Fission Yeast *cdc31p* Is a Component of the Half-bridge and Controls SPB Duplication

Anne Paoletti,^{*†} Nicole Bordes,^{*} Raphaël Haddad,^{*‡} Cindi L. Schwartz,[§]
Fred Chang,[¶] and Michel Bornens^{*}

^{*}Institut Curie, UMR144 CNRS, 75248 Paris Cedex 05 France; [§]Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309-0347; and [¶]Department of Microbiology, Columbia University, New York, New York 10032

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The fission yeast spindle pole body (SPB) is a nucleus-associated organelle that duplicates once each cell cycle during interphase. Duplicated SPBs serve as the poles of an intranuclear mitotic spindle after their insertion into the nuclear envelope in mitosis (Ding *et al.*, Mol. Biol. Cell 8, 1461–1479). Here, we report the identification and characterization of *Schizosaccharomyces pombe* *cdc31p*, a member of the conserved calcium-binding centrin/CDC31 family. Immunofluorescence and immunoelectron microscopy show that *cdc31p* is a SPB component localized at the half-bridge structure of the SPB. *cdc31* is an essential gene and $\Delta cdc31$ cells and *cdc31* conditional mutant cells arrest in mitosis with a monopolar mitotic spindle organized from a single SPB. EM analysis demonstrates that mutant *cdc31* cells fail to duplicate the SPB. In addition, *cdc31p* exhibits genetic interactions with the SPB component *sad1p* and is required for *sad1p* localization. Finally, *cdc31* mutant can undergo single or multiple rounds of septation before the exit from mitosis, suggesting that *cdc31p* activity or SPB duplication may be required for the proper coordination between the exit from mitosis and the initiation of septation.

INTRODUCTION

To accurately control the segregation of chromosomes between sister cells, eukaryotes have developed a very sophisticated apparatus, the mitotic spindle. In this apparatus, microtubules are most often focused on two poles organized by a specialized organelle, the centrosome. This organelle, which both nucleates and anchors microtubule (MTs), duplicates only once in a cell cycle. During duplication, new centrosomal structures usually appear in close vicinity to the preexisting ones. In animal cells for instance, new centrioles form perpendicularly to the proximal end of the preexisting pair of centrioles. This feature is also true for budding yeast centrosome, a layered organelle embedded in the nuclear envelope, also called spindle pole body (SPB): SPB duplication proceeds through the formation of a cytoplasmic satellite next to a substructure of the SPB lying on the nuclear

envelope called the half-bridge (Byers and Goetsch, 1974; see Adams and Kilmartin, 2000 for a review).

In fission yeast, the SPB is a nuclear-associated organelle composed of a layered cytoplasmic part and an electron dense nuclear part separated by the nuclear envelope. An electron dense particle, lying on the nuclear envelope beside the layered cytoplasmic part of the SPB, has been proposed to be analogous to budding yeast half-bridge. Fission yeast SPB duplication is thought to occur in the cytoplasm in late G2 when the single SPB structure is replaced by two smaller structures connected by an electron dense bridge. No intermediate state in SPB duplication such as the formation of a satellite has been described to date. At the onset of mitosis, the SPBs are inserted into the nuclear envelope and organize the assembly of an intranuclear spindle (Ding *et al.*, 1997).

Molecular mechanisms that underline centrosome duplication process are still poorly understood. However, molecular characterization of centrosomes has revealed that key components controlling centrosome functions have been conserved despite the large structural diversity of centrosomes among eukaryotes. For instance, a gamma-tubulin containing complex controlling microtubule nucleation has been characterized both in yeast and in animal cells (for reviews see Tassin and Bornens, 1999 and Knop *et al.*, 1999; see also Vardy *et al.*, 2002). Molecular mechanisms controlling the centrosome duplication process may have also been

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[†] Corresponding author. E-mail address: paoletti@curie.fr.

Present address: [†] Institut de Biochimie et Génétique cellulaires, UMR 5095 CNRS 1, rue Camille Saint-Saëns 33077 Bordeaux cedex.

Abbreviations used: HU, hydroxyurea; MTs, microtubules; MTOC, microtubule organizing center; SPB, spindle pole body.

conserved because orthologues of genes required for SPB duplication in budding yeast also appear to regulate centrosome duplication in *Schizosaccharomyces pombe* or in animal cells (Baum *et al.*, 1986; Winey *et al.*, 1993; West *et al.*, 1998; Middendorp *et al.*, 2000).

One of the budding yeast genes implicated in SPB duplication, *CDC31*, encodes a Ca^{2+} -binding protein of the centrin family (Baum *et al.*, 1986). It is a component of the half-bridge structure of the SPB (Spang *et al.*, 1993). Analysis of thermosensitive mutants has demonstrated that this essential gene is required in an early stage of SPB duplication because formation of the satellite does not occur in these mutants (Byers, 1981; Schild *et al.*, 1981). ScCdc31p association with the half-bridge depends on another half-bridge component Kar1p (Biggins and Rose, 1994; Spang *et al.*, 1995). How ScCdc31p mediates the formation of the satellite at the end of the half-bridge remains completely unknown.

Human centrin 3 (*CTN3* gene in human genome database) belongs to the same subfamily of centrins as ScCdc31p and is concentrated at the centrosome in the distal lumen of centrioles (Middendorp *et al.*, 1997). Functional experiments have shown that expression of HsCen3p has a dominant negative effect on centrosome duplication both in yeast and in *Xenopus* eggs (Middendorp *et al.*, 2000), suggesting the HsCen3p participate in centrosome duplication in human cells and shares a common function with ScCdc31p.

Here, we report the characterization of *ScCDC31/HsCen3* orthologue in fission yeast. We demonstrate that *S. pombe* cdc31p is a component of the half-bridge of the SPB required for SPB duplication. In addition, our data suggest that cdc31p and sad1p, another SPB component required for bipolar spindle formation, may act in the same pathway. Finally, because *cdc31* cells blocked in mitosis proceed to septation and display a multiseptation phenotype, we propose that cdc31p activity or SPB duplication may be required for the proper coordination between the exit from mitosis and the initiation of septation.

MATERIALS AND METHODS

Sequence Comparison and Dendrogram Construction

Sequence comparison and dendrogram construction were performed using ClustalW (<http://clustalw.genome.ad.jp/>) and Boxshade servers (http://ludwig-sun1.unil.ch:8080/software/BOX_form.html).

Yeast Strains and Genetic Methods

Standard *S. pombe* genetic techniques and cultures were performed as described at <http://www.bio.uva.nl/pombe/handbook/>. All *S. pombe* strains were isogenic to 972 and are listed in Table 1. Yeast transformations were performed by electroporation (Kelly *et al.*, 1993) or by the lithium acetate-DMSO method for integration of linear DNA (Bahler *et al.*, 1998b).

Cloning of *cdc31*, *HsCen3*, and *ScCDC31*

To clone *cdc31*, a 780-base pair (bp) fragment of DNA containing *cdc31* ORF was amplified by PCR from genomic DNA (forward oligo: cacactcgagGTCATGTAAATACTCACAC; reverse oligo: ttgatccGTAAATTAGCATGTGTTCTCC).

After purification of the fragment (PCR purification kit, Qiagen, Valencia, CA), fragment ends were digested by *XhoI* and *BamHI* (sites added on oligos) and ligated at same sites in pREP41X (Fors-

burg, 1993) to generate a LEU2+ *pnmt**-*cdc31* plasmid (pAP 80). Absence of PCR-induced mutations was checked by sequencing. Then, an *XhoI-BamHI* fragment from pAP80 was subcloned at *XhoI* and *BglII* sites of pSLF273 and pSLF173 (Forsburg and Sherman, 1997) to generate *ura4+* *pnmt**-*cdc31* and *pnmt*-*cdc31* plasmids (pAP101 and pAP123), respectively.

To generate an in frame N-terminal fusion of GFP with *cdc31p* a *NotI-BamHI* fragment including *cdc31* ORF was amplified from genomic DNA using oligonucleotides ggaagaattgcggccgcATGTTT-GCTAACGCACGGG and ttgatccGTAAATTAGCATGTGT-TCTCC. This fragment was ligated to *NotI* and *BglII* sites in pSGP573 (generous gift from S. Forsburg) to obtain pAP81. Finally, *nmt* promoter was replaced by *nmt** promoter from pREP41X plasmid using the *PstI* and *XhoI* sites to obtain a *ura4+* *pnmt**-GFPcdc31 plasmid (pAP83). *pnmt*-HA₃-*cdc31* plasmid (pAP158) was obtained by subcloning a *NotI-SacI* fragment from pAP81 into pSLF173.

For expression of *HsCen3* and *ScCDC31* in fission yeast, an *Sall-BamHI* fragment from pUF10 (gift from E. Schiebel) containing *ScCDC31* complete ORF was subcloned in pSLF273 at *XhoI* and *BglII* sites to obtain plasmid *pnmt**-*ScCDC31* (pAP103). A *XhoI-BamHI* fragment encoding HsCen3p was amplified by PCR from HsCen3 pBS-KS plasmid (Middendorp *et al.*, 1997) using oligos cacactcgagat-gagtttagctctgagaagt and ttgatccctaaatgtcaccagtcataatagc and ligated in pSLF273 at *XhoI* and *BglII* sites to obtain *pnmt**-HsCen3 plasmid (pAP100).

All fragments amplified by PCR were checked by sequencing (Genomexpress, Meylan, France).

Deletion of *cdc31*, Tetrad Analysis, and Germination of Random Spores

Deletion of *cdc31* ORF was achieved by homologous recombination according to Bahler *et al.* (1998b): KanMX cassette was amplified by PCR from kanMX4 plasmid using a forward oligonucleotide corresponding to 80 bp right upstream of *cdc31* ATG codon and a reverse oligonucleotide corresponding to 80 bp right downstream of *cdc31* stop codon and transformed into the diploid strain FC584. Stable transformants were then selected to obtain a Δ *cdc31/cdc31+* strain (AP54). Presence of the Δ *cdc31* allele was confirmed by PCR.

To analyze the progeny of the Δ *cdc31/cdc31+* strain, sporulation was induced on ME plates (BIO 101, Carlsbad, CA) for 2 d and tetrads dissected using an automated tetrad disector (MSM, Singer Instruments, Somerset, UK). Spores were allowed to germinate and form colonies at 25°C on YE5S medium. Finally, colonies were replicated on YE5S plates containing 50 mg/l G418. Or, to generate random spores, parental diploid cells were digested by overnight treatment with 5 μ l/ml glusulase (NEN, Boston, MA) in H₂O. Germination was then allowed by incubation of spores in YE5S liquid medium at 25°C for 15 h.

Production of Conditional *cdc31* Mutants

A point mutation leading to an E-to-K substitution at position 147 of *cdc31p* was first introduced in *cdc31* sequence by double PCR: in a first round, a 660-bp 5' fragment was amplified by PCR from pAP80 using oligos cacactcgagGTCATGTAAATACTCACAC and GTC-GATGTTTTTCATTAAGCTC. A 140-bp 3' fragment containing the mutation and overlapping the 5' fragment on 20 base pairs was amplified by PCR using oligos GAGCTTAATGAAAACATCGAC-GATCAGAAATTGGAAGC and ggatccGTAAATTAGCATGTGT-TCTCC. The full-length *E147K-cdc31* fragment was obtained in second round of PCR using the purified 5' and 3' fragments and oligos ctgagGTCATGTAAATACTCACAC and ggatccGTAAATTAGCATGTGTTCTCC and cloned at *XhoI-BamHI* sites in pREP41X to obtain pRH3. Presence of the mutation was checked by sequencing.

The mutated construct or wild-type *cdc31* were then cloned in integrative plasmid JK148 (Keeney and Boeke, 1994) together with *nmt** promoter and *nmt* termination sequence from pREP41X in two steps: A *PstI-BamHI* fragment from pAP80 containing

Table 1. List of strains used

Strain	Genotype	Origin
FC418	<i>ade6-M210 ura4-D18 leu1-32 h-</i>	Fred Chang
FC420	<i>ade6-M216 ura4-D18 leu1-32 h+</i>	Fred Chang
MFP7	<i>ade1-D25 ade6-M210 S65T-GFP::cam1 leu1-32 ura4D18 h-</i>	Moser <i>et al.</i> (1997)
AP91	<i>ade6-M210 ura4-D18 leu1-32 h- + pSLF172 (ura4+)</i>	This study
AP131	<i>ade6-M210 ura4-D18 leu1-32 h- + pREP41X (LEU2)</i>	This study
AP634	<i>ade6-M210 ura4-D18 leu1-32 h- + pREP42X (ura4+)</i>	This study
AP137	<i>ade6-M210 ura4-D18 leu1-32 h- + pAP83 (pnmt*-GFPcdc31 ura4+)</i>	This study
AP257	<i>ade6-M210 ura4-D18 leu1-32 h- + pAP100 (pnmt*-HsCen3 ura4+)</i>	This study
AP258	<i>ade6-M210 ura4-D18 leu1-32 h- + pAP101 (pnmt*-cdc31 ura4+)</i>	This study
AP260	<i>ade6-M210 ura4-D18 leu1-32 h- + pAP103 (pnmt*-ScCDC31 ura4+)</i>	This study
AP360	<i>ade6-M210 ura4-D18 leu1-32 h + pAP123 (pnmt*-cdc31 ura4+)</i>	This study
AP555	<i>ade6-M210 ura4-D18 leu1-32 h- + pAP158 (pnmt*-HA₃cdc31 ura4+)</i>	This study
FC584	<i>ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 h +</i>	Fred Chang
AP54	<i>cde31::kanMX/cde31⁺ ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 h+/h-</i>	This study
AP274	<i>cdc31::kanMX ade6-M210 leu1-32 ura4D18 + pAP101 (pnmt*-cdc31 ura4+) h-</i>	This study
AP390	<i>cdc31::kanMX ade6-M210 leu1-32 ura4D18 h- + pRH20 (pnmt*-cdc31 leu1+) integrated</i>	This study
AP388	<i>cdc31::kanMX ade6-M210 leu1-32 ura4D18 h- + pRH19 (pnmt*-E147Kcdc31 leu1+) integrated</i>	This study
IH274	<i>sad1-1 leu1-32 ura4-D18 h-</i>	Iain Hagan
AP635	<i>sad1-1 leu1-32 ura4-D18 h- + pREP42X</i>	This study
AP633	<i>sad1-1 leu1-32 ura4-D18 h- + pAP123 (pnmt*-cdc31 ura4+)</i>	This study
AP681	<i>sad1-1 leu1-32 ura4-D18 h- + pAP158 (pnmt*-HA₃cdc31 ura4+)</i>	This study
AP600	<i>sad1-1 leu1-32 ura4-D18 ade6-M216 h+</i>	This study
AP607	<i>sad1-1 leu1-32 ura4-D18 ade6-M210 h + + pRH19 (pnmt*-E147Kcdc31 leu1+) integrated</i>	This study
AP662	<i>sad1-1 cdc31::kanMX ade6-M210 leu1-32 ura4D18 h + + pRH19 (pnmt*-E147Kcdc31 leu1+) integrated</i>	This study
AP666	<i>cdc31::kanMX ade6-M210 leu1-32 ura4D18 h- + pRH19 (pnmt*-E147Kcdc31 leu1+) integrated</i>	This study
AP843	<i>alp16::kanMX leu1-32 ura4-D18 his7 (ade6) h + + pAP158</i>	This study
AP846	<i>cut12-1 leu1-32 ura4-D18 his2 h+ + pAP158</i>	This study
AP890	<i>alp4-1891 ade6-M216 ura4-D18 leu1-32 h- + pAP158</i>	This study
AP891	<i>alp4-1891 ade6-M216 ura4-D18 leu1 32 h- + pAP123</i>	This study
AP892	<i>alp4-1891 ade6-M216 ura4-D18 leu1-32 h- + pREP42X</i>	This study
AP893	<i>alp6-719 ade6-M216 leu1-32 ura4-D18 h- + pAP158</i>	This study
AP896	<i>cam1-E14 ade6-M210 ura4-D18 leu1-32 ade1 D25 h- + pAP158</i>	This study
AP837	<i>alp4-1891 ade6-M216 ura4-D18 leu1-32 h +</i>	This study
AP899	<i>alp4-1891 leu1-32 ura4-D18 ade6-M216 h- + pRH19 (pnmt*-E147Kcdc31 leu1+) integrated</i>	This study
AP900	<i>alp4-1891 cdc31::kanMX ade6 leu1-32 ura4-D18 + pRH19 (pnmt*-E147Kcdc31 leu1+) integrated</i>	This study
AP901	<i>cdc31::kanMX ade6 leu1-32 ura4-D18 h- + pRH19 (pnmt*-E147Kcdc31 leu1+) integrated</i>	This study

nm⁺promoter and cdc31 was cloned at similar sites in pJK148. Then a *SacI-SacI* fragment from pAP80 or pRH3 containing the wild-type or mutated 3' of cdc31 and nm⁺ termination sequence was cloned in the resulting plasmid to obtain pRH19 and pRH20.

Plasmids pRH19 and pRH20 were linearized in leu1 sequence using *NruI* and transformed into AP274 strain (Δ cdc31 + pAP101). Stable integrants were selected. Finally, pAP101 plasmid (ura4+) was lost spontaneously after three rounds of growth in patches on plates of minimum medium containing adenine and uracil, resulting in strains AP388 (pnmt*-E147Kcdc31 mutant) and AP390 (pnmt*-cdc31 mutant).

Production of *sad1-1 pnmt*-E147Kcdc31* and *alp4-1491 pnmt*-E147Kcdc31* Double Mutants

Strain IH274 was first crossed to strain FC420 to obtain an *h+* *sad1-1* strain (strain AP600). Strain AP600 was then crossed to strain AP388 (Δ cdc31 + pnmt*-E147Kcdc31 integrated). No double mutant was recovered but strain AP607 (*sad1-1* + pnmt*-E147Kcdc31 integrated) was selected and further crossed to strain AP388 transformed by pAP101 (pnmt*-cdc31 ura4+ plasmid) to complement cdc31 function. Finally, strains AP662 and AP666 (*sad1-1* Δ cdc31 + pnmt*-E147Kcdc31 integrated and Δ cdc31 + pnmt*-E147Kcdc31 integrated) were obtained from two strains selected from this cross submitted to two successive incubations on EMM plates containing 1 mg/ml

5FOA, 50 mg/l uracil, and 225 mg/l adenine at 25°C to induce the loss of pAP101 plasmid.

Strain DH1891 (*alp4-1891 leu1-32 h-*; generous gift from T. Toda) was crossed to strain FC420 to obtain an *h+* *alp4-1891* strain (AP837). Strain AP837 was then crossed to AP388 transformed by pAP101 to obtain strains AP899 (*alp4-1891* + pnmt*-E147Kcdc31 integrated), AP900 (*alp4-1891* Δ cdc31 + pnmt*-E147Kcdc31 integrated), and AP901 (Δ cdc31 + pnmt*-E147Kcdc31 integrated) after incubation on 5FOA as described above.

Analysis of Growth after Serial Dilutions and Growth in Liquid Culture

Growth of mutant strains AP388, AP390, AP607, AP662, AP666, AP899, AP900, and AP901 was evaluated after serial 1/10 dilutions of exponentially growing cultures at OD_{595 nm} 0.3. Drops of 5 μ l were deposited on plates of minimum medium containing uracil, adenine, 0.250 ml/l YE-phloxin B solution (BIO101, Carlsbad, CA) with or without 5 μ g/ml thiamine and incubated at 30 or 36°C until the colony formed. Growth of strains overexpressing cdc31p or HA₃cdc31p (AP360, AP555, AP634, AP633, AP635, AP681, AP843, AP846, AP890, AP893, and AP 896) was evaluated similarly.

For cytological studies, pnmt*-E147Kcdc31 cells were grown in exponential phase in liquid EMM supplemented with adenine and uracil at 25°C and shifted to 36°C in presence of 0.5 μ g/ml thiamine.

Western Blots

Anti-HsCen3p Igs were produced as follows: 6 Histidine-tagged HsCen3p was produced in bacteria, purified on nickel columns, and injected to rabbits. For purification of specific Igs, 6 Histidine-tagged HsCen3p was coupled to sepharose (Aminolink plus coupling gel, Pierce). Igs adsorbed on the column in PBS buffer were eluted in 100 mM glycine, pH 2.7, immediately neutralized by addition of 1 M Tris, pH 8.9, dialyzed, and concentrated to 1 mg/ml in PBS.

Total extracts were prepared as follows: exponentially growing cells were centrifuged and concentrated in 250 μ l PBS containing 2 mM EDTA, 1 mM PMSF, 10 μ g/ml aprotinin, and 1 μ g/ml pepstatin and leupeptin. Two hundred fifty microliters acid-washed glass beads (Sigma, St. Louis, MO) were added and tubes submitted to 15 min of vortexing at maximum speed on a IKA-vibrax VXA shaker. After addition of 250 μ l of 2 $\times$ sample buffer (125 mM Tris, pH 6.8, containing 6% SDS, 10% 2-mercaptoethanol and 20% glycerol), extracts were boiled for 5 min, centrifuged at 10,000 $\times$ g for 15 min, and supernatants were recovered.

Protein concentration was assayed by Coomassie blue staining. Equal amounts of extracts were loaded on 12% SDS-PAGE and blotted on nitrocellulose according to Towbin *et al.* (1979). Proteins were fixed on nitrocellulose by a 15-min incubation in TBS containing 0.2% glutaraldehyde. cdc31p was revealed using anti-HsCen3p affinity-purified Igs (1:250), peroxidase-coupled anti-rabbit Igs (1:10,000; Jackson ImmunoResearch, West Grove, PA) and a chemoluminescent revelation kit (Pierce, Rockford, IL).

Immunofluorescence and Microscopy

For immunofluorescence, cells were fixed by plunging cells in -20°C methanol after filtration on a HVLP 0.45- μ m filter (Millipore, Bedford, MA). Cells were incubated in methanol for 5 min before rehydration in PEM buffer (0.1 M NaPipes, pH 6.8, 1 mM EGTA, 1 mM MgCl_2). Cells were further processed as described (Snell and Nurse, 1993). Anti-HsCen3p affinity-purified Igs were used (1:100) together with a Cy3-conjugated anti-rabbit secondary antibody (1:1000; Jackson ImmunoResearch). Tubulin was stained using mAb TAT1 (1:10; generous gift of K. Gull) and an Oregon Green 488-coupled anti-mouse antibody (1:200; Molecular Probes, Eugene, OR).

For γ -tubulin, sad1p, MTs, or mid1p stainings, cells were fixed with 4% formaldehyde for 1 h by addition of a 2:1 mix of 16% EM-grade formaldehyde (Electron Microscopy Sciences, Ft. Washington, PA) and PEM buffer and further processed as described (Snell and Nurse, 1993). Sad1 serum was diluted 1:3 and TAT1 1:10. γ -tubulin mAb (Sigma) was diluted 1/100.

Anti-mid1p antibody was obtained by monthly injection of a rabbit (BabCo, Tucson, AZ) with 150 μ g GST-mid1 (aa 309–505) fusion protein produced in XL1blue bacteria from pGex-Dm1 plasmid (Sohrmann *et al.*, 1996; generous gift from V. Simanis) and purified by SDS-PAGE from the Triton-insoluble fraction. Serum was affinity-purified by retroelution on a His₃-tagged mid1p fragment (aa 300–506) as described in Paoletti and Chang (2000), except that elution was performed at pH 2.2. Purified Igs were diluted 1:5. Secondary antibodies were respectively Cy3-conjugated anti-rabbit secondary antibody (1:1000, Jackson ImmunoResearch) and Oregon Green 488-coupled anti-mouse antibody (1:200, Molecular Probes).

Cell wall and septum staining was performed as reported for calcofluor staining in Paoletti *et al.* (2000), using 2 μ g/ml fluostain I (Sigma). For nuclei staining, cells were fixed in formaldehyde 4% for 5 min, permeabilized in PEM containing 1% Triton X-100, and stained with DAPI (1 μ g/ml). Two microliters of stained cells was mounted between slide and coverslip and readily observed.

Images were acquired with a DMZ Leica microscope and a Hamamatsu CCD camera. For electron microscopy cells were prepared as previously described (Giddings *et al.*, 2001). Briefly, cells were rapidly frozen by high-pressure freezing (BAL-TEC HPM-010, Technotrade International, Manchester, NH), freeze-substituted at -90°C in 2% osmium tetroxide plus 0.1% uranyl acetate in acetone followed by embedding in Epon-Araldite. Serial sections (60 nm)

were cut on an Ultracut E microtome (Leica Microsystems, Bannockburn, IL.), poststained with uranyl acetate and lead citrate, and imaged in a Philips CM10 electron microscope operating at 80 kV. For immunolocalization, cells were frozen as above and then freeze substituted in 0.25% glutaraldehyde plus 0.05% uranyl acetate in acetone and then embedded in Lowicryl HM20 resin and UV-polymerized at -35°C . Embedded cells were sectioned as above and immunostained as previously described using anti-HsCen3p Igs (1:100) and secondary antibodies conjugated to 10-nm colloidal gold (BB International, Cardiff, UK). Immunostained sections were poststained and imaged as above.

Synchronization of *pnmt*^{*}-E147K *cdc31* Mutant

To synchronize *pnmt*^{*}-E147K *cdc31* mutant, cells were grown exponentially at 30°C in EMM medium complemented with adenine and uracil and shifted at 36°C in presence of 0.5 μ g/ml thiamine and 11 mM hydroxyurea (HU; Sigma) for 3 h. Cells were then filtered on 0.2- μ m HVLP filters (Millipore) to remove HU, resuspended in fresh medium containing thiamine equilibrated at 36°C (see $t = 0$ in Figure 10), and further incubated at 36°C . The percentage of cells with a bipolar or monopolar spindle was determined from MT staining on 400 cells at each time point. Percentage of cells with a monopolar spindle and a septum or short separated cell with a monopolar spindle was determined on 200 cells presenting a monopolar spindle according to mid1p staining. Percentage of cells with a normal septum or multiple or abnormal septa was determined on 400 cells stained with fluostain I.

RESULTS

Identification of an *S. pombe* Protein Belonging to the Centrin 3/ScCdc31p Subfamily of Centrins

Analysis of centrin in animal cells has revealed the existence of two distinct subfamilies of centrins (Middendorp *et al.*, 1997). In budding yeast however, a single centrin gene, named *CDC31* is present. Similarly, in the fission yeast genome database, we found a single ORF encoding a putative member of the centrin family (SPCC1682–04; GenBank accession no. CA20670). Closer sequence comparison between the protein encoded by the ORF and centrins (Figure 1A) and dendrogram construction (Figure 1B) clearly identified this protein as a member of the centrin 3/ScCdc31p subfamily. We have therefore named the *S. pombe* gene *cdc31* (for clarity, *cdc31* always refers to *S. pombe* gene. *Spcdc31* is only used when there is possible confusion with budding yeast *CDC31* gene noted *ScCDC31*).

An immunoblot of total *S. pombe* protein extract with an anti-HsCen3p affinity-purified antibody revealed a protein of 23 kDa, suggesting that the antibody was able to cross-react with the product of *S. pombe cdc31* gene (Figure 1C). To test whether this cross-reacting protein was *cdc31p*, we placed the *cdc31* gene alone, or in frame with the GFP coding sequence, under the control of the *nmt*^{*} thiamine-sensitive promoter (*pnmt*^{*}-*cdc31* and *pnmt*^{*}-GFP*cdc31* plasmids; see MATERIAL AND METHODS). Plasmids were transformed into wild-type cells and expression of *cdc31p* or GFP*cdc31* fusion protein was induced by removal of thiamine from the medium for 16 h. Anti-HsCen3p Igs revealed an increased 23-kDa band in extracts from cells transformed with the *pnmt*^{*}-*cdc31* plasmid. In the case of the *pnmt*^{*}-GFP*cdc31* plasmid, a 50-kDa band, corresponding to the expected molecular weight of the GFP*cdc31* fusion protein was observed as well as a degradation pattern underneath (Figure 1C).

These results indicate that *cdc31* gene encodes a 23-kDa protein that is recognized by the anti-HsCen3p Igs.

Figure 1. *cdc31* encodes a 23-kDa protein of the centrin 3/ScCdc31p subfamily of centrins. (A) Alignment of *cdc31p* with human centrins, ScCdc31p, and human calmodulin. Identical residues are boxed in black. Similar residues are shaded in gray. *cdc31p* presents 58.6% and 58.3% identity with HsCen3p and ScCdc31p, respectively, 50.0% and 51.1% identity with HsCen1p and HsCen2p, and 38.2% identity with human calmodulin. (B) Dendrogram of centrins and calmodulins. *cdc31p* clusters with the HsCen3p/ScCdc31p subfamily of centrins. (C) Western blot analysis of *cdc31p* using affinity-purified anti-HsCen3p Igs. 1, Wild-type cells; 2, wild-type cells overexpressing *cdc31p* from *pnmt⁺-cdc31* plasmid; 3, wild-type cells expressing a GFP*cdc31* fusion protein. Molecular weights (kDa) are shown on the left.

cdc31p Is a Component of the Half-bridge of the Spindle Pole Body

To determine the subcellular localization of *cdc31p*, we performed immunofluorescence experiments on wild-type cells fixed in -20°C methanol using anti-HsCen3p Igs and antitubulin mAb TAT1 (Figure 2A). Cdc31p was present in one or two bright juxta-nuclear spots. Interphase cells exhibited a single *cdc31p* spot associated with one bundle of microtubules, whereas mitotic cells exhibited two bright spots at the poles of the mitotic spindle. In postmitotic cells presenting a postanaphase array of MTs, a single spot was associated with each nucleus. In addition, an irregular background was consistently observed all over the cell, suggesting that the protein may also be present in other cellular compartments as reported for centrins in animal cells (Paoletti *et al.*, 1996). No specific staining

was found at the equatorial MTOCs during septation nor at interphase MTOCs on the nuclear envelope.

This data suggested that the protein was concentrated at the SPB. To further test the colocalization of *cdc31p* with the SPB, we stained a strain expressing a *cam1*-GFP fusion protein that localizes to the SPB (Moser *et al.*, 1997) with anti-HsCen3p Igs. *cdc31p* and *cam1*-GFP clearly colocalized at the SPB (Figure 2B).

Finally, to determine the ultrastructural localization of the protein, we performed immunogold labeling of *cdc31p* on wild-type cells. Clusters of gold particles were found on adjacent serial sections in association with an electron-dense appendage corresponding to the half-bridge of the SPB (Figure 3, A2–A4, and B3 and B4). No gold particle was detected on the SPB-layered structure (Figure 3, arrows in A–C). *cdc31p* was also detected in the bridge connecting the two duplicated SPBs

Figure 2. *cdc31p* localizes to the SPB at all phases of the cell cycle. (A) Top: Triple staining of wild-type cells for *cdc31p* (red), MTs (green), and DNA (blue). Bar, 5 μ m. Bottom: Series of similarly stained cells at different cell cycle stages. From left to right: early G2, early mitosis, early and late anaphase, and postmitotic cell. (B) Staining of a GFP-cam1 strain for *cdc31p*. *cdc31p* staining (left) and GFP fluorescence (right) after formaldehyde fixation and processing for immunofluorescence are shown. Arrows point at SPBs. Bar, 5 μ m.

(Figure 3C) and on the side of spindle poles during mitosis (Figure 3D). Interestingly, gold beads were distributed throughout the half-bridge appendage rather than on the nuclear facing surface. Therefore, *cdc31p* association with the half-bridge is unlikely to depend on a membrane spanning protein as reported for *ScCDC31* in budding yeast (Spang *et al.*, 1993, Biggins and Rose, 1994; see DISCUSSION).

cdc31 Is an Essential Gene Required for Bipolar Spindle Formation

To determine the function of *cdc31* in fission yeast, we deleted in a diploid strain one copy of the whole gene and replaced it by homologous recombination by the *kanMX* cassette conferring resistance to G418 (strain AP54; Figure 4A and Table 1). After sporulation, tetrads were dissected and spores allowed to germinate and form colonies on YE5S plates at 25°C. Only two spores per tetrad gave rise to colonies. These colonies were not resistant to G418, indicating that none of the Δ *cdc31* segregants were viable (Figure 4B). We thus conclude that *cdc31* is an essential gene.

Random spores were then produced from the diploid strain and allowed to grow in liquid YE5S medium for 15 h, fixed in -20°C methanol, and processed for immunofluorescence with anti-HsCen3p Igs and antitubulin mAb TAT1. Two populations of cells were observed: *cdc31+* cells with a *cdc31p* staining on the SPB and Δ *cdc31* cells with no *cdc31p* SPB staining. The Δ *cdc31* cells exhibited abnormal aster or V-shaped microtubule structures likely representing monopolar spindles and hypercondensed chromosomes (Figure 4C). This phenotype suggested that *cdc31* is necessary for bipolar spindle formation.

HsCen3p Expression in Fission Yeast Is Toxic

We next investigated the ability of genes of the centrin 3/Cdc31p subfamily of centrins to complement *cdc31* deletion in *S. pombe*. We found that neither *ScCdc31p*, nor *HsCen3p* expression, could complement *cdc31* deletion in a plasmid loss assay (our unpublished results).

Then, we examined the effect of overexpressing *Spcdc31p* or expressing *HsCen3p* or *ScCdc31p* in fission yeast from the medium strength *nmt** promoter. *cdc31p* overexpression from *nmt** promoter did not affect growth (Figure 5, A and B), nor from the full-strength *nmt* promoter (see Figure 9C). *ScCdc31p* expression had no effect either. In contrast, *HsCen3p* production inhibited colony formation. Analysis of cells expressing *HsCen3p* by immunofluorescence revealed an accumulation of cells with monopolar spindles (Figure 5C). This suggests that *HsCen3p* has a dominant negative effect on SPB duplication in fission yeast as reported in budding yeast (Figure 5A; Midden-dorp *et al.*, 2000). These results also indicate that *ScCdc31p* and *HsCen3p* cannot carry out *S. pombe* *cdc31p* function.

Construction of *cdc31* Conditional Mutants

To further characterize *cdc31* function in fission yeast, we generated conditional *cdc31* mutants. These mutants were obtained by stable integration in a Δ *cdc31* strain of plasmids in which wild-type *cdc31* or mutated *E147Kcdc31* were placed under the control of the repressible *nmt** promoter (*E147K* mutation is analogous to the mutation present in *cdc31-2* thermosensitive mutant in *Saccharomyces cerevisiae*; Biggins and Rose, 1994).

Growth of these mutant strains (AP390 and AP388 hereafter named *pnmt*-cdc31* and *pnmt*-E147Kcdc31* mutants) was analyzed on plates after serial dilutions of exponentially growing liquid cultures (Figure 6A). In thiamine-free medium, both strains grew similarly to wild-type cells at 30 or 36°C. But both strains were thiamine sensitive: *pnmt*-cdc31* mutant turned pink on minimum medium containing phloxin and thiamine, and *pnmt*-E147Kcdc31* mutant was not able to form colonies.

Figure 3. *cdc31p* is a component of the half-bridge. Postembedding immunogold localization of *cdc31p* in wild-type cells. (A–C) Serial sections through unduplicated (A1–A4 and B1–B4) or duplicated (C1–C3) SPBs. Gold particles are clustered on the half-bridge on sections adjacent to the SPB-layered structure pointed by black arrows or on the bridge between the duplicated SPBs. Position of the nuclear envelope is highlighted by double black lines in A1, B1, and C1. Orientation of cytoplasm (Cyt) and nucleus (Nu) is always as in A1. Bars, 100 nm. (D) Section through a mitotic spindle. Gold particles are located on the side of the spindle poles. Bar, 100 nm. Note that the gold particles are distributed throughout the half-bridge electron-dense structure rather than on the nuclear facing surface of it.

For this strain, any residual growth was abolished when cells were grown at 36°C in the presence of thiamine.

Expression levels of the mutant protein E147Kcdc31 were then analyzed by Western blotting. At 30°C in absence of thiamine E147Kcdc31p was slightly less expressed than *cdc31p* in wild-type cells. Depletion of E147Kcdc31p could readily be observed 2–4 h after addition of thiamine at 36°C. Almost no

protein was detected from 6 h on. Comparable levels of expression were observed in *pnmt**-*cdc31* mutant.

In conclusion, E147K point mutation, does not confer a thermo-sensitive phenotype when expressed at endogenous levels, and depletion of *cdc31p* by addition of thiamine in *pnmt**-*cdc31* mutant is not sufficient to stop growth. However, combining E147K mutation and depletion effectively abolishes *cdc31* function.

Figure 4. Deletion of *cdc31* is lethal and induces monopolar spindle formation. (A) *cdc31* ORF was replaced in a diploid strain by the kanMX cassette conferring resistance to G418. A scheme of *cdc31* exons and intron is represented. Position of their ends relative to *cdc31* ATG codon is indicated in base pairs. (B) Analysis of tetrads from a $\Delta cdc31/cdc31^+$ strain (AP54). Four spores from five tetrads were deposited vertically. Only two spores per tetrad form colonies on YE5S (left), which cannot grow in presence G418 (replica on the right side). (C) Random spores from the $\Delta cdc31/cdc31^+$ strain were grown in YE5S, fixed in methanol, and stained for *cdc31p*, MTs, and DNA. Arrows point at spores depleted in *cdc31p* presenting monopolar spindles. Bar, 5 μ m.

Two other strains carrying point mutations in *cdc31* (A62T and P108S; corresponding to *cdc31-1*, *cdc31-5* mutants in *S. cerevisiae*; Biggins and Rose, 1994) behaved similarly to *pnmt*-E147Kcdc31* mutant (unpublished data) and displayed similar phenotypes (see below).

Chromosome Segregation Defects and Monopolar Spindle Formation in Conditional *cdc31* Mutant

Because the growth of *pnmt*-E147Kcdc31* mutant was completely abolished in thiamine at 36°C, we choose these ex-

Figure 5. HsCen3p is toxic in *S. pombe*. (A and B) Wild-type cells carrying *pnmt*-cdc31*, *pnmt*-HsCen3*, or *pnmt*-ScCDC31* plasmids or an empty vector (pSLF172) were grown on EMM plates with (A) or without thiamine (B). HsCen3p prevents colony formation when the medium strength *nmmt** promoter is induced. (C) Wild-type cells carrying *pnmt*-HsCen3* plasmids grown for 24 h at 30°C in absence of thiamine and stained for tubulin. Arrowheads point at cells with monopolar spindles. Bar, 5 μ m.

Figure 6. Analysis of *Spcdc31* conditional strains. (A) Growth of *pnmt⁺-cdc31* (Δ *cdc31* + *pnmt⁺-cdc31* plasmid integrated, strain AP390) and *pnmt⁺-E147Kcdc31* (Δ *cdc31* + *pnmt⁺-E147Kcdc31* plasmid integrated, strain AP388) mutants at 30 or 36°C, in permissive (–thiamine) or restrictive (+thiamine) conditions. Wild-type strain: AP131. Serial 1:10 dilutions were spotted on EMM plates containing adenine, uracil, and phloxin B. (B) Western blot analysis of *cdc31p* or *E147Kcdc31p* expression levels in mutant strains. Total extracts from wild-type cells (wt) are compared with total extracts from *pnmt⁺-cdc31* or *pnmt⁺-E147Kcdc31* cells grown at 30°C in absence of thiamine (t = 0) or for 2–12 h at 36°C in presence of thiamine. (C and D) Accumulation of abnormal nuclei under restrictive conditions in *pnmt⁺-E147Kcdc31* strain: nuclei morphology was monitored 2–12 h after shift to 36°C in presence of thiamine. Cells with hypercondensed chromosomes (arrowhead), cut phenotype (*), missegregated nuclei (arrows), or disrupted nuclei start accumulating 6 h after shift to 36°C in presence of thiamine. Bars, 5 μ m.

perimental conditions to further analyze the function of *cdc31* gene. Cells were grown in exponential phase in absence of thiamine and shifted to 36°C in presence of thiamine for 2–12 h, fixed in formaldehyde, and stained with DAPI. Various abnormal nuclear structures were observed after 6 h. These include hypercondensed chromosomes, nuclei cut by a septum, missegregated nuclei, and cells with disrupted nuclei (Figure 6C). These events started to accumulate 6 h after the shift at 36°C in presence of thiamine and affected the whole population (>90%) after 12 h.

Thus, *pnmt⁺-E147Kcdc31* mutants exhibit strong defects in chromosome segregation. We then looked at SPBs and spindle formation in *pnmt⁺-E147Kcdc31* mutant. For that purpose, cells were fixed after 8 h at 36°C in presence of thiamine and double-stained for MTs and nuclei or γ -tubulin and nuclei (Figure 7). We could observe that 30% of cells presented

monopolar spindles and hypercondensed chromosomes, indicating that they were blocked in mitosis (Figure 7A). In these cells, a single dot of gamma-tubulin was observed (Figure 7B).

These data show that *cdc31* mutant cells arrest in mitosis with a monopolar spindle, consistent with Δ *cdc31* spore germination experiments, and further suggest that a single SPB structure is present at the spindle pole.

Absence of SPB Duplication in *pnmt⁺-E147Kcdc31* Mutants

To test whether this spindle assembly defect may be a consequence of a failure in SPB duplication, ultrastructural analysis of the mutant cells was performed after 8 h of growth at 36°C in presence of thiamine. Cells presenting a monopolar

Figure 7. Depletion of E147Kcdc31p induces monopolar spindle formation. *pnmt*-E147Kcdc31* cells (strain AP388) were double-stained for MTs and DNA (A) or γ-tubulin and DNA (B) after growth at 30°C in absence of thiamine (left) or after 8 h of growth at 36°C in presence of thiamine (right). In repressive conditions cells with hypercondensed chromosomes and monopolar spindles are observed (arrows). Cells with hypercondensed chromosomes present a single spot of γ-tubulin (arrows), suggestive of a failure in SPB duplication. Bars, 5 μm.

spindle were analyzed by serial sectioning ($n = 9$). All exhibited a single SPB structure (Figure 8, A1–A6). Some cells exhibited MTs that emanated from the SPB, reached the opposite surface of the nucleus, and induced deformations in the nuclear envelope. In addition, some cells exhibited an incomplete septum ($n = 4$, Figure 8A), which could cut the nucleus ($n = 2$). One cell exhibited three incomplete septa. This suggested that some cells had proceeded to septum formation while blocked in mitosis.

In conclusion, this analysis clearly indicates that *cdc31p* is required in an early stage of SPB duplication and further suggests that the control of septation initiation is perturbed in the mutant.

Genetic Interactions between *sad1* and *cdc31*

We also looked at the distribution of *sad1p*, another SPB component required for bipolar spindle formation (Hagan and Yanagida, 1995) using anti-*sad1p* antibody. *pnmt*-E147Kcdc31* or wild-type cells were grown at 30°C for 18 h in presence of thiamine and triple-stained for *sad1p*, MTs, and DNA. Surprisingly, *sad1p* was found in multiple spots in *pnmt*-E147Kcdc31* cells, instead of being concentrated in a single spot at the SPB like in wild-type cells (Figure 9A). These data suggest that *cdc31p* is required for the localization or concentration of *sad1* protein at the SPB.

Next, we compared the growth rates of single or double *sad1-1* and *pnmt*-E147Kcdc31* mutants in permissive conditions (30°C without thiamine). We found that the double mutant grew more slowly and exhibited more cell death compared with the single mutants, indicating a synthetic effect between the mutations (Figure 9B).

Finally, we compared the effect of overexpressing a nonfunctional dominant negative HA₃*cdc31* fusion protein in wild-type cells and in *sad1-1* mutant grown at permissive temperature. We found that HA₃*cdc31p* expression at low levels in presence of thiamine (*nmt* full-strength promoter) severely affected colony number and size in *sad1-1* mutant, whereas it had no effect on wild-type cells. Moreover, high expression in absence of thiamine was lethal in *sad1-1* strain and only mildly perturbed growth in a wild-type background, indicating that HA₃*cdc31p* is hyper-toxic in *sad1-1* strain (Figure 9C). Immunofluorescence analysis of *sad1-1* cells expressing HA₃*cdc31p* revealed the presence of cells blocked in mitosis with monopolar spindles (Figure 9D).

To check whether this effect was specific to *sad1-1* strain or could be observed in any SPB mutant, HA₃*cdc31p* was also expressed in *alp4-1891*, *alp6-719* (Vardy and Toda, 2000), *alp16Δ* (Fujita *et al.*, 2002), *cam1-E14* (Moser *et al.*, 1997), and *cut12-1* (Bridge *et al.*, 1998) strains. None of the strains grown at permissive temperature was affected by low levels of expression. At high levels of expression, HA₃-*cdc31* was slightly more toxic than in wild-type cells but not lethal like in *sad1-1* strain (Table 2). In addition, double *alp4-1491 pnmt*-E147Kcdc31* (Figure 9C) and double *alp6-719 pnmt*-E147Kcdc31* mutants (our unpublished results) grew as well as single mutants.

These results indicate that *cdc31p* may interact directly or indirectly with *sad1p* and regulate its localization and function.

Mis-timing of Septum Formation before Mitosis Exit in *pnmt*-E147Kcdc31* Mutants

Because ultrastructural analysis suggested that septum formation may occur untimely in *pnmt*-E147Kcdc31* mutant (see above), we monitored septum formation.

First, cells were grown in exponential phase in absence of thiamine and shifted to 36°C in presence of thiamine for 2–12 h and stained for septa. Abnormal septa including multiple or misshaped septa started accumulating 8 h after shift and reached ~40% of the population after 12 h (Figure 10, A and B), indicating that abnormal septation events occur after the formation of monopolar spindles.

Second, to test if septation did occur in cells blocked in mitosis and evaluate the timing of septation events, cells were synchronized in S phase by incubation for 3 h in HU at 36°C in presence of thiamine. After HU washout, cells were further incubated at 36°C in presence of thiamine, fixed every 20 min,

Figure 8. Monopolar spindles are organized by an unduplicated SPB. (A) Electron microscopy of *pnm1^{*}-E147Kcdc31* mutant (strain AP388) grown for 8 h at 36°C in presence of thiamine. Note that nuclear MTs emanating from the single SPB induce deformations in the nuclear envelope on the opposite side of the nucleus (arrowheads). Note also the presence of an incomplete septum (sept). nu, nucleolus. Bar, 200 nm. (A1–A6) Serial sections through the SPB. A4 corresponds to the white inset in A. White arrows point at the single SPB. Bar, 100 nm.

and stained for MTs, DNA, and mid1p, a protein that colocalizes with the contractile ring in mitosis until it starts contracting in late anaphase. Synchronized cells performed a normal first round of mitosis and septation (Figure 10C, $t = 80$ and 100 min, and D and E). Cells assembled monopolar spindles in the second round of mitosis and proceeded to septation 20–40 min later (Figure 10C, $t = 180$ and 220 min, and D and E) despite a sustained mitotic block as judged by the presence of long septated cells with monopolar spindles and of short cells with monopolar spindles after sister cell separation. In these cells, hypercondensed chromosomes were missegregated or “cut” by the septum. In addition, these cells maintained a strong mid1p staining in the septum region or at the new end, a situation never observed in the first round of normal mitosis and septation (see $t = 100$ in Figure 10C), nor in wild-type cells (Sorhmann *et al.*, 1996; Paoletti *et al.*, 2000). Additional rings or strands of mid1p were also sometimes observed next to the

septum (see left cell $t = 220$), suggesting that multiseptation events that accumulate after the second round of mitosis (see Figure 10C) may arise from continuous induction of septation in cells blocked in mitosis that form supernumerary cytokinetic rings.

Our results indicate that *cdc31p* is required for the proper coordination between mitotic exit and septation.

DISCUSSION

cdc31 Encodes a Half-bridge Component of the SPB Required for SPB Duplication

Centrins are a conserved family of centrosomal proteins that have been implicated in centrosome duplication (Baum *et al.*, 1986; Middendorp *et al.*, 2000; Salisbury *et al.*, 2002). Here, we show that fission yeast has one centrin-like gene that

Figure 9. *sad1p* staining redistributes into several spots in *pnmt⁺-E147Kcdc31* mutant and *sad1* interacts genetically with *cdc31*. (A) Triple staining of wild-type cells (left; strain AP131) and *pnmt⁺-E147Kcdc31* mutant (strain AP388) grown for 18 h at 30°C in presence of thiamine for *sad1p* (top), MTs (middle), and DNA (bottom). In restrictive conditions, a splitting of *sad1p* staining is observed in cells presenting monopolar spindles (arrows). Maximum projections of z series throughout the cells are shown. Bars, 5 μ m. (B) Growth of *sad1-1 pnmt⁺-E147Kcdc31* double mutant (AP662, *sad1-1* $\Delta cdc31$ + *pnmt⁺-E147Kcdc31* plasmid integrated) compared with simple mutants (AP666, $\Delta cdc31$ + *pnmt⁺-E147Kcdc31* plasmid integrated and AP607, *sad1-1* + *pnmt⁺-E147Kcdc31* plasmid integrated) and of *alp4-1891 pnmt⁺-E147Kcdc31* double mutant (AP900) compared with simple mutants (AP899 and AP901) at 30°C in absence of thiamine (permissive conditions). *sad1-1 pnmt⁺-E147Kcdc31* double mutant is pinker in presence of phloxin and shows reduced growth compared with the two simple mutants. (C) Hypertoxicity of HA₃cdc31p in *sad1-1* strain. *sad1-1*, *alp4-1891*, and wild-type strains transformed with *pnmt-cdc31* and *pnmt-HA₃cdc31* plasmids (full-strength nmt promoter), and pREP42X as a control were grown at 25 or 30°C in presence or absence of thiamine. High levels of expression of *cdc31p* do not affect growth. In wild-type and *alp4-1891* cells, HA₃cdc31p has a dominant negative effect when expressed at high levels only. In *sad1-1* strain, low levels of expression of HA₃cdc31p are sufficient to strongly reduce growth, and high levels of expression are lethal. (D) Double staining of *sad1-1* cells expressing HA₃cdc31p for tubulin and DNA. HA₃cdc31p expression was induced for 24 h at 25°C. Arrow: monopolar spindle; arrowhead: bipolar spindle. Bar, 5 μ m.

encodes a component of the SPB and is essential for SPB duplication: *cdc31* mutant arrests in mitosis with a single unduplicated SPB and a monopolar mitotic spindle. *cdc31p* is the first identified *S. pombe* protein required for SPB duplication.

cdc31p resides in a cytoplasmic electron dense appendage on the side of the SPB called the half-bridge by functional analogy with *S. cerevisiae* half-bridge or bridge, which connects the two duplicated SPBs (Byers and Goetsch, 1974; Ding *et al.*, 1997). The comparison of fission yeast *cdc31p* and ScCdc31p localizations highlights the structural differences between the

half-bridges in the two yeasts: whereas the ScCdc31p resides on a thin layer tightly associated with the nuclear envelope, fission yeast *cdc31p* is distributed throughout a thick electron-dense appendage, which transiently dissociate from the nuclear envelope in early mitosis when duplicated SPBs are inserted into the nuclear envelope (Ding *et al.*, 1997).

It has been shown that ScCdc31p association with the half-bridge depends on another half-bridge component, Kar1p, which is directly anchored to the nuclear envelope via its C-terminal domain (Biggins and Rose, 1994; Spang *et al.*, 1995). Because *cdc31p* distribution is not restricted to the nuclear side

Table 2. Growth of SPB mutant strains expressing HA₃cdc31p

Strain	+Th	-Th
wild type	+++	++
<i>sad1-1</i>	+/-	-
<i>cut12-1</i>	++	+/-
<i>alp4-1891</i>	+++	+
<i>alp6-719</i>	+++	+
<i>alp16Δ</i>	+++	+
<i>cam1-E14</i>	+++	+

Serial dilutions of wild type, *sad1-1*, *cut12-1*, *alp16Δ*, *alp4-1891*, *alp6-719* and *cam1-E14* strains expressing HA₃cdc31p under the control of *pnmt* promoter were spotted on plates of minimum medium with or without thiamine and incubated at 30°C until colony formed. Th, thiamine.

of the half-bridge, it seems unlikely that cdc31p anchoring to the half-bridge is mediated by a membrane-associated protein like Kar1p in *S. pombe*. Accordingly, no gene homologous to *KAR1* has been described in *S. pombe* genome. It will be of great interest in the future to determine how cdc31p association with the half-bridge is mediated in *S. pombe*.

A number of other mutants in which bipolar spindle formation is compromised because of perturbations of SPB function have been described previously in fission yeast. These include mutants in components of the fission yeast gamma-tubulin complex *alp4p* and *alp6p* (Vardy *et al.*, 2002), calmodulin (Moser *et al.*, 1997), *cut11p* (West *et al.*, 1998), *cut12p* (Bridge *et al.*, 1998), and *sad1p* (Hagan and Yanagida, 1995). *pnmt*-E147Kcdc31* is the first mutant in which spindle defects result from a complete lack of SPB duplication. Because we observed that in *cdc31* mutant, the single SPB was properly inserted into the nuclear envelope, we can also infer from our data that SPB duplication is not a prerequisite for SPB insertion into the nuclear envelope in fission yeast.

Conservation of a Network of Genes Controlling Centrosome Duplication

Our data on *cdc31p* indicate that the key factors controlling the initiation of SPB duplication have been conserved between the two distantly related yeasts and further illustrate the important function of the centrin 3/Cdc31p family in the control of centrosome reproduction. However, our attempts to complement *cdc31* deletion by the expression of HsCen3p or ScCdc31p failed. Moreover, HsCen3p expression was toxic, as reported previously for *HsCen3* expression in budding yeast (Middendorp *et al.*, 2000). This suggests that partners of the three proteins may have diverged more than centrin during evolution.

How members of the centrin 3/Cdc31p family may trigger centrosome duplication remains poorly understood. In *S. cerevisiae*, a network of SPB duplication genes including *CDC31*, *KAR1*, and two ubiquitin like genes, *DSK2* and *RAD23* acting upstream of *CDC31* (Biggins *et al.*, 1996) has been described. Recent data also indicate that this network may be regulated by the *PKC1* pathway (Khalfan *et al.*, 2000). As mentioned above, no gene homologous to *KAR1* has been identified in *S. pombe* genome, but *S. pombe* genes homologous to *DSK2* and *RAD23*

have been characterized (*dph1* and *rhp23*, respectively; He *et al.*, 1998; Elder *et al.*, 2002). These genes are not essential and direct evidence for their implication in SPB duplication is lacking. However, the fact that overexpression of *dph1* induces monopolar spindle formation like *DSK2* (He *et al.*, 1998) suggests that *dph1* and *DSK2* may share a conserved function in SPB duplication.

Our studies suggest that *sad1p* is a potential downstream target of *cdc31p*. *sad1p* is similar to *C. elegans Unc-84* (Malone *et al.*, 1999). A *sad1-1* mutant exhibits a monopolar spindle phenotype like *cdc31* mutant (Hagan and Yanagida, 1995). Ultrastructural localization of *sad1p* is not known, although *sad1p* is likely anchored to the nuclear envelope because it contains a putative transmembrane domain and overexpression leads to an accumulation on the nuclear envelope (Hagan and Yanagida, 1995). We observed that *sad1p* localization at the SPB is compromised in *pnmt*-E47Kcdc31* mutant, whereas *cdc31p* is still concentrated at the SPB in the *sad1-1* mutant (unpublished data). This suggests that *cdc31p* may control the accumulation of *sad1p* at the SPB. We also found that the two genes display extensive genetic interactions. In budding yeast, there is no *sad1* homologue, and no gene acting directly downstream of *CDC31* has been identified, although *CDC31* interacts genetically with SPB satellite components such as *Spc29p* (Adams and Kilmartin, 1999; Elliott *et al.*, 1999).

An Additional Function for *cdc31* in Regulating the Septation Initiation Network?

In budding yeast, it has been shown that ScCDC31p has additional functions independent of SPB duplication (Sullivan *et al.*, 1998; Ivanovska and Rose, 2001). In particular, ScCDC31p is required for KIC1 kinase activity, which plays a role in the maintenance of cellular integrity. In *pnmt*-E147Kcdc31* mutant, corresponding to *cdc31-2* allele in *S. cerevisiae*, we did not observe any cell lysis phenotype nor in two other mutants corresponding, respectively, to *cdc31-1* and *cdc31-5* (*pnmt*-A42Tcdc31* and *pnmt*-P108Scdc31*; unpublished data).

In *pnmt*-E147Kcdc31* mutant, we observed cells displaying multiple or aberrant septation events as well as cells containing both mitotic spindles and septa. In synchronous cultures, cells blocked in mitosis with a monopolar spindle proceeded to septation in less than an hour. Cells even maintained a mitotic state after full separation of sister cells.

This suggests that spindle checkpoints controlling mitotic exit are functioning normally in *pnmt*-E147Kcdc31* strain, but that septation is allowed to occur before mitosis completion. Accordingly, the strain is not hypersensitive to thiabendazole (our unpublished results) as reported for strains defective in spindle checkpoints (He *et al.*, 1997, 1998). This situation also differs from what has been described in APC mutants *cut9-665* and *lid1-6* in which delayed septation events are preceded by *cdc2p* inactivation (Chang *et al.*, 2001).

Induction of septation depends on a signaling cascade called the SIN (septation initiation network; see Le Goff *et al.*, 1999; Balasubramanian *et al.*, 2000; Sawin, 2000; and McCollum and Gould, 2001 for reviews). Activation of the cascade occurs in mitosis when the small GTP-binding protein *spg1p* switches from the GDP to the GTP-bound form (Schmidt *et al.*, 1997). However, activation of the last steps in the SIN requires the inactivation of *cdc2p* at the exit of mitosis, ensuring that septation does not take place before chromosome segregation is

Figure 10. Septation defects in *pnmt**-*E147Kcdc31* cells. (A and B) Accumulation of abnormal septa under restrictive conditions in *pnmt**-*E147Kcdc31* strain (AP388). Septation was monitored 2–12 h after shift to 36°C in presence of thiamine. Misorganized and multiple septa accumulate from 8 h on. Bar in A, 5 μ m. (C–E) Synchronized *pnmt**-*E147Kcdc31* cells proceed to septation before mitosis exit. *pnmt**-*E47Kcdc31* mutant (AP388) was synchronized by growth for 3 h at 36°C in presence of thiamine and HU. On HU washout, cells were further incubated at 36°C in presence of thiamine, fixed, and stained for MTs, mid1p, and DNA at 20-min intervals (C and D) or stained for septum at same time points (E). In D, the percentage of cells with a bipolar spindle (squares), of nonseptated cells with a monopolar spindle (circles), or of septated cells or short cells after sister cell separation containing a monopolar spindle (diamonds) was determined according to MTs and mid1p staining. The percentage of cells presenting normal (squares) or abnormal and multiple septa (diamonds) is represented in E. Cells undergo a first normal round of mitosis ($t = 80$ min) and cytokinesis ($t = 100$ min). In the second round of mitosis, cells assemble monopolar spindles and proceed to septation 20–40 min later, before spindle breakdown ($t = 180$ and $t = 220$). Note the strong mid1p staining in the septum region in septated cells or at the new end in short separated cells containing monopolar mitotic spindles (right cells at $t = 180$ and bottom cells at $t = 220$). Note also the two supernumerary mid1p rings in the left cell at $t = 220$ min on each side of the septum. Bar in C, 2 μ m.

achieved (Guertin *et al.*, 2000; Chang *et al.*, 2001). This requirement seems to be abolished in the *pnmt⁺-E147Kcdc31* mutant.

Because several components of the SIN network including the small GTP-binding protein spg1 (Schmidt *et al.*, 1997), the GAPs controlling its activity (Cerutti and Simanis, 1999; Li *et al.*, 2000) and downstream kinases of the SIN (Sohrmann *et al.*, 1998; Sparks *et al.*, 1999; Guertin *et al.*, 2000) are permanently or transiently located at the SPB, one intriguing possibility is that the localization of SIN components is defective in *cdc31* mutant and allows the last steps of SIN to occur before *cdc2* inactivation.

Untimely activation of septation before mitosis exit as well as a multiseptation phenotype have recently been reported for *alp4-1891* mutant (Vardy *et al.*, 2002), and a novel role for the γ -tubulin complex in inhibiting the SIN activation during mitosis has been proposed. However, because γ -tubulin is still associated with the spindle pole in *pnmt⁺-E147Kcdc31* mutant, it seems unlikely that the septation defects are caused solely through the γ -tubulin complex.

It will be important to determine whether the septation defects result from an independent function of *cdc31* in regulating the initiation of septation or are a consequence of the absence of SPB duplication. Characterization of the behavior of various components of the SIN in *pnmt⁺-E147Kcdc31* strain and of additional alleles of *cdc31* will help to distinguish these possibilities.

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