

Requirement for Bbp1p in the Proper Mitotic Functions of Cdc5p in *Saccharomyces cerevisiae*

Chong J. Park,* Sukgil Song,*[†] Thomas H. Giddings, Jr.,[‡] Hyeon-Su Ro,*[§]
Krisada Sakchaisri,* Jung-Eun Park,* Yeon-Sun Seong,* Mark Winey[‡], and
Kyung S. Lee*^{||}

*Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; and [‡]Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado 80309-0347

Submitted July 2, 2003; Revised November 7, 2003; Accepted November 21, 2003
Monitoring Editor: Tim Stearns

The polo-box domain of the budding yeast polo kinase Cdc5p plays an essential role for targeting the catalytic activity of Cdc5p to spindle pole bodies (SPBs) and cytokinetic neck-filaments. Here, we report the isolation of Bbp1p as a polo-box interacting protein by a yeast two-hybrid screen. Bbp1p localizes to the periphery of the central plaque of the SPB and plays an important role in SPB duplication. Similarly, Cdc5p localized to the cytoplasmic periphery of the SPB. In vitro binding studies showed that Cdc5p interacted with the N-terminal domain of Bbp1p (Bbp1p Δ C), but apparently not with Mps2p, a component shown to form a stable complex with Bbp1p. In addition, Bbp1p, but likely not Mps2p, was required for proper localization of Cdc5p to the SPB. The C-terminal coiled-coil domain of Bbp1p (Bbp1p^{243–385}), which is crucial for both the homodimerization and the SPB localization, could target the localization-defective Cdc5p Δ C to the SPB and induce the release of Cdc14p from the nucleolus. Consistent with this observation, expression of *CDC5 Δ C-BBP1^{243–385}* under *CDC5* promoter control partially complemented the *cdc5 Δ* defect. These data suggest that Bbp1p Δ C interacts with the polo-box domain of Cdc5p, and this interaction is critical for the subcellular localization and mitotic functions of Cdc5p.

INTRODUCTION

Polo protein kinases seem to play pivotal roles in regulating various cellular and biochemical events at multiple stages of M phase. Members of the polo subfamily of protein kinases have been isolated from species as divergent as budding yeast and mammals. They are characterized by the presence of a distinct region of homology in the C-terminal noncatalytic domain, termed the polo-box (Clay *et al.*, 1993). Studies in budding yeast have shown that mutations in the polo-box domain of both the mammalian polo-like kinase Plk1p or the budding yeast polo kinase homologue Cdc5p disrupt the ability of these enzymes to localize to the spindle pole body (SPB) (the functional equivalent of the mammalian centrosomes) and cytokinetic neck-filaments (Lee *et al.*, 1998; Song *et al.*, 2000). Subsequent studies in cultured mammalian cells have shown that the polo-box domain of Plk1p is required for the localization of this enzyme to centrosomes, kinetochores, and midbody (Seong *et al.*, 2002). These data suggest that the polo-box is critical in targeting the catalytic activity of the polo kinases to specific subcellular locations and that the role of the polo-box is likely conserved between budding yeast and mammalian cells.

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E03-07-0461. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E03-07-0461.

Present addresses: [†]College of Pharmacy, Chungbuk National University, 48 Gaesin-dong, Cheongju, Chungbuk, South Korea; [§]Laboratory of Integrative Biotechnology, Korea Research Institute of Bioscience and Biotechnology, 52 Oun-Dong, Yusong, Taejeon, South Korea.

^{||}Corresponding author. E-mail address: kyunglee@pop.nci.nih.gov.

The SPB of budding yeast is a multiple-layered structure with the central plaque embedded in the nuclear envelope. The outer plaque is important for organizing the cytoplasmic microtubules, whereas the inner plaque structure is important for the assembly of nuclear microtubules. Both direct biochemical purification of the SPB components and various genetic analyses of mutants defective in SPB function have led to the identification of many SPB components (Adams and Kilmartin, 2000; Schramm *et al.*, 2001). *BBP1* has been isolated as a dosage suppressor of the growth defect associated with temperature-sensitive mutations in *SPC29* (Schramm *et al.*, 2000), whose encoded protein plays a critical role in SPB duplication (Elliott *et al.*, 1999). Immunoelectron microscopy (EM) studies have shown that Bbp1p localizes to the central plaque periphery and the cytoplasmic side of the SPB (Schramm *et al.*, 2000). Studies with the temperature-sensitive *bbp1-1* mutant revealed that these cells are often defective in inserting a duplication plaque into the nuclear envelope at the restrictive temperature, whereas cells with already duplicated SPBs exhibit one non-functional SPB with defective microtubule structures (Schramm *et al.*, 2000). Coimmunoprecipitation and two-hybrid analyses suggest that Bbp1p forms a heterodimeric complex with Mps2p (Schramm *et al.*, 2000; Winey *et al.*, 1991), a protein that localizes to the SPB periphery and nuclear envelope (Munoz-Centeno *et al.*, 1999).

In addition to the role of SPB in microtubule organization and chromosome segregation, it is now apparent that the SPB also plays an important role in recruiting many regulatory components critical for mitotic exit (for reviews, see Bardin and Amon, 2001; Bettignies and Johnston, 2003). These regulatory components form an intricate signaling network, termed mitotic exit network, which leads to release

Table 1. Strains used in this study

Strain	Genotype	Source
KLY1546	<i>MATa his3-11,15 leu2-3, 112 trp1-1 ura3</i>	Laboratory stock ^a
KLY969	<i>MATa leu2-3, 112, trp1-289, ura3-52</i>	Laboratory stock ^b
JB811	<i>MATa prb1 pep4-3 trp1 leu2-3,112 ura3-52</i>	D. Kellogg
K699	<i>MATa ade2-1 trp1 -1 leu2-3, 112 his3-11,15 ura3 can1-100 ssd1-d</i>	K. Nasmyth
KKY921-2B	<i>MATa cdc5-1, ura1, leu2-3, 112 trp1-289</i>	A. Sugino
KLY961	KKY921-2B <i>URA1::GAL1-2xEGFP-CDC5ΔDB</i>	See text
SKY1710	K699 <i>bbp1Δ::KanMX6 + YCplac33-BBP1</i>	See text
SKY1716	SKY1710 + <i>YCplac22 vector</i>	See text
SKY1717	SKY1710 + <i>YCplac22-BBP1</i>	See text
SKY1718	SKY1710 + <i>YCplac22-BBP1(1-295)</i>	See text
SKY1719	SKY1710 + <i>YCplac22-BBP1(243-385)</i>	See text
SKY1720	SKY1710 + <i>YCplac22-BBP1(295-385)</i>	See text
SKY1721	SKY1710 + <i>YCplac22-BBP1(1-243)</i>	See text
IAY18	<i>MATa spe42Δ::LEU2 TRP1::SPC42-GFPx3 ade2-1 trp1-1 leu2-3, 112 his3-11, 15 ura3, GAL psi+ ssd1-d2</i>	J. Kilmartin
KLY1989	IAY18 <i>ADE2::GAL1-HA-GST-BBP1(1-295)</i>	See text
KLY1991	IAY18 <i>ADE2::GAL1-HA-GST-BBP1</i>	See text
KLY1993	IAY18 <i>ADE2::GAL1-HA-GST-BBP1(243-385)</i>	See text
KLY1995	IAY18 <i>ADE2::GAL1-HA-GST-BBP1(1-243)</i>	See text
KLY1997	IAY18 <i>ADE2::GAL1-HA-GST-BBP1(295-385)</i>	See text
ESM988-1	<i>MATa ura3-52 leu2Δ1 trp1Δ63 his3Δ200 SPC42-RFP::KanMX6</i>	E. Schiebel
KLY2761	KLY1546 <i>bbp1Δ::HIS3 TRP1::bbp1-1</i>	See text
KLY2770	KLY1546 <i>mps2-1</i>	See text
KLY3546	KLY1546 <i>CDC5-GFP::HIS3MX</i>	See text
KLY3791	KLY2761 <i>CDC5-GFP::KanMX6</i>	See text
KLY3729	KLY2770 <i>CDC5-GFP::HIS3MX</i>	See text
KLY5336	KLY1546 <i>BBP1-GFP::KanMX</i>	See text
KLY5334	KLY2761 <i>bbp1-1-GFP::KanMX</i>	See text
KLY3685	KLY1546 <i>URA3::SPC42-GFP</i>	See text
KLY3692	KLY2761 <i>LEU2::SPC42-GFP</i>	See text
KLY4323	KLY1546 <i>TRP1::CDC14-5xGFP</i>	See text
KLY4426	KLY2761 <i>URA3::CDC14-5xGFP</i>	See text
KLY3721	KLY969 <i>cdc5Δ::KanMX6 + YCplac33-GAL1-GFP-PLK1</i>	See text
SMY6-4b	<i>MATα mps2-1 ura3-52 his3Δ200 leu2-3, 112</i>	M. Winey
SAY801	<i>MATa CDC14-5xGFP:TRP1</i>	A. Toh-e
KLY4079	SAY801 <i>URA3::GAL1-CDC5</i>	See text
KLY4085	SAY801 <i>URA3::GAL1-CDC5</i>	See text
KLY4091	SAY801 <i>URA3::GAL1-CDC5ΔC-BBP1(243-385)</i>	See text
KLY4096	SAY801 <i>URA3::GAL1-CDC5ΔC</i>	See text

^a KLY1546 is in W303-1A genetic background.

^b KLY969 is a segregant of KKY902 (Kitada *et al.*, 1993).

of Cdc14p phosphatase from the nucleolus. Released Cdc14p dephosphorylates the Cdh1/Hct1 of the anaphase promoting complex (APC) to stimulate APC-dependent degradation of mitotic cyclins (Visintin *et al.*, 1998), resulting in the inactivation of the Cdc28/Clib2 complex. Tem1 GTPase plays a critical role in mediating this process by interacting with the downstream effector Cdc15p (Asakawa *et al.*, 2001; Ro *et al.*, 2002). It has been shown that Cdc5p functions upstream of Tem1 by phosphorylating and negatively regulating Bfa1p (Geymonat *et al.*, 2003; Hu *et al.*, 2001), which forms a two-component GTPase-activating protein (GAP) with Bub2p to negatively regulate Tem1 (Geymonat *et al.*, 2002). In addition, Cdc5p has been shown to trigger Cdc14p release from the nucleolus during early anaphase through the FEAR pathway (Stegmeier *et al.*, 2002; Yoshida *et al.*, 2002), leading to early release of Cdc14p to promote mitotic exit. As with Cdc5p, Tem1p, Bfa1, and Bub2p are also shown to localize to the SPB (Pereira *et al.*, 2000), emphasizing the importance of the SPB in regulating mitotic exit. In addition, it has been shown that Nud1p, an SPB component important for cytoplasmic microtubule organization, also plays a role in mitotic exit by promoting the

Tem1-Cdc15 interaction (Gruneberg *et al.*, 2000). This observation suggests that components at the SPB can also contribute to other cellular events by interacting with other SPB-associating proteins.

In this article, we demonstrate that, in a polo-box-dependent manner, Cdc5p interacts with Bbp1p. Bbp1p-dependent localization of Cdc5p to the SPB can induce mitotic exit and rescue the growth defect associated the *cdc5Δ* mutation. Reexamination of the *bbp1-1* mutant revealed that, in addition to the role of Bbp1p in SPB duplication, Bbp1p is required for proper mitotic progression. Our data suggest that Bbp1p contributes to the Cdc5p-dependent mitotic events by promoting the Cdc5p localization to the SPB.

MATERIALS AND METHODS

Strain and Plasmid Construction

Yeast strains and plasmids used in this study are listed in Tables 1 and 2. All deletion and epitope-tagged strains constructed in this study were confirmed by PCR. To perform immuno-EM studies, a *Bam*HI-*Sph*I fragment containing *GAL1* promoter-controlled 2 × (*EGFP*)-*CDC5ΔDB* (Song *et al.*, 2000) was first cloned into a pUC19 derivative bearing *URA1* (pSK906) at the corresponding

Table 2. Plasmids used in this study

Name	Description ^a	Source
pEG202-NLS	2 μ , <i>HIS3</i> , <i>LexA</i> DBD	Origene Technologies
pJG4-5	2 μ , <i>TRP1</i> , Transcriptional AD	Ausubel <i>et al.</i> (1995)
pET21b	<i>T7</i> , <i>His6</i>	Promega
pGEX-KG	GST fusion expression vector	Guan and Dixon (1991)
pRS305	<i>LEU2</i>	Sikorski and Hieter (1989)
pRS306	<i>URA3</i>	Sikorski and Hieter (1989)
pRS313	<i>CEN</i> , <i>HIS3</i>	Sikorski and Hieter (1989)
YCplac111	<i>CEN</i> , <i>LEU2</i>	Gietz and Sugino (1988)
YCplac22	<i>CEN</i> , <i>TRP1</i>	Gietz and Sugino (1988)
YCplac33	<i>CEN</i> , <i>URA3</i>	Gietz and Sugino (1988)
pSK910	<i>URA1</i> , <i>GAL1-2xEGFP-CDC5ΔDB</i>	This study
pSK1405	pEG202-NLS, <i>CDC5ΔDB</i>	This study
pSK1408	pEG202-NLS, <i>CDC5ΔDBΔC</i>	This study
pSK1390	pEG202-NLS, <i>CDC5ΔN</i>	This study
pSK1403	pEG202-NLS, <i>CDC5ΔN/FAA</i>	This study
pSK1883	pJG4-5, <i>BBP1(1–385)</i>	This study
pSK1882	pJG4-5, <i>BBP1(1–295)</i>	This study
pSK1884	pJG4-5, <i>BBP1(243–385)</i>	This study
pSK1901	pEG202-NLS, <i>BBP1(1–385)</i>	This study
pSK1900	pEG202-NLS, <i>BBP1(1–295)</i>	This study
pSK1902	pEG202-NLS, <i>BBP1(243–385)</i>	This study
pKL2497	pGEX-KG, <i>BBP1(1–385)</i>	This study
pKL2498	pGEX-KG, <i>BBP1(1–295)</i>	This study
pKL2500	pGEX-KG, <i>BBP1(1–243)</i>	This study
pKL2499	pGEX-KG, <i>BBP1(243–385)</i>	This study
pKL2501	pGEX-KG, <i>BBP1(295–385)</i>	This study
pKL2496	pGEX-KG, <i>MPS2</i>	This study
pKL1053	pET21b, <i>CDC5</i>	This study
pSK1868	YCplac111, <i>GAL10-FLAG-YFP-BBP1(1–385)</i>	This study
pSK1867	YCplac111, <i>GAL10-FLAG-YFP-BBP1(1–295)</i>	This study
pSK1870	YCplac111, <i>GAL10-FLAG-YFP-BBP1(1–243)</i>	This study
pSK1869	YCplac111, <i>GAL10-FLAG-YFP-BBP1(243–385)</i>	This study
pSK1871	YCplac111, <i>GAL10-FLAG-YFP-BBP1(295–385)</i>	This study
pSK1873	YCplac22, <i>GAL1-HA-GST-BBP1(1–385)</i>	This study
pSK1874	YCplac22, <i>GAL1-HA-GST-BBP1(243–385)</i>	This study
pSK1875	YCplac22, <i>GAL1-HA-GST-BBP1(1–243)</i>	This study
pSK1878	YCplac22, <i>BBP1(1–385)</i>	This study
pSK1877	YCplac22, <i>BBP1(1–295)</i>	This study
pSK1880	YCplac22, <i>BBP1(1–243)</i>	This study
pSK1879	YCplac22, <i>BBP1(243–385)</i>	This study
pSK1881	YCplac22, <i>BBP1(295–385)</i>	This study
pSK1910	<i>ADE2</i> , <i>GAL1-HA-GST-BBP1(1–385)</i>	This study
pSK1909	<i>ADE2</i> , <i>GAL1-HA-GST-BBP1(1–295)</i>	This study
pSK1912	<i>ADE2</i> , <i>GAL1-HA-GST-BBP1(1–243)</i>	This study
pSK1911	<i>ADE2</i> , <i>GAL1-HA-GST-BBP1(243–385)</i>	This study
pSK1913	<i>ADE2</i> , <i>GAL1-HA-GST-BBP1(295–385)</i>	This study
pCJ159	pRS306, <i>SPC42-GFP</i>	This study
pCJ204	pRS305, <i>SPC42-GFP</i>	This study
pCJ259	pUC19- <i>URA3</i> , <i>CDC14-5xGFP</i>	This study
pKL2701	YCplac111, <i>EGFP-CDC5</i>	This study
pKL2078	YCplac111, <i>YFP-CDC5ΔC-BBP1^{243–385}</i>	This study
pKL2422	pRS315, <i>YFP-CDC5ΔC-MPS2</i>	This study
pCJ232	YCplac111, <i>YFP-CDC5ΔC</i>	This study
pKL2420	pRS315, <i>YFP-CDC5ΔC-BBP1</i>	This study
pCJ107	pRS313, <i>MPS2</i>	This study
pCJ238	YCplac33- <i>GAL1</i> , Δ <i>CEN4</i>	This study
pCJ241	pCJ238, <i>GAL1-CDC5</i>	This study
pCJ240	pCJ238, <i>GAL1-CDC5ΔC-BBP1^{243–385}</i>	This study
pCJ242	pCJ238, <i>GAL1-CDC5ΔC</i>	This study
pKL2071	YCplac111, <i>GAL10-YFP-CDC5ΔC-BBP1^{243–385}</i>	This study
pCJ231	YCplac111, <i>GAL10-YFP-CDC5ΔC</i>	This study

^a 2 μ indicates high-copy plasmids; *CEN* indicates low-copy plasmids.

sites. The resulting acentromeric *URA1:GAL1-2x(EGFP)-CDC5 Δ DB* plasmid (pSK910) was integrated into the strain KKY921-2B (a gift of A. Sugino, Osaka University, Osaka, Japan) to generate strain KLY961. Complete deletions of the *BBP1* (*bbp1 Δ ::KanMX6*) and *CDC5* (*cdc5 Δ ::KanMX6*) open reading frames

(ORFs) were generated by the one-step gene disruption method (Longtine *et al.*, 1998). To generate strains expressing full-length or truncated forms of *HA-GST-BBP1* fusion proteins under the *GAL1* promoter control, *EcoRI-SphI* fragments containing indicated *GAL1-HA-GST-BBP1* fusions were inserted

into the corresponding sites of an acentromeric *ADE2*-bearing plasmid pASZ11 (Stotz and Linder, 1990). The resulting constructs were digested with *Bst*XI and then integrated into strain IAY18 (a gift of J. Kilmartin, Medical Research Council, Cambridge, United Kingdom) at the *ADE2* locus. To generate strains expressing a *CDC5-GFP* fusion under endogenous *CDC5* promoter control (strains KLY3546, KLY3791, and KLY3729), a *GFP::KanMX6* fragment obtained by PCR by using pFA6a-KanMX6 (Longtine *et al.*, 1998) as a template was integrated into strains KLY1546 (wild-type), KLY2761 (*bbp1-1*), or KLY2770 (*mps2-1*), respectively. Strains KLY2761 and KLY2770 were derived from strain YCS64 (a gift of E. Schiebel, The Paterson Institute for Cancer Research, Manchester, United Kingdom) and strain Wx193-7b (Winey *et al.*, 1991), respectively, by backcrossing the respective alleles repeatedly into the KLY1546 background. *Cdc5p-GFP* seemed to be fully functional, because strain KLY3546 did not exhibit any detectable defects (our unpublished data). Strains KLY5336 and KLY5334 were created by C-terminally tagging the *BBP1* locus and the *bbp1-1* gene in the *TRP1* locus, respectively, with a *GFP::KanMX6* fragment. Strains KLY3685 and KLY3692 were generated by integrating pRS306 (Sikorski and Hieter, 1989) or pRS305 (Sikorski and Hieter, 1989)-based *SPC42-GFP* plasmid into strains KLY1546 (wild-type) and KLY2761 (*bbp1-1*), respectively. Both pRS305- and pRS306-based *SPC42-GFP* constructs were generated by inserting an *Eco*RI-*Not*I or a *Xho*I-*Not*I fragment bearing *SPC42-GFP* into the corresponding sites in pRS306 or pRS305, respectively. To generate strain KLY4323, a pRS304-based *CDC14-5×GFP* plasmid (a gift of A. Toh-e, University of Tokyo, Tokyo, Japan) digested with *Stu*I was integrated into the *CDC14* locus of strain KLY1546. To generate strain KLY4426, a PCR fragment containing *URA3* was first inserted into the *Sna*BI site of pRS304-*CDC14-5×GFP*. The resulting plasmid pCJ259 was digested with *Eco*NI and integrated into strain KLY2761 at the *CDC14* locus. To study the ability of *Cdc5p*, *Cdc5pΔC-Bbp1p*²⁴³⁻³⁸⁵, and *Cdc5pΔC* to induce delocalization of *Cdc14p-GFP*₅ from the nucleolus, DNA fragments containing *GAL1-CDC5*, *GAL1-CDC5ΔC-BBP1*²⁴³⁻³⁸⁵, or *GAL1-CDC5ΔC* were first cloned into an *URA3*-based, *GAL1* promoter-controlled, acentromeric plasmid pCJ238 (*GAL1:URA3*) at the *Bam*HI and *Sph*I sites. The resulting constructs were digested with *Stu*I to integrate into strain SAY801 (a gift of A. Toh-e) at the *URA3* locus. Cells were cultured under induction conditions in the presence of 15 μg/ml nocodazole for 3 h, fixed with 3.7% formaldehyde, and then subjected to fluorescent microscopy to determine the localization of *Cdc14p-GFP*₅ in the nucleolus.

To construct plasmids for two-hybrid analyses, genes were amplified by PCR by using genomic DNA from strain S288C as template. For the tests of interaction between *Cdc5p* and *Bbp1p*, full-length *BBP1* was fused to the B42 transcriptional activation domain (AD) in pJG4-5 (Ausubel *et al.*, 1995) as a hemagglutinin (HA)-fusion protein (HA-tag derived from the vector), whereas *CDC5*, *CDC5ΔC*, *CDC5ΔN*, and *CDC5ΔN/FAA* were cloned in-frame to the LexA DNA-binding domain (DBD) in pEG202-NLS (Origene Technologies, Rockville, MD) (Song and Lee, 2001). For the tests of intramolecular interaction in *Bbp1p*, full-length or partial genes digested with *Bsp*EI and *Xho*I were cloned into pJG4-5 digested with the corresponding enzymes. The same fragments were also cloned into pEG202-NLS digested with *Bsp*EI and *Nco*I (end-filled) after the *Xho*I site was end-filled to allow blunt-end ligation. To construct plasmid pKL1053, which expresses full-length *Cdc5p* fused to both N-terminal T7 and C-terminal 6×His (His6) epitope tags, a *Bam*HI-*Hind*III fragment comprising the entire *CDC5* ORF was ligated into pET21b (Novagen, Madison, WI) after digesting with the corresponding enzymes. The baculovirus His6-HA-*Cdc5p*-Flag expression construct (Y.-W.C. and K.S.L., unpublished data) will be described elsewhere. To construct full-length or partial *BBP1* fused to glutathione S-transferase (GST), various *BBP1* fragments digested with *Bsp*EI (end-filled) and *Hind*III were cloned into pGEX-KG (Guan and Dixon, 1991) digested with *Xba*I (end-filled) and *Hind*III. To construct plasmid pKL2496, an *MPS2* fragment digested with *Xba*I and *Sal*I was inserted into pGEX-KG digested with the corresponding enzymes. Constructs expressing yellow fluorescent protein (YFP)-fused *BBP1* under control of the *GAL1* promoter control were generated by inserting full-length or truncated *BBP1* fragments digested with *Bsp*EI and *Sph*I into a YCplac111-*GAL1-Flag-YFP* (pSK913) vector digested with the corresponding enzymes. To generate pSK1873, pSK1874, and pSK1875, the pSK865 construct bearing *GAL1-HA-GST-CDC5* was first digested with *Bss*HIII and *Sph*I to eliminate *CDC5* and then ligated with the respective *BBP1* fragments digested with the corresponding enzymes. To investigate the ability of full-length or truncated forms of *BBP1* to complement the *bbp1Δ* defect, *Eco*RI-*Sph*I fragments containing various *BBP1* ORF sequences were inserted into the corresponding sites in YCplac22. These constructs bear the same endogenous *BBP1* promoter and 3'-untranslated region sequences flanking the inserted fragments. To express full-length *CDC5* as an enhanced green fluorescence protein (EGFP) fusion, a *Ppu*MI fragment containing the EGFP ORF was inserted into the *Ppu*MI site of the *CDC5* genomic DNA cloned at the *Xba*I site of YCplac111. To express *Flag-YFP-CDC5ΔC-BBP1*²⁴³⁻³⁸⁵ (pKL2078), *Flag-YFP-CDC5ΔC-BBP1* (pKL2420), or *Flag-YFP-CDC5Δ-MPS2* (pKL2422) under endogenous *CDC5* promoter control, a *Bss*HIII-*Nhe*I fragment containing *BBP1*²⁴³⁻³⁸⁵, *BBP1*, or *MPS2* was C-terminally fused to *Flag-YFP-CDC5ΔC* (aa 1-500) after digesting YCplac111-*Flag-YFP-CDC5ΔC* or pRS315-*Flag-YFP-CDC5ΔC*, respectively, with *Bss*HIII and *Nhe*I. pCJ107 was generated by inserting the *Bam*HI-*Xba*I fragment containing the full-length *MPS2* into pRS313 (Sikorski and Hieter,

1989) digested with the corresponding enzymes. To construct plasmids pCJ241, pCJ240, and pCJ242, *Bam*HI-*Sph*I fragments bearing *GAL1-CDC5*, *GAL1-CDC5ΔC-BBP1*²⁴³⁻³⁸⁵, or *GAL1-CDC5ΔC* were cloned into an *URA3*-based acentromeric vector (pCJ238) digested with the corresponding enzymes. Detailed maps for the constructs described here can be provided upon request.

Growth Conditions and Media

Yeast cell culture and transformations were carried out by standard methods (Sherman *et al.*, 1986). For cell cycle synchronization, *MATa* cells were arrested with 5 μg/ml α mating pheromone (Sigma-Aldrich, St. Louis, MO) for 2.5 h at 23°C, and then released into fresh growth medium. To select against cells containing *URA3* plasmids, cells were streaked onto synthetic minimal medium (SDM) supplemented with 1 g/l 5-fluoro-orotic acid (FOA) (Boeke *et al.*, 1984).

Two-Hybrid Assays

Quantitative β-galactosidase assays were performed as described previously (Ausubel *et al.*, 1995) according to manufacturer's protocol (Origin Technologies, Rockville, MD).

Immunoblotting

Cell lysates were prepared in TED buffer [40 mM Tris-Cl, pH 7.5, 0.25 mM EDTA, 1 mM diethiothreitol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (Pefabloc; Boehringer Mannheim, Indianapolis, IN), 10 mg/ml pepstatin A (Sigma-Aldrich), 10 mg/ml leupeptin (Sigma-Aldrich), and 10 mg/ml aprotinin (Sigma-Aldrich)] with an equal volume of glass beads (Sigma-Aldrich) as described previously (Song *et al.*, 2000). Total cellular proteins were separated by 10% SDS-PAGE (Ausubel *et al.*, 1995). Western blot analyses of total lysates were carried out with anti-HA.11 (Babco, Richmond, CA), anti-LexA (Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLAG (Sigma-Aldrich), anti-T7 (Novagen), anti-GST (BD Biosciences Clontech, Palo Alto, CA), anti-*Cdc5p* (Santa Cruz Biotechnologies), and anti-*Cdc28p* (a gift of R. Deshaies, California Institute of Technology, Pasadena, CA) as described previously (Song and Lee, 2001) using the enhanced chemiluminescence detection system (Pierce Chemical, Rockford, IL).

Preparation of Recombinant Proteins and In Vitro Protein-Protein Interaction Studies

Recombinant T7-*Cdc5p*-His6, GST, GST-*Bbp1p*, GST-*Bbp1p*¹⁻²⁹⁵, GST-*Bbp1p*¹⁻²⁴⁵, GST-*Bbp1p*²⁴³⁻³⁸⁵, GST-*Bbp1p*²⁹⁵⁻³⁸⁵, and GST-*Mps2p* fusion proteins were expressed from plasmids pKL1053, pGEX-KG, pKL2497, pKL2498, pKL2500, pKL2499, pKL2501, and pKL2496 in the *Escherichia coli* BL21 strain. T7-*Cdc5p*-His6 was partially purified using a Ni-NTA column (QIAGEN, Valencia, CA) according to the manufacturer's protocol, and GST or GST-fused proteins were purified using glutathione-Sepharose beads (Sigma-Aldrich). To investigate the interaction between *Cdc5p* and *Bbp1p* or *Mps2p*, cellular lysates prepared from Sf9 cells expressing His6-HA-*Cdc5p*-Flag were added to either bead-bound GST- or bead-bound GST-fusion proteins and then incubated in a binding buffer (1× phosphate-buffered saline containing 0.5% NP-40) for 1 h at 4°C. The resin was then washed five times with the binding buffer. Bound proteins were eluted by boiling in SDS-PAGE sample buffer and then analyzed by immunoblotting after SDS-PAGE. The same membrane was stained with Coomassie to detect GST and GST-fused proteins. To further investigate the interaction between *Cdc5p* and *Bbp1p* or *Mps2p*, T7-*Cdc5p*-His6 partially purified from *E. coli* was added to either bead-bound GST-*Bbp1p* or bead-bound GST-*Mps2p* and then incubated in a binding buffer as described above. Bound *Cdc5p* was separated by SDS-PAGE and detected by immunoblotting using anti-T7 (Novagen). Ligands precipitated were detected with anti-GST (BD Biosciences Clontech) antibody.

Cell Staining and Immunofluorescence Microscopy

Indirect immunofluorescence was performed as described previously (Lee *et al.*, 1998). Briefly, cells were fixed with 3.7% formaldehyde, and microtubules were visualized using YOL1/34 rat anti-tubulin antibody (Accurate Chemical & Scientific, Westbury, NY) and goat anti-rat CY3 antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Localization of GFP- or YFP-fused proteins was examined after fixing cells as described above. Similar localization patterns were observed with unfixed cells (our unpublished data). DNA was stained with 4',6'-diamidino-2-phenylindole (DAPI).

Immuno-EM

Immuno-EM was performed using high-pressure frozen and freeze-substituted cells as described by Giddings *et al.* (2001). Serial thin sections were viewed on a Philips CM10 electron microscope (Philips Electronic Instruments, Mahwah, NJ), and images were captured on film or with a Gatan digital camera and viewed with the Digital Micrograph Software package (Gatan, Pleasanton, CA). GFP-fused *Cdc5p* was detected with a polyclonal anti-GFP antibody (Zeng *et al.*, 1999) and 10-nm colloidal gold-conjugated secondary antibodies (Ted Pella, Redding, CA).

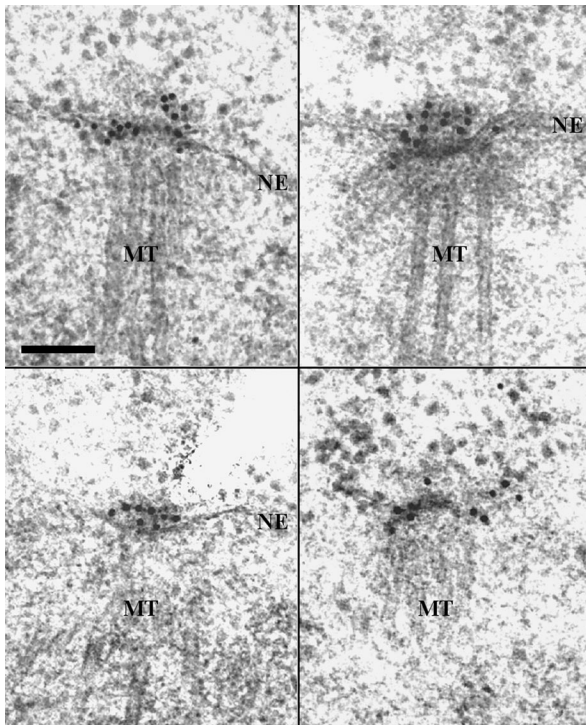


Figure 1. Immuno-EM localization of GFP-Cdc5p. A *cdc5-1* mutant strain integrated with a *GAL1*-promoter-controlled $2\times(EGFP)-CDC5$ at the *URA1* locus (KLY961) was cultured in YEP-raffinose overnight and then shifted to YEP-galactose for 1 h to induce the expression of $2\times(EGFP)-CDC5$. The cells were harvested and prepared for immuno-EM by high-pressure freezing and freeze-substitution (see MATERIALS AND METHODS). Shown are four representative images of ~ 13 SPBs examined. The 10-nm gold particles (black dots) indicate the EGFP₂-Cdc5p signals. NE, nuclear envelope; MT, nuclear microtubule. Bar, 0.1 μ m.

RESULTS

Localization of GFP-Cdc5p

Studies have shown that Cdc5p localizes to the SPBs and the cytokinetic neck-filaments (Shirayama *et al.*, 1998; Song *et al.*, 2000). To develop a better understanding of the localization and function of Cdc5p at the SPB, we attempted to localize the GFP-fused Cdc5p within the SPB by carrying out immuno-EM with affinity-purified anti-GFP antibody. The expression level of a Cdc5p-GFP fusion protein under the endogenous *CDC5* promoter control did not yield reliable signals (our unpublished data). Thus, strain KLY961, expressing *GAL1-2\times(EGFP)-fused CDC5\Delta DB* lacking the destruction box (Song *et al.*, 2000) in the *cdc5-1* background, was cultured under induction conditions for 1 h before fixation. Strong EGFP₂-Cdc5p ΔDB signals were manifest at or near the SPB under these conditions. Among 13 cells examined, EGFP₂-Cdc5p ΔDB was most commonly detected at the cytoplasmic side or the periphery of the central plaque or over the outer plaque (11/13 cells; Figure 1). In two cases, however, the EGFP₂-Cdc5p ΔDB signal was also evident at the inner plaque of the spindle pole body (our unpublished data). These observations suggest that Cdc5p primarily localizes to the cytoplasmic periphery of the SPB. Whether the less frequent EGFP₂-Cdc5p ΔDB signals at the nuclear side of the SPB are suggestive of a fraction of Cdc5p localizing to this location or are due to the artifact of overexpressed Cdc5p is not clear at present.

Isolation of Bbp1p as a Polo-Box-interacting Protein

We have previously shown that the C-terminal domain of Cdc5p (Cdc5p ΔN), which contains the polo-box, plays a critical role in targeting the catalytic activity of Cdc5p to the SPB and the bud-neck (Song *et al.*, 2000). In an effort to identify the polo-box-binding proteins at these subcellular structures, we carried out a yeast two-hybrid screening by using a DBD fused with the C-terminal domain of Cdc5p (Cdc5p ΔN ; pSK1390) as the bait. Strain EGY48 (α -type), which bears the bait and a *LexAop-LEU2* reporter, was transformed with *GAL1-B42-HA*-fused cDNA library constructed in pJG4-5 (*TRP1*) vector (Origene Technologies). Approximately 2×10^6 transformants were selected on a minimal medium lacking Trp, His, and Leu. Putative Cdc5p-interacting clones were retransformed into strain EGY48 and then mated with strain RFY206 (α -type) harboring a *lacZ* reporter plasmid (pSH18-34) for quantitative β -galactosidase assays. One of these clones encoding Bbp1p exhibited a strong interaction with Cdc5p ΔN , and also with the full-length Cdc5p (Figure 2A). Consistent with the polo-box-dependent localization of Cdc5p to the SPB, the N-terminal polo-box domain (Cdc5p ΔC) or Cdc5p ΔN bearing conserved FAA triple mutations in the polo-box (Song *et al.*, 2000) failed to interact with Bbp1p under the same conditions (Figure 2A).

BBP1 is predicted to encode a protein of 45 kDa, which contains no currently recognizable functional motifs except for two predicted coiled-coil regions (for review, see Lupas, 1996) at amino acids 243–290 and 305–385. Bbp1p localizes at the cytoplasmic side of the central plaque periphery of the SPB (Schramm *et al.*, 2000) and plays an important role in inserting a duplication plaque into the nuclear envelope and assembling a functional inner plaque (Schramm *et al.*, 2000). Similar subcellular localizations and also the observed polo-box-dependent binding between Cdc5p and Bbp1p in two-hybrid suggest that they may directly interact. To test this possibility, bead-bound full-length or truncated forms of GST-Bbp1p purified from bacterial cells were incubated with Sf9 cell lysates containing His6-HA-Cdc5p-Flag and then bound Cdc5p was analyzed as described in MATERIALS AND METHODS. To investigate the specificity of the Cdc5p–Bbp1p interaction, Mps2p (Winey *et al.*, 1991), which was shown to form a stable complex with Bbp1p (Schramm *et al.*, 2000), was also examined. GST-Bbp1p^{1–385}, GST-Bbp1p^{1–295}, and GST-Bbp1p^{1–245} interacted with Cdc5p, whereas GST alone, GST-Bbp1p^{243–385}, and GST-Bbp1p^{295–385} did not (Figure 2B). In addition, GST-Mps2p did not interact with Cdc5p under the same conditions (Figure 2B). In a second experiment, we observed that purified GST-Bbp1p, but not GST-Mps2p, could also interact with bacterially expressed, partially purified T7-Cdc5p-His6 (Figure 2C). These data together with the two-hybrid results suggest that the polo-box domain of Cdc5p interacts directly with the N-terminal domain of Bbp1p and that this interaction is likely specific. However, we failed to coimmunoprecipitate Cdc5p and Bbp1p from growing yeast cell lysates under various conditions (our unpublished data), suggesting that the interaction between Cdc5p and Bbp1p is likely transient.

The C-Terminal Coiled-Coil Domain of Bbp1p Is Sufficient for Localization and Homo-dimerization but Not for Function

Coiled-coil domain has been implicated in protein–protein interactions (Lupas, 1996). Because the coiled-coil domain of Bbp1p (Bbp1p^{243–385}) does not seem to interact with the C-terminal domain of Cdc5p, we tested whether it is responsible for the localization of Bbp1p to the SPB by using various GFP-fused Bbp1p constructs. Similar to the endog-

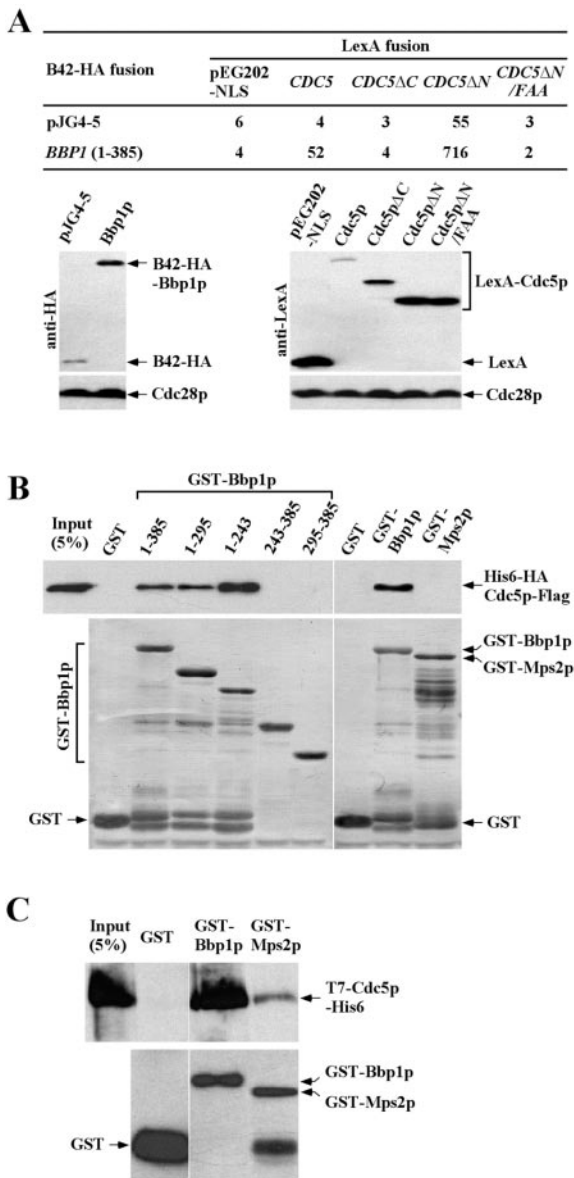


Figure 2. Physical interactions between Cdc5p and Bbp1p. (A) Two-hybrid assays were conducted as described in MATERIALS AND METHODS with plasmids that expressed full-length or truncated forms of *BBP1* or *CDC5* as AD or DBD fusions, respectively. Cdc5pΔN/FAA possesses the FAA mutations, which disrupt the function of the polo-box domain of Cdc5p (Song *et al.*, 2000). Numbers indicate the Miller units of β -galactosidase activity averaged from two independent experiments. Immunoblotting (bottom) indicated the expression levels of various constructs with Cdc28p as loading control. *CDC5*, pSK1405; *CDC5ΔC*, pSK1408; *CDC5ΔN*, pSK1390; *CDC5ΔN/FAA*, pSK1403; and *BBP1 (1-385)*, pSK1883. (B and C) In vitro interaction studies were carried out using various full-length or truncated forms of GST-Bbp1p or GST-Mps2p as ligands. Sf9 cell lysates expressing recombinant His6-HA-Cdc5p-Flag (B) or bacterially expressed, purified, recombinant T7-Cdc5p-His6 (C) were incubated with various ligands as described in MATERIALS AND METHODS. After SDS-PAGE, the amounts of bound His6-HA-Cdc5p-Flag or T7-Cdc5p-His6 were determined by immunoblotting with either an anti-FLAG antibody (B, top) or an anti-T7 antibody (C, top), whereas the amounts of GST, GST-Bbp1p, or GST-Mps2p ligands were determined by Coomassie staining (B, bottom) or immunoblotting with an anti-GST antibody (C, bottom). Input, 5% of His6-HA-Cdc5p-Flag or T7-Cdc5p-His6 that was incubated with GST, GST-Bbp1p, or GST-Mps2p. 1-385, pKL2497; 1-295, pKL2498; 1-243, pKL2500; 243-385, pKL2499; 295-385, pKL2501; GST-Bbp1p, pKL2497; and GST-Mps2p, pKL2496.

enous Bbp1p tagged with GFP at its C-terminal end (Figure 5B), expression of both the full-length YFP-Bbp1p¹⁻³⁸⁵ and YFP-Bbp1p²⁴³⁻³⁸⁵ (C-terminal domain containing the two predicted coiled-coil regions of Bbp1p) under the *GAL10* promoter control efficiently localized to the SPB. In contrast, both YFP-Bbp1p¹⁻²⁹⁵ bearing the first coiled-coil region or YFP-Bbp1p²⁹⁵⁻³⁸⁵ containing only the second coiled-coil region failed to localize to the SPB (Figure 3A). These data indicate that the C-terminal domain of Bbp1p (Bbp1p²⁴³⁻³⁸⁵) is sufficient for the localization of Bbp1p to the SPB and that both of the predicted coiled-coil regions most likely are required to form a functional localization domain of Bbp1p.

It has been suggested that Bbp1p forms a homo-dimer (Schramm *et al.*, 2000). Thus, we carried out two-hybrid analysis to investigate whether the C-terminal coiled-coil regions of Bbp1p are important for homo-dimerization. Both full-length Bbp1p¹⁻³⁸⁵ and Bbp1p²⁴³⁻³⁸⁵ exhibited interactions with Bbp1p¹⁻³⁸⁵ or Bbp1p²⁴³⁻³⁸⁵. Under the same conditions, Bbp1p¹⁻²⁹⁵ lacking the second coiled-coil region did not interact (Figure 3B). In addition, immunoprecipitation of either Bbp1p¹⁻³⁸⁵ or Bbp1p²⁴³⁻³⁸⁵, but not Bbp1p¹⁻²⁴³, from yeast cellular lysates coprecipitated Bbp1p²⁴³⁻³⁸⁵ (Figure 3C). These results suggest that the C-terminal two coiled-coil repeats in Bbp1p²⁴³⁻³⁸⁵ are necessary and sufficient for homo-dimerization of Bbp1p. Because Bbp1p²⁴³⁻³⁸⁵ is sufficient to localize to the SPB, the homo-dimerization of Bbp1p may likely be a critical step for the subcellular localization of Bbp1p to the SPB.

We then examined whether the C-terminal coiled-coil regions of Bbp1p are sufficient for the function of Bbp1p. A *bbp1Δ* mutant, kept viable by the presence of a *URA3*-based centromeric *BBP1* (SKY1710), was additionally transformed with full-length or truncated forms of *BBP1*. Transformants were then streaked on a minimal selection medium supplemented with 5-FOA to select against the *URA3*-based *BBP1* plasmid. Cells expressing full-length Bbp1p¹⁻³⁸⁵ grew well on the 5-FOA-containing plate (Figure 3D). In contrast, cells expressing any of the truncated forms of Bbp1p did not support the viability of the *bbp1Δ* mutant (Figure 3D). These data suggest that Bbp1p²⁴³⁻³⁸⁵-dependent localization is not sufficient for the function of Bbp1p, and that the N-terminal domain of Bbp1p may have an uncharacterized role critical for fulfilling the function of Bbp1p.

*Bbp1p*²⁴³⁻³⁸⁵ Functions Dominant-Negatively to Induce a Cell Cycle Arrest

We then examined the phenotype associated with overexpression of full-length and various truncated forms of Bbp1p. Expression of either full-length Bbp1p¹⁻³⁸⁵ or Bbp1p²⁴³⁻³⁸⁵ under the *GAL1* promoter control strongly inhibited cell growth (Figure 4A). Expression of Bbp1p¹⁻²⁹⁵ or Bbp1p¹⁻²⁴³ also induced a weak but significant growth inhibition (Figure 4A), even though the expression levels of these proteins were much lower than those of others (Figure 4B). These observations suggest that overexpression of either the full-length or the truncated forms of *BBP1* is detrimental for cell growth.

To closely monitor the arresting phenotype associated with Bbp1p¹⁻³⁸⁵ or Bbp1p²⁴³⁻³⁸⁵ expression, strain KLY1546 bearing control vector, *GAL10-YFP-BBP1*¹⁻³⁸⁵ or *GAL10-YFP-BBP1*²⁴³⁻³⁸⁵ were cultured in YEP-raffinose, arrested with α -factor, and then released into YEP-galactose to induce the protein expression. Cells harboring the control vector proceeded through the cell cycle normally (Figure 4C). Expression of *GAL10-YFP-BBP1*¹⁻³⁸⁵ resulted in a delayed cell cycle progression without a uniformed arresting phenotype (our unpublished data), suggesting that overex-

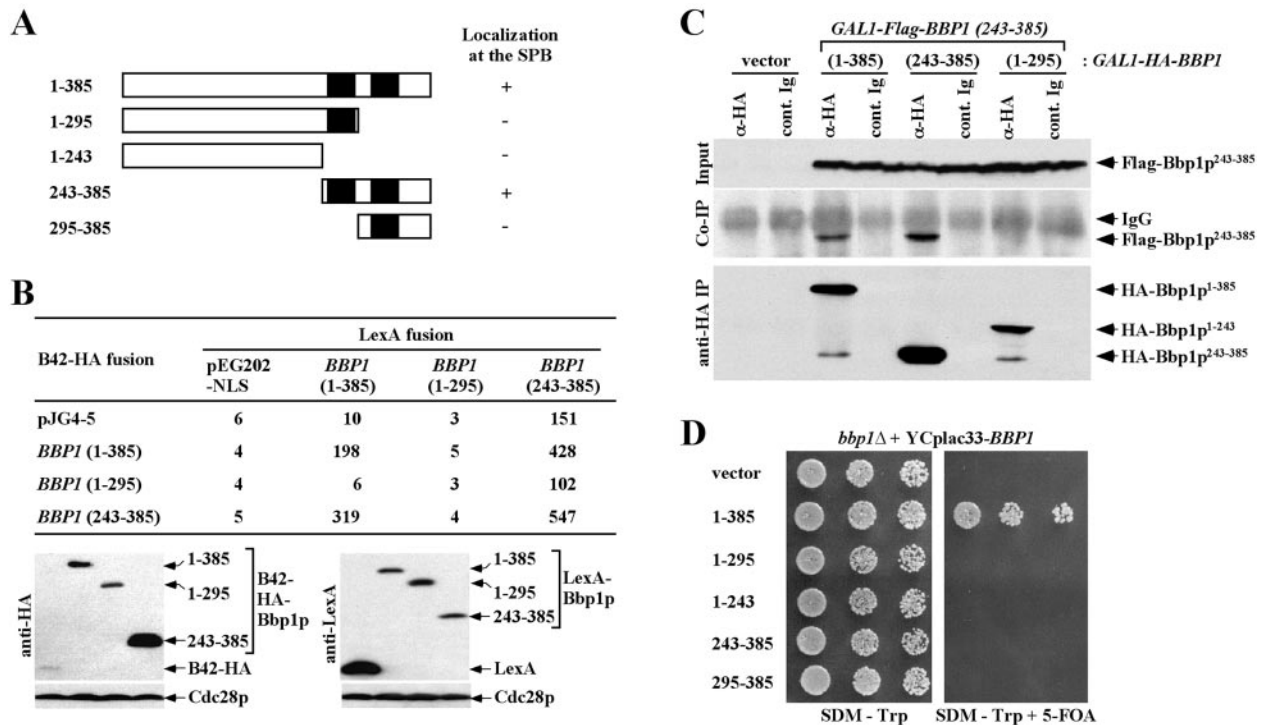


Figure 3. Functional domain analysis of Bbp1p. (A) Structures of the Bbp1p truncations used in these analyses (see MATERIALS AND METHODS) and the ability of these constructs to localize to the SPB. To determine the domain of Bbp1p responsible for the SPB localization, N-terminally YFP-tagged full-length and various truncated forms of Bbp1p as indicated were expressed under control of the *GAL10* promoter for 1 h. Filled box, the predicted coiled-coil domain; +, SPB localization; -, no detectable localization. 1-385, pSK1868; 1-295, pSK1867; 1-243, pSK1870; 243-385, pSK1869; and 295-385, pSK1871. (B) Intramolecular interactions of Bbp1p in two-hybrid assays. Two-hybrid assays were carried out as described in Figure 2A with full-length or truncated forms of *BBP1* as indicated. Numbers indicate the Miller units of β -galactosidase activity. Immunoblotting (bottom) indicate the expression levels of various constructs with Cdc28p as loading control. LexA fusion: 1-385, pSK1901; 1-295, pSK1900; 243-385, pSK1902. B42-HA fusion: 1-385, pSK1883; 1-295, pSK1882; and 243-385, pSK1884. (C) Bbp1p²⁴³⁻³⁸⁵ is sufficient for homo-dimerization. Equivalent amounts of protein from cells expressing indicated constructs under induction conditions for 2 h were subjected to immunoprecipitation with rabbit polyclonal anti-HA antibody to precipitate HA-Bbp1p¹⁻³⁸⁵, HA-Bbp1p²⁴³⁻³⁸⁵, or HA-Bbp1p¹⁻²⁴³. The immunoprecipitates were resolved by SDS-PAGE and analyzed by immunoblotting with anti-FLAG antibody to detect coprecipitated Flag-Bbp1p²⁴³⁻³⁸⁵ or with mouse monoclonal anti-HA.11 antibody to determine the level of immunoprecipitated HA-Bbp1p. Control Ig, rabbit control Ig. (D) Both N-terminal and C-terminal domains of Bbp1p are required for the function of Bbp1p. Strain SKY1710 (*bbp1* Δ + YCplac33-*BBP1*) was transformed with full-length or various truncated forms of *BBP1*. Transformants selected on SDM lacking tryptophan were cultured overnight, serially diluted, and spotted onto either SDM-Trp or SDM-Trp supplemented with 5-FOA to select against *URA3*-containing YCplac33-*BBP1* plasmid. Plates were incubated at 30°C for 3 d. Vector, SKY1716; 1-385, SKY1717; 1-295, SKY1718; 1-243, SKY1721; 243-385, SKY1719; and 295-385, SKY1720.

pression of *YFP-BBP1*¹⁻³⁸⁵ is detrimental for growth. Under the same conditions, expression of *GAL10-YFP-BBP1*²⁴³⁻³⁸⁵ induced a drastic arrest with a large-budded morphology (Figure 4C). Greater than 90% of these cells ($n = 110$) possessed one nucleus in the mother cell 150 min after α -factor release (Figure 4D), suggesting an arrest before nuclear division. Interestingly, in most of these cells, a single YFP-Bbp1p²⁴³⁻³⁸⁵ fluorescent dot was apparent in the bud, and these dots were not associated with DAPI-stained DNA mass (Figure 4, D and E). When the cells were treated with α -factor to mark the mother cells and then released into YEP-galactose medium as described above, the localization of YFP-Bbp1p²⁴³⁻³⁸⁵ to the daughter-side of the SPB was manifest in >91% ($n = 180$) of the population (Figure 4F). In addition, most of these fluorescent signals (87%; $n = 123$) were colocalized with Spc42-RFP (Figure 4G), a daughter-side SPB marker (Pereira *et al.*, 2001). These observations suggest that the newly synthesized YFP-Bbp1p²⁴³⁻³⁸⁵ has been incorporated into the daughter-side of the SPB, and this SPB is impaired in properly segregating sister chromatids. Consistent with this view, microtubule structures emanating from the daughter-side of the SPB were much

weaker than those from the mother cells (Figure 4E). A similar phenotype has been observed in the *bbp1-1* mutant (Schramm *et al.*, 2000). These observations suggest that expression of the localization-competent Bbp1p²⁴³⁻³⁸⁵ interfered with the function of Bbp1p at the daughter-side SPB and that this interference led to a failure in bipolar spindle formation and chromosome segregation.

Bbp1p Is Required for Proper Localization of Cdc5p at the SPB

Our data suggest that Bbp1p interacts with Cdc5p directly and that Bbp1p localizes to the SPB through the coiled-coil region. Interestingly, the expression level of *BBP1* peaks during the G₁ phase of the cell cycle (Spellman *et al.*, 1998), whereas *CDC5* is expressed at the late stages of the cell cycle (Kitada *et al.*, 1993; Hardy and Pautz, 1996). These temporal regulations of Bbp1p and Cdc5p during the cell cycle suggest that Cdc5p may require Bbp1p to localize to the SPB. To test this possibility, the temperature-sensitive *bbp1-1* mutant that expresses Cdc5p-GFP under endogenous *CDC5* promoter control was used to examine the efficiency of Cdc5p-

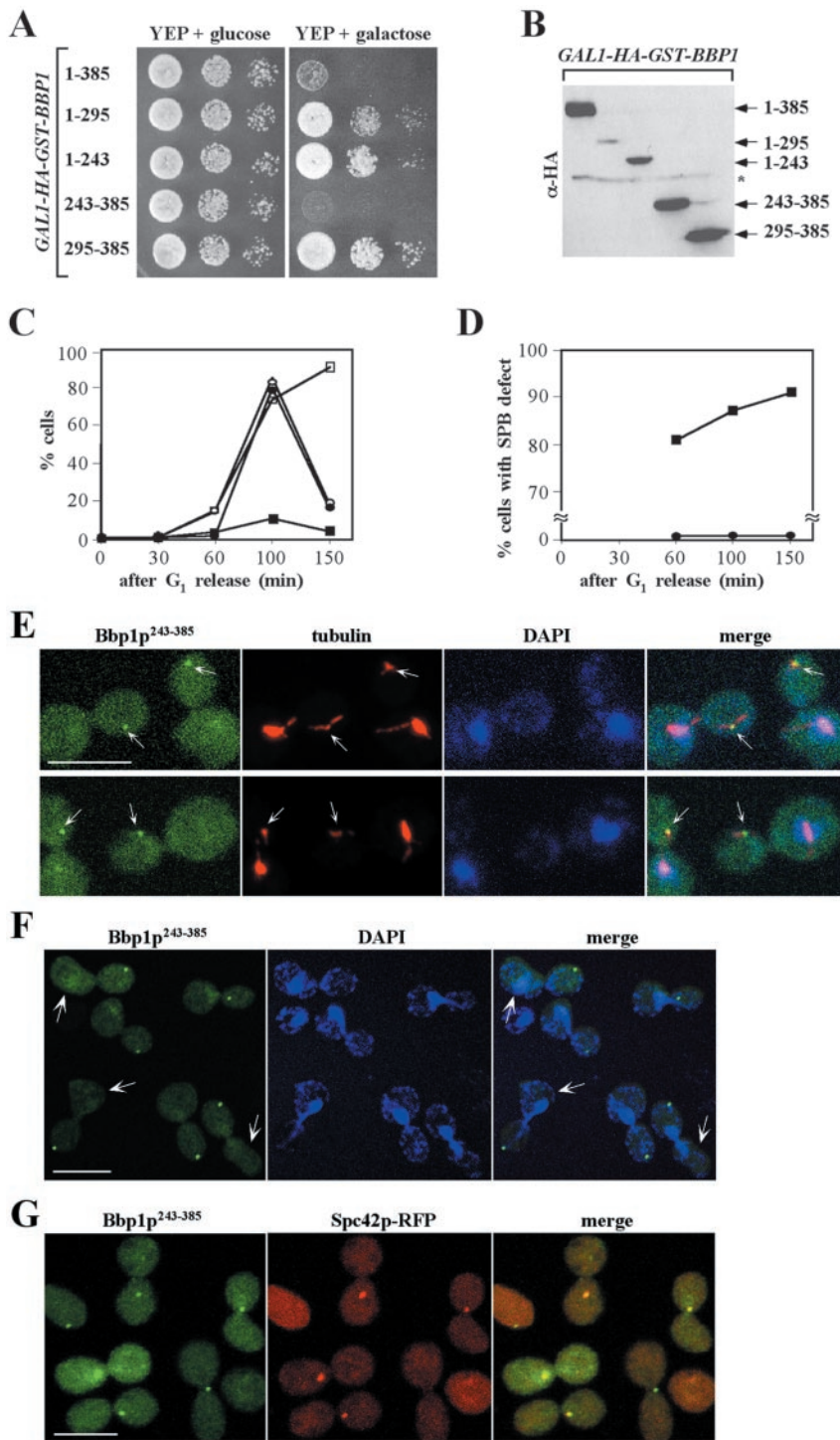


Figure 4. Overexpression of *BBP1*²⁴³⁻³⁸⁵ results in a cell cycle arrest with an aberrant daughter-side SPB. (A) Inhibition of cell growth by overexpression of full-length *BBP1* (*Bbp1p*¹⁻³⁸⁵) or *BBP1*²⁴³⁻³⁸⁵. Wild-type strain K699 expressing GST-*Bbp1p*¹⁻³⁸⁵ or GST-*Bbp1p*²⁴³⁻³⁸⁵ under control of the *GAL1* promoter was cultured in YEP-raffinose overnight, serially diluted, and spotted onto either YEP-galactose (YEPG) or YEP-glucose (YEPD) plates. Plates were then incubated at 30°C for 3 d. 1-385, strain KLY1991; 1-295, strain KLY1989; 1-243, strain KLY1995; 243-385, strain KLY1993; and 295-385, strain KLY1997. (B) To determine the expression levels of GST-*Bbp1p*¹⁻³⁸⁵ or GST-*Bbp1p*²⁴³⁻³⁸⁵ used in A, total protein prepared from cells cultured under induction conditions for 2 h was subjected to immunoblotting with anti-HA antibody. Asterisk indicates a nonspecific cross-reacting protein. (C and D) To determine the arresting phenotype induced by overexpression of *YFP-BBP1*²⁴³⁻³⁸⁵, wild-type strain KLY1546 was transformed with a centromeric *GAL10-YFP-BBP1*²⁴³⁻³⁸⁵ plasmid (pSK1869) or control YCplac111 vector. Transformants were arrested in G₁ by α -factor treatment in YEP-raffinose for 4 h and then released into YEP-galactose to induce the expression of *YFP-BBP1*²⁴³⁻³⁸⁵. (C) Percentages of large-budded cells (open circle, control vector; open square, *YFP-BBP1*²⁴³⁻³⁸⁵) and cells exhibiting divided nuclear morphology (closed circle, control vector; closed square, *YFP-BBP1*²⁴³⁻³⁸⁵) were determined at the indicated time points after induction. Approximately 200 cells were counted at each time point. (D) Among the large-budded cells, the percentages of cells with single YFP-*Bbp1p*²⁴³⁻³⁸⁵ fluorescent dot signals at the daughter-side SPB were determined at the indicated time points. Closed circle, YCplac111; closed square, pSK1869. (E) Representative spindle morphologies of cells with *GAL1-YFP-BBP1*²⁴³⁻³⁸⁵ expression. Cells harvested at the 100-min time point in C were subjected to indirect immunofluorescence microscopy, after visualizing tubulin (red) with an anti-tubulin antibody as described in MATERIALS AND METHODS. DNA was stained with DAPI. Arrows indicate YFP-*Bbp1p*²⁴³⁻³⁸⁵ signals with apparently defective SPB with significantly weakened microtubule structures. Bar, 5 μ m. (F) Localization of YFP-*Bbp1p*²⁴³⁻³⁸⁵ at the daughter-side of the SPB. Cells bearing *GAL1-YFP-BBP1*²⁴³⁻³⁸⁵ were treated with α -factor to mark the mother cells and then released into YEP-galactose medium for 1.5 h to examine the YFP-*Bbp1p*²⁴³⁻³⁸⁵ fluorescent signals. Arrows indicate cells with remnants of the shmoo morphology. Bar, 5 μ m. (G) Colocalization of YFP-*Bbp1p*²⁴³⁻³⁸⁵ with Spc42-RFP. The Spc42-RFP cells (ESM988-1) harboring *GAL1-YFP-BBP1*²⁴³⁻³⁸⁵ were cultured under induction conditions for 1.5 h, fixed, and then subjected to fluorescence microscopy. Bar, 5 μ m.

GFP localization at the nonpermissive temperature. As a comparison, Cdc5p localization was also determined in an isogenic wild-type and the temperature-sensitive *mps2-1* mutant. To enrich Cdc5p and to eliminate the cell cycle-dependent alterations in Cdc5p localization, these strains were arrested in M phase with nocodazole at 23°C for 3 h before shifting the temperature to 34°C, a nonpermissive temperature for both the *bbp1-1* and *mps2-1* mutants, for 3 h. At 23°C, Cdc5p-GFP efficiently localized to the SPB in all three strains. At 34°C, however, Cdc5p-GFP localization was

markedly decreased in the *bbp1-1* mutant, whereas both the wild-type and the *mps2-1* mutant exhibited an efficient Cdc5p-GFP localization to the SPB (Figure 5A). These data indicate that *Bbp1p* is required for the localization of Cdc5p to the SPB. These observations further suggest that *bbp1-1p*, but not *Bbp1p*, incorporated into the SPB may have become nonfunctional upon exposure to a restrictive temperature. To examine this possibility, cells expressing *Bbp1p*-GFP or *bbp1-1p*-GFP under endogenous *BBP1* promoter control (KLY5336 or KLY5334, respectively) were arrested with no-

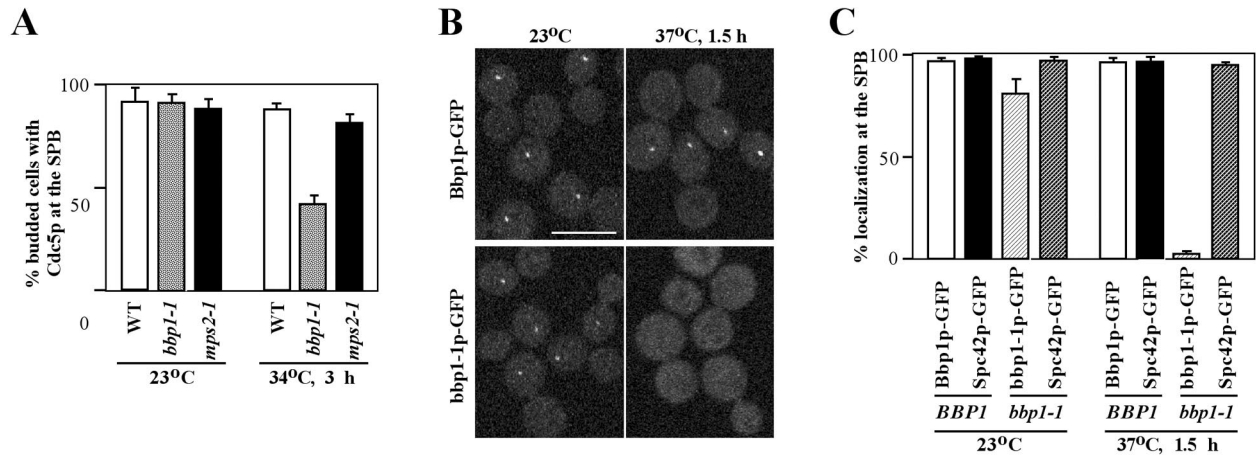


Figure 5. Requirement of Bbp1p, but not Mps2p, for proper localization of Cdc5p at the SPB. (A) Strain KLY3546 (*BBP1*), KLY3791 (*bbp1-1*), or KLY3729 (*mps2-1*), which expresses Cdc5p-GFP under endogenous *CDC5* promoter control was cultured overnight at 23°C and then shifted to 34°C in the presence of 15 μ g/ml of nocodazole for 3 h. Among the large-budded cells, the fraction of cells with Cdc5p-GFP signals at the SPB were determined by counting >200 cells for each sample. These results are obtained from three independent experiments. Error bars indicate SD. WT, strain KLY3546; *bbp1-1*, strain KLY3791; *mps2-1*, strain KLY3729. (B and C) To examine the temperature-dependent localization of Bbp1p and *bbp1-1p* to the SPB, strains KLY5336 and KLY5334, which expresses Bbp1p-GFP or *bbp1-1p*-GFP under endogenous *BBP1* promoter control, respectively, were arrested with nocodazole for 2.5 h. Cells were then shifted to 37°C for 1.5 h, fixed, and then examined by confocal microscopy (B). Localization of Spc42p-GFP in the *BBP1* wild-type (KLY3685) or the *bbp1-1* mutant (KLY3692) was also examined under the same conditions. More than 200 cells were counted in three independent experiments. Error bars indicate SD. Bar, 5 μ m.

codazole at 23°C for 2.5 h before shifting the cultures to nonpermissive temperatures. The localization of *bbp1-1p*-GFP, but not Bbp1p-GFP, to the SPB was almost completely disrupted upon culturing at 37°C for 1.5 h (Figure 5, B and C); localization of *bbp1-1p*-GFP to the SPB was greatly diminished at 34°C, but weak fluorescent signals were still detectable in ~25% of the population. In contrast, the localization efficiency of Spc42p-GFP in either the *BBP1* wild-type or the *bbp1-1* mutant remained unchanged under the same conditions (Figure 5C), suggesting that, unlike Cdc5p, Spc42p does not require Bbp1p for its localization to the SPB. We were not able to examine the Mps2p localization under these conditions because of a failure in generating a detectable Mps2p-GFP fluorescent signal (our unpublished data). Thus, even though we cannot rule out the possibility that Mps2p also contributes to the Cdc5p localization to the SPB, a drastic delocalization of *bbp1-1p* from the SPB may have resulted in delocalization of Cdc5p. Because Cdc5p interacts with Bbp1p in yeast two-hybrid and in vitro binding analyses, we speculate that the Bbp1p-dependent localization of Cdc5p to the SPB is likely through a direct protein-protein interaction.

Bbp1p Is Required for Proper M-Phase Progression

Proper subcellular localization of Cdc5p is critical for the mitotic functions of Cdc5p (Lee *et al.*, 1998). Because the *bbp1-1* mutant is impaired in Cdc5p localization to the SPB, it may have a previously uncharacterized mitotic defect. To examine this possibility, strains KLY3685 (isogenic wild-type) and KLY3692 (*bbp1-1*) arrested with nocodazole for 3 h at 23°C were shifted to 37°C to impair the *bbp1-1p* function (Figure 5, B and C) and then released into fresh medium at 37°C to monitor the mitotic progression. Flow cytometry analyses revealed that strain KLY3685 regenerated a distinct G₁ population 40 min after nocodazole release (Figure 6A). Under the same conditions, strain KLY3692 exhibited an ~10-min delay in generating G₁ population (Figure 6A).

Consistent with this observation, the *bbp1-1* mutant was delayed in both achieving the nuclear division and generating unbudded G₁ cells (Figure 6B). In comparison with the isogenic wild-type strain KLY3685, delocalization of Cdc14p-GFP₅ from the nucleolus was also delayed in the *bbp1-1* mutant (Figure 6C). Together, these data suggest that, in addition to the role of Bbp1p in SPB duplication, Bbp1p may also be required for proper mitotic progression. Because Cdc5p localization to the SPB is partially impaired in *bbp1-1*, the improper localization of Cdc5p may have contributed to the delayed mitotic progression observed in the *bbp1-1* mutant.

Bbp1p^{243–385}, but Not Mps2p, Can Replace the Function of the C-Terminal Domain of Cdc5p

To further investigate whether the Bbp1p-dependent localization of Cdc5p to the SPB is important for the mitotic functions of Cdc5p, we asked whether the localization domain of Bbp1p (Bbp1p^{243–385}) would substitute the C-terminal domain of Cdc5p, a region critical for subcellular localization of Cdc5p. To this end, Bbp1p^{243–385} or MPS2p was C-terminally fused to the localization-defective, nonfunctional GFP-Cdc5p Δ C and expressed under endogenous *CDC5* promoter control. In the wild-type strain KLY1546, GFP-Cdc5p localized to the SPB and weakly to the bud-neck, whereas GFP-Cdc5p Δ C lacking the polo-box domain did not yield any distinct localization signals (Figure 7A). As expected, both GFP-Cdc5p Δ C-Bbp1p^{243–385} and GFP-Cdc5p Δ C-Mps2p localized to the SPB at similar levels, but they failed to localize to the bud-neck under the same conditions (Figure 7A). These observations suggest that either Bbp1p^{243–385} or Mps2p can effectively target the Cdc5p Δ C to the SPB.

Next, we asked whether these chimera constructs could rescue the growth defect associated with the *cdc5* Δ mutation when expressed under the *CDC5* promoter control. Introduction of *CDC5* fully complemented the growth defect of the *cdc5* Δ mutant, whereas introduction of *CDC5* Δ C led to the

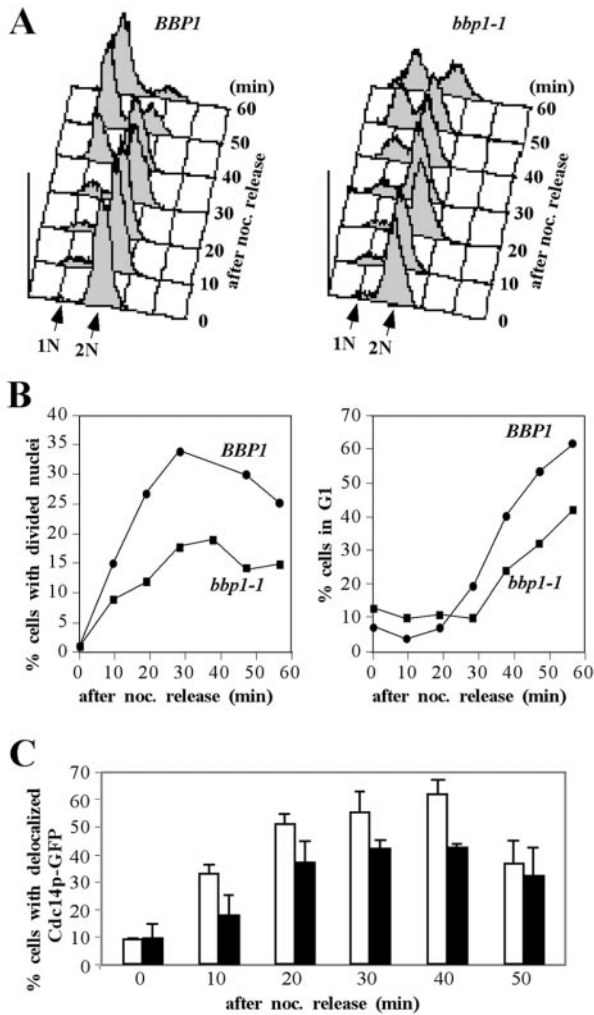


Figure 6. Loss of *BBP1* function leads to a delayed mitotic progression. (A) Strains KLY3685 (*BBP1*) and KLY3692 (*bbp1-1*) were arrested in G₁ with 5 μ g/ml of α -factor for 3 h at 23°C and then released into 15 μ g/ml of nocodazole-containing YEP-glucose at 23°C for 120 min to rearrest cells in early M phase. The resulting cells were shifted to 37°C for 1.5 h to cripple the *bbp1-1* function before releasing into prewarmed YEP-glucose medium containing α -factor at 37°C. Samples were harvested at the indicated time points after nocodazole release and then subjected to flow cytometry analysis. 1N, cells with 1N DNA content; 2N, cells with 2N DNA content. (B) Aliquots of the same samples in A were fixed with formaldehyde and stained with DAPI to determine the percentages of cells with divided nuclei morphologies (left) or unbudded G₁ cells (right) after nocodazole release. Approximately 200 cells were counted at each time point. (C) *BBP1* is required for normal release of Cdc14p from the nucleolus. Strain KLY4323 (*BBP1*) and KLY4426 (*bbp1-1*) expressing a *CDC14-5*×*GFP* fusion protein under endogenous *CDC14* promoter control were arrested with 15 μ g/ml nocodazole for 2.5 h at 23°C. Cultures were then shifted to 37°C for 1.5 h before release into prewarmed YEP-glucose medium at 37°C. Percentages of cells with delocalized Cdc14p-GFP₅ were determined at the indicated time points after nocodazole-release. Approximately 200 cells were counted at each time point. Error bars indicate SD. White bars, strain KLY4323; black bars, strain KLY4426.

generation of nonviable microcolonies (Figure 7B). Interestingly, *CDC5* Δ -*BBP1*²⁴³⁻³⁸⁵ rescued the *cdc5* Δ defect significantly, whereas *CDC5* Δ -*MPS2* did not (Figure 7B). As with *BBP1*²⁴³⁻³⁸⁵, provision of *CDC5* Δ -*BBP1*²⁴³⁻³⁸⁵ did not rescue the *bbp1-1* defect (Figure 7C). However, provision of *CDC5* Δ -

BBP1 or *CDC5* Δ -*MPS2* partially complemented the *bbp1-1* or the *mps2-1* defect, respectively (Figure 7, C and D). When expressed in a wild-type background, none of these constructs seemed to induce a dominant-negative growth defect (our unpublished data), suggesting that the encoded fusion proteins do not interfere with the function of Cdc5p, Bbp1p, or Mps2p in vivo. In addition, both Cdc5p Δ -Bbp1p²⁴³⁻³⁸⁵ and Cdc5p Δ -Mps2p immunoprecipitated from yeast cellular lysates exhibited autophosphorylation and transphosphorylation activities similar to those of Cdc5p (Figure 7E). Together, these data suggest that the C-terminal coiled-coil domain of Bbp1p (Bbp1p²⁴³⁻³⁸⁵) can substitute for the function of the C-terminal domain of Cdc5p by targeting the catalytic activity of Cdc5p (Cdc5p Δ) to the SPB and that Bbp1p, but not Mps2p, can direct Cdc5p to the proper locations at the SPB.

Cdc5p Δ -*Bbp1p*²⁴³⁻³⁸⁵ Promotes the Mitotic Exit

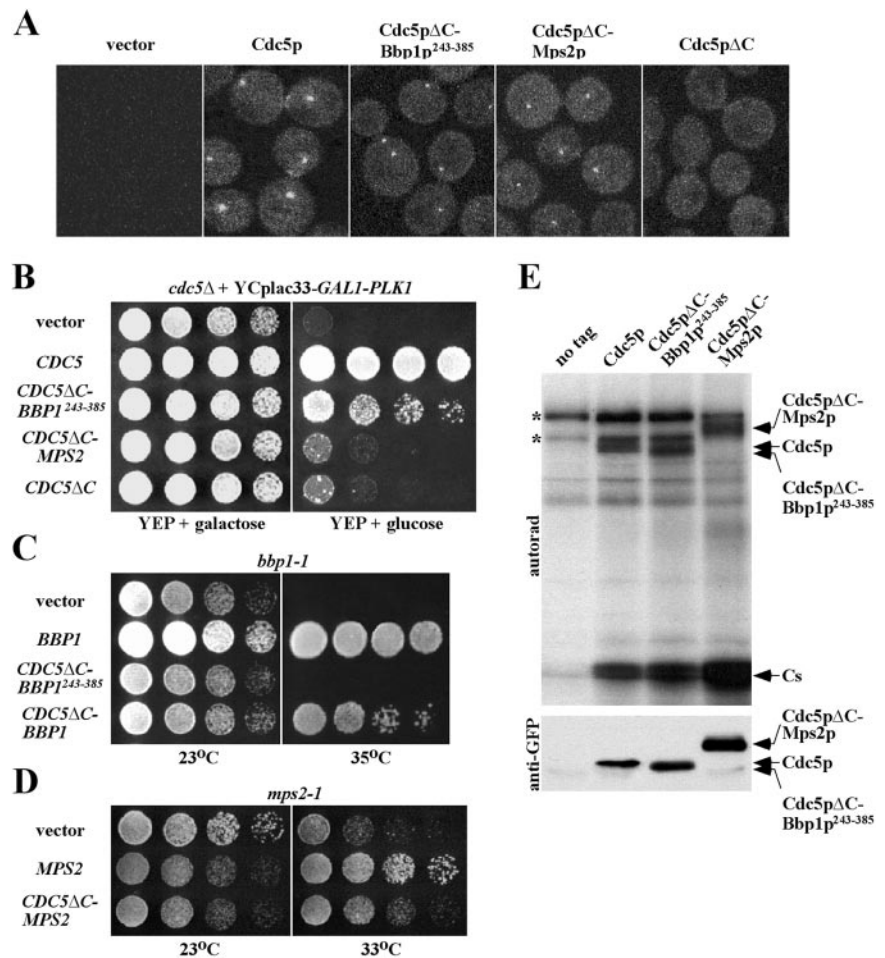
One of the critical mitotic events that requires the function of Cdc5p is mitotic exit, a late mitotic event that requires the inactivation of Cdc28p/Clb2p activity. Thus, we examined whether Bbp1p²⁴³⁻³⁸⁵-dependent targeting of Cdc5p Δ to the SPB is sufficient for inducing the Cdc14p release from the nucleolus. Strain SAY801 expressing *CDC14-5*×*GFP* under endogenous *CDC14* promoter control was additionally integrated with the control *GAL1* vector, *GAL1*-*CDC5*, *GAL1*-*CDC5* Δ -*BBP1*²⁴³⁻³⁸⁵, or *GAL1*-*CDC5* Δ at the *URA3* locus. The resulting transformants were cultured under induction conditions for 2.5 h in the presence of nocodazole and then the percentages of cells with released Cdc14p from the nucleolus were determined. Expression of *CDC5* resulted in Cdc14p release in 75% of the population, whereas expression of control vector did not yield any significant Cdc14p release. Under the same conditions, expression of *CDC5* Δ -*BBP1*²⁴³⁻³⁸⁵ induced Cdc14 release in 58% of the population (Figure 8A), whereas expression of *CDC5* Δ induced it in only 24% of the population even with a higher protein expression level (Figure 8, A and B). As with the localization of GFP-Cdc5p Δ -Bbp1p²⁴³⁻³⁸⁵ or GFP-Cdc5p Δ under endogenous *CDC5* promoter control, expression of *GAL10*-GFP-*CDC5* Δ -*BBP1*²⁴³⁻³⁸⁵ exhibited strong fluorescent signals at the SPB, whereas expression of *GAL10*-GFP-*CDC5* Δ yielded a largely diffused signal in the nucleus (Figure 8C). These observations indicate that Bbp1p²⁴³⁻³⁸⁵-dependent targeting of Cdc5p to the SPB is sufficient to induce the Cdc14p release from the nucleolus and promote the mitotic exit.

DISCUSSION

Bbp1p as a Polo-Box-binding Protein at the SPB

It has been suggested that the polo-box plays an essential role for protein-protein interactions to target the catalytic activity of Cdc5p to specific subcellular structures. In an attempt to identify the polo-box-interacting proteins, we carried out a yeast two-hybrid screening and isolated Bbp1p as a potential polo-box-binding protein at the SPB. In vitro binding and in vivo localization studies showed that Cdc5p interacted with Bbp1p, but not with Mps2p, a protein suggested to form a heterodimer with Bbp1p (Schramm *et al.*, 2000). These observations suggest that Cdc5p specifically interacts with Bbp1p, and likely that this interaction is direct. In support of this opinion, a Cdc5p Δ fused with Bbp1p²⁴³⁻³⁸⁵, but not with Mps2p, could support the SPB localization-dependent Cdc5p function in inducing Cdc14p release from the nucleolus. This observation suggests that Bbp1p²⁴³⁻³⁸⁵ can function as a substitute for the polo-box by targeting the catalytic domain of Cdc5p (Cdc5p Δ) to Cdc5p

Figure 7. Bbp1p²⁴³⁻³⁸⁵-dependent localization of Cdc5pΔC to the SPB remedies the *cdc5Δ* defect. (A) Bbp1p²⁴³⁻³⁸⁵-dependent targeting of the localization-defective Cdc5pΔC to the spindle poles. Wild-type strain KLY1546 transformed with control YCplac111 vector, GFP-CDC5 (pKL2701), GFP-CDC5ΔC-BBP1²⁴³⁻³⁸⁵ (pKL2078), GFP-CDC5ΔC-MPS2 (pKL2422), or GFP-CDC5ΔC (pCJ232) was grown in the presence of 15 μg/ml of nocodazole for 3 h at 23°C, fixed, and then subjected to fluorescent microscopy to examine the localization of the GFP-fused proteins. (B) To examine the ability of various constructs in A to complement the *cdc5Δ* defect, strain KLY3721 (*cdc5Δ* + *GAL1-EGFP-PLK1*) transformed with each plasmid was cultured overnight, serially diluted, and spotted onto either YEP-galactose (YEPG) or YEP-glucose (YEPD) plates, and then incubated at 30°C for 3 d. (C and D) To examine the ability of CDC5ΔC-BBP1, CDC5ΔC-BBP1²⁴³⁻³⁸⁵, or CDC5ΔC-MPS2 to complement the *bbp1-1* or *mps2-1* defect, strains KLY2761 (*bbp1-1*) or SMY6-4b (*mps2-1*) were transformed with the indicated plasmids. The resulting transformants were cultured overnight, serially diluted, spotted onto YEP-glucose plates, and then incubated at the indicated temperatures for 3 d. BBP1, pSK1878; CDC5ΔC-BBP1²⁴³⁻³⁸⁵, pKL2078; CDC5ΔC-BBP1, pKL2420; MPS2, pKL1135; and CDC5ΔC-MPS2, pKL2422. (E) To measure the kinase activities associated with Flag-YFP-Cdc5p, Flag-YFP-Cdc5pΔC-Bbp1p²⁴³⁻³⁸⁵, or Flag-YFP-Cdc5pΔC-Mps2p, a protease-negative JB811 strain transformed with pKL2772, pKL2078, or pKL2422, respectively, were arrested with nocodazole for 3 h, and then harvested. Equal amounts of protein prepared from each transformant were subjected to anti-FLAG immunocomplex kinase assays. Reaction mixtures were separated in SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and then exposed to detect the kinase activities (top). The same blot was subjected to immunoblotting with anti-GFP antibody to determine the amount of Flag-YFP-Cdc5p or the corresponding chimera proteins in each immunoprecipitate (bottom). Asterisks indicate nonspecific phosphorylation bands. Cs, casein.



substrates at the SPB, which are essential for mitotic exit. It has been shown that Nud1p interacts with Bfa1p and Bub2p and influences the interactions between Tem1p and Cdc15p, a critical step in activating the mitotic exit network (Gruneberg *et al.*, 2000). Because Cdc5p interacts with Bfa1p (Hu *et al.*, 2001), Nud1p may also be important for targeting the Cdc5p to the SPB. However, as with the nonfunctional Cdc5pΔC-Mps2p fusion, Cdc5pΔC-Nud1p also failed to complement the *cdc5Δ* defect, even though it could efficiently target the Cdc5pΔC to the SPB (J.-E.P. and K.S.L., unpublished data). These observations suggest that interactions between Cdc5p and its specific binding partner(s) at the SPB most likely are critical to carry out Cdc5p-dependent mitotic functions.

Bbp1p localized to the SPB through the C-terminal coiled coil domain (Bbp1p²⁴³⁻³⁸⁵), and this domain was sufficient for homo-dimerization in yeast two-hybrid and coimmunoprecipitation studies. These data suggest that the C-terminal domain-dependent homodimerization of Bbp1p is most likely a prerequisite for the SPB localization. However, Bbp1p²⁴³⁻³⁸⁵ is not sufficient to rescue the *bbp1Δ* mutation, suggesting that the N-terminal domain of Bbp1p (Bbp1pΔC) is also critical for the function of Bbp1p. Our data showed that Bbp1pΔC interacts with the polo-box domain of Cdc5p

in both yeast two-hybrid and in vitro binding studies. Thus, it is possible that at least one of the important roles that Bbp1pΔC plays is to target Cdc5p to the SPB, thus contributing to the Cdc5p-dependent mitotic functions at the SPB (Figure 9). In this scenario, besides its role in SPB duplication, Bbp1p plays an additional role in Cdc5p-dependent mitotic events. Because SPB duplication must precede the mitotic events, the timely interaction between Bbp1p and Cdc5p later in the cell cycle may ensure the order of these two events.

In the *bbp1-1* mutant, Cdc5p-GFP localization to the SPB was diminished, but not eliminated, at a nonpermissive temperature. In addition, these cells exhibited a mitotic delay, but still continued to progress through mitosis at 37°C. These observations suggest that the *bbp1-1* mutant is not deprived of the Cdc5p function at the SPB under these conditions. Several explanations can account for these observations. One possibility is that because the SPB structure is already matured in mitosis, the temperature sensitivity of the *bbp1-1* allele could not have been rapidly exhibited. Alternatively, it is possible that SPB component(s) other than Bbp1p play a role in localizing Cdc5p to the SPB (Figure 9). These components may either cooperate with Bbp1p to localize Cdc5p to the periphery of the central plaque, or inde-

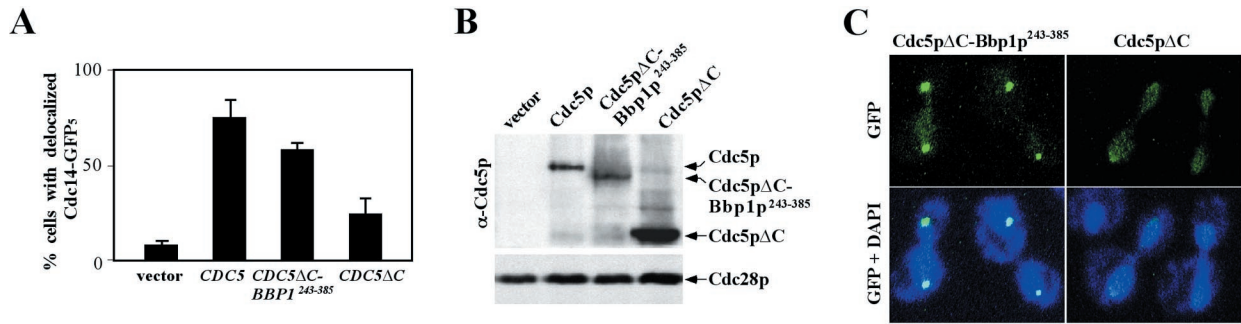


Figure 8. Cdc5pΔC-Bbp1p²⁴³⁻³⁸⁵ can induce the Cdc14p release from the nucleolus. (A) Strain SAY801 (Cdc14p-GFP₅) integrated with *GAL1* vector (pCJ238), *GAL1-CDC5* (pCJ241), *GAL1-CDC5ΔC-BBP1*²⁴³⁻³⁸⁵ (pCJ240), or *GAL1-CDC5ΔC* (pCJ242) was cultured in YEP-raffinose overnight and then transferred to YEP-galactose medium containing 15 μg/ml of nocodazole for 2.5 h before fixation with formaldehyde to assess the subcellular localization of Cdc14p-GFP₅ by GFP fluorescence. The percentages of cells with Cdc14p-GFP₅ at the nucleolus were determined by counting >200 cells for each sample. Data were obtained from three independent experiments. Error bars indicate SD. Vector, strain KLY4079; *CDC5*, strain KLY4085; *CDC5ΔC-BBP1*²⁴³⁻³⁸⁵, strain KLY4091; and *CDC5ΔC*, strain KLY4096. (B) Expression levels of the above constructs were examined after transforming each plasmid into the protease-negative JB811 strain. Total cellular protein prepared from each transformant was subjected to immunoblotting with an anti-Cdc5p antibody. The levels of Cdc28p are shown as loading controls. (C) To determine the localization of Cdc5pΔC-Bbp1p²⁴³⁻³⁸⁵ or Cdc5pΔC, strain KLY1546 transformed with *GAL10-YFP-CDC5ΔC-BBP1*²⁴³⁻³⁸⁵ (pKL2071) or *GAL10-YFP-CDC5ΔC* (pCJ231) was cultured under induction conditions for 2.5 h, fixed, and then subjected to fluorescent microscopy.

pendently target Cdc5p to other parts of the SPB to dictate specific functions at those sites.

The Role of Cdc5p at the SPB

Our data showed that Cdc5p primarily localizes to the outer plaque of the SPB, and a fraction of Cdc5p may also localize to the nuclear side of the SPB. Many components functioning in the mitotic exit network localize to the SPB, suggesting that Cdc5p localization to the SPB is probably important for promoting mitotic exit. Consistent with this view, we observed that targeting Cdc5pΔC to the SPB by tethering with the Bbp1p localization domain (Bbp1p²⁴³⁻³⁸⁵) was sufficient to induce mitotic exit.

Does Cdc5p play any other roles at the SPB? Close examination of Cdc5p localization during the cell cycle revealed that Cdc5p localizes to the SPB as early as G1 phase (C.J.P.

and K.S.L., unpublished data). Because mitotic exit occurs late in M phase, this finding suggests that Cdc5p may play additional roles earlier in the cell cycle. In other eucaryotic organisms, it has been shown that polo is required for centrosome maturation and bipolar spindle formations (Sunkel and Glover, 1988; Llamazares *et al.*, 1991; Ohkura *et al.*, 1995; Lane and Nigg, 1996). In addition, polo seems to provide sufficient activity to induce microtubule nucleation in salt-stripped centrosomes *in vitro* (de Carcer *et al.*, 2001). Thus, it may be of interest to investigate whether Cdc5p activity is required for proper microtubule function, perhaps through the interaction with SPB component(s) critical for this event. However, because of the multiplicity of Cdc5p function, it is difficult to study specific Cdc5p functions without interfering with other Cdc5p-dependent processes. Identification of additional Cdc5p-interacting proteins and generation of *cdc5* mutants defective in SPB localization or a specific protein-protein interaction at the SPB will likely be a critical step in dissecting the roles of Cdc5p at the SPB.

ACKNOWLEDGMENTS

We are grateful to Doug Kellogg, John Kilmartin, Kim Nasmyth, Elmar Schiebel, Akio Sugino, and Akio Toh-e for strains. We also thank Geum-Yi Kim, Young-Wook Cho, and Satoshi Asano for technical support, and Susan Garfield for helping with confocal microscopy. This work was supported in part by National Institutes of Health grant R01 GM-51312 (to M.W.).

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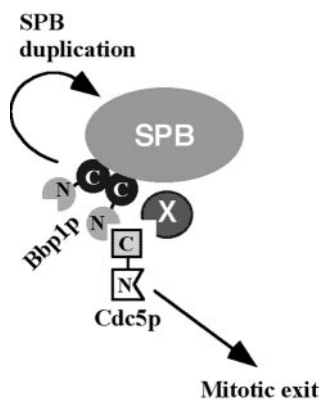


Figure 9. Model proposing the dual role of Bbp1p at the SPB. Bbp1p localizes to the SPB through the homo-dimerized C-terminal domain. In an early stage of the cell cycle, Bbp1p plays a critical role in SPB duplication. When Cdc5p is expressed late in the cell cycle, the N-terminal domain of Bbp1p interacts with the polo-box domain of Cdc5p, thus promoting the localization and mitotic functions of Cdc5p at the SPB. X indicates a hypothetical component, which may also contribute to the Cdc5p localization to the SPB (see text).

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