Coordinated Spindle Assembly and Orientation Requires Clb5p-dependent Kinase in Budding Yeast

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Abstract. The orientation of the mitotic spindle along a polarity axis is critical in asymmetric cell divisions. In the budding yeast, *Saccharomyces cerevisiae*, loss of the S-phase B-type cyclin Clb5p under conditions of limited cyclin-dependent kinase activity (*cdc28-4 clb5*Δ cells) causes a spindle positioning defect that results in an undivided nucleus entering the bud. Based on time-lapse digital imaging microscopy of microtubules labeled with green fluorescent protein fusions to either tubulin or dynein, we observed that the asymmetric behavior of the spindle pole bodies during spindle assembly was lost in the *cdc28-4 clb5*Δ cells. As soon as a spindle formed, both poles were equally likely to interact with the bud cell cortex. Persistent dynamic interactions with the bud ultimately led to spindle translocation across the bud neck. Thus, the mutant failed to assign one spindle pole body the task of organizing astral microtubules towards the mother cell. Our data suggest that Clb5p-associated kinase is required to confer mother-bound behavior to one pole in order to establish correct spindle polarity. In contrast, B-type cyclins, Clb3p and Clb4p, though partially redundant with Clb5p for an early role in spindle morphogenesis, preferentially promote spindle assembly.

Key words: spindle polarity • astral microtubules • cell cycle • cyclin • *Saccharomyces cerevisiae*

Introduction

Development of a spindle in *Saccharomyces cerevisiae* is initiated upon progression through START, before the G1/S transition (Byers, 1981; Hoyt and Geiser, 1996; Lew et al., 1997). As cells proceed through START, the yeast microtubule organizing center, the spindle pole body (SPB)[1] is duplicated. SPBs are embedded in the nuclear envelope throughout the cell cycle and are responsible for organizing astral microtubules in the cytosol, as well as the mitotic spindle within the nucleus. After duplication, SPBs separate to assemble a spindle. Before anaphase, the spindle positions at the bud neck with one SPB directed towards the mother and the other towards the daughter cell. As the spindle assembles, astral microtubules dynamically interact with the cell cortex of the mother or daughter cell to ultimately establish spindle orientation along the mother–daughter polarity axis (Yeh et al., 1995; Carminati and Stearns, 1997; Shaw et al., 1997b). Thus, spindle assembly and orientation are normally tightly linked processes contributing to the asymmetric nature of the spindle pathway. Asymmetry is also reflected in the fact that the newly synthesized spindle pole is destined for the daughter cell (Vallen et al., 1992).

The kinetics of spindle assembly has been previously characterized by time-lapse video-enhanced differential interference contrast (DIC) microscopy (Yeh et al., 1995). This study identified a two-step process. SPB separation initially occurs rapidly, creating a 1-μm long spindle, followed by a second slower phase to produce a 2-μm long spindle oriented along the mother–bud axis. Overall, this process takes ~30 min, followed by an additional period in which spindle length remains constant until onset of anaphase. This study, however, could not correlate these kinetics with astral microtubule behavior during spindle assembly.

A separate study in which astral microtubules were visualized by labeling with dynein fused to green fluorescent protein (GFP) provided further support to the notion that the spindle pathway is inherently asymmetric. The asymmetry was revealed by sequential association of the dynein fusion, first with astral microtubules emanating from the daughter-bound SPB ($SPB_{daughter}$), then, once spindle poles were ~1 μm apart, with astral microtubules emanating from the mother-bound pole (SPB_{mother}). Temporal as-

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[1]*Abbreviations used in this paper:* DIC, differential interference contrast; GFP, green fluorescent protein; SPB, spindle pole body.

sociation of dynein–GFP reflected astral microtubule organization by the SPBs rather than a consequence of microtubule orientation into the bud. Thus, the dynein–GFP label provides valuable information on SPB polarity and astral microtubule behavior in a variety of processes, including spindle orientation and karyogamy (Shaw et al., 1997b; Maddox et al., 1999).

Genetic analysis has implicated B-type cyclin function in spindle assembly. Strains containing multiple *CLB* deletions (e.g., *clb1-4*Δ, clb3-5Δ), fail to form a bipolar spindle (Surana et al., 1991; Fitch et al., 1992; Schwob and Nasmyth, 1993). Yet, due to functional redundancy, the relative contribution of individual Clbs in the various aspects of the spindle pathway has not been precisely determined.

We have previously described a spindle positioning defect associated with loss of the S-phase cyclin Clb5p under conditions in which the cyclin-dependent kinase Cdc28p is partially impaired (*cdc28-4 clb5*Δ cells). The *cdc28-4* allele, hypomorphic at permissive temperature, confers a sensitized environment for the genetic analysis of loss of individual cyclins (Segal et al., 1998). This strategy circumvents the problem of functional redundancy among the Clbs (Fitch et al., 1992; Richardson et al., 1992; Schwob and Nasmyth, 1993). The positioning defect in *cdc28-4 clb5*Δ cells ultimately perturbed spindle dynamics at the metaphase to anaphase transition, resulting in a terminal phenotype characterized by an undivided nucleus migrating into the bud. The Clb5p requirement for correct spindle positioning, however, was restricted to a temporal window at the G1/S boundary, coincident with the normal time of Clb5p-associated Cdc28p kinase activation (Segal et al., 1998).

In the present study, we have investigated the primary defect that results in nuclear mispositioning in *cdc28-4 clb5*Δ cells. We show that spindle polarity is normally established at the time of SPB separation and that Clb5p kinase is required to correctly coordinate these two processes. Based on time-lapse microscopy of microtubules labeled with GFP fusions to either tubulin or dynein, the asymmetric behavior of SPBs during spindle assembly was lost in the *cdc28-4 clb5*Δ cells: the mutant failed to assign one SPB the task of organizing astral microtubules towards the mother cell. At the same time, the kinetics of spindle assembly was altered in these cells. The combination of both defects results in symmetric spindles with astral microtubules from both poles initially interacting with the bud. Thus, Clb5p-associated kinase coordinates spindle assembly and orientation to confer mother-bound behavior to one SPB to establish correct spindle polarity. In contrast, Clb3p and -4 were not required for polarity establishment; these B-type cyclins contributed to spindle morphogenesis by promoting spindle assembly. These results indicate that both establishment of spindle polarity and spindle assembly are differentially subjected to cell cycle control.

Materials and Methods

Yeast Strains, Genetic Procedures, Media, and Growth Conditions

Strains MYT1010, *MATa/α cdc28-4/cdc28-4 GAL1:CLB5-TRP1/trp1 HIS3:GFP:TUB1-URA3/ura3*; MYT1416, *MATa/α cdc28-4/cdc28-4 clb5::ARG4/clb5::ARG4 GAL1:CLB5-TRP1/trp1 HIS3:GFP:TUB1-URA3/ura3;* and MYT2426, *MATa/α cdc28-4/cdc28-4 clb3::TRP1/clb3::TRP1 clb4::HIS2/clb4::HIS2 GAL1:CLB5-LEU2/leu2 HIS3:GFP:TUB1-URA3/ura3*, or isogenic versions carrying a *MET3:CLB5* construct instead of the *GAL1:CLB5* plasmid were previously described (Segal et al., 1998). Deletion of *DHC1* was constructed as previously described (Li et al., 1993). Standard yeast media and genetic procedures were used (Sherman et al., 1986). Yeast cultures were grown at 23°C unless indicated.

Digital Imaging Microscopy in Live Cells Expressing GFP–TUB1

Cells were grown to ~5×10^6 cells/ml in selective 3% galactose/0.1% dextrose medium and collected by filtration for a 2-h shift on selective glucose medium at 23°C to repress *CLB5* expression. Cells were then mounted in the same medium containing 25% gelatin to perform time-lapse recordings at room temperature as described (Shaw et al., 1997a; Maddox et al., 1999). In brief, a total of five fluorescence images were acquired at a Z-distance of 0.75 μm between each plane. A single bright-field image was taken in the middle focal plane. This acquisition regime was repeated at 30- or 60-s intervals. Images were processed as previously described (Shaw et al., 1997a,b; Maddox et al., 1999) using Metamorph software (Universal Imaging). Quantitation of oriented astral microtubules organized by the SPB_{mother} or $SPB_{daughter}$ was carried out by scoring single digital images corresponding to cells at metaphase with ~2-μm spindles observed in 22 independent time-lapse series.

Still cell images (see Figs. 4 and 5) were captured using 100% incident light intensity and 500-ms exposures to optimize visualization of astral microtubules. Spindle measurements in digital images were carried out as previously described (Segal et al., 1998).

Digital Imaging Microscopy in Live Cells Expressing DHC1–GFP

Recordings were performed in cells transformed with pKBY701. This construct expresses a dynein–GFP fusion under the control of the *GAL1* promoter (Shaw et al., 1997b). Strains MY1010GD, *MATa/α cdc28-4/cdc28-4 MET3:CLB5-TRP1/trp1 [GAL1:DHC1:GFP]*; or mutant MY1416GD, *MATa/α cdc28-4/cdc28-4 clb5::ARG4/clb5::ARG4 MET3:CLB5-TRP1/trp1[GAL1:DHC1:GFP]* were grown to midlog phase in selective dextrose lacking methionine (for expression of *CLB5* under the control of the *MET3* promoter). Cells were then transferred to selective galactose medium supplemented with methionine (to induce *DHC1–GFP* expression and repress *CLB5*) for 2 h at room temperature. Recordings and image processing were performed as described (Shaw et al., 1997b).

Results

Astral Microtubule Behavior during Spindle Assembly

We have previously assigned a role for Clb5p-dependent kinase at an early step of the spindle pathway. Loss of Clb5p under conditions of limiting Cdc28p activity (*cdc28-4 clb5*Δ at permissive temperature) resulted in a spindle positioning defect before anaphase (Segal et al., 1998).

Dynamic astral microtubule interactions play a critical role in the establishment of spindle orientation (Carminati and Stearns, 1997; Shaw et al., 1997b). To determine the possible contribution of Clb5p to this process, we undertook a detailed analysis of astral microtubule behavior throughout spindle assembly by time-lapse microscopy in live cells.

Recordings were carried out in parental *cdc28-4* and mutant *cdc28-4 clb5*Δ diploids expressing a GFP–Tub1 fusion (Straight et al., 1997) to visualize both astral microtubules and spindle structures. Fig. 1 shows a representative recording ($n = 15$) of parental *cdc28-4* cells. A budded cell oriented astral microtubules towards and into the bud (Fig. 1, 9 min, arrowhead) while the SPBs remained side

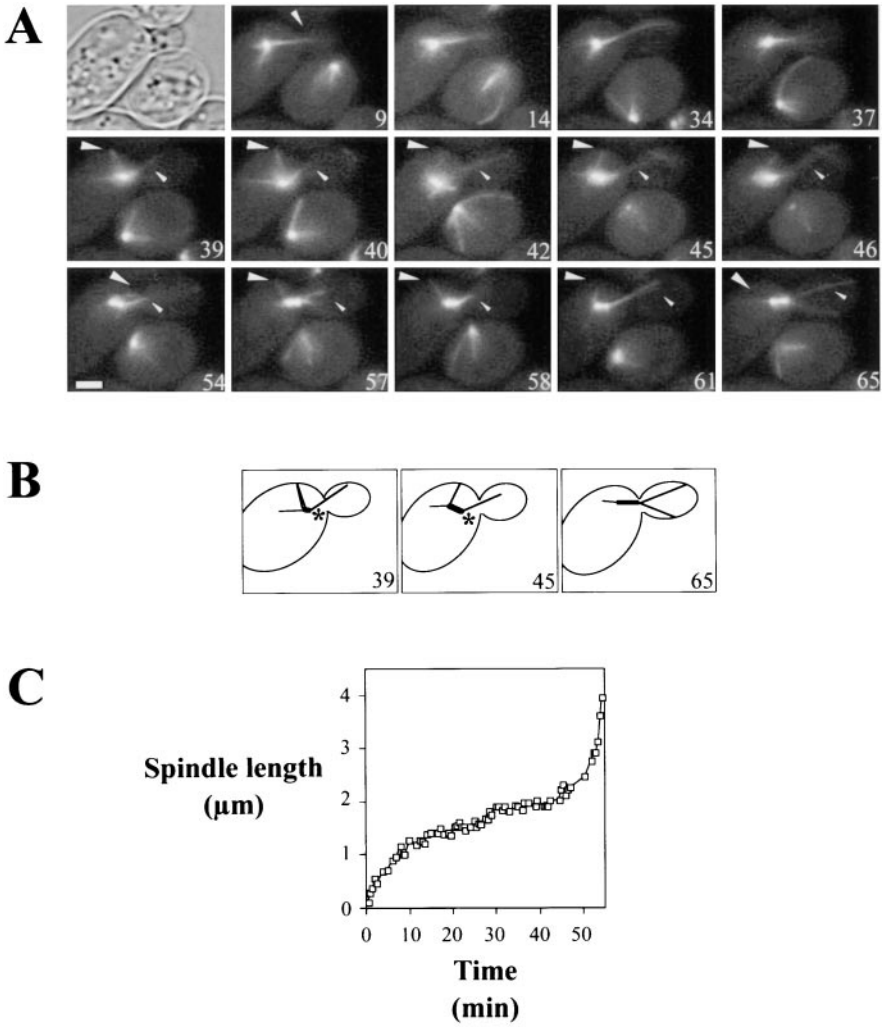

Figure 1. Spindle assembly in a *cdc28-4* diploid cell expressing a GFP–Tub1 fusion. A, Time-lapse series showing formation of a mitotic spindle in a *cdc28-4* cell. Astral microtubule interactions with the bud and with the vicinity of the neck cortex (organized by the $SPB_{daughter}$ and SPB_{mother}, respectively) contribute to spindle orientation. Selected frames from a 65-min time-lapse experiment are shown. Numbers correspond to the time elapsed in minutes relative to bud emergence. Arrowheads are described in the text. Bar, 2 μm. B, Cartoon of microtubule structures and cell outline for the indicated frames. Asterisk indicates $SPB_{daughter}$. C, Kinetics of spindle assembly in a *cdc28-4* cell. A representative time-lapse series was plotted from onset of SPB separation to initiation of spindle elongation at anaphase. For statistical information, see Table I.

by side facing the bud neck (Fig. 1, 9–37 min). During SPB separation, astral microtubules emanating from one SPB dynamically interacted preferentially with the bud cortex ($SPB_{daughter}$; Fig. 1, 39–40 min, small arrowhead). At the same time, astral microtubules from the SPB_{mother} first interacted with the mother cortex in the vicinity of the bud neck (Fig. 1, 39–40 min, large arrowhead). Therefore, spindle polarity is already evident during SPB separation. As spindle development progressed, the astral microtubules of the SPB_{mother} dynamically interacted with the mother cell cortex at points progressively further away from the bud neck (Fig. 1, 42–58 min, large arrowhead). As a result of these interactions, the SPB_{mother} was pushed away from the bud neck, promoting rotation of the spindle into the mother cell as the spindle assembled, thus ultimately orienting the spindle along the mother–bud axis (Fig. 1, 58–65 min). Once oriented, mobility of the spindle was restricted along the mother–bud axis. This sequence of events suggests that the initial astral microtubule interactions in the vicinity of the neck may be important for correct orientation of the SPB_{mother}.

The kinetics of SPB separation and spindle formation were consistent with the previous analysis of DIC time-lapse series that identified a two-step process (Yeh et al., 1995). From our data (Fig. 1 C; Table I), an ~1-μm spindle (1.2 ± 0.2 μm) was formed initially. This step took ~12 min. During this first phase, astral microtubules emanating from the SPB_{mother} interacted primarily with the cortex near the bud neck. Then, SPB separation proceeded to produce an ~2-μm spindle oriented along the mother–daughter axis (Fig. 1 C; Table I). During this second phase, microtubules emerging from the SPB_{mother} grew into the mother cell and interacted further away from the neck. Once spindles became oriented along the mother–bud axis, astral microtubules from the SPB_{mother} grew exclusively into the mother cell (100%, $n = 39$), whereas astral microtubules from the $SPB_{daughter}$ mainly grew into the bud (88%, $n = 42$).

We conclude that spindle polarity is specified soon after, or concomitant with, SPB separation, leading to differential astral microtubule interactions: either with the bud cortex or the bud neck region, respectively. Thus, the resulting asymmetric behavior of the two SPBs is a critical determinant of spindle polarity and orientation.

Spindle Assembly and Orientation in cdc28-4 clb5Δ Cells

In contrast to parental cells, in *cdc28-4 clb5*Δ diploids, astral microtubules from both SPBs dynamically interacted

Table I. Analysis of Spindle Morphogenesis in Parental and clb− Mutants

Strain	Onset of spindle assembly	Spindle Assembly‡		Preanaphase spindle length
		~1 μm	~2 μm	
	*min**	*min*	*min*	*μm*
cdc28-4	37 ± 5, *n* = 15	12 ± 3, *n* = 15	19 ± 5, *n* = 13	2.0 ± 0.2, *n* = 24
cdc28-4 clb5	34 ± 7, *n* = 22	7 ± 3, *n* = 26	7 ± 3, *n* = 26	2.2 ± 0.5, *n* = 31
cdc28-4 clb3 clb4	72 ± 9, *n* = 6	11 ± 2, *n* = 6	12 ± 4, *n* = 8	2.3 ± 0.4, *n* = 11

*Timing was relative to bud emergence.
‡~1 μm, Time from initiation of spindle assembly until length remained constant for at least 5 min; ~2 μm, additional time to produce a preanaphase spindle.

with the bud cortex as soon as SPB separation occurred (Fig. 2). Astral microtubules from both SPBs at onset of SPB separation were more evident in cells expressing a dynein–GFP fusion, since the strong fluorescence of Tub1–GFP label associated with the spindle makes visualization of astral microtubules particularly difficult (Fig. 2, 39 min, and Fig. 3 C). Astral microtubules from both SPBs entering the bud can be clearly seen in the time-lapse series shown in Fig. 2 at 42 min after bud emergence. The interactions with the bud cortex continued throughout the process of spindle assembly (90%, *n* = 22; Fig. 2, cell A). Overall, both poles seemed equally likely to establish dy-

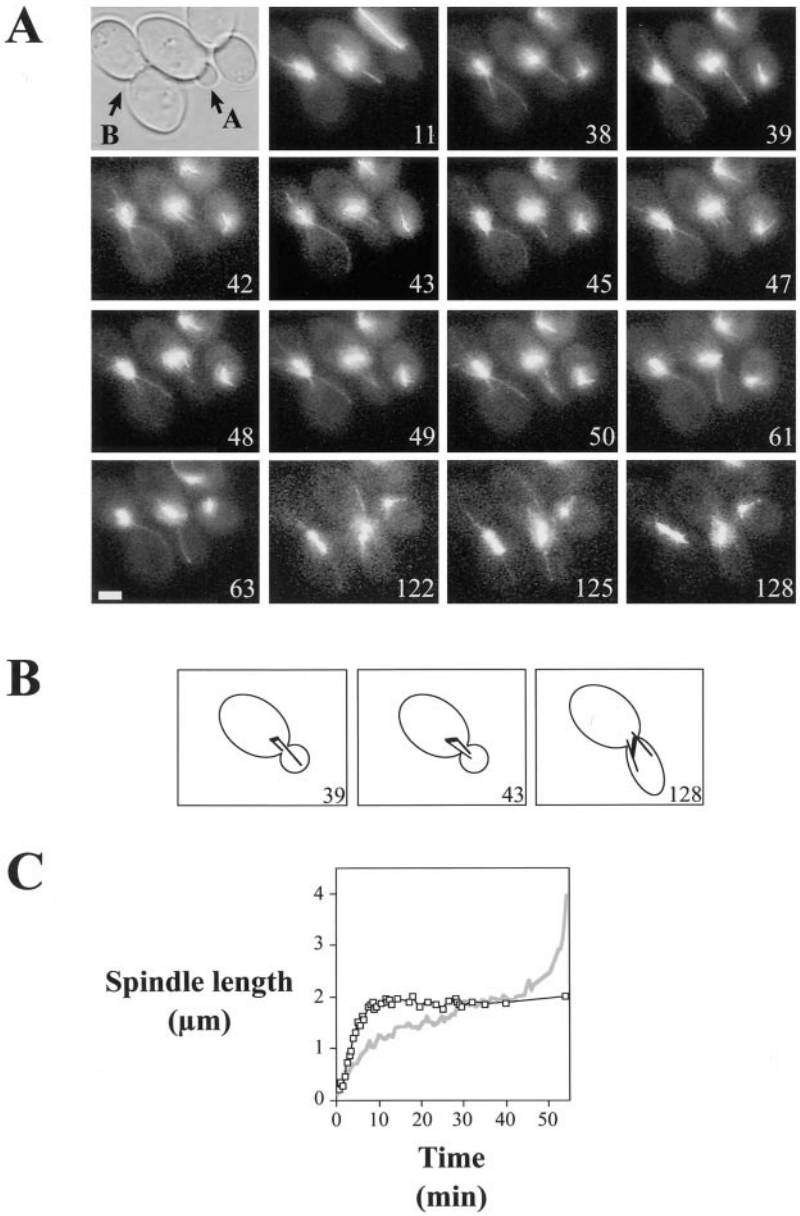

Figure 2. Spindle assembly in a *cdc28-4 clb5*Δ diploid cell expressing a GFP–Tub1 fusion. A, Time-lapse series showing formation of a mitotic spindle (cell A) in a *cdc28-4 clb5*Δ cell. During spindle assembly both SPBs organize astral microtubules interacting with the bud cortex. Microtubules from both poles entering the bud are seen at 42 min after bud emergence. A second cell already blocked late at metaphase is shown (cell B). Notice the large bud size of this cell (top) already in the first frame. In this cell, microtubules from both poles are directed towards the bud (visible in frames 49 and 50). Selected frames from a 145-min time-lapse experiment are shown. Numbers correspond to the time elapsed in minutes relative to bud emergence for cell A. Bar, 2 μm. B, Cartoon of microtubule structures and cell outline for the indicated frames (cell A). C, Kinetics of spindle assembly in a *cdc28-4 clb5*Δ cell (□) from onset of SPB separation. For reference, the kinetics of the parental cell shown in Fig. 1 C has been overlaid (gray line). For statistical information, see Table I.

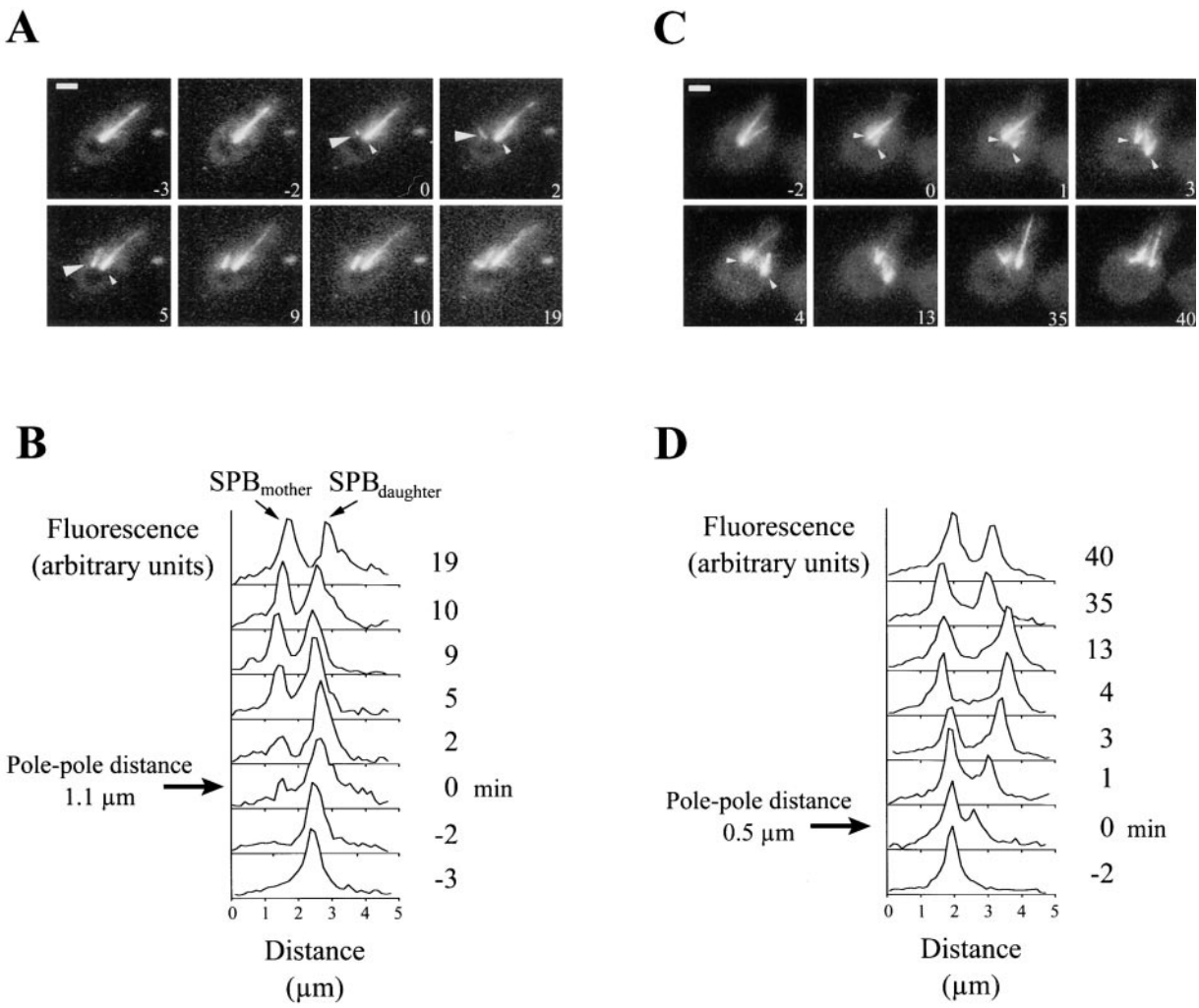

Figure 3. Dynein–GFP accumulation at the SPBs and astral microtubule behavior during spindle assembly. A and B, SPBs and astral microtubules visualized with a dynein–GFP fusion during spindle assembly in a *cdc28-4* cell. A, Initially the $SPB_{daughter}$ is labeled. The second SPB (large arrowhead, 0–5 min) acquires fluorescence once SPB separation has occurred. Small arrowhead, $SPB_{daughter}$. B, Plot showing line scans through the axis of the spindle corresponding to each time frame in A. Pole to pole distance at time 0 was 1.1 μm and increased to 1.3 μm by 19 min. C and D, Symmetric behavior of SPBs during spindle assembly in a *cdc28-4 clb5*Δ cell. C, Both SPBs are already labeled at onset of SPB separation (arrowheads, 0–4 min). Microtubules from both poles enter the bud as the spindle forms, corresponding to the behavior observed in cells expressing the GFP–Tub1 fusion. D, Plot showing line scans through the axis of the spindle corresponding to each time frame in B. Pole to pole distance was 0.5 μm at 0 min and increased to 2.0 μm by 4 min. Poles appear closer at time points 35–40 min due to spindle rotation away from the axis of measurement. Numbers indicate time elapsed in minutes relative to the first frame in which the two SPBs become visible. Bars, 2 μm.

namic interactions with the daughter cell cortex >40 min after SPB separation. The dynamic nature of these interactions with the bud was evident from the fact that assembled spindles were abnormally mobile and initially tended to orient orthogonally relative to the mother–bud axis. This reflected net force between both SPBs and the bud cortex. Due to these pulling forces, spindles eventually translocated across the neck into the bud (Fig. 2, 122 min), as has been previously described (Segal et al., 1998).

The kinetics of SPB separation was dramatically affected in this mutant. Contrary to the two-step spindle assembly observed in parental *cdc28-4* diploids (Fig. 1 C) or wild-type cells (not shown; Yeh et al., 1995), *cdc28-4 clb5* mutants appeared to assemble a spindle in one step (Fig. 2 C). While parental cells completed spindle assembly in ~30 min, the mutant formed ~2-μm spindles in seven minutes (Table I).

After spindle assembly, most cells failed to proceed with spindle elongation. During the block, spindles tended to become aligned with respect to the mother–bud axis. This was due to the restrictions in orientation imposed by occasional transits of the spindle across the bud neck. However, polarity was still disrupted since both poles continued to interact with the bud cortex (Fig. 2, cell B). After a prolonged block, astral microtubules directed towards the daughter cell cortex seemed functional, but those growing into the mother cell became abnormally long and curved. It is possible that this behavior resulted from lack of initial

interactions between these mother-bound microtubules and the neck cortex. This corresponded to the terminal phenotype previously described for this mutant.

These data suggest that Clb5p-dependent kinase ensures that SPBs become asymmetric regarding their ability to promote specific astral microtubule interactions either with the mother or daughter cell cortex in tight coordination with spindle assembly. In addition, our results suggest a direct or indirect role for Clb5p in regulating the kinetics of spindle assembly.

Dynein–GFP Labeling and Spindle Symmetry

Differential association of dynein fusion proteins with each SPB throughout the cell cycle has demonstrated the inherent asymmetry of the SPBs during spindle morphogenesis (Yeh et al., 1995; Shaw et al., 1997b). The fact that both SPBs in *cdc28-4 clb5*Δ cells appeared to organize astral microtubules that interacted with the bud cortex during spindle assembly suggested that the inherent polarity of the spindle might be disrupted in *cdc28-4 clb5*Δ cells. To address this issue, parental and *cdc28-4 clb5*Δ mutant cells expressing a dynein–GFP fusion were studied by time-lapse digital imaging microscopy. As previously reported (Shaw et al., 1997b), otherwise wild-type diploids displayed the characteristic lag in dynein–GFP acquisition to the SPB_{mother} (delayed acquisition in ten out of ten events; not shown). Parental *cdc28-4* diploids behaved like wild-type cells (delayed acquisition 11 out of 13 events; Fig. 3, A and B). In other words, the SPB_{mother} was not labeled until the spindle was at least 1 μm long (1.1 ± 0.2 μm, n = 11). At this time, the SPB_{mother} was weakly labeled (Fig. 3 A, 0 min) as in wild-type cells. Label gradually increased until it reached comparable intensities to the $SPB_{daughter}$ (Fig. 3 B).

This asymmetric behavior, however, was lost in *cdc28-4 clb5*Δ diploids. Both SPBs were labeled and visible as soon as SPBs separated (no lag in eight out of eight events, pole to pole distance 0.45 ± 0.1 μm; Fig. 3, C and D, 0 min). The absence of any observable lag in the acquisition of dynein–GFP correlated with the fact that both SPBs promoted interactions with the bud cortex.

Previous digital imaging microscopy studies using the same fusion have indicated that astral microtubules mediate dynein–GFP labeling of the SPB (Shaw et al., 1997b; Maddox et al., 1999). Thus, dynein–GFP label acts as a "read out" for the delayed presence of microtubules emerging from the SPB_{mother}. Such built-in delay in astral microtubule organization, relative to SPB separation, may constitute the basis for correct spindle orientation, as previously suggested by Shaw et al. (1997b).

Consistent with dynein not being a direct mediator of daughter-bound polarity, deletion of *DHC1*, the gene encoding dynein heavy chain (Eshel et al., 1993; Li et al., 1993), did not suppress the polarity defect observed in *cdc28-4 clb5*Δ cells. Astral microtubule behavior was examined after a four-hour shift of a *cdc28-4 clb5*Δ *dhc1*Δ *GAL1-CLB5* strain to glucose. Initial symmetric astral microtubule interactions with the bud occurred in *cdc28-4 clb5*Δ *dhc1*Δ as in *cdc28-4 clb5 DHC1* cells (Fig. 4). In addition, 10% of cells displayed a combination of the polarity defect of *cdc28-4 clb5*Δ and the astral microtubule

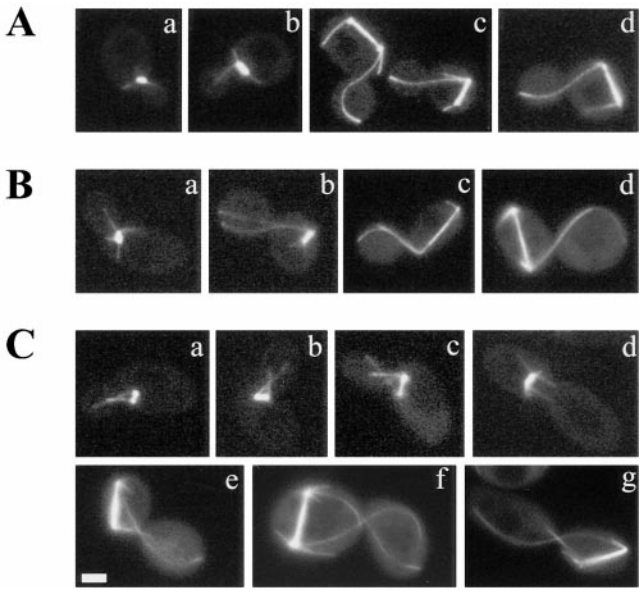

Figure 4. Definition of spindle polarity is not affected in *dhc1*Δ mutants. Cells were grown on 3% galactose–0.1%glucose synthetic medium followed by a 4-h shift to glucose synthetic medium to repress *GAL1-CLB5* expression necessary for viability of *cdc28-4 clb5* diploids. Cells were examined microscopically and scored for defects in apparent polarity definition after spindle assembly, irrespective of position or orientation (i.e., astral microtubule interactions with the bud from both poles). Images of cells expressing the GFP–Tub1 fusion were captured as previously described (Segal et al., 1998). Percentages represent the average of two independent counts of 500 cells containing a spindle for the indicated strains. A, Representative stages in *dhc1*Δ diploids. Spindle polarity is evident as soon as a spindle forms (a and b), as well as in cells undergoing anaphase without the spindle translocating across the neck (c and d). Anaphases in the mother occurred in 12% of cells under the experimental conditions. 98% of cells carrying a spindle exhibited astral microtubules from a single pole interacting with the bud. B, Representative stages in a *cdc28-4 dhc1*Δ diploid. a, Cell with a short spindle. One pole is interacting with the bud. Also, prominent interactions with the neck are evident. b, Cell containing a short spindle away from the bud neck. c and d, Mispositioned anaphase spindles. Astral microtubules from a single pole interacted with the bud in 95% of cells. C, Representative stages in a *cdc28-4 clb5 dhc1* diploid. a–c, As a spindle forms, both poles interact with the bud as described in Fig. 2. d, Cell with spindle mispositioned in the bud (20% of cells). e–g, Mispositioned anaphase spindles. Both poles still interact with the bud (10% of cells). Bar, 2 μm.

behavior characteristic of *dhc1*Δ mutants. Comparable behavior of astral microtubules from both SPBs was observed even in cells with spindles positioned in the mother (Fig. 4 C). In contrast, *cdc28-4 dhc1*Δ cells displayed correct spindle polarity regardless of spindle positioning, i.e., only a single SPB seemed to associate with the bud via abnormally long astral microtubules.

Overall, the *dhc1*Δ mutation seemed to exacerbate the polarity defect of *cdc28-4 clb5* cells. Presumably, due to the decreased microtubule dynamic instability characteristic of *dhc1*Δ cells (Carminati and Stearns, 1997), misdirected microtubule attachments were even harder to rectify after spindle assembly. The mutation also increased

the proportion of spindles remaining in the mother cell (irrespective of orientation of astral microtubules into the bud), consistent with dynein's importance in spindle translocation. However, a new spindle-dynamic behavior resulted in this situation. Occasional anaphases in the mother cell stalled, apparently, as a consequence of lack of functional attachments into the mother cell (not shown).

Taken together, these results suggest that the Clb5p-dependent kinase effect on inherent spindle polarity was not mediated by dynein activity. However, the resulting genetic interaction emphasizes the contribution of microtubule dynamic instability as a factor in the establishment of correct spindle orientation. The *dhc1*Δ mutation, however, did not alter the fact that both SPBs ultimately displayed the characteristic daughter-bound behavior of the *cdc28-4 clb5*Δ mutant, even though translocation across the neck was partially suppressed.

Contribution of Clb3p and Clb4p to Spindle Morphogenesis

A redundant function early in the spindle pathway has been genetically assigned to Clb5p, Clb3p, and Clb4p. A triple *clb3 clb4 clb5* mutant fails to form a bipolar spindle and arrests with a 2C DNA content (Schwob and Nasmyth, 1993). Nevertheless, the precise role played by each of these cyclins in spindle development has not been addressed.

Using our genetic approach of sensitizing cells to cyclin deficiencies with a mutant Cdc28p kinase, we constructed a strain carrying a *cdc28-4* allele in combination with disruptions at the *CLB3* and *CLB4* loci and expressing the GFP–Tub1 fusion (Segal et al., 1998). The diploid mutant was viable at permissive temperature and displayed comparable temperature sensitivity to a parental *cdc28-4* diploid (data not shown). However, elimination of Clb3p and Clb4p had a profound effect on the cell cycle timing of spindle development. Fluorescence microscopy of cells from an asynchronous culture indicated that a high proportion of large budded cells was apparently delayed for spindle assembly. The percentage of large budded cells that had not initiated spindle assembly was 21 ± 2% for parental cells, whereas in *cdc28-4 clb3*Δ *clb4*Δ diploids, it was 54 ± 6%. Yet, in the *cdc28-4 clb3 clb4* cells, astral microtubules projecting from the unseparated SPBs were oriented correctly, one bundle towards the mother and one towards the bud, a behavior normally characteristic of cells that have already assembled a spindle (Fig. 5). The fact that *cdc28-4 clb3 clb4* diploids can establish correct spindle polarity, even though SPB separation is delayed, was confirmed by time-lapse microscopy (Fig. 6). The timing of SPB separation was significantly delayed relative to bud emergence (72 min, compared with 37 min in *cdc28-4* cells or 34 min in *cdc28-4 clb5* cells; Table I). Yet, astral microtubule behavior followed a cell cycle pattern comparable to that of parental *cdc28-4* cells (Fig. 1). Since attachments appeared to orient before SPB separation (Fig. 5 C and Fig. 6, 41–73 min), spindle orientation along the mother–bud axis could be rapidly established upon assembly (Fig. 6, 88 min). Kinetics of spindle formation in this mutant, while delayed with respect to bud emergence, was more comparable to that of parental cells (Fig. 6 C; Table I). Formation of a 2-μm long spindle took place over a period of ~20 min. Then, spindle length continued to increase until onset of anaphase. As a result, anaphase occurred with a slight delay, relative to bud emergence (110 min vs. 92 min in parental cells).

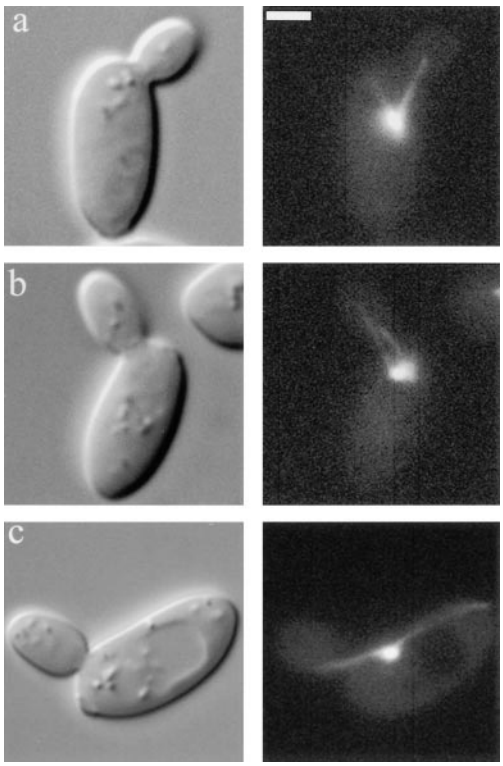

Figure 5. Definition of spindle polarity in a *cdc28-4 clb3*Δ *clb4*Δ diploid. Comparison of astral microtubule behavior in *cdc28-4* (a), *cdc28-4 clb5*Δ (b), and *cdc28-4 clb3*Δ *clb4*Δ (c) cells expressing the GFP–Tub1 fusion. Notice that in a and b, SPB separation has already started, whereas in c, SPBs remain side by side. Pairs of DIC and single fluorescence images are shown. Bar, 2 μm.

The distinct contributions of Clb3p, Clb4p, and Clb5p, respectively, in spindle morphogenesis suggests that nuclear and astral microtubules are regulated differentially by the cell cycle machinery. In addition, Clb5p may be directly or indirectly responsible for the two-step kinetics of spindle assembly.

Discussion

Clb5p-dependent Kinase Contributes to Spindle Polarity

Our analysis of spindle development by time-lapse digital imaging microscopy indicates that the inherent asymmetry of SPBs can dictate the correct orientation of astral microtubules so that they interact with the bud or mother cell cortex, respectively, during spindle assembly (Figs. 1 and 3 A). This asymmetric behavior was lost in *cdc28-4 clb5*Δ cells, causing astral microtubules, emanating from both SPBs, to primarily orient towards the bud (Figs. 2 and 3 C). Therefore, Clb5p-associated kinase is required to impart polarity to the spindle during assembly, by regulating,

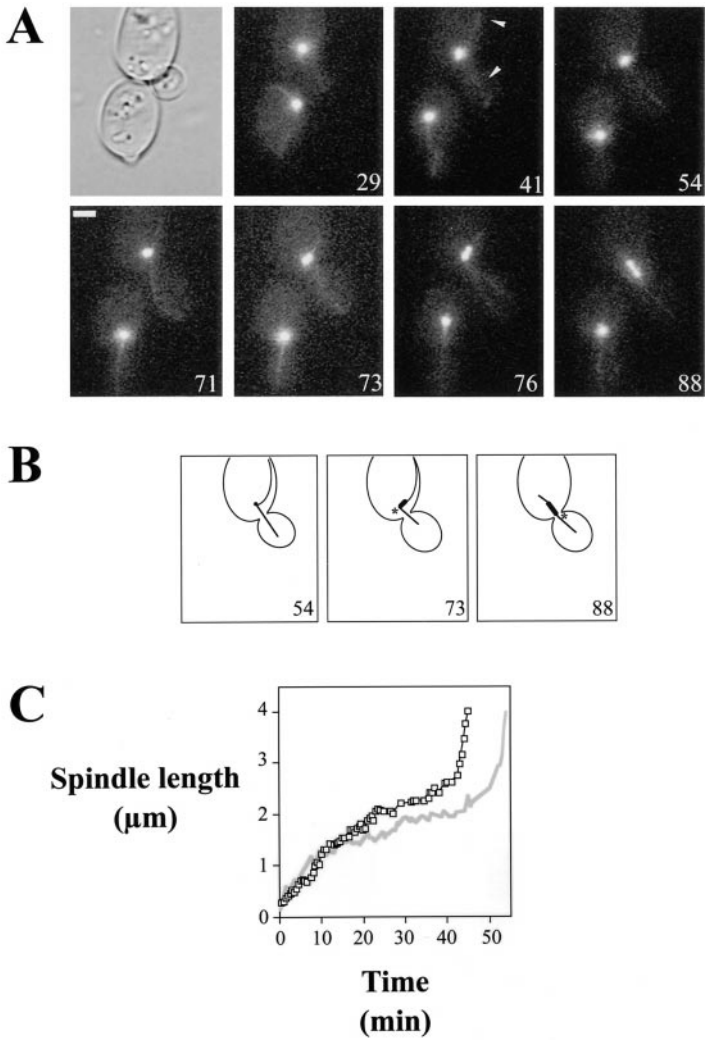

Figure 6. Spindle assembly and orientation in a *cdc28-4 clb3*Δ *clb4*Δ cell expressing the GFP–Tub1 fusion. A, Time-lapse series showing delayed spindle assembly in a *cdc28-4 clb3*Δ *clb4*Δ cell. SPB separation started at 73 min after bud emergence. Spindle orientation along the mother–bud axis was achieved by 88 min, before anaphase. Though astral microtubules are difficult to distinguish in this series, frame 41 corresponds approximately to the cell cycle stage of the cell shown in Fig. 5 C, suggesting that attachments to the mother and bud cell cortex occurred approximately on schedule (arrowheads), even though SPBs remained side by side for an additional 32 min. Numbers indicate time elapsed in minutes relative to bud emergence for the upper cell. Bar, 2 μm. B, Cartoon of microtubule structures and cell outline for the indicated frames. Asterisk indicates $SPB_{daughter}$. C, Kinetics of spindle assembly from pole separation to initiation of anaphase in a *cdc28-4 clb3 clb4* cell. For reference, the kinetics of the parental cell shown in Fig. 1 C has been overlaid (gray line). For statistical information, see Table I.

at least, SPB_{mother} function. This event must occur within a restricted temporal window (Segal et al., 1998), possibly related to changes in astral microtubule dynamic properties during the cell cycle. Before spindle development, astral microtubules display fast turnover rates (Carminati and Stearns, 1997; Shaw et al., 1997b; Tirnauer et al., 1999). Failure within this restricted period to specify spindle polarity appears to compromise spindle orientation, suggesting that misoriented astral microtubule contacts might be difficult to rectify once microtubule turnover rates decrease later in the cell cycle. Alternatively, Clb5p activity may only ensure correct spindle polarity if present before SPB separation.

Two additional B-type cyclins, Clb3p and Clb4p, were dispensable for correct spindle polarity (Figs. 5 and 6). However, these cyclins were important for correct timing of spindle assembly (Fig. 6; Table I).

Spindle Assembly and Orientation Are Tightly Linked Processes

Our studies of spindle assembly in cells expressing a Tub1–GFP fusion enabled us to correlate astral microtubule behavior with spindle assembly. Our conclusions, while consistent with previous studies dealing separately with either kinetics of SPB separation (Kahana et al., 1995; Yeh et al., 1995) or astral microtubule behavior (Carminati and Stearns, 1997; Shaw et al., 1997b), convey an integrated view of spindle assembly and initial orientation as tightly linked processes.

After bud emergence, side by side SPBs orient facing the bud neck, a process that depends on a microtubule-based search mechanism (Shaw et al., 1997b). Both in wild-type (not shown) and parental *cdc28-4* diploids (Fig. 1), we observed that formation of a short, <1.2-μm spindle was accompanied by interactions between one pole (SPB_{mother}) and the vicinity of the neck via astral microtubules. Individual spindles spent a variable time (5–15 min) at this stage before proceeding to the second phase of spindle assembly (not shown). During the second phase, astral microtubules from the SPB_{mother} continued to grow into the mother cell and interacted with points further away from the neck as the spindle oriented along the mother–bud axis and reached a constant size of ~2 μm.

Interestingly, dynein–GFP label is first acquired by the SPB_{mother} when the poles were ~1 μm apart (Fig. 3 B). As label gradually increased, the distance between the poles remained approximately constant (Fig. 3, A and B). This

suggests that acquisition of dynein–GFP label by the SPB_{mother} occurs coincident with the transition between the first and second phase of spindle assembly.

In view of these results, it is striking that *cdc28-4 clb5*Δ cells displayed a spindle polarity defect, as well as altered kinetics of spindle assembly (Fig. 2). The simultaneous labeling by dynein–GFP at onset of SPB separation and the perturbed kinetics of spindle assembly might represent independent defects resulting from loss of Clb5p-dependent kinase. Yet, it is tempting to suggest that both defects are somehow related. The wild-type program of dynein–GFP acquisition and the kinetics of SPB separation may indicate that spindle orientation (i.e., astral microtubule organization and/or interactions with the neck) and spindle morphogenesis can cross-talk at the transition between the first and second step of spindle assembly.

It is unlikely that the spindle polarity and morphogenesis defects in *cdc28-4 clb5*Δ cells are a consequence of Clb5p normally antagonizing Clb3p and Clb4p function. First, these three cyclins have been suggested to play a redundant function in the spindle pathway (Schwob and Nasmyth, 1993). Second, *CLB3* is a high dosage suppressor of the *cdc28-4 clb5*Δ lethality and spindle positioning defect (Segal et al., 1998). In addition, our kinetic studies of spindle assembly in the context of S-phase checkpoint activation by 0.1 M hydroxyurea (Clarke et al., 1999; Clarke, D.J., M. Segal, and S.I. Reed, unpublished results), do not favor a relationship between the biphasic kinetics of spindle assembly discussed here and events associated with completion of DNA replication.

Previously, we have reported that in *cdc28-4 clb5*Δ cells, nuclear division in the bud was blocked (Segal et al., 1998). The combination of defects in spindle morphogenesis described in the present study may account for this observation. Whether the inability of spindles to elongate results from the triggering of a checkpoint or from a mechanical defect remains to be determined.

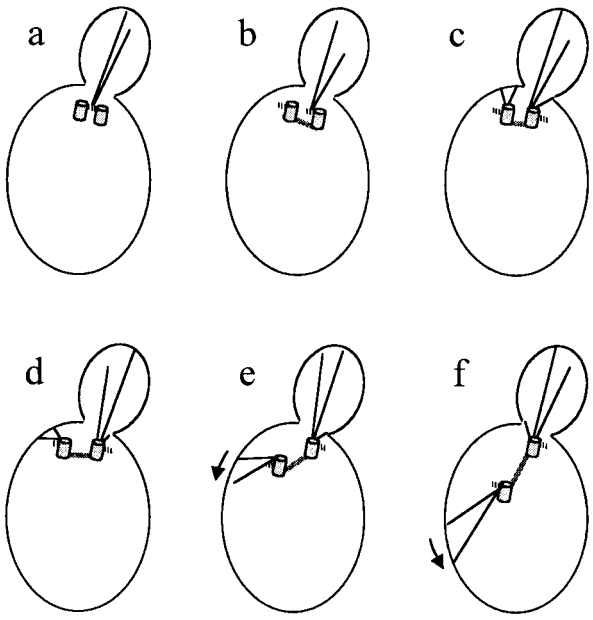

Figure 7. A model for coordinated spindle assembly and orientation. After bud emergence, the duplicated SPBs orient facing the bud neck via astral microtubules emanating from the bridge (a). Once oriented, one pole, the $SPB_{daughter}$, inherits these astral microtubules as the bridge divides between the two SPBs (b). A "fast" phase of spindle assembly occurs and, coordinate with this event, astral microtubules initiate interactions from each pole with the mother cortex at the neck area (c). These interactions may initiate a transition to a second, slower phase of spindle formation. While spindle assembly continues, astral microtubules from the SPB_{mother} are crucial to impart correct orientation to the spindle along the mother–bud axis (d–f). Spindle morphogenesis in *cdc28-4 clb5* cells is deregulated in step (c), leading to inherent symmetry of the spindle already at this point.

A Model for Clb5p Role in Spindle Morphogenesis

At present, our understanding at the molecular level of structures and events associated with astral microtubule organization is too limited to suggest a defined target(s) for Clb5p kinase relevant to SPB asymmetry. After SPB duplication, astral microtubules emerge from the bridge region (Byers and Goetsch, 1975; Byers, 1981). Throughout the rest of the cell cycle, however, astral microtubules seem to organize from the outer plaques of the SPBs. In addition, a model to explain establishment of asymmetry upon SPB separation must incorporate the fact that it is the old SPB inherited from the previous cell cycle that is destined to the mother cell (Vallen et al., 1992). Interestingly, it was a Kar1–LacZ fusion (mistargeted to the outer plaque) that revealed the asymmetric nature of SPBs. Wild-type Kar1, which localizes to the half-bridge, associates with both SPBs throughout the cell cycle (Spang et al., 1995). Thus, the mechanistic implications for the differential association of this protein fusion remain unclear.

The asymmetric acquisition of a dynein–GFP fusion by the SPBs correlates with the promotion of asymmetric dynamic contacts towards the bud and mother cell. This suggests that timing of microtubule organization by SPBs is crucial for microtubule orientation, as anticipated by Shaw et al. (1997b). Thus, dividing spindle morphogenesis into two temporally distinct steps might ensure the asymmetric behavior of SPBs. The first step would commit the $SPB_{daughter}$ to interact with the bud cortex. The second step would impart mother-bound behavior to the remaining pole (Fig. 7). Initially, astral microtubules emanating from the bridge interact with the bud cell cortex (Fig. 7 a). Based on time-lapse microscopy, astral microtubules interact continuously with the bud before and during SPB separation. This may indicate that, as the poles separate, the microtubules initially present on the bridge are inherited by a single pole, the $SPB_{daughter}$ (Fig. 7 b). Regardless of the mechanistic details, however, this event cannot solely regulate spindle polarity. As SPBs separate, microtubule organization at the respective outer plaques must be delayed until a temporal window, when initial contacts with the mother cell at the neck area, would be favored. Since one pole is initially committed to be the $SPB_{daughter}$, the delay in microtubule organization could actually be imparted to both poles with the net result of ensuring correct fate to the SPB_{mother} (Fig. 7 c).

Dynein–GFP labeling is consistent with such a model. The intensity of label remains constant through initial orientation of the microtubule bundle into the bud and the

early steps of SPB separation. Thus, the bundle of microtubules associated with the bud seems to be inherited by the $SPB_{daughter}$. As a short spindle forms, delayed acquisition is only evident on the SPB_{mother}. Microtubule organization by the $SPB_{daughter}$, however, is not revealed because of the already existing label at this pole.

The pattern of dynein–GFP labeling in a *cdc28-4 clb5*Δ cell (Fig. 3 C) suggests that the initial partition of microtubules associated with the bud occurs correctly since only one pole seemed to retain contacts to the bud cortex at onset of SPB separation (Fig. 3 C, 0 min). However, this mutant presumably organized microtubules at the outer plaque of the SPBs prematurely and/or uncoordinated with respect to SPB separation. The net result is that the SPB_{mother} lacks the normal lag in dynein–GFP acquisition that reflects correct fate. Thus, both poles become equally likely to be daughter-bound. This model can explain how Clb5p regulates correct asymmetry at a point in which two poles are already present. It is possible that Clb6-dependent kinase in this strain may be sufficient to bring about clipping of the bridge with correct timing relative to bud emergence. This event also requires B-type cyclin-dependent Cdc28p activity since it is blocked in a *cdc4* mutant, which arrests before Clb-dependent kinase activation at the restrictive temperature (Byers, 1981; Schwob et al., 1994). Yet, Clb6p activity may be unable to regulate outer plaque function unless grossly overexpressed (Segal et al., 1998). Thus, Clb5p/Cdc28p kinase activity may be necessary to inhibit or delay astral microtubule organization from the SPB outer plaque until the cell is permissive for mother cortex interaction.

It is not known what triggers initial organization of astral microtubules at the outer plaque or how the process might be coordinated during SPB separation. Yet this, or a closely related event, remains a likely target in imparting Clb5p-dependent SPB asymmetry. For example, association of the γ-tubulin complex to the outer plaque target, Spc72p (Knop and Schiebel, 1998), may be subjected to regulation by cyclin-dependent kinases. Cortical cues are important in establishment of correct spindle orientation (Lee et al., 1999; Miller et al., 1999). Their contribution to the *cdc28-4 clb5* phenotype, however, is mostly reflected in the different penetrance of the nuclear positioning defect in haploids and diploids (Segal et al., 1998; Segal, M., K. Bloom, and S.I. Reed, manuscript in preparation). However, our genetic analysis does not support a primary role for known cortical cues in mediating the initially symmetric behavior of spindles in *cdc28-4 clb5*Δ cells described here (Segal, M., K. Bloom, and S.I. Reed, unpublished results).

Development of a multicellular organism entails the ability to generate a variety of cell types beginning from a single cell. Diversity arises primarily from asymmetric divisions, resulting in daughter cells that differ in developmental fates (Rhyu and Knoblich, 1995). The strategy, which relies on regulating the orientation of cell divisions in response to positional cues, has been characterized in a variety of systems, including the early divisions of *Caenorhabditis elegans* (Rhyu and Knoblich, 1995) and mammalian neurogenesis (Hyman and White, 1987). The mechanism for spindle orientation shares common features with the yeast system (Chenn and McConnell, 1995; Rhyu and Knoblich, 1995; Skop and White, 1998). The role of Clb5p in the definition of inherent spindle polarity in yeast suggests that the cell cycle machinery may also play a crucial role in early development, by regulating centrosome asymmetry. In turn, alternative modes of spindle orientation might contribute to specify symmetric versus asymmetric divisions and, eventually, cell fate.

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