The budding yeast PP2A<sup>Cdc55</sup> protein phosphatase prevents the onset of anaphase in response to morphogenetic defects

Elena Chirolí, Valentina Rossio, Giovanna Lucchini, and Simonetta Piatti

Introduction

Balanced chromosome partitioning during anaphase relies on the prior establishment of sister chromatid cohesion, which takes place concomitantly to DNA replication. Sister chromatids are tethered together by cohesin, which is displaced from chromosomes through cleavage of its Mcd1 subunit by the separase protease. Separase is in turn inhibited, up to this moment, by securin. Budding yeast cells respond to morphogenetic defects by a transient arrest in G2 with high securin levels and unseparated chromatids. We show that neither securin elimination nor forced cohesin cleavage is sufficient for anaphase in these conditions, suggesting that other factors contribute to cohesion maintenance in G2. We find that the protein phosphatase PP2A bound to its regulatory subunit Cdc55 plays a key role in this process, uncovering a new function for PP2A<sup>Cdc55</sup> in controlling a noncanonical pathway of chromatid cohesion removal.

Results

The morphogenesis checkpoint prevents sister chromatid separation independently of Pds1

High levels of a truncated version of the budding yeast p21-activated kinase Cla4 (Cla4t) activate the morphogenesis checkpoint by
inhibiting endogenous Cla4 and its paralogue Ste20 (Chiroli et al., 2003), which share essential functions in bud neck formation, septin ring assembly, and cytokinesis (Johnson, 1999). Upon CLA4t overexpression from the GAL1 promoter, haploid yeast cells arrest with wide bud necks, replicated chromosomes, undivided nuclei, short metaphase spindles, and high levels of the securin Pds1 (Chiroli et al., 2003). In addition, they markedly delay activation of the Polo kinase Cdc5 (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200609088/DC1), suggesting that they arrest in G2.

As PDS1 deletion is sufficient to allow anaphase in most mutants arresting in mitosis, we asked whether it could bypass the G2 arrest caused by high Cla4t levels. Elutriated G1 cells of a pds1Δ strain with four copies of the GAL1-CLA4t construct integrated in the genome (4X GAL1-CLA4t pds1Δ) were released into the cell cycle in the presence of galactose. As expected, DNA replication (Fig. 1 A) and bipolar spindle formation (Fig. 1 B) took place normally in these conditions, whereas bud neck formation was abnormal because of CLA4t overexpression (not depicted). Surprisingly, pericentromeric chromosomal sequences marked by a tet operator array that binds TetR-GFP (Michaelis et al., 1997) could not separate in these cells (Fig. 1 B), indicating that sister chromatid separation did not occur. Nuclear division and spindle elongation did not take place throughout the course of the experiment (Fig. 1, B and C), similar to 4X GAL1-CLA4t cells under the same conditions (Fig. 1, A, B, and C). Thus, deletion of PDS1 is not sufficient to bypass the G2 arrest caused by high levels of Cla4t.

As shown in Fig. 1 (D–F), latrunculin-A (Lat-A), which activates the morphogenesis checkpoint by depolymerizing the actin cytoskeleton, induced, like Cla4t, a securin-independent G2 arrest. In fact, cells released from a G1 arrest in the presence of Lat-A did not bud (Fig. 1 E) but replicated DNA (Fig. 1 D) and formed bipolar spindles (Fig. 1, E and F). However, neither wild-type nor pds1Δ cells underwent sister chromatid separation, nuclear division, or spindle elongation (Fig. 1, E and F). In contrast, the same events took place promptly in the morphogenesis checkpoint–defective swe1Δ cells, which also exited mitosis and entered a new round of DNA replication, as indicated by the appearance of 4C DNA contents (Fig. 1 D). Altogether, these data indicate that the morphogenesis checkpoint appears to prevent the onset of anaphase independently of securin.

CLA4t overexpression does not impair securin-mediated nuclear import of separase

Besides its inhibitory function, securin also has a positive role in separase activation in several eukaryotic systems, prompting us to test whether Cla4t overproduction might impair Pds1 interaction with the Esp1 separase and/or Esp1 nuclear import. Wild-type, 4X GAL1-CLA4t, and 4X GAL1-CLA4t swe1Δ cells expressing HA-tagged Pds1 (Pds1-HA) and myc-tagged Esp1 (Esp1-myc18) were grown in raffinose, arrested in G1 by α-factor, and released in the presence of galactose, followed by the analysis of Pds1 and Esp1 nuclear localization and physical interaction. As shown in Fig. 2 A, budding was delayed in 4X GAL1-CLA4t cells compared with wild type, but kinetics of Pds1-HA and Esp1-myc18 nuclear accumulation were similar in the two strains.
Thus, Pds1 can still act as an Esp1 molecular chaperone in the presence of high levels of Cla4t. Accordingly, similar levels of Esp1-myc18 were immunoprecipitated with Pds1-HA from both wild-type and 4X GAL1-CLA4t cell extracts (Fig. 2 B).

**Cohesin cleavage is not sufficient for execution of anaphase in the presence of high Cla4t levels**

Although lack of securin did not allow chromatid separation upon morphogenesis checkpoint activation, ectopic cohesin cleavage could be expected to trigger nuclear division in the same conditions. We engineered 4X GAL1-CLA4t cells to express a Mcd1–tobacco etch virus (TEV) variant, where the Esp1 cleavage site at position 268 is replaced by the recognition sequence for the TEV protease (Uhlmann et al., 2000). We then introduced in the same cells the TEV protease coding sequence under the control of the GAL1 promoter. These cells grow normally under uninduced conditions because the Mcd1-TEV variant can be cleaved by separase at position 180, whereas it is cleaved and fully removed from chromosomes upon TEV induction even if separase is inactive. Small G1 cells of this strain were elutriated and released in the presence of galactose to trigger expression of both Cla4t and TEV. Remarkably, nuclear division did not take place (Fig. 3 A), suggesting that cohesin cleavage might be insufficient to allow chromosome segregation in 4X GAL1-CLA4t cells. Conversely, as previously reported (Uhlmann et al., 2000), cohesin cleavage by the TEV protease was sufficient to trigger anaphase in cells depleted for Cdc20 (Fig. 3 B), the APC regulatory subunit essential for Pds1 proteolysis and anaphase onset (Peters, 2006). Thus, cohesin cleavage seems to be sufficient to trigger anaphase in metaphase-arrested cells but not in cells arrested in G2 by the morphogenesis checkpoint.

Because it was formally possible that the lack of nuclear division in 4X GAL1-CLA4t MCD1-TEV cells was due to inefficient cohesin cleavage, we analyzed the kinetics of cohesin cleavage by the TEV protease in 4X GAL1-CLA4t versus wild-type cells after release from G1 in the presence of galactose. Full length of Mcd1-TEV tagged with 3 HA epitopes at the C terminus (Mcd1-HA3) and its cleavage product by separase (at position 180) were detectable in both strains in cycling cells and at time 0 (Fig. 3 C). Upon galactose addition, kinetics of TEV production, as well as appearance of the TEV-induced Mcd1-HA3 cleavage product (at position 268), were similar in the two strains. However, disappearance of full-length Mcd1 and its separase-induced cleavage product, which can both be cleaved by TEV, was slower in 4X GAL1-CLA4t than wild-type cells (Fig. 3 C). This might be due to delayed activation of the Polo/Cdc5 kinase, which stimulates Mcd1 cleavage (Alexandru et al., 2001), in 4X GAL1-CLA4t versus wild-type cells. In spite of that, most, if not all, Mcd1-HA3 was cleaved by 3 h in 4X GAL1-CLA4t cells, but nuclear division occurred only in a small fraction of them (Fig. 3 C). In contrast, >75% of wild-type cells had accomplished nuclear division under the same conditions. Therefore, other mechanisms besides cohesin-mediated sister chromatid cohesion likely contribute to prevent chromosome segregation when the morphogenesis checkpoint is active.

**The spindle is functional under morphogenesis checkpoint activation**

Because mitotic Cdks regulate spindle assembly and microtubule dynamics, the morphogenesis checkpoint might delay nuclear division through spindle misfunction. Upon bipolar attachment of sister kinetochores to microtubules, spindle forces overwhelm centromeric cohesion, leading to precocious separation of sister
centromeres before anaphase (Goshima and Yanagida, 2000), thus providing a readout for spindle function. We found that sister centromeres of chromosome 15 could separate concomitantly with spindle formation in the presence of Lat-B (Fig. 4 B), suggesting that spindle forces are normal.

Because kinetochore inactivation by the ndc10-1 mutation prevents kinetochore–microtubule attachment without affecting spindle formation and elongation (Goh and Kilmartin, 1993), we also asked whether spindle elongation could take place in ndc10-1 cells under morphogenesis checkpoint activation. We induced morphogenetic defects by using a temperature-sensitive cdc24 mutation, which alters a guanine-nucleotide exchange factor for the GTPase Cdc42 that is required for budding (Johnson, 1999). Upon release of synchronized G1 cells at 37° C, cdc24 cells arrested in G2 as unbudded with undivided nuclei and short metaphase spindles. Lack of kinetochore attachment in cdc24 ndc10-1 cells was sufficient to allow spindle elongation (Fig. 4 A), suggesting that spindle dynamics is not affected by morphogenetic defects. Therefore, residual sister chromatid cohesion, rather than a misfunctional spindle, is likely responsible for preventing chromosome segregation in the absence of Mcd1 upon morphogenesis checkpoint activation.

The phosphatase PP2ACdc55 prevents sister chromatid separation upon morphogenesis checkpoint activation

Cdc55 is one of the two regulatory subunits of yeast protein phosphatase PP2A and was previously implicated in maintaining sister chromatid cohesion in response to spindle defects (Minshull et al., 1996). This prompted us to test whether CDC55 deletion could allow sister chromatid separation in Cla4t-overexpressing cells. Elutriated G1 cells of a 4X GAL1-CLA4t cdc55 Δ strain carrying the tetO/tetR-GFP constructs for monitoring sister chromatid separation were released into the cell cycle in the presence of galactose.

As shown in Fig. 5 A, deletion of CDC55 partially rescued the cytokinetic defects caused by high Cla4t levels, indicated by reaccumulation of a small fraction of cells with 1C DNA contents at the end of the first cell cycle. Most cells, however, displayed abnormal bud necks characteristic of 4X GAL1-CLA4t cells. In spite of that, they underwent efficient sister chromatid separation.

Figure 3. Mcd1 cleavage is not sufficient for nuclear division upon Cla4t overexpression. (A) GAL1-CLA4t cells expressing Mcd1-TEV and GAL1-TEV (ySP5871) were grown in YEPR at 25°C. Elutriated small G1 cells were released in YEPRG at 25°C (time 0). At the indicated time points, cell samples were analyzed for DNA contents (top left), budding, and nuclear division (top right). Micrographs represent cells at the end of the experiment. (B) MET3-CDC20 MCD1-TEV GAL1-TEV cells (ySP5870) were grown in raffinose medium lacking methionine. Elutriated G1 cells were released in YEPR containing 2 mM methionine (time 0). Cell samples were analyzed as in A. (C) MCD1-TEV (ySP4448) and GAL1-CLA4t MCD1-TEV (ySP5871) cells were grown in YEPR at 25°C, arrested in G1 by α-factor, and released in YEPRG at 25°C at time 0. Cells were collected at the indicated times for Western blot analysis with anti-HA (Mcd1) and anti-myc (TEV) antibodies (left), FACS analysis of DNA contents (not depicted), and kinetics of nuclear division and bipolar spindle formation (right). Swi6 was used as loading control.

Figure 4. Spindle dynamics is not affected by the morphogenesis checkpoint. (A) cdc24 (ySP305) and cdc24 ndc10-1 (ySP6207) cell cultures were arrested in G1 by α-factor and released at 37°C (time 0). Cells were analyzed at the indicated times for DNA contents (not depicted), budding, spindle formation/elongation, and nuclear division (graphs). Micrographs represent cells at 150 min after release. (B) Wild-type cells with tagged CEN15 (ySP1717) were arrested in G1 by α-factor and released in the presence of Lat-B (time 0). Cells were analyzed at 1-h intervals for DNA contents (not depicted), CEN15 separation, tubulin immunostaining, and nuclear division.
and nuclear division (Fig. 5, B and C), suggesting that Cdc55 prevents anaphase onset when p21-activated kinases are inactive.

Nuclear division could also be induced in 4X GAL1-CLA4t cells by expressing a mutant form of the Pph21 catalytic subunit (Pph21-L369D; Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200609088/DC1) that was shown to preferentially fail to interact with Cdc55 (Jiang, 2006). Therefore, chromatid cohesion upon morphogenesis checkpoint activation requires the protein phosphatase PP2A bound to Cdc55.

The catalytic and structural PP2A subunits can form mutually exclusive complexes with either one of the regulatory subunits Cdc55 and Rts1 (Evans and Hemmings, 2000). PP2ARts1 and its human counterpart have recently been shown to prevent precocious dissociation of centromeres both in mitosis and in meiosis I (Kitajima et al., 2006; Riedel et al., 2006). In an experiment similar to the one described for cdc55Δ, we found that pericentromeric sequences could not separate in the majority of 4X GAL1-CLA4t rts1Δ cells (Fig. 5 B). When pericentromeric regions did split (~25% of the cells), GFP dots were always found very close to each other (Fig. 5 C) and nuclear division was negligible (Fig. 5 B), suggesting that PP2ARts1 plays a minor role, compared with PP2ACdc55, in controlling chromatid cohesion under these circumstances.

Because Cdc55 and Rts1 compete for binding to the other PP2A subunits, sister chromatid separation in the absence of Cdc55 could be ascribed to increased levels of the PP2ARts1 complex. To investigate this possibility, we asked whether 4X GAL1-CLA4t cells lacking both Cdc55 and Rts1 could undergo anaphase. Elutriated G1 cells of the 4X GAL1-CLA4t cdc55Δ rts1Δ strain released in the presence of galactose progressed into the cell cycle very slowly, as a result of budding and replication defects (Fig. 5, A and B). In spite of that, those that could finish chromosome replication underwent efficient dissociation of sister chromatids and nuclear division (Fig. 5 B), suggesting that anaphase onset in 4X GAL1-CLA4t cells lacking Cdc55 is not due to increased levels of PP2ARts1 activity.

We then asked whether PP2ACdc55 also controls sister chromatid cohesion in other conditions that activate the morphogenesis checkpoint. Wild-type and cdc55Δ cells were arrested in G1 by α-factor and then released in the presence of Lat-A. In these conditions, neither wild-type nor cdc55Δ cells budded throughout the course of the experiment (Fig. 5 D).

Figure 5. PP2ACdc55 prevents sister chromatid separation upon activation of the morphogenesis checkpoint. (A–C) Strains with the indicated genotypes (ySP5115, ySP5112, and ySP5165) were grown at 30°C in YEP. Elutriated G1 cells were released in YEPG at 25°C at time 0. Cell samples were analyzed at the indicated times for DNA contents (A), budding, sister chromatid separation, and nuclear division (B). (C) Micrographs represent sister chromatid separation at 285 min (4X GAL1-CLA4t cdc55Δ) and 300 min (4X GAL1-CLA4t rts1Δ). (D) Wild-type [wt; ySP3575] and cdc55Δ [ySP5068] cells were grown in YEPD at 30°C, arrested in G1 by α-factor, and released at 16°C. Cells were analyzed at the indicated times for DNA contents (histograms), budding, sister separation, nuclear division, and spindle formation/elongation (graphs). (E) Wild-type (ySP3575) and cdc55Δ (ySP5068) cells were arrested in G1 by α-factor and released at 16°C. Cells were analyzed at the indicated times for budding, sister chromatid separation, spindle formation/elongation, and nuclear division.
As expected, wild-type cells accumulated with 2C DNA contents, unsevered sister chromatids, undivided nuclei, and short metaphase spindles (Fig. 5, D and E). Strikingly, sister chromatids separated efficiently in cdc55Δ cells under the same conditions, thus allowing spindles to elongate and nuclei to divide (Fig. 5, D and E). Finally, because CDC55 deletion causes by itself morphogenetic defects and Swe1 stabilization at low temperatures (Healy et al., 1991; Yang et al., 2000), we asked whether the cdc55Δ mutant could separate sister chromatids at 16°C. At this temperature, cdc55Δ cells showed prominent morphogenetic defects (not depicted), but nevertheless could split chromatids and divide nuclei, albeit with a delay compared with wild-type cells (Fig. 5 F).

To directly compare the effects of cohesin inactivation and lack of PP2AIdc55 on sister chromatid separation of cells with morphogenetic defects, we used the temperature-sensitive scc1-73 allele, which inactivates Mcd1 and advances sister chromatid separation relative to wild type at the restrictive temperature (Michaelis et al., 1997). G1-arrested cdc24 cells either lacking CDC55 or carrying the scc1-73 allele were released at 37°C. cdc24 scc1-73 cells could efficiently separate chromosome V arm sequences, although with a delay compared with scc1-73 cells, but did not elongate spindles or divide nuclei (Fig. 6 A). In contrast, cdc24 cdc55 cells underwent complete chromosome segregation under the same conditions (Fig. 6 A). Accordingly, the distance between separating chromatids at 150 min after release was significantly higher in cdc24 cdc55 cells than in cdc24 scc1-73 cells (Fig. 6 B). Therefore, some residual chromatid cohesion likely persists even when cohesin is inactivated and PP2AIdc55 plays a crucial role in controlling sister chromatid separation when the morphogenes checkpoint is activated.

Mcd1 cleavage does not occur in cdc55Δ cells undergoing anaphase under morphogenesis checkpoint activation

Although ectopic cohesin cleavage did not allow nuclear division during morphogenesis checkpoint activation, CDC55 deletion might still allow anaphase onset in these conditions through cohesin cleavage. To test this possibility, cdc24, cdc24 swe1Δ, and cdc24 cdc55Δ cells were arrested in G1 by α-factor and then released at 37°C, followed by analysis of cell cycle parameters (Fig. 7, A and B) and Mcd1 cleavage by separate (Fig. 7 C). As expected, cdc24 cells arrested with 2C DNA contents, unseparated sister chromatids, and metaphase spindles, whereas most cdc24 swe1Δ cells underwent anaphase and spindle elongation and eventually exited mitosis and rereplicated their chromosomes, accumulating DNA contents higher than 2C (Fig. 7, A and B), suggesting that lack of Swe1 overrides cells’ ability to sense morphogenetic defects. Interestingly, cdc24 cdc55Δ cells could also undergo anaphase in the same conditions, albeit with a delay compared with cdc24 swe1Δ cells, but remained mostly arrested with 2C DNA contents. The Mcd1 cleavage product, which was readily apparent in cdc24 swe1Δ cells and preceded sister chromatid separation, was mostly negligible in cdc24 cdc55Δ cells (Fig. 7 C). Nevertheless, chromatin staining of Mcd1 after chromosome spreading revealed that cohesin remained bound to chromatin in wild-type cells (not depicted) but had dissociated from the chromosomes in nuclei of cdc55Δ cells that underwent anaphase (Fig. 7 D). Thus, sister chromatid separation and Mcd1 dissociation from chromosomes in cdc55Δ cells under morphogenesis checkpoint activation do not seem to correlate with separate-dependent cleavage of cohesin. Accordingly, the Mcd1 cleavage product was not detectable in 4X GAL1-CLA4t cdc55Δ cells undergoing anaphase in the presence of galactose, similar to 4X GAL1-CLA4t cells (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200609088/DC1), and Mcd1 disappeared from the nuclei of 4X GAL1-CLA4t cdc55Δ cells in anaphase (Fig. S3 E). Mcd1 displacement from chromatin did not correlate with increased Mcd1 phosphorylation, which could instead be detected as electrophoretic mobility shift in nocodazole-arrested cells (Fig. S3 D). It is interesting to note that SWE1 deletion in Cla4t-overexpressing cells caused rapid Pds1 and Clb2 proteolysis, as well as appearance of the Mcd1 cleavage product, whereas Pds1 and Clb2 remained mostly stable upon deletion of CDC55 (unpublished data).

Unlike in cdc55Δ cells under morphogenesis checkpoint activation, sister chromatid separation in nocodazole-treated cdc55Δ cells was accompanied by Pds1 degradation Mcd1 cleavage, although with a delay compared with the spindle checkpoint-defective mad2Δ cells (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200609088/DC1).

Figure 6. Inactivation of PP2AIdc55, but not of cohesin, allows nuclear division in the presence of morphogenetic defects. Strains with the indicated genotypes (ysp601, ysp818, yp6236, yp6241, and yp6214) were arrested in G1 by α-factor at 25°C and released at 37°C (time 0). Cells were analyzed at the indicated times for DNA contents (not depicted), budding, sister chromatid separation, spindle formation/elongation, and nuclear division (A). Distances between separated chromatids were measured at time point 150 min (n = 180) with MetaMorph software.

A

B

C

D

E

F

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Therefore, PP2ACdc55 contributes to maintaining sister chromatid cohesion in nocodazole by impinging on the same targets of the spindle assembly checkpoint, as recently suggested by others (Yellman and Burke, 2006). In contrast, PP2ACdc55 likely prevents sister chromatid separation in G2 through a different mechanism.

Sister chromatid dissociation induced by lack of PP2ACdc55 does not require the Cdc14 phosphatase, Polo kinase, and condensin but can be reversed by topoisomerase II inhibition

PP2ACdc55 has recently been shown to prevent Cdc14 early anaphase release from the nucleolus through Net1 dephosphorylation (Queralt et al., 2006). Cdc14 can in turn trigger Pds1 proteolysis in nocodazole-arrested cells (Visintin et al., 1998), and this mechanism has been proposed to be responsible for the precocious dissociation of sister chromatids in nocodazole-treated cdc55Δ cells (Yellman and Burke, 2006). We therefore asked whether Cdc14 was released from the nucleolus in cdc55Δ cells with morphogenetic defects and necessary for their onset of anaphase. Wild-type and cdc55Δ cells were arrested in G1 by α-factor and released in the presence of Lat-B. In situ immunostaining of Cdc14 showed that anaphase took place in cdc55Δ cells before Cdc14 release from the nucleolus (Fig. 8 A).

In addition, analysis of cdc55Δ anaphase cells 150 min after release revealed that a high fraction of them (68.3%; n = 120) had undergone anaphase with Cdc14 in the nucleolus (Fig. 8 B), suggesting that premature Cdc14 release is not responsible for sister chromatid separation in these cells.

To test whether Cdc14 was required for the onset of anaphase in cdc55Δ mutants with morphogenetic defects, we inactivated Cdc14 in cdc24 cdc55Δ cells with the temperature-sensitive
cdc14-3 allele. As a control for Cdc14 inactivation, we analyzed the subcellular localization of the Swi5 transcription factor, whose nuclear import in telophase is strictly dependent on its dephosphorylation by Cdc14 (Visintin et al., 1998). Cell cultures of cdc24 cdc55Δ and cdc24 cdc55Δ cdc14-3 strains expressing a myc-tagged Swi5 protein were synchronized in G1 by α-factor and released at 37°C to analyze, over time, budding kinetics, Swi5 localization, and nuclear division. Swi5 was cytoplasmic in both strains throughout most of the cell cycle. However, although it was imported into the nucleus of cdc24 cdc55Δ telophase cells, it always remained in the cytoplasm of cdc24 cdc55Δ cdc14-3 cells, indicating that Cdc14 had been inactivated (Fig. 8 C). Lack of Cdc55 allowed a fraction of cdc24 cells to divide nuclei irrespective of Cdc14 function (Fig. 8 C), indicating that Cdc14 is dispensable for the onset of anaphase in these conditions. Accordingly, Cdc14 was also insufficient to promote sister chromatid separation in CLA4t-overexpressing cells carrying the dominant TAB6-1 allele, which encodes a hyperactive Cdc14 variant with reduced affinity to its inhibitor Net1 (Shou et al., 2001; Fig. 8 D).

Although we did not detect any increase in Mcd1 phosphorylation in cdc55Δ versus wild-type cells overproducing Cla4t (Fig. S3), it was still possible that PP2Acα5 could prevent sister chromatid separation by countering the Cdc5-mediated phosphorylation of a small fraction of Mcd1 or other cohesin subunits. However, inactivation of Cdc5 with the cdc5-2 temperature-sensitive allele did not prevent anaphase in cdc24 cdc55Δ cells (Fig. 9 A), suggesting that Cdc5 is not required for this process.

Timely sister chromatid segregation, especially of ribosomal DNA and chromosome sequences far from centromeres, depends on condensin and DNA topoisomerase II (DiNardo et al., 1984; Holm et al., 1985; Bhalla et al., 2002; D’Amours et al., 2004; Sullivan et al., 2004). We therefore tested the effects of the temperature-sensitive ycg1-10 and top2-4 mutations, affecting condensin and DNA topoisomerase II, respectively, on the unscheduled anaphase of cdc24 cdc55Δ cells. Although inactivation of Ycg1 had no significant effect, inactivation of topoisomerase II in cdc24 cdc55Δ top2-4 cells mostly prevented anaphase (Fig. 9 A), suggesting that the presence of topological linkages prevents sister chromatid separation under these conditions. Consistently, the presence of the top 2-4 allele could partially rescue the cold sensitivity of cdc55Δ cells (Fig. 9 B), which is presumably due to unscheduled sister chromatid separation in the presence of morphogenetic defects.

We then asked whether morphogenetic defects could arrest the cell cycle in a stage where topological linkages are not resolved, using an assay that allows detection of accumulation of catenated forms of a circular minichromosome (Koshland and Hartwell, 1987). Unlike top2-4 mutants, however, neither cdc24 (Fig. 9 C) nor GAL1-CLA4t cells (not depicted) accumulated minichromosome topoisomers. Although we cannot exclude the possibility that the behavior of natural chromosomes is different from that of minichromosomes, the delay of nuclear division caused by the morphogenetic checkpoint does not seem to be accompanied by lack of decatenation.

**CDC55 overexpression delays sister chromatid separation independently of Pds1**

If PP2Acα5 acts as an inhibitor of sister chromatid separation, increasing its dosage might delay the onset of anaphase. We therefore introduced into the genome of otherwise wild-type cells multiple copies of a galactose-inducible GAL1-CDC55 construct. Parental and transformed strains growing in raffinose were arrested in G1 with α-factor and released in the presence of galactose. We then monitored separation of the tetO array located 13 kb away from CEN5, as well as spindle formation and elongation (Fig. 10). **CDC55 overexpression did not affect bipolar spindle formation but delayed sister chromatid separation, nuclear division, and spindle elongation,**
that PP2ACdc55 prevents dissociation of sister chromatids along telomeric and telomeric regions (unpublished data), suggesting a delay of Cdc55 at both pericentromere onset of anaphase in CDC55-overexpressing cells. High levels of PP2ACdc55 prevents dissociation of sister chromatids along their length.

If PP2A Cdc55 acted as anaphase inhibitor independently of securin, we could expect that simultaneous loss of Pds1 and Cdc55 might have additive effects, allowing precocious separation of sister chromatids during the unperturbed cell cycle. Indeed, concomitant deletion of CDC55 and PDS1 turned out to be lethal (unpublished data).

Discussion

The control of sister chromatid separation by the morphogenesis checkpoint

It has been well established that morphogenetic defects, such as lack of actin polarization or budding, cause a G2 arrest in budding yeast because of the inhibitory phosphorylation of Cdk1 on tyrosine 19 by the Swe1 kinase (Lew, 2003). This inhibitory phosphorylation likely involves only a small pool of mitotic Cdk1. In fact, the morphogenesis checkpoint arrests the cell cycle after spindle formation, whereas complete inactivation of all mitotic Ckds by mutations or SWE1 overexpression prevents spindle pole body separation and bipolar spindle assembly (Crasta and Surana, 2006). We show here that the morphogenetic checkpoint prevents sister chromatid separation independently of securin because pds1Δ cells treated with Lat-A or overexpressing the dominant-negative CLA4t allele do not attempt anaphase. Our data also indicate that morphogenesis checkpoint activation does not delay separate association to securin and its nuclear import, which depends on Pds1 phosphorylation by Ckds (Agarwal and Cohen-Fix, 2002; Uhlmann, 2003), consistent with only a minor pool of mitotic Ckds being inactivated under these conditions.

Inactivation of Mcd1 through the temperature-sensitive scc1-73 allele or its ectopic cleavage also turned out to be insufficient for anaphase and chromosome segregation under morphogenesis checkpoint activation, raising the possibility that either spindle function is compromised or residual cohesion persists on chromosomes after Mcd1 inactivation. Because in our assays spindle forces seem normal, we favor the second interpretation. Whether residual cohesion depends on other cohesion subunits or on other proteins remains to be established. Cohesin-independent chromatid linkages have been reported for repetitive sequences (D’Amours et al., 2004; Dynek and Smith, 2004; Sullivan et al., 2004), and a role for condensin in chromatin cohesion has been recently described (Lam et al., 2006). Swel-mediated phosphorylation of mitotic Ckds could prevent the release of these linkages in addition to inhibiting securin degradation. Although a direct role for mitotic Ckds in dismantling sister chromatid cohesion has not been reported so far, Ckds are required at different levels for Polo kinase activation, which in turn contributes to dissociation of sister chromatids by phosphorylating the cohesin Mcd1 and enhancing its susceptibility to cleavage by separase (Alexandru et al., 2001). In addition, in higher eukaryotic cells Polo and Aurora B kinases promote the prophase pathway of cohesin dissociation from chromosome arms that is independent of securin degradation and relies on phosphorylation of the SA2 cohesin subunit (Sumara et al., 2002; Hauf et al., 2005). In budding yeast, mitotic Ckds activate the Polo kinase through several mechanisms, including transcription (Spellman et al., 1998), phosphorylation (Mortensen et al., 2005), and inhibition of proteolysis (Zachariae et al., 1998). It is therefore not surprising that Cdc5 activation is dramatically delayed in response to the morphogenesis checkpoint.
The failure to timely activate Cdc5 could contribute to the lack of sister separation in these conditions but cannot be the only culprit. In fact, Cdc5 inactivation leads to inefficient separation of telomeric regions but has no or little effect on that of centromeric and arm sequences (Alexandru et al., 2001). In addition, Cdc5 is not required for the onset of anaphase of cdc24 cdc55Δ cells. If the failure to separate sister chromatids when the morphogenetic checkpoint is active were merely due to delayed Cdc5 activation, anaphase should be resumed by ectopic Mcd1 cleavage, which we show not to be the case. Therefore, sister chromatid cohesion seems to be maintained by the morphogenesis checkpoint through a previously unanticipated mechanism that does not depend only on securin stabilization and Polo kinase inactivation.

PP2ACdc55 and the control of sister chromatid separation

We find that inactivation of the protein phosphatase PP2ACdc55 is sufficient to allow sister chromatid separation when the morphogenesis checkpoint is activated. Unlike upon deletion of SWE1, which completely abolishes the cell’s ability to respond to morphogenetic defects, this is not achieved through switch off of checkpoint signaling, because lack of PP2ACdc55 activates by itself the checkpoint and induces Swe1 stabilization by causing morphogenetic defects (Jiang, 2006). In agreement with a critical function for PP2ACdc55 in controlling sister separation when the morphogenesis checkpoint is active, deletion of CDC55 turned out to be lethal for cla4 and cdc12 mutants (unpublished data), whose morphogenesis defects are known to activate the checkpoint (Lew, 2003).

Recently, PP2A bound to Rts1/B56, the other regulatory subunit, has been found to protect centromeric cohesion during mitosis and meiosis I, in both yeast and human cells (Kitajima et al., 2006; Riedel et al., 2006). In our experimental conditions, PP2ARts1 seems to have only a minor role, perhaps restricted to centromeric regions, in preventing chromatid dissociation.

Cdc55 was previously implicated in maintaining sister chromatid cohesion in response to activation of the spindle assembly checkpoint (Minshull et al., 1996), suggesting that PP2ACdc55 acts as anaphase inhibitor in several conditions. However, in nocodazole-treated cdc55Δ cells, sister chromatid separation is accompanied by Mcd1 proteolytic cleavage (Yellman and Burke, 2006; this study), whereas we could not find evidence for such event in cdc55Δ cells undergoing anaphase in the presence of morphogenetic defects. In agreement with our data, Cdc55 has recently been shown to prevent chromatid separation independently of securin degradation and Mcd1 cleavage in cells with telomeric DNA lesions (Tang and Wang, 2006).

How could PP2ACdc55 prevent sister chromatid separation in G2? For instance, it could regulate a pathway of cohesin removal similar to the prophase pathway of higher eukaryotic cells, although so far Mcd1 cleavage by separase seems to be the only necessary and sufficient event for cohesin removal from yeast chromosomes (Uhlmann, 2003). If PP2ACdc55 were to inhibit cohesin dissociation independently of Mcd1 cleavage, its inactivation could allow anaphase in the absence of separase. In contrast to recently published data (Tang and Wang, 2006), we find that both the esp1-1 mutation (Ciosk et al., 1998) and
overexpression of nondegradable Pds1 (Cohen-Fix et al., 1996) prevent cdc55Δ cells from undergoing anaphase (unpublished data), suggesting that separase is still required for sister chromatid separation in the absence of PP2A Cdc55. It should be noted, however, that separase has additional functions that are unrelated to its role in Mccl cleavage (Sullivan et al., 2001; Stegmeier et al., 2002; Sullivan and Uhlmann, 2003; Papi et al., 2005). Interestingly, Cdc55 has recently been shown to interact physically with Esp1 and to prevent the early anaphase release of Cdc14 by causing dephosphorylation of its inhibitor Net1 (Queralt et al., 2006). This raises the possibility that lack of PP2A Cdc55 causes the unscheduled activation of Cdh1/ APC, and thereby Pds1 degradation, by promoting Cdc14 release. Although this could partly explain the separation of sister chromatids in nocodazole-treated cdc55Δ cells, we show here that nuclear division of cdc55Δ cells when the morphogenesis checkpoint is active is independent of Cdc14 function, suggesting that PP2A Cdc55 must have other roles, besides inhibiting Cdc14 dissociation from Net1, before the onset of anaphase. Therefore, a more direct role of PP2A Cdc55 in controlling sister chromatid separation in G2 must be invoked.

**Putative targets of PP2A Cdc55 in the control of anaphase**

The importance of PP2A Cdc55 as anaphase inhibitor is underscored by the synthetic lethality of pds1Δ cdc55Δ double mutants, where sister chromatid separation could be so premature as to cause lethal chromosome missegregation. In addition, CDC55 overexpression delays chromatid dissociation independently of securin. In agreement with a crucial function as anaphase inhibitor, PP2A Cdc55 phosphatase activity decreases at the onset of anaphase (Queralt et al., 2006).

An obvious candidate for being dephosphorylated by PP2A Cdc55 to prevent sister chromatid dissociation was Mcd1, especially in light of recent data indicating that the other PP2A complex, PP2A Bn/B56, prevents precocious loss of centromere cohesion by counteracting Mcd1 phosphorylation by Polo kinase (Kitajima et al., 2006; Riedel et al., 2006). However, as discussed above, PP2A Cdc55 might target other proteins beside Mcd1. For instance, it could dephosphorylate other cohesin subunits and prevent cohesin unloading through a pathway analogous to the vertebrate prophase pathway. Despite the efforts, we could not detect any difference in the electrophoretic mobility of other cohesin subunits, such as Scc3 and Pds5, in cdc55Δ versus wild-type cells (unpublished data). Alternatively, PP2A Cdc55 could regulate other chromatin-bound proteins, such as the condensin complex. It is worth mentioning that the human condensin HCP-6 interacts with and is dephosphorylated by PP2A bound to the B subunit (Yeong et al., 2003). Finally, another putative target of PP2A Cdc55 might be Esp1, which interacts physically with Cdc55 (Queralt et al., 2006). Although separase has been proposed to down-regulate PP2A Cdc55 activity, separase regulation of PP2A Cdc55 can also be envisaged.

In summary, a crucial role for PP2A Cdc55 in maintaining sister chromatid cohesion in response to several stress conditions is emerging, making it a key factor for preserving genome stability.
nuclear division when the morphogenesis checkpoint is activated. Fig. S3 shows that Mcd1 falls off chromatin but its proteolytic cleavage is unde-
tectable in cdc55Δ cells overexpressing Clb4t. Fig. S4 shows that sister separation in the absence of Cdc55 upon nocodazole treatment correlates with Mcd1 cleavage. Table S1 describes the genotypes of strains used in this study. Online supplemental material is available at http://www.jcb .org/cgi/content/full/jcb.200609088/DC1.

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