Supplemental Materials:

Supplemental Figures:

Supplemental Figure 1: tem1Δ CDC15-UP Cells are Spindle Position Checkpoint Defective.

dyn1Δ (A2444), bub2Δ dyn1Δ (A2270), and tem1Δ CDC15-UP dyn1Δ (A23657) cells were grown to mid-exponential phase at 30°C and then incubated for 24 h at 14°C. Cells were fixed and the number of nuclei in cells was determined. Cells that were anucleate, multinucleate, or multi-budded with two nuclei in the mother cell body were counted as “bypassed”. Single budded cells with two nuclei in the mother cell body were counted as “arrested”.

Supplemental Figure 2: Separase is Not Required for Dbf2 activity in tem1Δ CDC15-UP Cells

(A,B) tem1Δ CDC15-UP (A22670) and tem1Δ esp1-1 CDC15-UP (A23716) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 µg/ml) in YEPRG medium at room temperature. Thirty minutes prior to release the cells were shifted to 37°C. When the arrest was complete (after 3 hours 30 minutes), cells were released into pheromone free YEPRG medium at 37°C. After 65 minutes, α-factor pheromone (10 µg/ml) was added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed squares, A), anaphase spindles (closed circles, A) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, B) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, B) was determined at the indicated times.

(C) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (B) was determined by quantitative autoradiography and quantitative Western blot, respectively. Shown is the specific Dbf2-associated kinase activity.

Supplemental Figure 3: Anaphase Entry is not Required for MEN Activity in the Absence of TEM1

(A, B, C) tem1Δ CDC15-UP (A22670) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 µg/ml) in YEPRG
medium. When the arrest was complete (after 2 hours 45 minutes), cells were released into YEPRG medium supplemented with nocodazole (15 µg/ml; (+) nocodazole) or solvent control (DMSO; (-) nocodazole). After 65 minutes, α-factor pheromone (10 µg/ml) was added to prevent entry into the subsequent cell cycle. The percentage of budded cells (A), DNA content (as assayed by flow cytometry, B) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, C) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, C) was determined at the indicated times.

(D) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (C) was determined as in Supplemental Figure 2C. Shown is the specific Dbf2-associated kinase activity.

Supplemental Figure 4: Cdc5 is Not Required for Tem1 SPB Localization

TEM1-GFP (A22556) and TEM1-GFP GAL-URL-3HA-CDC5 (A28411) cells containing a mCherry-Tub1 fusion protein were arrested in G1 with α-factor pheromone (5 µg/ml) in YEPRG medium. URL, which stands for ubiquitin-arginine-LacZ, acts as a destabilizing module that permits rapid degradation of appended proteins (Bachmair et al. 1986). Two hours prior to release, glucose was added to a final concentration of 2% (to repress expression of GAL-URL-3HA-CDC5). When the arrest was complete (after 2 hours 45 minutes), cells were released into pheromone free YEPD medium. Cell cycle stage was determined based on spindle morphology and correlated with Tem1 localization at SPBs (n ≥ 100 cells for each cell cycle stage). Representative images of anaphase cells are shown. Tem1 is shown in green, microtubules in red and DNA in blue.

Supplemental Figure 5: Cdc15-SPB localizes to the SPB constitutively throughout the cell cycle

pMET3-CDC15-eGFP-CN6 (CDC15-SPB; A26486) cells containing a mCherry-Tub1 fusion protein were grown to log phase in YEPRG medium supplemented with 8 mM methionine and imaged after a brief paraformaldehyde fixation. Cell cycle stage was determined based on spindle morphology and correlated with Cdc15-SPB localization at SPBs (n ≥ 100 cells for each cell cycle stage).

Supplemental Figure 6: Dbf2-Mob1 Kinase Activity is Not Sufficient for Cdc14 Release Prior to Anaphase
(A, B) Wild-type (A2747) and pMET3-CDC15-eGFP-CN67 (CDC15-SPB; A26418) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPD medium supplemented with 8 mM methionine. When the arrest was complete (after 2 hours 40 minutes), cells were released into pheromone free YEPD medium supplemented with 8 mM methionine. After 65 minutes, α-factor pheromone (10 μg/ml) was re-added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed squares, A), anaphase spindles (closed circles, A), 3HA-Cdc14 released from the nucleolus (open circles, A) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, B) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, B) was determined at the indicated times. (C) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (B) was determined as in Supplemental Figure 2C. Shown is the specific Dbf2-associated kinase activity.

Supplemental Figure 7: Removal of the C-terminal 274 Amino Acids of Cdc15
Results in Constitutive Cdc15 SPB Targeting and Tem1 and Cdc5-Independent Activation of the MEN

(A) tem1Δ GAL-GFP-CDC15 (A25662), tem1Δ GAL-GFP-CDC15 cdc5-as1 (A25661), tem1Δ GAL-GFP-CDC15(1-750) (A25596), and tem1Δ GAL-GFP-CDC15(1-750) cdc5-as1 (A25594) cells containing a mCherry-Tub1 fusion protein were arrested in G1 as in Figure 4A. Cells were released into pheromone free YEPRG medium supplemented with 5 mM CMK (cdc5-as1 inhibitor) and imaged after a brief paraformaldehyde fixation. Cell cycle stage was determined based on spindle morphology and correlated with Cdc15 localization at SPBs (n ≥ 100 cells for each cell cycle stage).

(B, C) GAL-GFP-CDC15 (A24698) and GAL-GFP-CDC15 cdc5-as1 (A24695) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 μg/ml) in YEPRG medium. When the arrest was complete (after 2 hours 50 minutes), cells were released into pheromone free YEPRG medium. After 70 minutes, α-factor pheromone (10 μg/ml) was re-added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed squares, B), anaphase spindles (closed circles, B) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, C) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, C) was determined at the indicated times.
(D) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (C) was determined as in Supplemental Figure 2C. Shown is the specific Dbf2-associated kinase activity.

(E, F) GAL-GFP-CDC15(1-750) (A21924) and GAL-GFP-CDC15(1-750) cdc5-as1 (A24508) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were examined at the same time and in the same manner as strains described in Supplemental Figure 7B - D. The percentage of cells with metaphase spindles (closed squares, E), anaphase spindles (closed circles, E) and the amount of Dbf2-associated kinase activity (Dbf2 kinase, F) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, F) was determined at the indicated times.

(G) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (F) was determined as in Supplemental Figure 2C. Shown is the specific Dbf2-associated kinase activity.

**Supplemental Figure 8: Overexpression of Cdc15(1-750) hyperactivates Dbf2-Mob1 but does not Result in the Premature Release of Cdc14 from the Nucleolus**

(A, B, C) GAL-GFP-CDC15 (A21922) and GAL-GFP-CDC15(1-750) (A21924) cells containing 3HA-Cdc14 and 3MYC-Dbf2 fusion proteins were arrested in G1 with α-factor pheromone (5 µg/ml) in YEPR medium. 45 minutes prior to release, galactose was added to induce expression of GAL-GFP-CDC15 and GAL-GFP-CDC15(1-750). When the arrest was complete (after 3 hours), cells were released into pheromone free YEPRG medium. After 85 minutes, α-factor pheromone (10 µg/ml) was re-added to prevent entry into the subsequent cell cycle. The percentage of cells with metaphase spindles (closed squares, A), anaphase spindles (closed circles, A) 3HA-Cdc14 released from the nucleolus (open circles, A), the amount of Dbf2-associated kinase activity (Dbf2 kinase, B) and immunoprecipitated 3MYC-Dbf2 (Dbf2 IP, B), and the amounts of GFP-Cdc15 and GFP-Cdc15(1-750) (α-GFP, C) was determined at the indicated times. Pgk1 was used as a loading control in Western blots.

(D) The amount of Dbf2-associated kinase activity and immunoprecipitated 3MYC-Dbf2 from (B) was determined as in Supplemental Figure 2C. Shown is the specific Dbf2-associated kinase activity.
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Supplemental Experimental Procedures

Yeast Strains
All strains are derivatives of W303 (A2587) and are listed in Table S1. Cdc15-eGFP and \textit{PGPD-CDC15} were constructed by standard PCR-based methods (Longtine et al. 1998; Janke et al. 2004).

Plasmid Construction
All plasmids used in this study are listed in Table S2.

pA1813: \textit{CDC5\Delta db} (Charles et al. 1998; Shirayama et al. 1998) was cloned under the control of the \textit{MET25} promoter using the following strategy. Approximately 1 kb of the \textit{MET25} promoter was amplified with primers (5'-aataAAGCTTCCGGATGCAAGGGTGTTCAATG-3') and (5'-aataCTGCAGGGGTTGTAATAGGATT-3') from A2587 genomic DNA (PCR product 1); the N-terminally truncated (70 amino acids) \textit{CDC5} ORF was amplified with primers (5'-aataCTGCAGAAACATCCACCTCCAATGCAAAACAAG-3') and (5'-CATGGCAATTTTGAATAGAATT-3') from A2587 genomic DNA genomic DNA (PCR product 2). PCR product 1 was digested with HindIII and PstI; PCR product 2 was digested with PstI and XbaI; plasmid YIplac211 was digested with HindIII and XbaI (Gietz and Sugino 1988). Fragments were three way ligated to yield: YIplac211-\textit{MET25-CDC5\Delta N70}.

pA1880: \textit{CDC15-eGFP-CN67} was cloned under the control of the \textit{MET3} promoter using the following strategy. \textit{PMET3} was amplified with primers (5'-TTACGCCAAGCTTGCATGCTGCAGGTCGACTCTAGAGGATGAAACTGAGTAAGATGCTCAGAATAC-3') and (5'-GAGTCAAGTTGACTCTATCGGATCGGCCATGACTTTTATGATTTAATTATATACATTATTCTTG-3') with a \textit{PMET3} containing plasmid as template; \textit{CDC15-eGFP} was amplified with primers (5'-ATGAACAGTATGGCCGATACC-3') and (5'-GCCACCAGGACGACCTCCACCAAGAACCTCCACCTAGTGGTTACTGACATTCCATCAATCATGACAGGCAA-3') with A20791 genomic DNA as PCR template; and \textit{CN67} was amplified with primers (5'-CTAGGGTGGAGGAGTTCTGGGAGGTTGCGTCTGTTGGGTGGCAGACTGATTCCGATTAAATG-3') and (5'-
TAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGGGAACCCCTAAAAGCTCA
TAGTAGCAG-3') with A2587 genomic DNA as template. Plasmid YCplac22 was
digested with BamHI (Gietz and Sugino 1988). Approximately equimolar amounts of
BamHI-digested plasmid YCplac22 and each of the three PCR products above were
cotransformed into yeast strain A2587. Homologous recombination between YCplac22
and the three PCR fragments generates the PMET3-CDC15-eGFP-CN67 allele.
Plasmids were recovered from resulting Trp+ colonies and sequence confirmed to
contain mutation-free PMET3-CDC15-eGFP-CN67. PMET3-CDC15-eGFP-CN67
was then subcloned into the SphI & KpnI sites of YIplac128 (Gietz and Sugino 1988).

Note that expression of the fusion protein shows cell-to-cell variability under non-
inducing conditions (as is evident by GFP signal intensity in fluorescence microscopy).
Even under these conditions, however, Cdc15-SPB protein levels remained high enough
in all or almost all cells to complement the temperature sensitive lethality of the cdc15-2
allele.

**Cell Cycle Staging by Spindle Morphology**
The stage of the cell cycle of individual cells was assessed by spindle morphology. G1
or S phase cells were defined as having unduplicated or newly duplicated spindle pole
bodies but lacking a spindle that spanned the DAPI-stained nucleus. Metaphase cells
were defined as having a thick, bar shaped spindles that spanned an undivided DAPI-
stained nucleus. Anaphase cells were defined as cells with separated DNA masses
connected by an elongated spindle.

**Spindle position checkpoint assay**
Cells were grown to mid-exponential phase at 30°C and then incubated for 24 h at 14°C.
Cells were fixed and the number of nuclei in cells was determined. Cells that were
anucleated, multinucleated, or multi-budded with two nuclei in the mother cell body were
counted as exhibiting a checkpoint bypass morphology. Single budded cells with two
nuclei in the mother cell body were counted as arrested.

**Supplemental References**


A

**tem1Δ CDC15-UP**

**tem1Δ CDC15-UP esp1-1**

B

C

**Dbf2 kinase**

**Dbf2 IP**

- **tem1Δ CDC15-UP**
- **tem1Δ CDC15-UP esp1-1**
A. % Budded Cells

B. Dbf2 kinase and Dbf2 IP

C. Western blots for (-) nocodazole and (+) nocodazole

D. Dbf2 Specific Activity
Percent anaphase cells

- CDC5
- GAL-URL-3HA-CDC5

DIC Tem1 tubulin DAPI merge

CDC5

GAL-URL-3HA-CDC5

Tem1 on SPB
Tem1 not on SPB
**CDC15-SPB**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Cdc15-SPB visible on SPB</th>
<th>Cdc15-SPB not visible on SPB</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1/S</td>
<td>80%</td>
<td>0%</td>
</tr>
<tr>
<td>Metaphase</td>
<td>80%</td>
<td>0%</td>
</tr>
<tr>
<td>Anaphase</td>
<td>80%</td>
<td>0%</td>
</tr>
</tbody>
</table>
A

% Cells with Cdc15 on SPB

G1/S Metaphase Anaphase

tem1Δ GAL-GFP-CDC15
tem1Δ GAL-GFP-CDC15 cdc5-as1
tem1Δ GAL-GFP-CDC15(1-750)
tem1Δ GAL-GFP-CDC15(1-750) cdc5-as1

B

GAL-GFP-CDC15

GAL-GFP-CDC15 cdc5-as1

Percent cells

Time (min)

C

Dbf2 kinase

Dbf2 IP

GAL-GFP-CDC15

GAL-GFP-CDC15 cdc5-as1

Time (min)

D

Dbf2 Specific Activity

Time (min)

E

GAL-GFP-CDC15(1-750)

GAL-GFP-CDC15(1-750) cdc5-as1

Percent cells

Time (min)

F

Dbf2 kinase

Dbf2 IP

Time (min)

G

Dbf2 Specific Activity

Time (min)
A. **GAL-GFP-CDC15**

- **GAL-GFP-CDC15(1-750)**

- Time (min)
- Percent Cells

B. **Dbf2 kinase**

- **Dbf2 IP**

C. **α-GFP**

- **α-Pgk1**

D. **Dbf2 Specific Activity**

- **GAL-GFP-CDC15**
- **GAL-GFP-CDC15(1-750)**

- **Cdc14 released**