). Whereas 100% of control cells lacked a septum, 97% of cells expressing *GAL-SIC1ΔNT* had an apparently complete septum at every level of the original budneck (Figure 1C; Supplementary Figure 4; new buds were seen in the same sections in 13 of the 30 cells expressing *Sic1ΔNT*).

These data indicated that inactivation of mitotic CDK before anaphase is sufficient to drive cytokinesis and septation, but does not induce cell separation. In budding yeast, cell separation requires enzymes that digest the primary septum, expression of which is dependent upon the action of the Ace2 transcription factor (Roncero and Sanchez, 2010). Ace2 is excluded from the nucleus by mitotic CDK until it is dephosphorylated by Cdc14 at the end of mitosis (Supplementary Figure 5A), but only accumulates in the daughter cell nucleus due to additional regulation by the Cbk1 kinase, which phosphorylates and inactivates a Nuclear Export Sequence in Ace2 in a daughter-specific manner (Colman-Lerner *et al*, 2001; Bourens *et al*, 2008; Mazanka *et al*, 2008). The mechanism allowing nuclear accumulation of Ace2 in the daughter cell nucleus is not active in our experiments due to the absence of nuclear division (Supplementary Figure 5B). To allow Ace2 to accumulate in the mother cell nucleus despite the lack of anaphase, we exploited a mutation in Ace2 (Ace2-G128E) that inactivates the nuclear export sequence (Bourens *et al*, 2008). Whereas expression of *GAL-ACE2* did not induce cell separation

following inactivation of mitotic CDK in nocodazole-arrested cells and expression of *GAL-ACE2-G128E* had no effect on its own (ASD and KL, unpublished data), the combined induction of *GAL-SIC1ΔNT* and *GAL-ACE2-G128E* allowed most cells to complete division and cell separation (Supplementary Figure 5C). Taken together, the above data indicate that inactivation of mitotic CDK before anaphase is sufficient to drive cytokinesis and septation, but does not cause cell separation due to the absence of nuclear Ace2.

Cln-CDK-dependent polarisation of the actin cytoskeleton interferes with cytokinesis when Clb-CDK is inactivated before release of Cdc14

To compare the kinetics of Clb-CDK inactivation in single cells with the behaviour of the actomyosin ring, we used a previously described cassette comprising a fusion of the nuclear import and export sequences of the Mcm2-7 DNA helicase (referred to here as NLS-NES-GFP), which is excluded from the nucleus by Clb-CDK until the end of mitosis (Liku *et al*, 2005). Cells expressing both NLS-NES-GFP and Myo1-Tomato were released from nocodazole arrest at 30°C to allow cells to complete mitosis and cytokinesis. Time-lapse (Supplementary Figure 6) and time-course (Figure 2A) analysis showed that the NLS-NES-GFP cassette only accumulated in the nucleus at the end of anaphase, a few minutes before contraction of the Myo1-Tomato ring was initiated, mirroring the behaviour of the endogenous Mcm2-7 proteins that enter the nucleus following inactivation of Clb-CDK (Labib *et al*, 1999; Nguyen *et al*, 2000).

We then monitored NLS-NES-GFP and Myo1-Tomato in nocodazole-arrested control or *GAL-SIC1ΔNT* strains, following transfer to fresh medium containing both galactose and nocodazole. In control cells, the NLS-NES-GFP cassette remained cytoplasmic and Myo1-Tomato remained at the budneck throughout the 2-h experiment (data not shown), reflecting the persistence of high Clb-CDK activity. In the cells expressing *GAL-SIC1ΔNT*, however, 91% of cells had inactivated Clb-CDK within 45 min as reflected by nuclear NLS-NES-GFP (Figure 2B). Nevertheless, 75% of these cells still retained Myo1-Tomato at the budneck, and cytokinesis was delayed by about 30–45 min under such conditions relative to inactivation of mitotic CDK (compare the NLS-NES-GFP data in Figure 2B with GFP-Ras2 in Figure 1A). Consistent with this view, about half of the cells formed a second Myo1-Tomato ring at the site of the new bud, before disappearance of the first ring at the original budneck (examples are shown in Figure 2B). It thus appears that contraction of the actomyosin ring is defective when mitotic CDK is inactivated in the absence of anaphase.

We measured the width of the Myo1-Tomato rings at the original budneck after transferring *GAL-SIC1ΔNT* cells to galactose-containing medium for 120', and found that inactivation of mitotic CDK caused the Myo1 rings to contract to 40% of their initial width ($0.6 \mu\text{m} \pm 0.2$ after 120', compared with $1.4 \mu\text{m} \pm 0.2$ at t0; $n = 50$ in both cases), whereas no change was observed in control cells ($1.4 \mu\text{m} \pm 0.2$ after 120'; $n = 50$). Therefore, it seems that contraction of the actomyosin ring is initiated following premature inactivation of mitotic CDK, but is not completed in the timely fashion. Accordingly, about 60% of cells retained a partially contracted Myo1-Tomato signal at the original budneck even

Figure 2 Contraction of the actomyosin ring is defective when Clb-CDK is inactivated before anaphase. **(A)** Cells expressing NLS-NES-GFP and Myo1-Tomato (YASD1614) were released from nocodazole arrest at 30°C and the indicated parameters were quantified. Images (i) to (vi) show representative cells at progressively later stages of cell division and entry into the next cell cycle (NLS-NES-GFP enters both daughter nuclei at the end of anaphase upon inactivation of mitotic CDK). Scale bars = 2 μm. **(B)** *GAL-SIC1ΔNT NLS-NES-GFP MYO1-TOMATO* (YASD1612) was arrested with nocodazole at 30°C as above and then switched to medium containing galactose. Representative cells are shown at the indicated time points. **(C)** *GAL-SIC1ΔNT 3GFP-RAS2 MYO1-TOMATO* (YASD1870) were treated as in **(B)** and images taken after 120'. Red arrows denote cells that retain a partially contracted Myo1 ring at the original budneck despite having divided their cytoplasm.

2 h after induction of *GAL-SIC1ΔNT* (Figure 2B), despite the fact that almost all cells had completed cytokinesis by this point (Figure 1A). Whereas cytokinesis is almost entirely

dependent upon Myo1 under these conditions (Figure 1B), our data indicated that cells can complete cytokinesis with a partially contracted actomyosin ring, and we confirmed this

in a similar experiment by monitoring GFP-Ras2 and Myo1-Tomato in the same cells (Figure 2C). These data are consistent with the findings of a recent paper, indicating that Myo1 can guide formation of a primary septum in budding yeast even without proper contraction of the actomyosin ring (Fang *et al*, 2010).

Inhibition of Clb-CDK induces the expression of G1 cyclins as well triggering cytokinesis, and it seemed possible that the reactivation of Cln-CDK might explain why cytokinesis is defective under such conditions. Cln-CDK inhibits the APC by phosphorylation of the Cdh1 activator, but we found that co-expression of Sic1 Δ NT with the non-phosphorylatable *CDH1-m11* allele (Lopez-Aviles *et al*, 2009) still led to slow induction of cytokinesis and defective contraction of the actomyosin ring (ASD and KL, unpublished data). Reactivation of Cln-CDK following inactivation of Clb-CDK also causes polarisation of the actin cytoskeleton to make a new bud (Figure 1A). When budding yeast cells inactivate mitotic CDK at the end of anaphase, budding never occurs before cytokinesis, but the mechanisms that restrain polarised growth until the following G1-phase are not understood. Our data suggested that inactivation of Clb-CDK before release of Cdc14 might lead to a competition between assembly of the actomyosin ring and re-polarisation of the actin cytoskeleton to a new bud site, helping to explain the observed defects in progression through cytokinesis. To examine directly the behaviour of the actin cytoskeleton following inactivation of Clb-CDK, we fixed cells expressing NLS-NES-GFP and treated them with Rhodamine-Phalloidin to stain the actin cytoskeleton. We first examined cells passing normally through cytokinesis following release from nocodazole arrest at 30°C (Figure 3A). The arrested cells had high mitotic CDK activity and this was associated with a depolarised actin cytoskeleton and cytoplasmic localisation of NLS-NES-GFP. Upon release from nocodazole arrest, an actin ring formed in binucleate cells with nuclear NLS-NES-GFP, and this was never associated with re-polarisation of actin to the site of a new bud (Figure 3Ai). Contraction of the actomyosin ring was followed by the transient accumulation of actin patches at the budneck (Figure 3Ai and ii), before subsequent re-polarisation of the actin cytoskeleton to the site of the new bud in the next cell cycle.

We then examined the behaviour of the actin cytoskeleton following premature inactivation of Clb-CDK by expression of *GAL-SIC1 Δ NT* in nocodazole-arrested cells (Figure 3B). Nuclear NLS-NES-GFP first appeared in cells with an actin ring at the budneck, analogous to the normal situation in late mitosis (except for the presence of only one nucleus due to the absence of anaphase). This population peaked after 45' expression of *GAL-SIC1 Δ NT*, but was then replaced by cells with a unique phenotype that is never normally seen. In addition to nuclear NLS-NES-GFP and an actin ring at the original budneck, these cells had polarised actin to the site of a new bud before completing cytokinesis (Figure 3Bi; see example from 60' time point in (ii)). This population peaked at 60', and then declined as actin disappeared from the ring and reappeared as patches around the old budneck. By 90', almost all cells had lost an actin ring at the old budneck (Figure 3B), despite the fact that Myo1 was still present at the original budneck in around 85% of cells at this point (Figure 2B). Overall, these data indicate that cells assemble an actomyosin ring following premature inactivation of

mitotic CDK, but in many cells the function of the ring is hindered by the contemporaneous diversion of the actin cytoskeleton to a new bud site.

The induction of polar growth at a new bud site during G1-phase is dependent upon the activation of Cln-CDK activity, which can be blocked by treating cells with α -factor mating pheromone. To investigate the impact of the premature induction of polar growth on cytokinesis, we arrested cells with nocodazole as above, and then compared the kinetics of cytokinesis following induction of *GAL-SIC1 Δ NT* in the presence or absence of mating pheromone. Clb-CDK was inhibited with similar kinetics in both cases, as reflected by the timing of entry of NLS-NES-GFP into the nucleus (Figure 4A), and Cdc14 remained in the nucleolus (Supplementary Figure 2B and C). As described above, inhibition of Clb-CDK led to re-activation of Cln-CDK and the rapid re-polarisation of the actin cytoskeleton to a new bud site (Figure 4Bi, $-\alpha$ -factor), before contraction of the actomyosin ring had occurred (Figure 4Biii, $-\alpha$ -factor). In contrast, re-budding was blocked following simultaneous inactivation of both Clb-CDK and Cln-CDK (Supplementary Figure 2C). Cells still re-polarised the actin cytoskeleton to form 'shmoos' under such conditions (Figure 4Bii), but this occurred more slowly than cytokinesis (Figure 4Bi, $+\alpha$ -factor; and Figure 5B discussed below), so that shmoos were never seen in cells that still had an actin ring (Figure 4Biii, $+\alpha$ -factor; n = total of 287 cells with actin rings). These data indicated that simultaneous inhibition of Clb-CDK and Cln-CDK induced formation of the contractile actomyosin ring without diversion of the actin cytoskeleton to a new site of polarised growth.

Actin rings appeared with similar kinetics following the inactivation of Clb-CDK alone, or both Clb-CDK and Cln-CDK (Figure 4Biii). However, actin rings (Figure 4Biii) and Myo1 rings (Figure 5A) disappeared more rapidly from the budneck when both Clb-CDK and Cln-CDK were inhibited, and this reflected the fact that cytokinesis proceeded more rapidly (Figure 5B). Similarly, time-lapse analysis indicated that inhibition of Clb-CDK led to efficient contraction of the actomyosin ring in conditions where the formation of new buds was inhibited (Figure 5C). These findings implied that cells must have a mechanism that normally blocks the premature reactivation of Cln-CDK when Clb-CDK is inactivated at the end of mitosis, in order to prevent re-polarisation of the actin cytoskeleton that would otherwise interfere with contraction of the actomyosin ring during cytokinesis.

Accumulation of cytoplasmic Cdc14 blocks the premature resumption of polarised growth and aids cytokinesis following inactivation of mitotic CDK

Release of Cdc14 phosphatase into the cytoplasm at the end of mitosis could provide a simple explanation for both the rapid onset and efficient progression of cytokinesis following inactivation of Clb-CDK. In addition to its established role in triggering the inactivation of mitotic CDK, Cdc14 might also help activate cytokinesis and prevent premature polarisation of the actin cytoskeleton, by directly dephosphorylating CDK target proteins and antagonising the premature action of Cln-CDK. Indeed, previous work showed that *GAL-CDC14* in an asynchronous culture blocked budding in the majority of cells (Visintin *et al*, 1998). Cdc14 returns to the nucleolus following the completion of cytokinesis, and growth control

Figure 3 Re-polarisation of the actin cytoskeleton to a new bud competes with contraction of the actomyosin ring when Clb-CDK is inactivated before anaphase. **(A)** Cells expressing NLS-NES-GFP (YASD1616) were released from nocodazole arrest at 30°C as above. (i) Samples were fixed at the indicated times before staining with rhodamine-phalloidin to visualise the actin cytoskeleton. (ii) Examples are shown of a cell before assembly of the actin ring (cell (a), 0'), a cell with an actin ring (cell (b), 30'; the actin ring is denoted by a white arrow), and a cell in which the actin ring has disassembled and been replaced with patches of actin at the budneck (cell (c), 45'). **(B)** *NLS-NES-GFP* (YASD1616) and *GAL-SIC1ΔNT NLS-NES-GFP* (YMP197) were grown as in Figure 1A to inactivate Clb-CDK in G2-M-phase, and the cells were then fixed and stained with rhodamine-phalloidin. (i) Quantification of the indicated parameters. (ii) Examples are shown from the indicated time points; scale bars = 2 μm.

would then be the major factor governing the activation of Cln-CDK and the establishment of polarised growth at the end of the next G1-phase.

We compared the kinetics of cytokinesis in nocodazole-arrested cells following expression of *GAL-SIC1ΔNT*, *GAL-CDC14*, or combined expression of *GAL-CDC14 GAL-SIC1ΔNT*. Ectopic expression of *GAL-CDC14* induced cytokinesis more quickly than *GAL-SIC1ΔNT*, and cytokinesis was even quicker following the combined expression of Cdc14 and Sic1ΔNT (Figure 6A; Supplementary Figure 7 shows that

expression of Sic1ΔNT is not altered by co-expression of Cdc14). As predicted, ectopic expression of Cdc14 in G2-M cells prevented re-budding and the assembly of a second Myo1 ring (images in Figure 6A, and ASD unpublished data), and speedier progression through cytokinesis was reflected by rapid loss of Myo1 from the budneck upon entry of NLS-NES-GFP into the nucleus (Figure 6B), analogous to the normal situation during late mitosis. These data suggest that the efficient onset of cytokinesis at the end of mitosis is achieved by combining inactivation of Clb-CDK with an

Figure 4 Inhibition of Cln-CDK prevents re-polarisation of the actin cytoskeleton from interfering with assembly of the actin ring, when Clb-CDK is inactivated before anaphase. (A) *NLS-NES-GFP MYO1-TOMATO* (YASD1614) and *GAL-SIC1ΔNT NLS-NES-GFP MYO1-TOMATO* (YASD1612) were arrested with nocodazole at 30°C. Each culture was then split in two and transferred to medium containing galactose plus nocodazole, with or without α-factor mating pheromone. (B) A similar experiment was performed with *NLS-NES-GFP* (YASD1616) and *GAL-SIC1ΔNT NLS-NES-GFP* (YMP197) and samples were fixed and stained with rhodamine-phalloidin as above. (i) For each sample, 100 cells were examined to determine the percentage in which the actin cytoskeleton was polarised to the site of a new bud (–α-factor) or a shmoo (+α-factor). (ii) Examples of post-cytokinesis cells with shmoos; scale bars=2μm. (iii) The percentage of cells with actin rings was determined for the same experiment.

increase in the pool of free Cdc14 phosphatase, which prevents the inappropriate and premature resumption of polarised growth.

Retention of Cdc14 in the cytoplasm is central to the mechanism by which MEN proteins promote the initiation of cytokinesis in budding yeast

As discussed above, previous studies indicated that the MEN contributes to the onset of cytokinesis independently of the inactivation of mitotic CDK, and our data suggested that a key feature of this regulation was that accumulation of cytoplasmic Cdc14 allows efficient functioning of the actin ring by preventing diversion of the actin cytoskeleton to the site of a

new bud. The question remained, however, whether the MEN proteins and Cdc14 are also required in other ways for the initiation of cytokinesis. Full activation of the MEN and Cdc14 normally occur during anaphase, and the bulk of Cdc14 phosphatase is not released from the nucleolus following premature inactivation of Clb-CDK in nocodazole-arrested cells (Supplementary Figure 2). Nevertheless, Dbf2 and Dbf20 relocate to the budneck under such conditions (Hwa Lim *et al*, 2003), and previous studies have indicated that MEN and a small pool of Cdc14 can be active before anaphase (Lopez-Aviles *et al*, 2009; Akiyoshi and Biggins, 2010; Hotz *et al*, 2012). We wanted to test, therefore, whether the MEN and Cdc14 contributed to the efficiency of

Figure 5 Inhibition of Clb-CDK without reactivation of Cln-CDK induces contraction of the actomyosin ring and the rapid completion of cytokinesis. **(A)** The proportion of cells with Myo1 rings at the budneck was determined for the experiment in Figure 4A. **(B)** A similar experiment was performed with *GAL-SIC1ΔNT 3GFP-RAS2* (YASD1759), to monitor completion of cytokinesis. **(C)** The experiment in Figure 4A was repeated and time-lapse video microscopy was used to monitor the relation between Clb-CDK inactivation and contraction of the actomyosin ring. Note that re-budding was inhibited in this experiment by illumination of the field of view with UV light. The times at which contraction of the Myo1 ring began and was completed are indicated by white and black circles, respectively.

cytokinesis following inactivation of Clb-CDK in nocodazole-arrested cells, independently of the need to block diversion of the actin cytoskeleton from the actin ring to a new bud site.

Whereas expression of *GAL-SIC1ΔNT* leads to contemporaneous cytokinesis and re-budding at normal growth temperatures such as 30°C (Figure 1) or 24°C (PJN and ASD, unpublished data), premature inactivation of Clb-CDK at 37°C induces cytokinesis but re-budding is largely inhibited (Figure 7A). This is consistent with previous studies showing that inactivation of Clb1-4 at 30 or 37°C induced re-expression of *CLN1-2* (Amon *et al*, 1993; Dahmann *et al*, 1995), but re-budding was blocked at 37°C (Dahmann *et al*, 1995), presumably as a result of the mild heat-shock. This meant that we could induce *GAL-SIC1ΔNT* at 37°C in strains with temperature-sensitive mutations in MEN proteins and Cdc14, and then examine the contribution of these factors to cytokinesis without competition for the actin cytoskeleton from a new bud.

We first compared the kinetics of cytokinesis following premature inactivation of Clb-CDK in control cells, *cdc14-1* and *cdc15-2*. Cells were arrested with nocodazole at 24°C and

then shifted to 37°C for 60' to inactivate Cdc14-1 and Cdc15-2 (confirmed by releasing an aliquot to medium lacking nocodazole and observing that all cells completed anaphase before arresting as binucleate cells). We then induced expression of *GAL-SIC1ΔNT* and found that about 60% of cells completed cytokinesis with similar kinetics in all three strains (Supplementary Figure 8; note that the effects of *GAL-SIC1ΔNT* are less efficient at 37°C and Clb-CDK was only inactivated in about 60–70% of cells, as shown below by the behaviour of NLS-NES-GFP in analogous experiments). To try and inactivate the MEN even more efficiently, we also examined a *cdc14-1 cdc15-2* double mutant, as we found that cells were still able to grow well at 24°C. We observed that cytokinesis was delayed by >30' in *cdc14-1 cdc15-2* cells relative to a control strain (Figure 7A). It thus appears that Cdc15 and Cdc14 still contribute to the efficiency of cytokinesis following premature inactivation of Clb-CDK, even under conditions where the premature resumption of polarised growth does not occur, and despite the small pool of free Cdc14 before anaphase. Similarly, we found that cytokinesis was delayed following premature inactivation of Clb-CDK in

Figure 6 An increase in the pool of free Cdc14 improves the efficiency of cytokinesis and actomyosin-ring contraction, when Clb-CDK is inactivated before anaphase. **(A)** *GAL-SIC1ΔNT 3GFP-RAS2* (YASD2047), *GAL-CDC14 3GFP-RAS2* (YASD2049) and *GAL-SIC1ΔNT GAL-CDC14 3GFP-RAS2* (YASD2041) were grown as in Figure 1A. Red arrows denote the budneck region of cells with divided cytoplasm. The scale bar corresponds to 5 μm. **(B)** Analogous experiments were performed with *GAL-SIC1ΔNT NLS-NES-GFP MYO1-TOMATO* (YASD1612), *GAL-CDC14 NLS-NES-GFP MYO1-TOMATO* (YASD2005) and *GAL-SIC1ΔNT GAL-CDC14 NLS-NES-GFP MYO1-TOMATO* (YASD2001).

dbf2-2 dbf20Δ cells (Figure 7B), consistent with a previous study showing that Dbf2 and Dbf20 are important for the onset of cytokinesis even when Clb-CDK was inactivated by stable Sic1 (Meitinger *et al*, 2010).

We examined the behaviour of Myo1 in the same set of strains, and observed contraction and disappearance of the Myo1 ring in about 70% of control cells within 75' of inducing *GAL-SIC1ΔNT*, whereas contraction of the Myo1 ring was delayed considerably in *cdc14-1 cdc15-2* and *dbf2-2 dbf20Δ* (Figure 7C and D). These data are consistent with previous studies, indicating that MEN proteins are important for the contraction of the actomyosin ring. Importantly,

however, we found that nuclear accumulation of NLS-NES-GFP was defective in both *cdc14-1 cdc15-2* and *dbf2-2 dbf20Δ* (Figure 7C and D). This did not reflect failure of Sic1ΔNT to inactivate mitotic CDK *per se*, as other events dependent upon Clb-CDK inactivation such as relocalisation of the Inn1 protein to the budneck still occurred in both control cells and *cdc14-1 cdc15-2* following inactivation of Clb-CDK (Supplementary Figure 9), analogous to previous observations of *dbf2-2 dbf20Δ* cells expressing stable Sic1 (Meitinger *et al*, 2010). Instead, our data indicated that both *cdc14-1 cdc15-2* and *dbf2-2 dbf20Δ* cells are defective in the action of cytoplasmic Cdc14, which is important for the regulation of the NLS-NES-GFP cassette. This is analogous to the previous observation that Cdc14 contributes to the efficient accumulation of the MCM2-7 DNA helicase in the nucleus during late mitosis (Zhai *et al*, 2010).

These findings suggested that accumulation of Cdc14 in the cytoplasm might be a critical aspect of the mechanism by which Dbf2/Dbf20 promote the onset of cytokinesis. To test this idea we took advantage of the *cdc14-BP1,2A* allele, which contains mutations of basic amino acids that are critical for the function of the carboxy-terminal Nuclear Localisation Sequence, which is normally phosphorylated and inactivated during late mitosis by Dbf2-Mob1 (Mohl *et al*, 2009). As shown in Figure 8Ai, combined expression of *GAL-SIC1ΔNT* and *GAL-cdc14-BP1,2A* largely restored cytokinesis in *dbf2-2 dbf20Δ* to the level seen in control cells, albeit with a slight delay. In contrast, expression of wild-type Cdc14 was less efficient at rescuing the cytokinesis defect of *dbf2Δ dbf2-2* (Figure 8Aii). These data indicate that the retention of cytoplasmic Cdc14 is a key feature of the mechanism by which the MEN promotes the onset of cytokinesis in budding yeast.

Discussion

Our data indicate that budding yeast cells induce cytokinesis at the end of mitosis by reversing the effects of mitotic CDK, similar to the situation in animal cells and probably most other eukaryotes. During anaphase, the MEN helps release Cdc14 from the nucleolus and then drives its accumulation in the cytoplasm, leading to efficient inactivation of the remaining pool of mitotic CDK. The MEN also helps initiate cytokinesis independently of CDK inactivation *per se* (Lippincott *et al*, 2001; Luca *et al*, 2001; Menssen *et al*, 2001; Meitinger *et al*, 2010, 2011), but our data indicate that a central feature of this regulation is the dephosphorylation of CDK target proteins by cytoplasmic Cdc14. Identifying the relevant targets of Cdc14 will be a key challenge for future studies of cytokinesis in budding yeast, probably involving multiple factors phosphorylated by mitotic CDK (and perhaps other kinases too).

We have identified a novel mechanism by which Cdc14 helps regulate cytokinesis in budding yeast, namely by preventing the premature polarisation of growth to a new bud site at the end of mitosis. Blocking polarised growth allows the efficient action of the contractile actomyosin ring by preventing diversion of the actin cytoskeleton to the site of a new bud. The relevant targets of Cdc14 remain to be determined, but one possibility is that Cdc14 directly counteracts the accumulation of Cln-CDK at the end of mitosis following inactivation of Clb-CDK, by blocking the positive

Figure 7 Defective cytokinesis following Clb-CDK inactivation in MEN mutants is associated with defects in Cdc14 function. **(A)** GAL-SIC1ΔNT 3GFP-RAS2 (YASD1870) and *cdc14-1 cdc15-2* GAL-SIC1ΔNT 3GFP-RAS2 (YASD1852) were arrested with nocodazole at 24°C in medium containing raffinose, before switching cells to 37°C for 60'. An aliquot of cells was released from nocodazole at this point to confirm that inactivation of Cdc14 and Cdc15 caused cells to arrest after anaphase (not shown). The remainder of the culture was switched to medium containing galactose and nocodazole, and incubation continued at 37°C for the indicated times. Scale bars = 5 μm. **(B)** *dbf2-2 dbf20Δ* GAL-SIC1ΔNT 3GFP-RAS2 (YASD2154) was compared with the control strain in a similar experiment. **(C)** An analogous experiment to that in **(A)** was performed with GAL-SIC1ΔNT NLS-NES-GFP MYO1-TOMATO (YASD1612) and *cdc14-1 cdc15-2* GAL-SIC1ΔNT NLS-NES-GFP MYO1-TOMATO (YASD1926). **(D)** *dbf2-2 dbf20Δ* GAL-SIC1ΔNT NLS-NES-GFP MYO1-TOMATO (YASD2111) was compared with the control strain (YASD2093) in a similar experiment.

feedback loop that allows Cln1-2 to stimulate their own expression (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Ogas *et al*, 1991). Another mechanism could be via direct reversal of Cln-CDK phosphorylations that promote polar growth or inhibit cytokinesis in other ways. Once cytokinesis has been completed and Cdc14 returns to the nucleolus, the changes in cell size put new blocks on the activation of Cln-CDK, preventing a new bud from forming until the end of the next G1-phase. We note that this role of Cdc14 would be somewhat analogous to the action of the SIN in fission yeast, which blocks polarised growth during cytokinesis by interfering with the action of the NDR family kinase Orb6 (Ray *et al*, 2010).

In mammalian cells, mitotic CDK and Polo kinase play opposing roles in regulating the ability of RhoA to drive the assembly of the cleavage furrow. Mitotic CDK phosphorylates the RhoGEF Ect2 and prevents its association with the Cyk4 protein, an interaction necessary for recruitment of Ect2 to its site of action at the central spindle (Yuce *et al*, 2005). In contrast, phosphorylation of Cyk4 by Polo stimulates its interaction with Ect2, following dephosphorylation of the latter at the CDK-mediated site (Petronczki *et al*, 2007; Wolfe *et al*, 2009). In budding yeast, the Cdc5 orthologue of Polo activates Rho1, in part through Polo-dependent phosphorylation of RhoGEFs (Yoshida *et al*, 2006). Interestingly, Cln-CDK also phosphorylates the RhoGEF

Tus1 to activate Rho1 during G1-phase (Kono *et al*, 2008). It will be important in future studies to determine whether Clb-CDK prevents Rho1 from activating the actomyosin ring before the end of mitosis in budding yeast, and whether such regulation controls the action of Cdc5 at the budneck. It seems likely that Clb-CDK will regulate cytokinesis via multiple targets, and we note that other key regulators of the actomyosin ring are known to be targets of Clb-CDK, such as the formin Bni1 that is a target of Rho1, and the IQGAP protein Iqg1/Cyk1 (Holt *et al*, 2009), although the significance of these phosphorylations for the regulation of cytokinesis remains to be explored in future studies.

Cytokinesis and septation are intimately related in budding yeast (Vallen *et al*, 2000), and the stability of the actomyosin ring during contraction is dependent upon formation of the primary septum by the chitin synthase Chs2 (VerPlank and Li, 2005). Previous work showed that the recruitment of Chs2 to the budneck is negatively regulated by Clb-CDK phosphorylation (Zhang *et al*, 2006), which serves to trap Chs2 in the endoplasmic reticulum until the end of mitosis (Teh *et al*, 2009). Moreover, the Inn1 and Cyk3 proteins activate cytokinesis (Korinek *et al*, 2000; Sanchez-Diaz *et al*, 2008), probably by promoting formation of the primary septum (Nishihama *et al*, 2009), and both factors move to the budneck at the end of mitosis in response to the inactivation of mitotic CDK. It thus seems likely that mitotic

Figure 8 Cytoplasmic Cdc14 largely rescues the cytokinesis defects of *dbf2-2 dbf20Δ*. (A) (i) *GAL-SIC1ΔNT 3GFP-RAS2* (YASD2047), *dbf2-2 dbf20Δ GAL-SIC1ΔNT 3GFP-RAS2* (YASD2154) and *dbf2-2 dbf20Δ GAL-SIC1ΔNT GAL-cdc14-BP1,2A 3GFP-RAS2* (YASD2179) were grown and processed as in Figure 7, to monitor progression through cytokinesis. (ii) Analogous experiments were performed with *dbf2-2 dbf20Δ GAL-SIC1ΔNT 3GFP-RAS2* (YASD2154), *dbf2-2 dbf20Δ GAL-SIC1ΔNT GAL-CDC14 3GFP-RAS2* (CC8336) and *dbf2-2 dbf20Δ GAL-SIC1ΔNT GAL-cdc14-BP1,2A 3GFP-RAS2* (YASD2179). (B) Representative images from the experiments in (A). Red arrows denote cells with divided cytoplasm at the budneck. Scale bars = 2 μ m.

CDK controls cell division by inhibiting septation as well as assembly of the actomyosin ring.

Not all targets of mitotic CDK are dependent upon Cdc14 for their dephosphorylation, and other phosphatases are able to reverse CDK phosphorylations in other species, explaining why Cdc14 orthologues are not universally required for mitotic exit and cytokinesis (Stegmeier and Amon, 2004; Mocciaro and Schiebel, 2010). The regulation of cytokinesis by mitotic CDK might still involve some similar principles in different species, however, even if the identity of the relevant phosphatases has changed over evolution. There are clear parallels (as well as differences) between the MEN in budding

yeast, and the SIN in fission yeast, despite the fact that the fission yeast Clp1/Flp1 phosphatase, orthologous to Cdc14, is not essential for septation. The fission yeast SIN mediates export of the Clp1/Flp1 phosphatase to the cytoplasm and this is important for a 'cytokinesis checkpoint', though not essential for cytokinesis *per se* (Cueille *et al*, 2001; Trautmann *et al*, 2001; Mishra *et al*, 2004; Chen *et al*, 2008). Moreover, it seems likely that the reversal of key CDK phosphorylations on cytokinesis proteins is an important early step in the assembly of the actomyosin ring in fission yeast, as mutations of putative CDK sites in the F-BAR protein Cdc15 induces premature recruitment of type II myosin and actin

'spots' to the mid-zone of cells in G2-phase, without inducing full assembly of an actomyosin ring (Roberts-Galbraith *et al*, 2010), and premature inactivation of CDK in metaphase caused a fraction of cells to undergo cytokinesis (He *et al*, 1997; Guertin *et al*, 2000; Dischinger *et al*, 2008). The SIN is required during mitosis for recruitment of Cdc15 to the mid-zone (Hachet and Simanis, 2008), probably by promoting the accumulation of cytoplasmic Clp1/Flp1 that dephosphorylates Cdc15 (Johnson *et al*, 2012).

As shown in previous studies, the MEN proteins and Cdc14 all localise briefly to the budneck at the end of mitosis (Frenz *et al*, 2000; Xu *et al*, 2000; Luca *et al*, 2001; Yoshida and Toh-e, 2001; Hwa Lim *et al*, 2003; Bembenek *et al*, 2005), and it seems clear that the MEN does indeed control septum formation independently of promoting the dephosphorylation of CDK targets. Dbf2-Mob1 controls the localisation of Hof1 at the budneck, and probably its degradation too (Blondel *et al*, 2005; Meitinger *et al*, 2011). A very recent report also showed that Dbf2 phosphorylates the Chitin Synthase II protein that makes the primary septum (Oh *et al*, 2012). Nevertheless, our data suggest that such regulation controls the septation machinery (and perhaps the disassembly of the contracted actomyosin ring) but does not represent the core mechanism that controls assembly and contraction of the actomyosin ring and the initiation of cytokinesis. We note that previous studies have shown that an increase in the pool of free Cdc14, or over-expression of Sic1, is partially able to suppress a wide range of mutations in MEN proteins (Jaspersen *et al*, 1998; Shou *et al*, 1999; Shou and Deshaies, 2002). Strikingly, low copy expression of the *cdc14-BP1,2A* allele from the native promoter is able to suppress deletion of the *CDC15* gene (Mohl *et al*, 2009), further indicating that cytoplasmic Cdc14 is the major output of the MEN pathway in budding yeast. One interesting possibility for future study would be that the MEN proteins control the recruitment of Cdc14 phosphatase to the budneck, analogous to their roles in releasing Cdc14 from the nucleolus and allowing it to enter the cytoplasm, helping thereby to regulate the dephosphorylation of key CDK targets that control cytokinesis.

Materials and methods

Yeast strains and growth

The strains used in this study are described in Supplementary Table 1 and are all based on the W303 background. Cells were grown in rich medium (1% Yeast Extract, 2% Peptone) supplemented with 2% sugar (glucose, raffinose, or galactose; producing medium called YPD, YPRaff and YPGal, respectively). We synchronised cells in G1-phase by incubation with 7.5 µg/ml α -factor for at least one generation time, and used 5 µg/ml nocodazole to arrest cells in G2-M-phase. To induce degradation of Myo1-

aid via the 'auxin degron' system (Nishimura *et al*, 2009), we added 500 µM indole-3-acetic acid and 500 µM 1-naphthaleneacetic acid to the cell culture medium.

Microscopy

Fluorescence and electron microscopy was performed as described previously (Sanchez-Diaz *et al*, 2008), except that time-lapse video microscopy was performed using a CellASIC ONIX Microfluidic Y2 Plate (Image Solutions (UK) Ltd.). To visualise the actin cytoskeleton, we treated cells with 3.7% formaldehyde and 0.1% Triton X-100 for 10' at room temperature, washed twice with PBS, and then fixed for a further 60' with 3.7% formaldehyde, before incubation with 0.05 U/µl rhodamine-phalloidin (Invitrogen) for 90'. Cells were then washed and processed for image capture. To study Ace2-GFP and Cdc14-GFP, we examined nine z-sections with a spacing of 0.375 µm. To visualise actin, Myo1 and NLS-NES-GFP, we used 21 z-sections with a spacing of 0.2 µm. Finally, for GFP-Ras2 we used 27 z-sections with a spacing of 0.15 µm.

Images were viewed using Imaris (Bitplane) or Image J (NIH) software, and for quantification we examined 100 cells per sample unless specified otherwise in the text. Fluorescence microscopy images of GFP-Ras2 were deconvolved using Huygens (SVI) according to the 'Quick Maximum Likelihood Estimation' method and a measured point spread function. To determine whether cells had divided their cytoplasm, we examined the GFP-Ras2 signal at the budneck in every optical section (z-level). The figures show a single optical section corresponding to the level of the budneck.

Flow cytometry

DNA content was measured by flow cytometry as described previously (Kanemaki *et al*, 2003).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We are grateful to Cancer Research UK for funding this work. We thank Joachim Li and Elmar Schiebel for plasmids, Asli Devrekanli, Magda Foltman, Masato Kanemaki, Clive Price and Frank Uhlmann for strains, and Steve Bagley, Achille Dunne and Gabriel Bretones for assistance with imaging software. ASD joined the University of Cantabria as a recipient of a Ramon y Cajal contract (call 2010) and now receives funding from the Cantabria International Campus and via grant BFU2011-23193 from the Spanish 'Ministerio de Economía y Competitividad' (co-funded by the European Regional Development Fund).

Author contributions: ASD performed all the experiments and made all the figures, with the following exceptions: PJN was responsible for Figures 1Ai, Bi and 4B, C and 7A, Supplementary Figures 2B, 3 and 7; SM performed the electron microscopy analysis in Figure 1C and Supplementary Figure 4; KL performed the experiment in Supplementary Figure 9, and helped to analyse and quantify all the microscopy data. ASD and KL designed all the experiments, and KL wrote the manuscript together with ASD.

Conflict of interest

The authors declare that they have no conflict of interest.

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