The budding yeast Ipl1/Aurora protein kinase regulates mitotic spindle disassembly

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Ipl1p is the budding yeast member of the Aurora family of protein kinases, critical regulators of genomic stability that are required for chromosome segregation, the spindle checkpoint, and cytokinesis. Using time-lapse microscopy, we found that Ipl1p also has a function in mitotic spindle disassembly that is separable from its previously identified roles. Ipl1–GFP localizes to kinetochores from G1 to metaphase, transfers to the spindle after metaphase, and accumulates at the spindle midzone late in anaphase. Ipl1p kinase activity increases at anaphase, and ipl1 mutants can stabilize fragile spindles. As the spindle disassembles, Ipl1p follows the plus ends of the depolymerizing spindle microtubules. Many Ipl1p substrates colocalize with Ipl1p to the spindle midzone, identifying additional proteins that may regulate spindle disassembly. We propose that Ipl1p regulates both the kinetochore and interpolar microtubule plus ends to regulate its various mitotic functions.

Introduction

The proper regulation of mitotic spindle dynamics is a fundamental aspect of chromosome segregation and is crucial for the maintenance of genetic information. During S phase, chromosomes replicate and linkage is established between the sister chromatids. The mitotic spindle forms, and microtubules attach to chromosomes via kinetochores, multiprotein complexes that assemble onto centromeric DNA. Once the sister kinetochores attach to microtubules from opposite poles, the linkage between the sister chromatids is dissolved and the chromosomes segregate, resulting in two daughter cells with identical genomes.

Mitotic spindles consist of two types of microtubules: those that bind to kinetochores (kinetochore microtubules) and those that interdigitate with each other (interpolar microtubules) (for review see Desai and Mitchison, 1997). The microtubule minus ends are bound to the microtubule organizing center, whereas the dynamic plus ends either capture kinetochores or interdigitate. In budding yeast, the mitotic spindle is formed after separation of the duplicated spindle pole bodies (SPBs)* (for review see Winey and O’Toole, 2001). During metaphase, cytoplasmic microtubules ensure that the nucleus migrates to the bud neck (nuclear migration) and the spindle aligns parallel to the mother-bud axis (spindle orientation) (for review see Segal and Bloom, 2001). Anaphase then occurs in two phases. In anaphase A, the sister chromatids move toward the SPBs, and in anaphase B a rapid phase of spindle elongation is followed by a second slow phase that drives the SPBs apart (Yeh et al., 1995; Straight et al., 1997). When the spindle has reached its maximal length of ~10 μm, it disassembles exclusively from the plus ends at the midzone, the site of overlap between the interpolar microtubules (Maddox et al., 2000). Proper regulation of microtubule dynamics is critical for chromosome segregation and is performed in part by motor and nonmotor microtubule-associated proteins.

The fidelity of spindle assembly is monitored by the spindle checkpoint that prevents cells from separating their sister chromatids until kinetochore attachment is complete and kinetochores are under tension (for review see Millband et al., 2002). Spindle checkpoint arrest is achieved through inhibition of the anaphase-promoting complex (APC), a multiprotein ubiquitin ligase that catalyses proteolysis of the mitotic inhibitor proteins Pds1 and Clb2 (for review see Peters, 2002). When the APC targets Pds1p for degradation, the Esp1 protease is liberated to cleave the cohesin Mcd1/Scc1p, resulting in sister chromatid separation (for review see Nasmyth, 2002).

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Shannon and Salmon, 2002; Stern, 2002). In mammals, the kinases can be subdivided into three families: Aurora A, B, and C (for review see Nigg, 2001). Aurora A localizes to centrosomes and is required to maintain the separation of centrosomes and to form a bipolar spindle (Glover et al., 1995). Aurora B exhibits a “chromosomal passenger” localization pattern, where it localizes to the chromosomes and kinetochores, transfers to the spindle, and eventually accumulates at the spindle midzone and midbody (Bischoff et al., 1998; Schumacher et al., 1998; Terada et al., 1998; Petersen et al., 2001; Murata-Hori et al., 2002). Aurora B is in a complex with the chromosomal passenger proteins INCENP (inner centromere protein) and Survivin/Bir1 (Kim et al., 1999; Adams et al., 2000; Kaitna et al., 2000; Speiotes et al., 2000; Morishita et al., 2001; Rajagopalan and Balasubramanian, 2002).

In budding yeast, there is a single essential Aurora protein kinase, Ipl1 (Chan and Botstein, 1993; Francisco et al., 1994). In ipl1 mutant cells, sister chromatids are pulled to the same spindle pole instead of opposite poles (Biggins et al., 1999; Kim et al., 1999). Experiments in vitro and in vivo suggest that this defect is due to an inability of ipl1 mutants to release monooriented kinetochore-microtubule attachments in order to make the correct bioriented attachments (Biggins et al., 1999; Tanaka et al., 2002). ipl1 mutants also fail to activate the spindle checkpoint when kinetochores are not under tension (Biggins and Murray, 2001). Like mammalian homologues, Ipl1 localizes to kinetochores and the mitotic spindle (Biggins et al., 1999; Biggins and Murray, 2001; He et al., 2001; Kang et al., 2001; Tanaka et al., 2002). Several Ipl1p/Aurora substrates have been identified in various organisms. CENP-A, the human histone centromeric H3 variant, is an Aurora B kinase substrate, and nonphosphorylatable CENP-A mutants have cytokinesis defects (Zeitlin et al., 2001). In budding yeast, three kinetochore proteins are substrates either in vivo and/or in vitro and are good candidates for essential Ipl1p targets: the INCENP homologue (Sli15p), the Dam1 protein, and the CBF3 component Ndc10p (Biggins et al., 1999; Kang et al., 2001; Cheeseman et al., 2002; Li et al., 2002).

Here we show that the Ipl1p kinase has a role in spindle disassembly that is independent from its previously identified functions. There is a dynamic relocation of Ipl1p in anaphase, and Ipl1p tracks the plus ends of depolymerizing microtubules. We propose that Ipl1p regulates microtubule plus ends to promote chromosome segregation and spindle disassembly.

Results

Ipl1 localization is similar to chromosomal passenger proteins

Ipl1p localizes as a distinct dot that separates into two discrete dots in medium budded metaphase cells and corresponds to precociously separated kinetochores (Goshima and Yanagida, 2000; He et al., 2000, 2001; Tanaka et al., 2000; Biggins and Murray, 2001; Pearson et al., 2001). In large budded cells, Ipl1p–GFP exhibits dynamic localization patterns. In some cells, Ipl1p–GFP is on the whole spindle but absent from the kinetochores that are clustered at the poles as reported previously (Tanaka et al., 2002). We also found two sites of Ipl1p localization that had not been seen before. Ipl1p–GFP is found at the spindle midzone and in small tufts that vary in shape near the spindle poles at telophase. This likely represents Ipl1p bound to the remnants of depolymerized microtubules (Winey et al., 1995). Since the Ipl1p–GFP fusion created a temperature-sensitive protein, we also localized endogenous Ipl1p. Immunofluorescence with anti-Ipl1p antibodies on chromosome spreads showed the same localization patterns as the Ipl1p–GFP fusion protein (Loidl et al., 1998) (Fig. S1 available at http://www.jcb.org/cgi/content/full/jcb.200209018/DC1). Therefore, Ipl1p localization is similar to Aurora B and other “chromosomal passenger” proteins.

Ipl1p localizes to kinetochores under tension

We found previously that Ipl1p localizes to kinetochores that are not under tension and that it is required for the spindle checkpoint at this time (Biggins and Murray, 2001). Once tension is established, Ipl1p likely needs to be inactivated to allow cell cycle progression. Although it was reported that Ipl1p no longer colocalizes with the kinetochore protein Ndc10p at metaphase (Tanaka et al., 2002), we always found Ipl1p localized in discrete dots. Therefore, we repeated their Ipl1p–YFP and Ndc10–CFP colocalization experiment. In asynchronously growing cells, Ipl1p–YFP and
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The dynamic localization of Ipl1p on spindles suggested that it might regulate spindle function. To test this, we performed live cell imaging of wild-type and ipl1–321 mutants containing Tub1–GFP. Cells were synchronized in G1 with α-factor and then released to 35°C to inactivate Ipl1–321p, and time-lapse images were captured every minute. The start of spindle elongation was used as a reference for anaphase initiation and examples of the time-lapse data for a wild-type (video 1 available at http://www.jcb.org/cgi/content/full/jcb.200209018/DC1) and an ipl1–321 mutant cell (video 2 available at http://www.jcb.org/cgi/content/full/jcb.200209018/DC1) are shown in Fig. 3 A. Spindle elongation was quantified by measuring the length of the spindle every minute after the initiation of anaphase B (Fig. 3 B). In wild-type cells, we observed biphasic spindle elongation (Yeh et al., 1995; Straight et al., 1997). Wild-type spindles reach ~8.4 μm in length ~14 min after the initiation of anaphase B and then disassemble. In ipl1–321 mutant cells, spindle elongation occurs with kinetics similar to wild-type cells. However, the spindle continues to grow to a length of ~10.4 μm, delaying spindle disassembly for ~6 min, a 42% increase in the duration of anaphase B. In two of the ten cells analyzed, the spindle is forced to bend when reaching the cell membrane (Fig. 3 A, A*). Since these phenotypes are not seen in wild-type cells and are similar to mutants in the motor protein Kip3 (Straight et al., 1998), we also analyzed spindle elongation and breakdown kinetics in ipl1–321, kip3Δ, and kip3Δ ipl1–321 mutants and found they exhibit similar delays in spindle breakdown (unpublished data). Therefore, Ipl1p and Kip3p may act in the same pathway to promote spindle disassembly.

The ipl1–321 mutant cells also exhibit a spindle orientation defect that we quantified by measuring the angle between the spindle axis and the mother-bud axis every minute starting at metaphase. In wild-type cells, it takes less than 6 min for the spindle to orient itself on the mother-bud axis, whereas it takes more than 11 min in ipl1–321 mutant cells (Fig. 3 A).

**The role of Ipl1p in spindle disassembly is an independent function**

Since Ipl1p is required for chromosome segregation and the spindle checkpoint, we tested whether the spindle disassembly delay was a consequence of defects in these functions. We showed previously that if bipolar spindle assembly occurs before Ipl1p inactivation, chromosome segregation is normal (Biggins and Murray, 2001). Therefore, we arrested cells containing Tub1–GFP in metaphase by depleting the Cdc20 protein by shifting the cells to the restrictive temperature to inactivate Ipl1p, and then released them into the cell cycle. Aliquots were taken every 5 min, and cells with a pole to pole distance corresponding to a late anaphase cell (equal or greater than 9 μm) were analyzed for the presence or absence of a spindle. Since tubulin is always at the SPB, the pole to pole distance can be measured regardless of whether a spindle is present. Spindle disassembly occurred in 68% of Cdc20-depleted cells compared with only 36% of the Cdc20-depleted ipl1–321 double mutant cells (Fig. 4 A). In addition, there was no defect in spindle elongation in either strain. This result suggests that the spindle breakdown defect in ipl1–321 mutants is independent from Ipl1p’s role in chromosome segregation.

The spindle disassembly defect in the ipl1–321 mutant cell population also occurred when chromosome segregation was defective. We analyzed this by synchronizing wild-type and ipl1–321 mutant cells containing Tub1–GFP in α-factor, releasing them to 37°C, and analyzing spindle disassembly as described above. Whereas 78% of the wild-type cells underwent spindle breakdown, only 40% of ipl1–321 mutant cells had disassembled their spindles (Fig. 4 B). There-
fore, the spindle disassembly defect occurs in ipl1–321 mutants when chromosome segregation is normal or defective.

Since mutants defective in the mitotic exit network exhibit a spindle breakdown delay (Stegmeier et al., 2002), we tested whether ipl1–321 mutant cells delayed mitotic exit by monitoring the destruction of Clb2p, the major mitotic B-type cyclin. Cells from the experiment described in Fig. 4A were collected every 5 min and then immunoblotted with anti-Clb2p antibodies (Fig. 4C). Clb2p degradation occurred with similar kinetics in wild-type and ipl1–321 mutant cells, indicating Ipl1p is not required for mitotic exit.

Ipl1p also has a function in the spindle checkpoint when kinetochore tension is not generated (Biggins and Murray, 2001) so we tested whether other spindle checkpoint genes are also required for spindle disassembly. We found that the dynamics of spindle elongation and breakdown in wild-type, mad1Δ, and mad2Δ strains containing Tub1–GFP were similar (Fig. S2 available at http://www.jcb.org/cgi/content/full/jcb.200209018/DC1). Therefore, a function in spindle disassembly is not a general property of all checkpoint proteins. Together, these data suggest that Ipl1p’s role in spindle disassembly is independent from its roles in chromosome segregation and the spindle checkpoint and identifies a previously unknown function for this protein kinase.

ipl1–321 mutant cells can stabilize fragile spindles

Since ipl1–321 mutants are defective in spindle breakdown, we tested whether they could stabilize fragile spindles that result from loss of sister chromatid cohesion in metaphase. When sister chromatid cohesion is released in the absence of APC function by a mutation in the Mcd1/Sccl cohesion protein, spindle elongation occurs in the presence of high levels of the Pds1 and Clb2 proteins (Michaelis et al., 1997). This leads to fragile spindles where the spindle elongates but breaks down abnormally fast, creating an “anaphase-like prometaphase” (Severin et al., 2001b). Therefore, we tested whether the addition of an ipl1–321 mutation could stabilize the fragile spindles. We used a cdc26Δ strain, which leads to temperature-sensitive inactivation of the APC, in combination with the cohesin mutation mcd1–1 to create fragile spindles. cdc26Δ mcd1–1 and cdc26Δ mcd1–1 ipl1–321 mutant cells containing Tub1–GFP were arrested in G1 using α-factor, released to the restrictive temperature, and monitored for budding, spindle formation, and spindle breakdown. As reported previously, 93% of cdc26Δ mcd1–1 mutants underwent spindle breakdown within 150 min of release from G1 (Fig. 4D) (Severin et al., 2001b). However, only 48% of cdc26Δ mcd1–1 ipl1–321 mutant cells had undergone spindle breakdown at this time, indicating that the ipl1–321 mutation stabilizes the fragile spindles.
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Ipl1p kinase activity increases when spindles disassemble

To analyze Ipl1p kinase activity when spindles disassemble, we developed a kinase assay using antibodies generated against a recombinant GST-Ipl1 fusion protein. The affinity-purified antibodies specifically recognize a single major band in yeast lysates that migrates just above 45 kD (Fig. S3 available at http://www.jcb.org/cgi/content/full/jcb.200209018/DC1). The antibodies were used to immunoprecipitate wild-type Ipl1p and the Ipl1–321 protein that has reduced kinase activity at high temperatures (Biggins et al., 1999). The majority of Ipl1p present in the yeast lysates (Fig. 5 A, pre) was depleted by the antibody (Fig. 5 A, post). The immunoprecipitates (Fig. 5 A, IP) were then incubated with the histone-fold domain of the kinetochore protein Cse4p in a kinase reaction in vitro. Cse4p was radiolabeled in the presence of wild-type Ipl1p but not the kinase inactive Ipl1–321 protein (Fig. 5 A, right), showing that the assay specifically reflects an Ipl1p-associated kinase activity.

We analyzed Ipl1p kinase activity as cells exited mitosis by releasing cells arrested in metaphase by Cdc20p depletion and taking time points every 10 min. Ipl1p was immunoprecipitated, and the kinase activity was monitored against the substrate Cse4p (Fig. 5 B). To determine the cell cycle position, we monitored the budding index and spindle disassembly (Fig. 5 C). We quantified the total kinase activity, which represents the protein and its corresponding kinase activity (Fig. 5 C). The Ipl1p kinase activity increases as cells leave metaphase, peaking just before spindle disassembly. Although we do not know whether this increase is due to regulation of the protein levels and/or specific activity, the data is consistent with Ipl1p kinase activity being required for spindle breakdown.

Ipl1p’s substrates localize to the spindle midzone, and Ipl1p follows the plus ends of the depolymerizing spindle microtubules

The novel midzone localization pattern for Ipl1p led us to test whether proteins that Ipl1p regulates also localize to the spindle midzone. First, we tested Cse4p which we have shown here is an Ipl1p substrate in vitro. Localization of a Cse4–GFP fusion showed that it does not transfer to the spindle (unpublished data). We next analyzed the localization of COOH-terminal GFP fusions to the Ndc10, Sli15, and Dam1 proteins (Fig. 6 A). Ndc10–GFP localized to the midzone in late anaphase cells in addition to the previously reported spindle and kinetochore localization (Lechner and Carbon, 1991; Goh and Kilmartin, 1993). We found that Sli15–GFP also accumulates at the spindle midzone and exhibits the same localization pattern as Ipl1p throughout the entire cell cycle (Fig. 6 A; unpublished data). Various labs have reported that Dam1p localizes to kinetochores throughout the cell cycle and to the mitotic spindle (Hofmann et al., 1998; He et al., 2001; Jones et al., 2001). Here we show that Dam1–GFP also localizes to the spindle midzone in anaphase cells. Therefore, the majority of known Ipl1p substrates localize to the spindle midzone, providing several potential candidates for Ipl1p regulation of spindle disassembly.

To learn more about how Ipl1p might regulate spindle disassembly, we analyzed the various Ipl1–GFP anaphase
localization patterns more precisely by performing time-lapse microscopy (video 3 available at http://www.jcb.org/cgi/content/full/jcb.200209018/DC1). At anaphase, Ipl1–GFP is distributed along the entire spindle length in a punctate pattern. Ipl1p then accumulates at the spindle midzone, splits into two distinct dots, and then travels back to the poles.

Since Ipl1p localizes to the spindle midzone very late in anaphase and then travels back to the poles, we tested whether it was following the plus ends of the depolymerizing microtubules. Live microscopy was performed on cells coexpressing Tub1–CFP (tubulin) and Ipl1–GFP. Although CFP and GFP have overlapping spectrums, the Ipl1–GFP and Tub1–CFP signals were easily discernible (Fig. 6 B). It was not possible to use the nonoverlapping spectrum of YFP because the Ipl1–YFP signal was not strong enough to perform time-lapse imaging of cells. We started imaging a cell when Ipl1p (Fig. 6 B, green) localized to the midzone of a long spindle (Fig. 6 B, red, 0'). After 1 min, the Ipl1p signal split and there was no longer any tubulin signal in the center of the spindle, indicating that the spindle is starting to break down. As the spindle depolymerized toward the poles, the Ipl1p signal always localized near the plus end of the microtubules (Fig. 6 B, 2'). At the end of spindle disassembly, the remaining tubulin at the pole colocalized with Ipl1p (Fig. 6 B, 3.5'). Therefore, Ipl1p accumulates at the spindle midzone in late anaphase and then follows the plus ends of the depolymerizing microtubules back to the poles, suggesting it may specifically regulate the microtubule plus ends.

Discussion

We found that the Ipl1/Aurora protein kinase has a role in spindle microtubule disassembly that is not a consequence of a delay in mitotic exit or a prior defect in chromosome segregation. In addition, it is unlikely to be due to a defect in the spindle checkpoint, since the mad1 and mad2 checkpoint mutants exhibited normal spindle disassembly. Ipl1p localizes to the spindle midzone in anaphase and tracks the plus ends of the depolymerizing microtubules, suggesting it may directly regulate the microtubule plus ends.
suggested that the plus ends of the interpolar microtubules may be regulated in a manner similar to kinetochores. In addition, we found that *ipl1* mutants have a spindle orientation defect that may also reflect a role in cytoplasmic microtubule plus-end function. Therefore, we propose that Ipl1p is a general microtubule plus-end regulator and that its function in spindle disassembly in anaphase is similar to its function in promoting biorientation in prometaphase.

**Ipl1p leaves the kinetochores after tension is established**

We show here that Ipl1p exhibits a localization pattern similar to chromosomal passenger proteins, making it likely that Ipl1p is an Aurora B homologue. The dynamic localization of Ipl1p reflects its various mitotic functions. During prometaphase, Ipl1p monitors kinetochore tension and promotes microtubule release of monooriented kinetochore attachments, thus ensuring that sister kinetochores establish biorientation before chromosome segregation (Biggins and Murray, 2001; Tanaka et al., 2002). The proposal that Ipl1p might delocalize from kinetochores at metaphase provided an attractive mechanism for inactivating the kinase when tension and biorientation are established (Tanaka et al., 2002). However, we found that both Ipl1–GFP and endogenous Ipl1p localize to kinetochores in metaphase. Our data agrees with the observation that a mammalian Aurora B–GFP fusion protein left kinetochores 0.5 min after the initiation of anaphase, well after tension was established (Murata-Hori et al., 2002). K. Tanaka and T.U. Tanaka recently found that Ipl1p is on metaphase kinetochores by chromatin immunoprecipitation, thus reconciling our data (personal communication). To understand how tension regulates Ipl1p, we are actively trying to elucidate the mechanisms that control Ipl1 protein stability and kinase activity, since these may be alternative modes of regulation at metaphase.

**Functions of the Ipl1/Aurora B protein kinase family**

We show that the localization of Ipl1p to the mitotic spindle is correlated with spindle disassembly in budding yeast. Since Aurora B localizes to the spindle midzone in all organisms, this may be another conserved function of the Ipl1/Aurora B protein kinase family. Alternatively, this may reflect similarity to the role of Aurora A, since it regulates spindle function and phosphorylates the Eg5 motor protein in frog egg extracts (Giet et al., 1999; Giet and Prigent, 2000). In other organisms, Aurora B is required for cytokinesis, and this may be coupled to defects in spindle microtubule depolymerization that have not been noticed previously. In human cells, cytokinesis is regulated by the phosphorylation of CENP-A by Aurora B (Zeitlin et al., 2001). We report here that the budding yeast histone variant Cse4p is also a good substrate for Ipl1p in vitro. Further analysis of the effects of Cse4p phosphorylation by Ipl1p may reveal more details about spindle disassembly and/or cytokinesis in budding yeast. We show that the dynamics of spindle elongation are not altered in *ipl1* mutant cells, unlike mutants in the Aurora–INCENP–Survivin complex in *Schizosaccharomyces pombe* (Morishita et al., 2001; Rajagopal and Balasubramanian, 2002). This difference may be due to the number of kinetochore microtubule-binding sites in each organism. In *S. pombe*, there are multiple binding sites, which results in lagging chromosomes if biorientation is not achieved. In budding yeast where there is a single microtubule binding site, defects in biorientation cannot generate lagging chromosomes. However, when a conditional dicentric chromosome is activated in budding yeast, thus creating a lagging chromosome, spindle elongation is delayed (Yang et al., 1997). Our study in budding yeast had the advantage that defects in biorientation do not interfere with spindle dynamics. To determine whether the spindle disassembly function of Ipl1p is conserved, spindle dynamics will need to be analyzed in situations where chromosome segregation is normal.

**How does Ipl1p regulate spindle disassembly?**

In support of a role for Ipl1p in direct regulation of microtubules, we found that *ipl1* mutants are able to alleviate the spindle fragility of *apc mcd1* mutant cells. However, since the mechanism that leads to fragile spindles is not known, it is not clear how Ipl1p stabilizes spindles. Although mutations in Ipl1p affect spindle breakdown, they do not do this by grossly altering the structure of the spindle midzone, since in the midzone all the microtubules we tested still localized in an *ipl1* mutant.

Our studies using live microscopy revealed a previously unidentified localization pattern for Ipl1p. At anaphase, Ipl1p is transported along the spindle to the midzone and then tracks the plus ends of the depolymerizing spindle microtubules back to the poles. To our knowledge, the only other protein in budding yeast that exhibits this localization pattern is the Ipl1p substrate Ndc10p and suggests regulated transport of these proteins on microtubules in anaphase, possibly by motor proteins (D. Bouck and K. Bloom, personal communication). The localization to the plus ends may indicate that Ipl1p directly destabilizes microtubules like catastrophe factors, such as the KINI family of motor proteins (Desai et al., 1999). However, although Ipl1p binds microtubules in vitro (Kang et al., 2001), we have not been able to induce microtubule depolymerization with bacterial Ipl1p in vitro (unpublished data). Therefore, Ipl1p may directly promote microtubule depolymerization in a manner that we have not yet detected, or it may instead control a microtubule-binding protein.
There are two nonessential proteins known to be involved in spindle microtubule disassembly in budding yeast: the motor protein Kip3 and the microtubule-associated protein Asc1 (Juang et al., 1997; Straight et al., 1998). Ipl1p and Kip3p may act in the same spindle disassembly pathway because the double mutant exhibits the same spindle breakdown defect as each single mutant. However, we have yet to obtain evidence that Ipl1p regulates Kip3p (unpublished data). There are other potential candidates for Ipl1p regulation that will need to be investigated, such as the midzone protein Stu2 that opposes the Kip3 protein and the Esp1p/Pds1p cell cycle regulation complex that is also found at the midzone and has a function stabilizing spindles during anaphase (Uhlmann et al., 2000; Jensen et al., 2001; Severin et al., 2001a). We also found that three known Ipl1p substrates are at the spindle midzone: Ndc10p, Sli15p, and Dam1p. It is interesting to note that Dam1p was originally identified for its role in regulating spindle dynamics (Hofmann et al., 1998; Jones et al., 1999). Therefore, several potential Ipl1p substrates localize to the spindle midzone, and it will need to be determined whether any of these candidates also promote spindle disassembly.

The spindle midzone: a kinetochore-like structure?
Several kinetochore proteins are now known to localize to the spindle midzone in anaphase, including Stu2p, Slk19p, and the motor protein Cin8 (Hoyt et al., 1992; Zeng et al., 1999; Kosco et al., 2001). Here we show four additional kinetochore proteins localizing to the midzone: the Ipl1/Aurora protein kinase, the INCENP homologue Sli15p, Dam1p, and Ndc10p. Since midzone staining is difficult to detect, it may have been overlooked in several other localization studies and many more kinetochore proteins may be present at the midzone. Most of the spindle midzone proteins have been implicated in the regulation of spindle dynamics in anaphase by either promoting spindle elongation or spindle disassembly. An intriguing possibility is that the microtubule plus ends at the spindle midzone are regulated in anaphase similarly to the kinetochore-microtubule attachments in prometaphase. Future work will determine whether the Ipl1/Aurora protein kinase and other spindle midzone proteins are global regulators of microtubule plus ends.

Materials and methods
Microbial techniques
Media and genetic and microbial techniques were essentially as described (Sherman et al., 1974; Rose et al., 1990). All experiments where cells were released from G1 arrest were performed by adding 1 μg/ml α-factor (stock: 10 mg/ml in DMSO; United Biochemical Research Inc.) at the permissive temperature for 3 h, washing the cells twice in α-factor-free medium, and resuspending them in fresh medium. α-Factor was added back to 1 μg/ml after cells had budded to prevent cells from entering the next cell cycle. Galactose induction was performed by growing cells in 2% raffinose and subsequently sporulated at 23°C. All strains containing the pGal-CDC20 constitutive expression plasmid were isogenic with the W303 strain background. Plasmids are indicated in parentheses.

Yeast strain construction
Yeast strains are listed in Table I and were constructed by standard genetic techniques. Diploids were isolated on selective medium and subsequently sporulated at 23°C. All strains containing the pGal-CDC20 construct were isogenic with the W303 strain background. Plasmids are indicated in parentheses.

| Strain | Genotype |
|--------|----------|
| SBY3   | MATα ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ |
| SBY97  | MATα ura3-1 TUB1-GFP:URA3 leu2-3,112 his3-11 pCUP1-GFP2-lacI12:HI53 trp1-1 lacO:TRP1 lys2Δ ade2-1 bar1Δ can1-100 ip1-321 |
| SBY130 | MATα ura3-1 TUB1-GFP:URA3 leu2-3,112 his3-11 pCUP1-GFP2-lacI12:HI53 trp1-1 lacO:TRP1 lys2Δ ade2-1 bar1Δ can1-100 |
| SBY322 | MATα ura3-1 leu2-3,112 his3-11 pCUP1-GFP2-lacI12:HI53 trp1-1 lacO:TRP1 lys2A ade2-1 bar1Δ can1-100 ip1-321 |
| SBY539 | MATα ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ NDC10-GFP:KAN |
| SBY554 | MATα ura3-1 pGAL-IPL1:URA3 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ |
| SBY556 | MATα ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 bar1Δ can1-100 IPI1-GFP:KAN |
| SBY617 | MATα ura3-1 leu2-3,112 his3-11 trp1-1 ade2-1 bar1Δ can1-100 CSE4-myc12:URA3 |
| SBY736 | MATα ura3-1 pGAL-myc13-IPL1:URA3 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ |
| SBY875 | MATα ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ Sli15p-GFP:HI53 |
| SBY943 | MATα ura3-1 TUB1-GFP:URA3 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 cdc20-Δ:LEU2 ipl1-321 (pGAL-low-CDC20-HIS3[CENI]) |
| SBY952 | MATα ura3-1 TUB1-GFP:URA3 leu2-3,112 his3-11 trp1-1 ade2-1 can1-100 cdc20-Δ:LEU2 (pGAL-low-CDC20-HIS3[CENI]) |
| SBY965 | MATα ura3-1 leu2-3,112 TUB1-GFP:LEU2 his3-11 pCUP1-GFP2-lacI12:HI53 trp1-1 lacO:URA3:TRP1 lys2Δ ade2-1 mdc1-1 cdc26-Δ:KAN |
| SBY1036 | MATα ura3-1 TUB1-CFP:URA3 leu2-3,112 his3-11 trp1-1 ade2-1 bar1Δ can1-100 IPL1-GFP:KAN |
| SBY1115 | MATα ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1Δ DAM1-GFP:TRPL |
| SBY1246 | MATα ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100 cdc20-Δ:LEU2 IPL1-YFP:HIS3 NDC10-CFP:KAN (pGAL-low-CDC20-HIS3[CENI]) |
| SBY1422 | MATα ura3-1 TUB1-GFP:URA3 leu2-3,112 his3-11 pCUP1-GFP2-lacI12:HI53 trp1-1 lacO:TRP1 lys2Δ ade2-1 bar1Δ can1-100 mad2-Δ:KAN |
| SBY1423 | MATα ura3-1 TUB1-GFP:URA3 leu2-3,112 his3-11 pCUP1-GFP2-lacI12:HI53 trp1-1 lacO:TRP1 lys2Δ ade2-1 bar1Δ can1-100 mad2-Δ:KAN |
| SBY2066 | MATα ura3-1 leu2-3,112 his3-11 LacI12:HI53 trp1-1 LacO:TRP1 lys2Δ bar1Δ ip1-321 mdc1-1 cdc26-Δ:KAN |

All strains are isogenic with the W303 strain background. Plasmids are indicated in parentheses.
Plasmid constructions
A clone that encoded Cse4p internally tagged with 12 copies of the myc epitope (pSB162) was used to PCR amplification of the myc tag from pSB162 (Biggins et al., 1999) using primers SB84 and SB85. The PCR product was digested with Spel and ligated into the XbaI site of pSB241 (CSE4, URA3 integrating vector). pSB241 was created by digesting a genomic clone containing CSE4 with EcoRI and PstI. To create the CSE4 fragment, pSB340 cut with AgeI, and the CSE4 fragment was ligated into pMAS27 (a gift from M. Shonn, TUTS University, Boston MA) cut with StuI at the URA3 locus. pSB554 was obtained by integrating pSB164 (pGAL-IPL1: URA3 [Biggins et al., 1999]) cut with StuI at the URA3 locus. pSB736 was obtained by integrating pSB257 (pGAL-myc12-IPL1:URA3) cut with StuI at the URA3 locus. Deletions in yeast genes and GFP, CFP, and myc epitope tags were made using the PCR-based integration system (Longtine et al., 1998). YFP epitope tags were made using pDH5, a gift from T. Davis (University of Washington, Seattle, WA). Specific primer sequences are available upon request. All deletions and epitope tags were confirmed by PCR.

Protein and immunological techniques
Protein extracts were made and immunoblotted as described (Minshull et al., 1996). Anti-Tub1p antibodies were obtained from Accurate Chemical and Scientific and used at a 1:1,000 dilution, anti-Cbf2p antibodies (a gift from A. Rudner, Harvard Medical School, Boston, MA) were used as described (Rudner et al., 2000), and anti-Ipl1p antibodies were used at 1:1,000. To generate anti-Ipl1p antibodies, GST-Ipl1p was injected into mice. GST purified from bacterial supernatant was added to 5 µg Ipl1p antibodies, GST-Ipl1p was injected into rabbits at Cocalico Biological Inc. For the fourth and fifth boosts, boiled protein was injected. The antibodies were affinity purified by coupling GST-Ipl1p to SulfoLink Coupling gel (Pierce Chemical Co.).

Microscopy
For live microscopy to analyze GFP fusion proteins, cells were grown in yeast media, pH 7.0, washed, and resuspended in 1/10 volume minimal medium with casamino acids. For live microscopy at RT, 1.5 µl of cell preparation was put on a 2% agarose pad containing minimal medium with casamino acids. The slide was then sealed with VALAP (1:1:1, vaseline: lanolin: paraffin) and imaged. For live microscopy at 35°C, prepared cells were mounted directly onto a heated stage (Bioptechs). Images were collected through an Olympus 1X17 60x objective with a CH350 CCD camera (Rooper Scientific) using the Softwox 2.5 (Applied Precision) software. The same software was used for deconvolution. At least 10 cells were analyzed for all reported experiments.

Chromosome spreads were performed as described (Loidl et al., 1991; Michaels et al., 1997). Lipid was obtained from Lipid. 9010 antibodies that recognize myc tag were used at a 1:500 dilution and obtained from Covance. Anti-Tub1p antibodies (Accurate Chemical and Scientific) were used at a 1:500 dilution. Anti-Ipl1p antibodies were used at 1:250 dilution. Alexafluor-594 and Alexafluor-488 secondary antibodies were obtained from Molecular Probes and used at 1:250 dilution.

Cse4 histone fold domain purification
The histone fold domain of CSE4 (encoding aa 121-229) was PCR amplified from Saccharomyces cerevisiae genomic DNA cloned and cloned into a T7 expression vector of the pCR7/CT/TOPO TA cloning kit (Invitrogen). The resulting expression plasmid (pT7Cse4) was transformed into BL21-CodonPlus (DE3)-RII cells (Stratagene), and 2 liters were induced with IPTG to 0.2 mM for 2.5 h at 37°C, and Cse4 was purified under denaturing conditions as described (Gerhardt et al., 2001). The Cse4 histone fold domain purity was verified by SDS-PAGE and dialyzed against water. A substantial portion of the protein was not soluble in water, but solubility was adequate for kinase assays in vitro.

Ipl1p kinase assays
40 ml cultures of mid-log cells were collected and resuspended in 500 µl lysis buffer (100 mM NaCl, 50 mM Tris, pH 7.5, 50 mM NaF, 50 mM β-glycerophosphate, pH 7.4, 2 mM EDTA, 2 mM EGTA, 0.1% Triton X-100), 2 mM NaVO4, 2 mM PMSF, 10 µg/ml LPC (leupeptin, pepstatin, and chymostatin; Chemicon), 1 mM DTT, 0.1 µg/ml microcystin (Calbiochem) were added fresh. All subsequent steps were performed at 4°C. Cells were lysed with glass beads in a beater (Biospec Products Inc.) for 30 s and then centrifuged for 10 min. 400 µl supernatant was added to 5 µl magnetic protein G beads (Dynal Biotech Inc.) and 4 µl anti-Ipl1p antibodies for 2 h. The beads were washed three times with 400 µl lysis buffer and once with 100 µl kinase buffer without ATP (50 mM Tris, pH 7.4, 1 mM DTT, 25 mM β-glycerophosphate, 5 mM MgCl2) and then resuspended in buffer with 10 µl ATP, 5 µg 1/P3 ATP and 5 µg Cse4p for 30 min at 30°C. 2× sample buffer was added, and reactions were separated on SDS-PAGE and subjected to autoradiography using a Phosphorimager Screen (Molecular Dynamics). Kinase assays were quantified using ImageQuant (Molecular Dynamics) software.

Online supplemental material
Videos 1–3 and Figs. S1–S3 are available at http://www.jcb.org/cgi/content/full/jcb.200209018/DC1. Video 1 displays wild-type cells expressing Tub1-GFP from metaphase to anaphase. Video 2 shows ipl1-321 cells expressing Tub1–GFP that are delayed in spindle disassembly. Video 3 shows Ipl1–GFP localization during late anaphase. Fig. S1 shows endogenous Ipl1 localization on chromosome spreads. Fig. S2 demonstrates that the spindle checkpoint mutants mad2Δ and cdc24Δ do not have spindle disassembly defects. Fig. S3 shows the Ipl1p antibody specificity.

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