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Evidence that the Ipl1-Sli15 (Aurora Kinase-INCENP) Complex Promotes Chromosome Bi-orientation by Altering Kinetochore-Spindle Pole Connections

Tomoyuki U. Tanaka,[1,2,4] Najma Rachidi,[1] Carsten Janke,[3] Gislene Pereira,[3] Marta Galova,[2] Elmar Schiebel,[3] Michael J.R. Stark,[1] and Kim Nasmyth[2]
[1]School of Life Sciences
University of Dundee
MSI/WTB complex
Dundee DD1 5EH
United Kingdom
[2]Research Institute of Molecular Pathology
Dr Bohr-Gasse 7, A-1030
Vienna
Austria
[3]The Beatson Institute for Cancer Research
CRC Beatson Laboratories
Glasgow G61 1BD
United Kingdom

Summary

How sister kinetochores attach to microtubules from opposite spindle poles during mitosis (bi-orientation) remains poorly understood. In yeast, the ortholog of the Aurora B-INCENP protein kinase complex (Ipl1-Sli15) may have a role in this crucial process, because it is necessary to prevent attachment of sister kinetochores to microtubules from the same spindle pole. We investigated *IPL1* function in cells that cannot replicate their chromosomes but nevertheless duplicate their spindle pole bodies (SPBs). Kinetochores detach from old SPBs and reattach to old and new SPBs with equal frequency in *IPL1*+ cells, but remain attached to old SPBs in *ipl1* mutants. This raises the possibility that Ipl1-Sli15 facilitates bi-orientation by promoting turnover of kinetochore-SPB connections until traction of sister kinetochores toward opposite spindle poles creates tension in the surrounding chromatin.

Introduction

The partitioning of complete copies of genomes during cell division is a crucial aspect of cellular reproduction. DNA replication produces two sister chromatids that are segregated to opposite poles of the cell prior to cytokinesis. In eukaryotic cells, sister chromatids are pulled poleward by microtubule-dependent forces. Each sister chromatid is attached, via kinetochores, to the plus ends of microtubules whose minus ends extend to opposite poles (Wittmann et al., 2001). The mechanism by which sister kinetochores attach to microtubules from opposite poles, known as bi-orientation or bipolar attachment, lies at the heart of the mitotic process, but is nevertheless poorly understood.

Sister chromatids are attached to each other by a multiprotein complex called cohesin from their production during DNA replication until their segregation to opposite poles at the onset of anaphase (Nasmyth et al., 2000). Sister chromatid cohesion resists the tendency of microtubules to pull chromatids apart during their bi-orientation on mitotic spindles, whereas its final destruction by a cysteine protease called separase triggers the segregation of sisters to opposite poles at the metaphase to anaphase transition (Uhlmann et al., 2000; Tomonaga et al., 2000; Hauf et al., 2001). A surveillance mechanism, called the spindle checkpoint, delays activation of separase until all sister chromatid pairs have bi-oriented on the spindle (Amon, 1999; Rudner and Murray, 1996). This checkpoint is not, however, an integral part of the mechanism that promotes bi-orientation, because mutations of checkpoint proteins such as Mad2 have little effect on the fidelity of bi-orientiation during mitosis in yeast (Li and Murray, 1991).

An important variation of the bi-orientation process occurs during the first meiotic division, when sister kinetochores are somehow fused together and thereby induced to attach to spindles from the same pole (Rieder and Cole, 1999; Toth et al., 2000). Here the goal is not the bi-orientation of sister chromatids, but rather the attachment of homologous sister kinetochore pairs to spindles from opposite poles. This process, called co-orientiation (Oestergren, 1951), has been studied in grasshopper spermatocytes, where chromosomes can be manipulated with a microneedle (Nicklas, 1997; Nicklas and Ward, 1994). The kinetochores of bivalent chromosomes repeatedly capture and release microtubules during meiosis I. Kinetochore-spindle pole connections are more stable when kinetochores attach to microtubules from different spindle poles than to those from the same pole. This is at least partly due to the tension produced when bivalents attach in the correct manner. Bi-orientation during mitosis might utilize a similar mechanism.

The attachment of kinetochores to only a single microtubule in the yeast *Saccharomyces cerevisiae* (Winey and O'Toole, 2001) may make its bi-orientation mechanism particularly easy to study. By marking individual centromeres with arrays of bacterial operators, which are bound by repressor proteins fused to green fluorescent protein (GFP), it has recently been possible to visualize this process directly (Goshima and Yanagida, 2000; He et al., 2000; Pearson et al., 2001; Tanaka et al., 2000). Bi-orientation causes operators situated 9 kb (or less), but not 13 kb, from centromeres to split during metaphase (He et al., 2000). Chromatin close to centromeres comes under sufficient tension during this process that it unravels down to a 10 nm fiber (or even less).

A cohesin component Scc1 (and therefore presumably sister chromatid cohesion), also known as Mcd1 or Rad21, is necessary to ensure bi-orientation (Tanaka et al., 2000; Sonoda et al., 2001). Cohesin could facilitate bi-orientation by two different mechanisms, which are not mutually exclusive. By holding sister centromeric chromatin together, it could ensure that kinetochores face in opposite directions and are therefore more likely to be captured by microtubules from opposite poles. Alternatively, by holding sister chromatids together,

[4]Correspondence: t.tanaka@dundee.ac.uk

cohesin could ensure that bi-orientation creates tension within centromeric chromatin, whose detection signals stabilization of the microtubular kinetochore-spindle pole connections.

The frequent monopolar attachment in Scc1-depleted yeast cells causes unequal segregation of the genome during mitosis, with the result that mother cells and buds inherit different amounts of DNA. Unequal chromosome segregation is also striking in mutants with defective *IPL1* or *SLI15* proteins (Biggins et al., 1999; Chan and Botstein, 1993; Kim et al., 1999). Unlike mutants with completely defective kinetochores, *ipl1* and *sli15* mutant cells clearly segregate chromosomes but do so in an asymmetric manner. In contrast, DNA replication, SPB duplication, spindle formation, and cytokinesis all appear unaffected in the mutants.

IPL1 is the only Aurora kinase in *S. cerevisiae*, whereas *SLI15* encodes an yeast ortholog of INCENP protein of animal cells. Ipl1 and Sli15 form a complex in yeast (Kim et al., 1999), as do Aurora B and INCENP in animal cells (Adams et al., 2000; Kaitna et al., 2000). The Aurora B-INCENP complex associates with chromosome arms during prophase, with the interface between sister centromeres during prometaphase and metaphase, with the midzone of the mitotic spindle during anaphase, and with the midbody during telophase (Cooke et al., 1987; Adams et al., 2001a). The complex is required for correct chromosome segregation but not for mitotic spindle formation (Mackay et al., 1998; Adams et al., 2000, 2001b; Giet and Glover, 2001; Kaitna et al., 2000; Oegema et al., 2001), suggesting that Ipl1-Sli15 is the yeast counterpart of Aurora B-INCENP.

In this paper, we investigate the role of Ipl1-Sli15 in bi-orientating chromosomes in yeast. Our findings suggest that sister kinetochores are frequently connected to the same pole in *ipl1* and *sli15* mutants. Whereas univalent kinetochores (produced by preventing centromere duplication) are connected to old and new spindle pole bodies (SPBs) by microtubules at random in wild-type cells, they are connected predominantly to old SPBs in *ipl1* and *sli15* mutants. Our findings are consistent with the notion that the activity of the Ipl1 protein kinase facilitates reorientation of kinetochore-SPB connections as long as tension is not generated within centromeric chromatin.

Results

Ipl1 and Sli15 Are Required for Bi-orientation

We used time lapse microscopy to monitor the motion of *CEN5* and SPBs, both marked by GFP (Tanaka et al., 2000), in wild-type and *ipl1-321* mutant cells (Biggins et al., 1999) after shifting them from 25°C (permissive temperature) to 35°C (restrictive temperature). In 40% of *ipl1-321* cells, sister *CEN5*s behave as they do in wild-type cells; that is, they split soon after separation of SPBs and formation of bipolar spindles (Figure 1A). However, in 60% of the mutant cells, sister *CEN5*s never separated and remained in the vicinity of one of the two SPBs (Figure 1B). Though they made short movements in its vicinity, these mono-oriented sister centromere pairs never changed their SPB partner and eventually moved poleward with it upon the initiation of anaphase (Figure 1C). In *ipl1* mutant cells arrested in metaphase by depletion of the APC activator Cdc20p (Nasmyth et al., 2000) at 35°C, neither mono-oriented nor bi-oriented chromosomes switched their mode of attachment during a 20 min window of observation (n = 30; data not shown). Mitotic chromosomes do not, therefore, readily switch between these two states in *ipl1* mutants. The motion of *CEN5* sequences in *ipl1-2* and *sli15-3* cells (Chan and Botstein, 1993; Kim et al., 1999) was similar to that in *ipl1-321* cells (data not shown and Figure 1D).

To confirm our assignment of *CEN5* and SPB GFP signals, we labeled *CEN5* and SPBs using yellow- (YFP) and cyan- (CFP) GFP variants, respectively, whose emissions can be distinguished using appropriate filters. Unseparated *CEN5*s located at one pole occurred much more frequently in *ipl1-321* cells than in *IPL1*+ cells (Figure 1E). Our data suggest that the Ipl1 kinase is necessary for ensuring that sister kinetochores always connect to opposite poles.

Both SPBs Are Equally Functional and Sister Chromatid Separation Is Normal in *ipl1* Mutants

To test whether the mono-orientation of chromosomes in *ipl1* mutant cells might be due to defective sister chromatid disjunction, we measured the separation during mitosis of sister DNA sequences situated near telomeres (395 kb from *CEN5*). Sister DNA sequences seldom if ever segregated to the same daughter cell in wild-type, but did so in 66% of *ipl1* mutant cells (Figure 2A). Despite this massive missegregation, the kinetics of sister chromatid separation (that is, the appearance of two GFP dots) were almost identical in mutant and wild-type cells (Figure 2B). These data are consistent with the prior observation that sister centromeres separate with similar kinetics in *mad2*Δ and *mad2*Δ *ipl1-321* double mutant cells when incubated in the presence of nocodazole (Biggins et al., 1999). They also agree with the finding that Scc1p disappears from chromosomes on schedule in *ipl1* mutants (Biggins et al., 1999).

In *ipl1* mutant cells that had just completed anaphase, mono-orientation was found more frequently in buds than in mother cells (Figure 2C). This raised the possibility that the mono-orientation of chromosomes in *ipl1* mutants could be caused by a failure of microtubules emanating from the SPB destined for mother cells to connect to kinetochores. To address this, we analyzed the segregation of two different chromosomes in the same cell. Chromosomes V and XV were marked by GFP dots with different intensities (Figures 2D–2F). In 99% or more of binuclear *IPL1*+ cells, both mothers and buds contained one strong and one weak GFP dot (Figure 2F, right); that is, they both inherited a single copy of chromosome V and XV. In 25% of binuclear *ipl1-321* cells, both copies of both chromosomes had segregated to the bud (Figure 2D). However, in a substantial fraction of cells (16%), both copies of chromosome V had segregated to one pole and both copies of chromosome XV had segregated to the opposite one (Figures 2D and 2F, left). This demonstrates that chromosomes can mono-orient at both mother and bud spindle poles in the same *ipl1* mutant cell. Chromosome missegregation in *ipl1* mutants cannot, therefore, be caused merely by a defect associated with a single SPB.

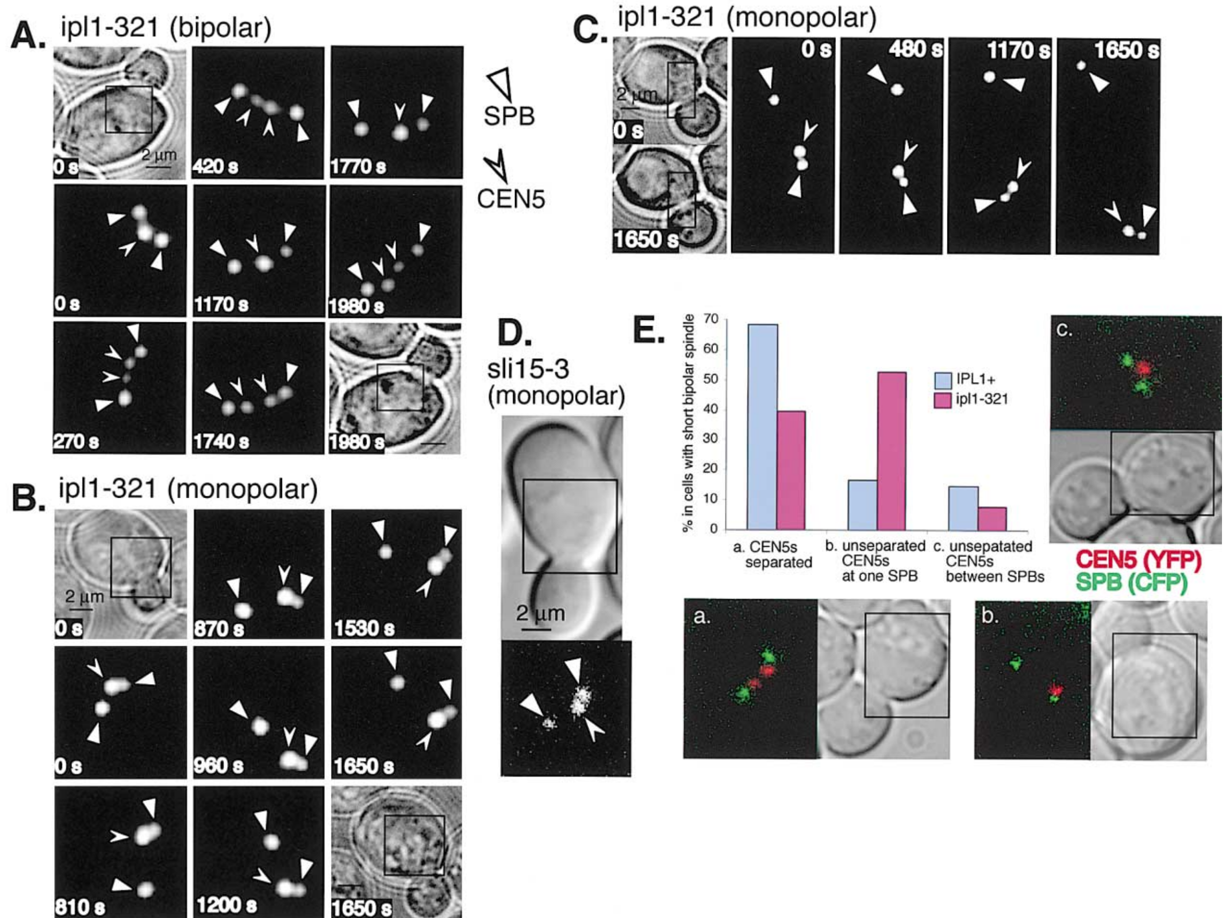

Figure 1. *ipl1* and *sli15* Mutants Frequently Show Mono-oriented Attachment of Sister Centromeres to Microtubules During Metaphase and Anaphase

(A–C) Bi-oriented attachment of *CEN5*s in metaphase (A) and mono-oriented attachment during metaphase (B) and anaphase (C) in *ipl1-321* cells. T1658 cells (*ipl1-321, tetR-GFP, tetOs* at 1.4 kb from *CEN5, SPC42-GFP*) incubated at 35°C. Timelapse images of bright field and GFP in representative cells. SPBs and *CEN5*s are indicated by arrowheads and barbed arrowheads, respectively. Rectangles in bright field images show corresponding frames of GFP images. The GFP signals from *CEN5* and SPBs can be distinguished by their different fluorescence intensities (a single SPB is brighter than a single *CEN5* but dimmer than two attached *CEN5*s) and by their different positions within cells (SPBs are usually closer to the cell periphery).

(D) Mono-oriented attachment during metaphase in a *sli15-3* cell. T1831 cell (*sli15-3, tetR-GFP, tetOs* at 1.4 kb from *CEN5, SPC42-GFP*) incubated at 35°C.

(E) Unseparated sister centromeres frequently found in the vicinity of one SPB during metaphase in *ipl1-321* cells. *IPL1+* (T2320) and *ipl1-321* (T2318) cells (*tetOs* at 1.4 kb from *CEN5, tetR-YFP, SPC42-CFP*) with short SPB distance were classified into three categories, based on the relative position of *CEN5*s to SPBs.

We repeated the above experiment with cells treated with nocodazole from the time of their release from G1 arrest until 90% of the cells had produced small to medium sized buds (by which time most cells should have completed SPB duplication). We then permitted cells to undergo anaphase in nocodazole-free medium. In most wild-type binuclear cells produced in this manner, both mothers and their buds inherited a single copy of chromosome V and XV (data not shown). Remarkably, transient incubation in nocodazole eliminated the preference of chromosomes to mono-orient to the bud SPB in *ipl1-321* cells; that is, both chromosomes missegregated to mothers as frequently as they did to their buds (Figure 2E). Moreover, in those cells in which both chromosomes had missegregated, they were as likely to have missegregated to different poles as to the same one. Thus, under these circumstances, chromatids are as likely to be captured by new SPBs as they are by old ones (note that this conclusion is valid even though nocodazole eliminates the asymmetric segregation of old SPBs; see below and Pereira et al., 2001). This experiment suggests that Ipl1 is required for bi-orientation even when a preference for a particular SPB has been eliminated. We also failed to detect defects in SPB morphology by electron microscopy in either mothers or buds in *ipl1-321* cells (C.J. and E.S., unpublished data).

Both Sister Kinetochores Are Able to Attach to Microtubules in *ipl1* Mutant Cells

Another explanation for mono-orientation of sister chromatids in *ipl1* mutants is that only a single kinetochore from each pair of sister chromatids is functional. If so,

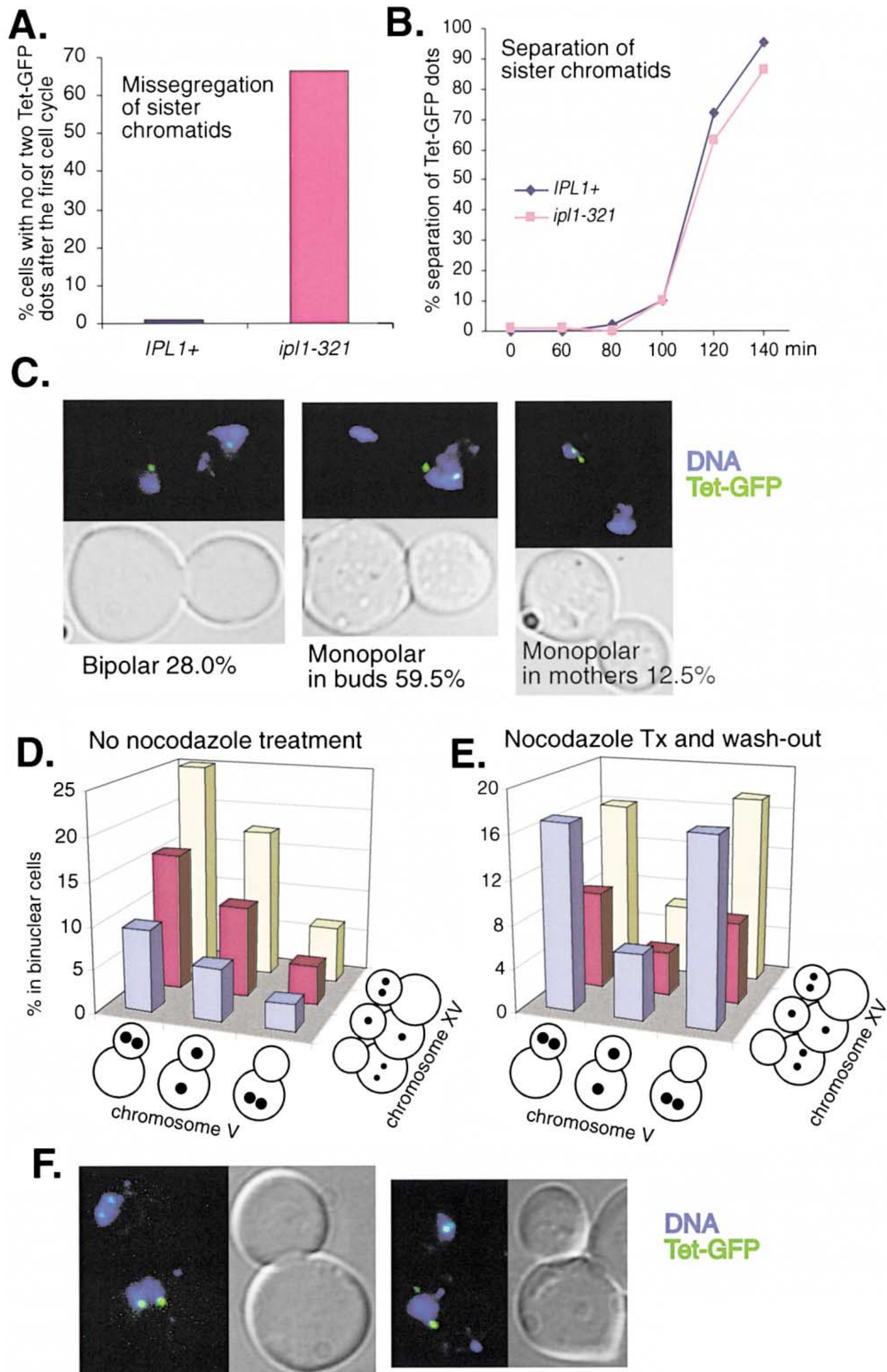

Figure 2. Sister Chromatids Separate Normally and Two SPBs Are Equally Functional in an *ipl1* Mutant

(A–C) *IPL1*+ (K7022) and *ipl1-321* (T1700) cells (*tetR-GFP, tetOs* at 395 kb from *CEN5*) were arrested by α factor for 2.5 hr at 25°C, washed out of α factor, and cultured at 37°C. Separation of GFP dots in the first cell cycle (B). Pattern of segregation of GFP dots in binuclear cells (i.e., ones that had undergone anaphase but not yet completed cytokinesis) of *ipl1-321* (C). Rate of missegregation of GFP dots (rate of cells with no dots or two dots) in cells which finished the first cytokinesis (A).

(D) *IPL1*+ (T2184) and *ipl1-321* (T2182) cells (*tetR-GFP, tetO×448* at 395 kb from *CEN5, tetO×112* at 395 kb from *CEN15*) were treated as in (A)–(C). The graph shows the pattern of segregation of GFP dots in binuclear *ipl1-321* cells in which four dots were found.

(E) *ipl1-321* (T2182) cells treated as in (A)–(C) except that nocodazole (15 μg/ml) was added after α factor was washed out. Nocodazole was then washed out by filtration when 90% cells showed bud emergence. The cells were cultured further at 37°C. Segregation of GFP dots were scored in binuclear cells as in (D).

(F) Examples of the segregation of GFP dots from (D).

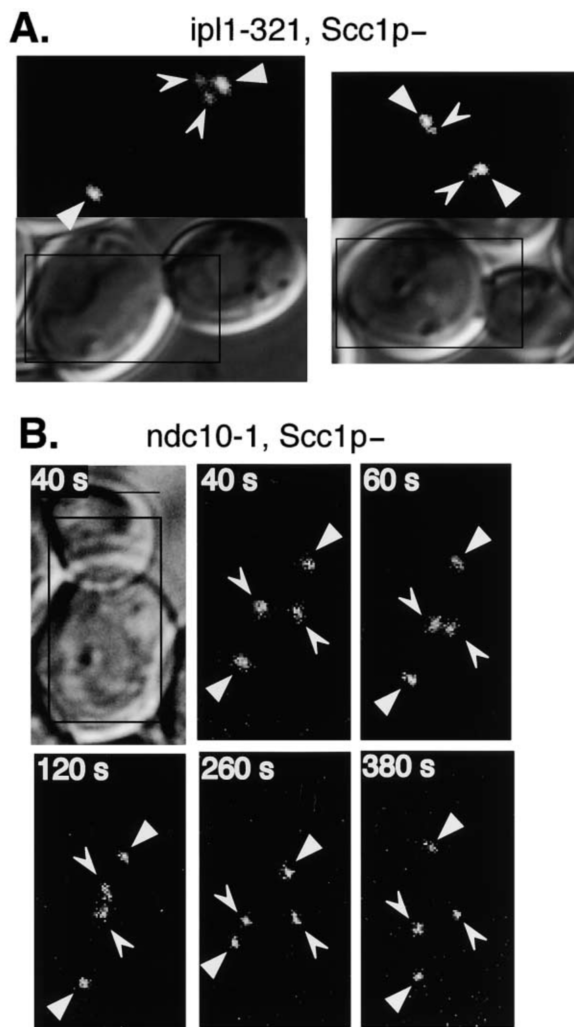

Figure 3. Both Sister Centromeres Are Able to Attach to Microtubules in an *ipl1* Mutant

(A) Both *CEN5*s stay in the vicinity of SPBs in either mono-oriented (left) or bi-oriented (right) manner in Scc1p-depleted *ipl1-321* cells. T1779 cells (*ipl1-321,GAL-SCC1, scc1Δ, tetR-GFP, tetOs* at 1.4 kb from *CEN5, SPC42-GFP*) incubated in YEP-Glc at 35°C.

(B) Both *CEN5*s separately move far away from SPBs in Scc1p-depleted *ndc10-1* cells. T1643 cells (*ndc10-1,GAL-SCC1, scc1Δ, tetR-GFP, tetOs* at 1.4 kb from *CEN5, SPC42-GFP*) incubated in YEP-Glc at 35°C.

abolition of sister chromatid cohesion should permit the chromatid with an active kinetochore to be drawn to a spindle pole, but should leave the chromatid lacking an active kinetochore within the middle of the nucleus. When we removed Scc1 from *ipl1-321* cells (using a strain whose sole *SCC1* gene is under control of the galactose-inducible *GAL1-10* promoter), both sister centromeres moved to the vicinity of SPBs after shift to the restrictive temperature, sometimes to the same pole but often to the opposite poles (Figure 3A). Importantly, once they had moved to a pole, they rarely if ever disengaged from that pole (that is, they remained within 0.6 μm of the SPB). In contrast, when Scc1 was depleted from *ndc10-1* cells that lack functional kinetochores (Goh and Kilmartin, 1993), sister centromeres drifted around in a random and uncoordinated fashion within the nucleus and never stayed for any length of time in the vicinity of either SPB (Figure 3B). This implies that mono-orientation in *ipl1* mutants is not caused by a failure to establish new kinetochores following DNA replication.

Chromosome Bi-orientation Can Be Maintained in *ipl1* and *sli15* Mutants

To test whether Ipl1 is dispensable for chromosome segregation once bi-orientation has been achieved, we arrested *IPL1+SLI15+, ipl1-321, ipl1-2,* and *sli15-3* cells in metaphase at 23°C by shutting off expression of Cdc20 (which was under control of the *GAL1-10* promoter). This permitted chromosome bi-orientation, because sister *CEN5* sequences were found separated in 70% of cells in all four strains. We then shifted the cells to 37°C and one hour later allowed them to undergo anaphase by inducing Cdc20 synthesis while still at 37°C. Shifting cells to the restrictive temperature neither altered the separation of sister *CEN5* sequences during the metaphase arrest nor greatly affected the fidelity of chromosome segregation during the subsequent anaphase (data not shown). In all four strains, sister sequences segregated to opposite poles in more than 94% of cells (Supplemental Data, Section S1 at http://www.cell.com/cgi/content/full/108/3/317/DC1). This raises the possibility that Ipl1-Sli15 might be required to establish, but not to maintain, bi-orientation.

Most Unreplicated Chromosomes Segregate with "Old" SPBs in *ipl1* and *sli15* Mutants

Our results are consistent with at least two different models. According to the first, Ipl1 is involved in a process that resolves sister kinetochores from each other and thereby enables their attachment to microtubules with opposing polarity. According to the second, it is part of a correction mechanism that facilitates bi-orientation by eliminating kinetochore-spindle pole connections that have not generated tension within centromeric chromatin. These two models are not incompatible and Ipl1 might indeed perform both functions. Nevertheless, if Ipl1 were merely required for sister kinetochore resolution, then it should not affect the stability or turnover of kinetochore-SPB connections in cells unable to replicate chromosomes.

Two properties of yeast kinetochores and SPBs provided an opportunity to investigate the stability or turnover of their interactions in cells unable to replicate their chromosomes. The first is the finding that centromeres are connected to SPBs even during G1 phase (Supplemental Data, Section S2). The second stems from the conservative nature of SPB duplication. "Old" SPBs from the previous cell cycle remain intact during SPB duplication in S phase. A satellite SPB forms adjacent to them, whose maturation into a fully new SPB induces formation of a bipolar spindle and hence the separation of old and new SPBs to opposite sides of the nucleus (Segal and Bloom, 2001). We have recently discovered that buds inherit the old SPB in 98% of cells (Pereira et al., 2001). The asymmetric distribution of old and new SPBs is unaffected by mutation of either *IPL1* or *SLI15* (see Figure 5D), by depletion of the replication initiation

factor Cdc6 (see below), or by their simultaneous inactivation (data not shown).

If Ipl1 were required for increasing the turnover of connections between SPBs and kinetochores, and if it also acted on unreplicated chromosomes produced by depletion of Cdc6, then Ipl1 should at least transiently disrupt the connection of unreplicated kinetochores to old SPBs and sometimes cause them to attach to microtubules from the new SPB. Indeed, previous studies have shown that unreplicated chromosomes are segregated to both poles in *IPL1+* cells lacking Cdc6 (Piatti et al., 1995; Stern and Murray, 2001). To test whether inactivation of Ipl1 causes unreplicated chromosomes to maintain their connection with old SPBs, we created *IPL1+ SLI15+*, *ipl1-321*, and *sli15-3* strains whose sole *CDC6* gene was under control of the *GAL1-10* promoter. These strains also expressed a Bfa1-GFP fusion protein that decorates SPBs that enter buds during anaphase, but not those that stay in their mothers (Pereira et al., 2001).

Cdc6-depleted G1 cells were incubated in glucose-containing medium (*GAL1-10* promoter is off) at 37°C. Measurement of cellular DNA contents by FACS showed that none of the strains replicated their DNA but that all nevertheless underwent cytokinesis (Figure 4A). *IPL1+ SLI15+* cells produced cells with a DNA content of approximately 0.5 C, whereas *ipl1-321* and *sli15-3* cells produced a population of cells with a bimodal distribution of DNA content. Half the cells inherited little or no DNA, whereas the other half inherited a 1 C DNA content. Figure 4A also shows FACS profiles of the equivalent *CDC6+* cells that replicated their DNA normally. DAPI staining of Cdc6-depleted cells, in which one SPB had moved to the bud, showed that equal amounts of DNA segregated to mothers and their buds in *IPL1+ SLI15+* cells but that the bulk of nuclear DNA segregated to buds in *ipl1-321* and *sli15-3* mutant cells (Figures 4B and 4C). Remarkably, the anaphase spindles of *ipl1-321* cells frequently looked normal despite the almost complete lack of chromosomes at the pole in mothers (Figure 4C) (Supplemental Data, Section S3). Thus, inactivation of either Ipl1 or Sli15 causes most unreplicated chromosomes of Cdc6-depleted cells to segregate into buds along with the old SPB.

As we have already argued, the lack of chromosomes associated with new SPBs within mother cells in *ipl1* mutants is unlikely to be due to defects in their new SPBs (see Figures 2D and 2E). The segregation of most unreplicated chromosomes exclusively with old SPBs in *ipl1* and *sli15* mutants but not in *IPL1+ SLI15+* cells is therefore consistent with the notion that the ability of unreplicated chromosomes to change the SPB to which they are connected (that is, to reorient) depends on the Ipl1-Sli15 complex. In *ipl1* and *sli15* mutants, unreplicated chromosomes appear to retain their connection to old SPBs, which they inherit from G1.

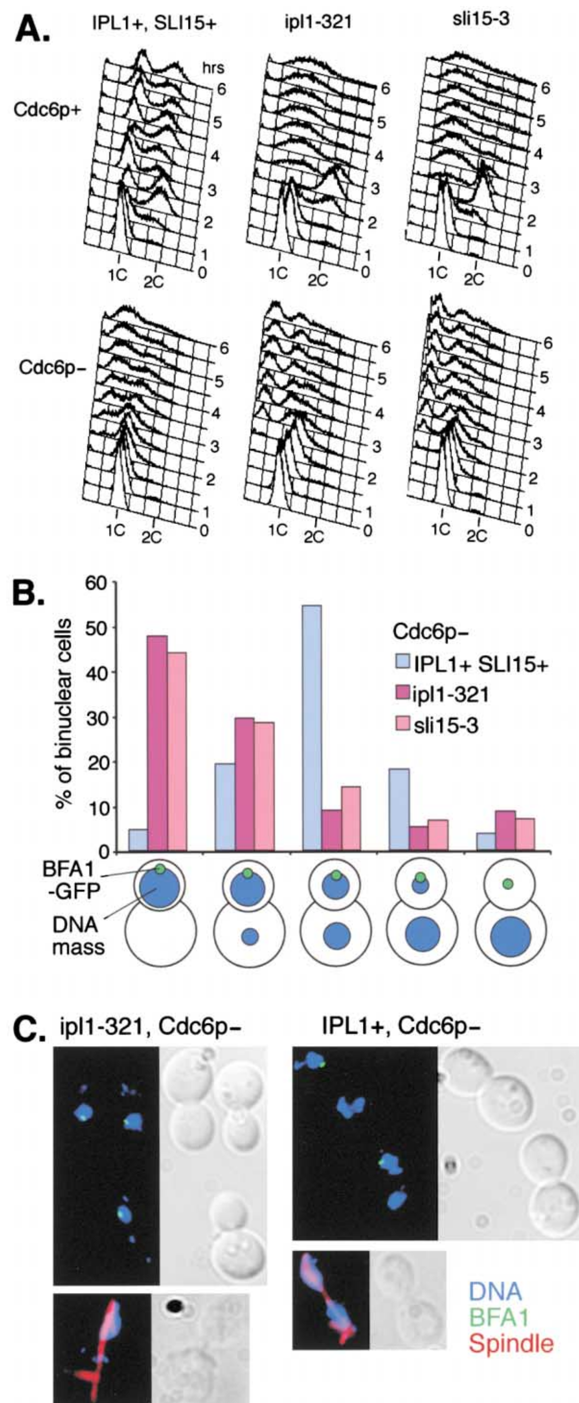

Figure 4. The Majority of Chromosomes Segregate toward Buds in *ipl1* and *sli15* Mutants when DNA Replication Is Inhibited

Small G1 cells of *IPL1+SLI15+* (T1995), *ipl1-321* (T1991) and *sli15-3* (T2021) strains (*GAL-CDC6, cdc6Δ, BFA1-GFP*) were collected by elutriation after Cdc6p was depleted by culturing cells in YEP-Raff for 90 min, and then incubated at 37°C either in YEP-RaffGal (Cdc6p+) or in YEP-Glc (Cdc6p−). DNA contents were analyzed by FACS (A). Pattern of DNA mass segregation was scored in cells in which Bfa1-GFP entered the buds after DAPI staining (B). Representative cells after DAPI staining (C, top) and indirect immunostaining of the spindle (C, bottom).

Ipl1 Is Required for the Reorientation of Minichromosomes Lacking Replication Origins in *CDC6+* Cells

To confirm the lack of reorientation of unreplicated kinetochores in *ipl1* and *sli15* mutants, we analyzed the segregation of a chromosome whose replication origin had

previously been removed (in cells whose other chromosomes were allowed to replicate normally). To do this, we constructed a minichromosome whose sole replication origin or autonomously replicating sequence (ARS) is flanked by recombination sites for the *Z. rouxii* R recombinase (Araki et al., 1992). The minichromosome was marked by the insertion of tet operators that bind Tet repressor-GFP and was placed in *IPL1*+ and *ipl1-321* strains in which the recombinase gene was transcribed from the galactose-inducible *GAL1-10* promoter (Figure 5A). The minichromosome was stably maintained in the absence of galactose. Addition of galactose to the medium caused, within 2.5 hr, efficient recombination between recombination sites and the appearance of minichromosomes lacking any ARS (Figure 5B, RS-ARS-RS). This led to a rapid increase in the number of cells born without the minichromosome. The same ARS was, in contrast, not lost from a control minichromosome containing only a single recombination site (Figure 5B, RS-ARS). In binuclear cells produced at 37°C, unreplicated minichromosomes segregated almost equally to mothers and their buds in *IPL1*+ cells but predominantly to buds in *ipl1* mutant cells (Figure 5C). Unreplicated minichromosomes were connected to SPBs in both *IPL1*+ and *ipl1-321* binuclear cells (Figures 5F, b and 5F, c; see below). These data imply that Ipl1 is required for the reorientation of kinetochore-SPB connections even in *CDC6*+ cells.

Cosegregation of Unreplicated Minichromosomes with Old SPBs in *ipl1* Mutants Is Reduced by Transient Disruption of Microtubules

To confirm that unreplicated minichromosomes cosegregate with old SPBs in *ipl1* mutant cells, we repeated the above experiments in a strain whose SPB protein Spc42 was fused to red fluorescent protein (RFP). Spc42-RFP fluorescence during cell cycles after stationary phase can be used to monitor movement and segregation of old SPBs (Pereira et al., 2001). Red fluorescent SPBs segregated to buds in most *IPL1*+ cells (Figure 5D). Meanwhile, green fluorescent unreplicated minichromosomes segregated with old (red fluorescent) and new SPBs with almost equal frequency (Figures 5D–5F). Inactivation of Ipl1 had little or no effect on the preferential segregation of old SPBs to buds, but greatly increased the frequency with which unreplicated minichromosomes cosegregated with old SPBs (Figures 5D–5F). Remarkably, the unreplicated minichromosomes tended to cosegregate with old SPBs even in those rare cases where old SPBs had segregated to the mother cell (16 out of 20 cells; Figure 5F, c) in the *ipl1* mutant. Ipl1 is therefore required for preventing the preferential cosegregation of unreplicated minichromosomes with old SPBs irrespective of whether this occurs in mothers or their buds.

We also treated cells with nocodazole from G1 until after SPB duplication. This greatly reduced the tendency of old SPBs to segregate into buds in both *IPL1*+ and *ipl1-321* cells (Figure 5D; see Pereira et al, 2001). Importantly it also reduced cosegregation of unreplicated minichromosomes with old SPBs in *ipl1* mutant cells (Figure 5E). These data along with those in Figure 2E suggest that kinetochores from *ipl1* mutant cells that have been disconnected from SPBs by nocodazole do not exhibit a strong bias to be connected to old SPBs upon nocodazole removal, whether or not they had been allowed to replicate. The similar effect on kinetochore orientation of either Ipl1 activity or transient nocodazole treatment is consistent with the notion that Ipl1 promotes turnover of kinetochore-SPB connections.

Delaying Centromere Replication in *ipl1* Mutant Cells Increases Mother Cell Mono-orientation

The tendency of sister kinetochores to mono-orient at old SPBs in *ipl1* mutants that have undergone DNA replication (Figures 2C and 2D) is something of an enigma. One explanation for the mono-orientation bias in favor of buds is that centromere replication occurs early during S phase (McCarroll and Fangman, 1988) and is completed before the new SPB (destined for the mother cell) has become operative. As a result, nascent kinetochores may be frequently connected to the old SPB by microtubules even in wild-type cells, and Ipl1 may be essential for inducing their reorientation. Crucially, this hypothesis predicts that delaying replication of a kinetochore might increase the chances that its progeny attach to new SPBs in *ipl1* mutants, assuming that kinetochores transiently disconnect from SPBs during their replication.

To test this, we compared the segregation in *ipl1* mutants of two minichromosomes that differ in the timing of their replication. Each minichromosome replicated by virtue of a single ARS, one of which replicates early and the other late during S phase (Friedman et al., 1996). In most *IPL1*+ cells, sister chromatids from both early- and late-replicating minichromosomes segregated to opposite poles (Figures 6A and 6B, a). In *ipl1-321* cells, the early-replicating minichromosome mono-oriented in 67% of the cells, with a strong bias in favor of buds (Figures 6A and 6B, b). This asymmetric segregation resembles that of authentic chromosomes (see Figures 2C and 2D). Although the overall frequency of mono-orientation of the late-replicating minichromosome was slightly higher than that of the early-replicating ones, the bias toward buds was almost absent (Figures 6A and 6B, c). These data are consistent with the notion that late replication does indeed increase the probability that nascent kinetochores will attach to new SPBs (Supplemental Data, Section S4).

Ipl1 and the Kinetochore Protein Ndc10 Have Different Localization Patterns During Metaphase and Anaphase

If the Ipl1-Sli15 kinase complex is involved in correcting mono-oriented chromosomes, then some mechanism must enable it to discriminate between mono- and bi-oriented kinetochores so that only the connections of the former are altered. A potential clue concerning the mechanism of discrimination emerged when we compared the localization during mitosis of Ipl1 and the kinetochore protein Ndc10 (each fused to a fluorescent protein or epitope tags).

Like other kinetochore proteins, Ndc10-GFP distributes to two lobes in cells with medium to large buds (Figure 7A bottom, 0 s). This bilobed distribution is due to the separation of sister centromeres caused by their traction toward opposite poles (Goshima and Yanagida,

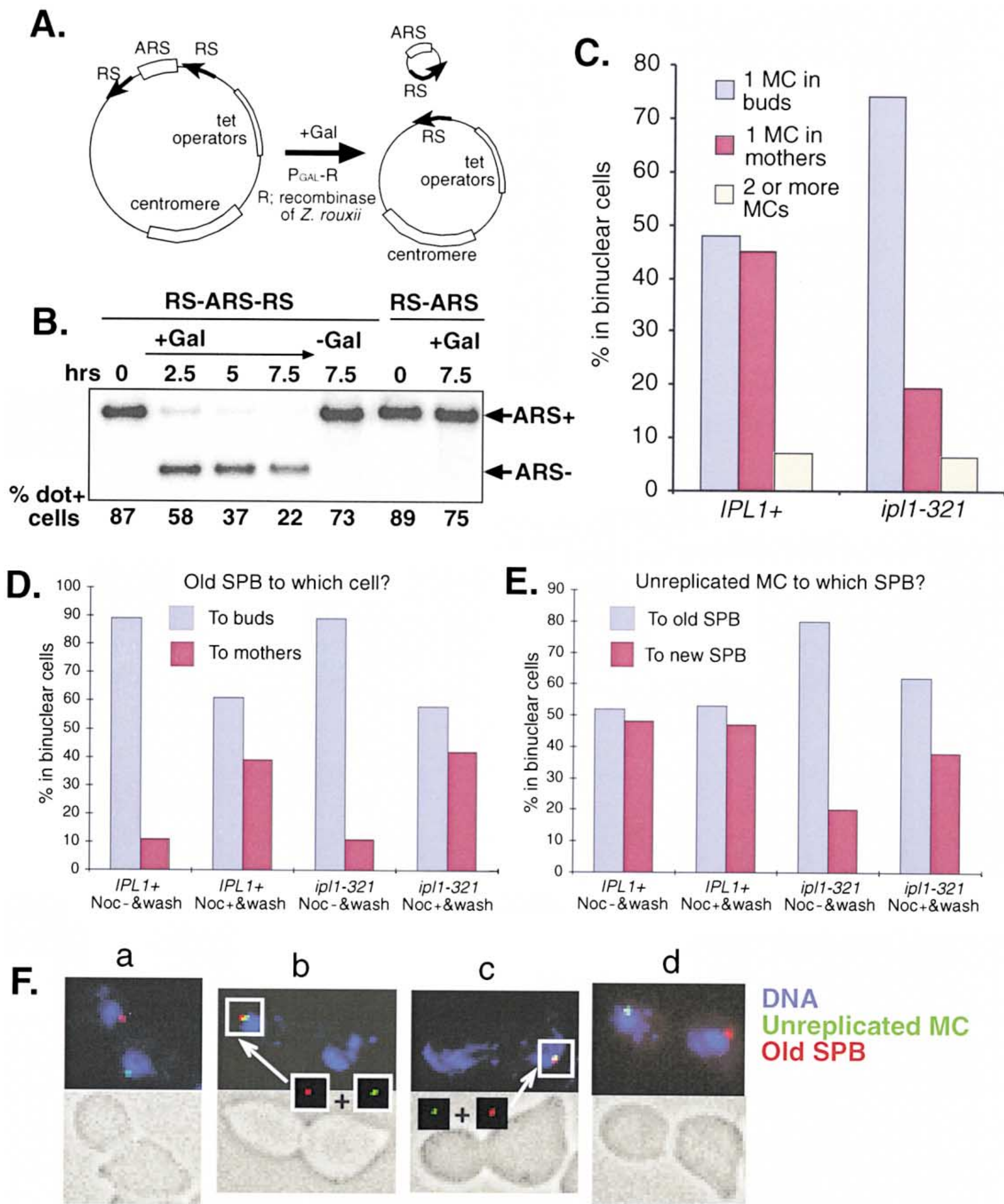

Figure 5. Unreplicated Minichromosomes Segregate with the Old SPB in an *ipl1* Mutant

(A) Scheme on how to flip out replication origins (ARS) from minichromosomes. RS; recombination site.

(B) Southern blot analysis and rate of cells containing minichromosomes. RS-ARS-RS (pT323); ARS flanked by two recombination sites (see A). RS-ARS (pT327); ARS flanked by only one recombination site. *TetR-GFP GAL-R×3* cells harboring pT323 (T2156) or pT327 (T2157) were incubated either in YEP-RaffGal (Gal+) or in YEP-Raff (Gal−) at 25°C. Similar results were obtained in *ipl1-321* cells (data not shown).

(C) Small G1 cells of *IPL1+* (T2156) and *ipl1-321* (T2163) strains (*TetR-GFP GAL-R×3 pT323*) were collected by elutriation after incubation for 7.5 hr in YEP-RaffGal, then cultured at 37°C. Segregation of minichromosomes were scored in binuclear cells. 1 MC; unreplicated minichromosome. Two overlapped minichromosomes were discerned by brighter GFP signals.

(D–F) *IPL1+* (T2448) and *ipl1-321* (T2444) cells (*TetR-GFP SPC42-RFP GAL-R×3 pT323*) were incubated in stationary phase for two days, then diluted in YEP-RaffGal, grown for 5 hr, arrested by α factor for 2.5 hr, washed out of α factor, incubated with or without nocodazole at 37°C, washed out of nocodazole when 90% cells showed bud emergence, and then further incubated at 37°C. Segregation of RFP (old SPB) and GFP (unreplicated minichromosome) signals were scored in binuclear cells which harbour an unreplicated minichromosome (D and E). Representative cells (F). Insets in (F, b) and (F, c) show RFP and GFP signals separately.

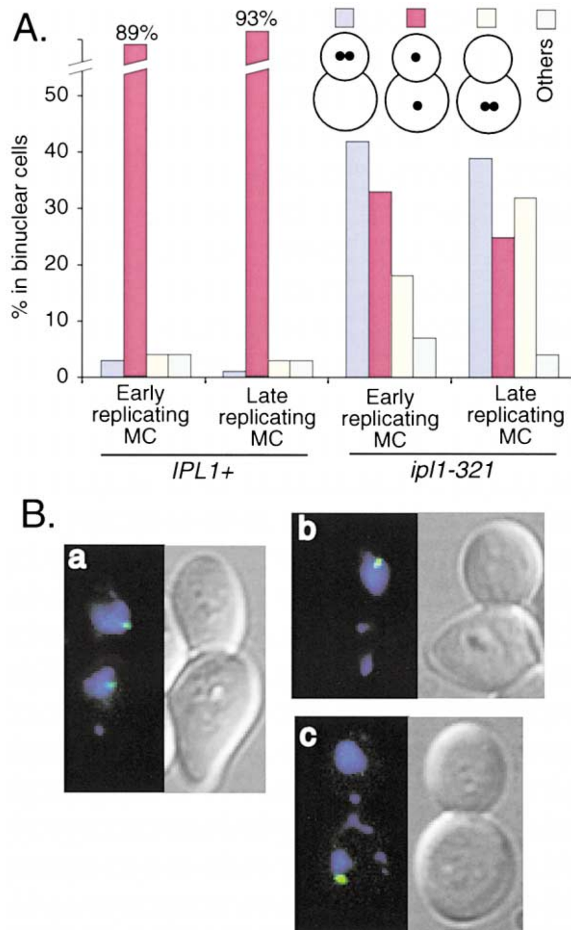

Figure 6. Delaying the DNA Replication of Minichromosomes Leads to More Mono-oriented Sister Kinetochores Segregating to Mothers in an *ipl1* Mutant

IPL1+ and *ipl1-321* cells (*TetR-GFP*) harboring an early-replicating minichromosome (pT331; T2170 and T2165, respectively) and a late-replicating minichromosome (pT334; T2171 and T2167, respectively) were synchronized and cultured as in Figures 2A–2C. Minichromosomes were visualized by insetion of *tet* operators. Pattern of segregation of minichromosomes was scored in binuclear cells (A). Representative cells (B). Two overlapped minichromosomes were discerned by the brighter GFP signal.

2000; He et al., 2000). Each lobe segregated to opposite spindle poles during anaphase (420–840 s; Ndc10 also localized along the anaphase spindle, albeit only weakly). Ipl1-GFP's distribution during mitosis clearly differed from that of Ndc10-GFP. It was confined to a small cloud within the nucleus during metaphase (Figure 7A top, 0–180 s), localized to the mitotic spindle at the onset of anaphase (240–300 s), and only accumulated near SPBs along with Ndc10 when the poles reached opposite ends of the cell (post 780 s; that is, during telophase; data not shown). Ipl1-GFP also formed a nuclear "cloud," while Ndc10-GFP clearly formed two lobes in cells arrested in metaphase by Cdc20 depletion (data not shown). We obtained similar results when we compared the localization of Ipl1-YFP and Ndc10-CFP in the same cells (Figure 7B, metaphase, anaphase/telophase). Ipl1-YFP and Ndc10-CFP colocalized at small patches in unbudded (Figure 7B; G1) and small-budded cells (S and early G2 phases; data not shown).

Meanwhile, Ipl1-myc colocalized with Ndc10-HA on spread chromosomes in the majority of cells (Figure 7C) (Supplemental Data, Section S5). These data suggest that the Ipl1-Sli15 complex localizes to the vicinity of kinetochores for much of the cell cycle, but is not confined to the vicinity of kinetochores during metaphase, when spindle forces pull sister kinetochores toward opposite poles. Our finding that bi-orientation alters the physical relationship between kinetochores and Ipl1 suggests at least two mechanisms by which bi-orientation could block the tendency of this kinase to alter kinetochore-SPB connections (Figure 7D and Discussion).

Discussion

A mechanism ensuring that sister kinetochores attach to microtubules from opposite poles (bi-orientation) is a crucial aspect of mitosis. Studies on meiotic cells have suggested that the traction of maternal and paternal kinetochore pairs to opposite spindle poles during meiosis I might be brought about by the selective stabilization of kinetochore-centrosome connections that produce tension (Nicklas, 1997; Nicklas and Ward, 1994). Bi-orientation during mitosis could operate using a similar principle. We describe here evidence that specific proteins perform this task. We show that the Ipl1-Sli15 complex, the yeast ortholog of the Aurora B-INCENP (Adams et al., 2000; Kaitna et al., 2000), is essential for bi-orientation and for the turnover of SPB-kinetochore connections.

Ipl1-Sli15 Increases Turnover of the Connections between Unreplicated Kinetochores and SPBs

Ipl1-Sli15 could in principle promote bi-orientation by two different mechanisms: either by modifying the structure of kinetochores so that sisters face in opposite directions (sister kinetochore resolution), or by altering kinetochore-SPB connections. We reasoned that if the latter were true, then it should be possible to assay Ipl1 activity in the complete absence of DNA replication, which would greatly simplify the process being studied. We found that most unduplicated centromeres cosegregate with old SPBs in *ipl1* and *sli15* mutants. No such bias is found in wild-type cells. The simplest interpretation of this observation is that old SPB-kinetochore connections inherited from G1 persist throughout the cell cycle in the absence of Ipl1 activity. Ipl1-Sli15 presumably promotes the turnover of these connections, possibly because they do not generate tension (see below), and as a result, unreplicated kinetochores connect with equal probability to microtubules from old and new SPBs. As predicted by this hypothesis, transient disruption of microtubules with nocodazole had a similar effect as Ipl1-Sli15 activity: it tended to abolish the tendency of chromosomes to cosegregate with old SPBs. These observations prove that even if Ipl1-Sli15 also has a role in sister kinetochore resolution, it has an equal if not more important role in regulating the turnover of SPB-kinetochore connections by microtubules.

It is likely that Ipl1-Sli15 has a similar role on replicated chromosomes, because even replicated kinetochores tend to attach to microtubules from old SPBs in *ipl1*

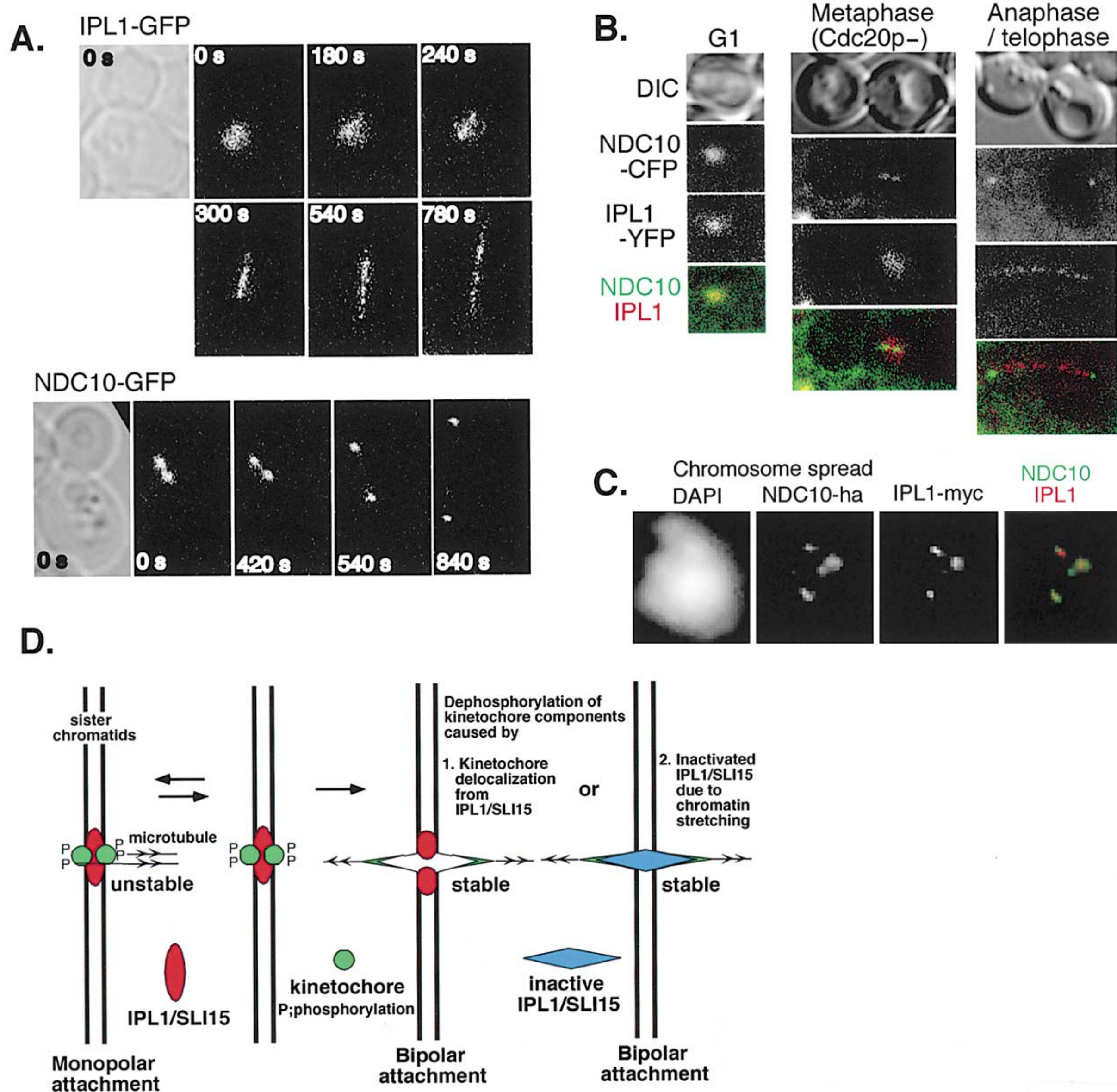

Figure 7. Ipl1 and Ndc10 Colocalize in G1, S, and G2 Phases but Show Different Localization Pattern in Metaphase and Anaphase

(A) Timelapse bright field and GFP fluorescence images of *IPL1-GFP* (T2035) and *NDC10-GFP* cells (T2093).

(B) Differential interference contrast (DIC), CFP, and YFP images of *NDC10-CFP IPL1-YFP* cells (T2376) (G1, Anaphase/telophase), and of *NDC10-CFP IPL1-YFP GAL-CDC20 cdc20Δ* cells (T2369) (metaphase). T2369 cells were cultured in YEP-Glc for 1.5 hr to deplete Cdc20.

(C) Immunostaining of spread chromosomes of *NDC10-HA3 IPL1-myc12* cells (T2058) by anti-HA and anti-myc antibodies.

(D) Model of how the Ipl1-Sli15 complex ensures bi-oriented microtubule attachment to sister kinetochores.

mutants. Our finding that this tendency is reduced when chromosome replication is delayed relative to SPB duplication suggests that centromeres usually replicate before the formation of functional "new" SPBs. Nascent kinetochores therefore attach to old SPBs, and this attachment is maintained in the absence of Ipl1-Sli15 activity.

It should be noted that we have not yet been able to visualize the turnover of kinetochore-spindle pole connections in *IPL1*+ cells in real time. We expected that this might be possible in Scc1-depleted cells where a lack of tension should cause Ipl1-Sli15 to remain active, which might make kinetochore-SPB disconnect and reconnect repeatedly (e.g., back-and-forth motion of a kinetochore to an SPB). In fact, we observed that centromeres that had connected to one of the two poles in *scc1* mutants tended to remain in the vicinity of that same pole for extended periods of time. They either do not detach very frequently or reattach very rapidly to the same pole due to the high microtubule density in the vicinity of that pole.

A Tension-Dependent Mechanism for Inactivating Ipl1-Sli15

If Ipl1 is to ensure bi-orientation through an error-correcting mechanism, its ability to increase the turn-

over of SPB-kinetochore connections must be turned off once the desired state (bi-orientation) has been achieved. Ipl1/Aurora B could in principle regulate SPB-kinetochore connections by three different mechanisms: by altering the stability of the attachment between kinetochores and microtubules, by changing dynamics of microtubules associated with mono-oriented kinetochores, or even by regulating the connections between microtubules and SPBs in a manner that is somehow influenced by the state of the kinetochore attached to that microtubule. Because it is neither immediately obvious how the third mechanism could function nor how changing microtubule dynamics would automatically facilitate turnover of SPB-kinetochore connections, we currently favor the first of these mechanisms.

The localization of Aurora B-INCENP in the chromatin, sandwiched between sister kinetochores but not at kinetochores themselves (e.g., Cooke et al., 1987; Oegema et al., 2001), suggests one mechanism by which Ipl1/Aurora B could regulate connections between kinetochores and microtubules. The compaction of centromeric chromatin when not under tension might ensure that the Ipl1/Aurora B kinase remains in close contact with those kinetochore proteins whose phosphorylation by this kinase increases turnover of the connections between SPBs and kinetochores. Bi-orientation would pull sister kinetochores away from inner centromere chromatin and therefore from Aurora B-INCENP, which would permit dephosphorylation of these key kinetochore proteins by the PP1 phosphatase (Glc7 in yeast). Glc7/PP1 is known to counteract Ipl1/Aurora B in yeast and animal cells (Francisco et al., 1994; Sassoon et al., 1999; Hsu et al., 2000; Murnion et al., 2001). Unlike Aurora B, PP1 colocalizes with kinetochores during metaphase, at least in animal cells (L. Trinkle-Mulcahy, A. Lamond, and J. Swedlow, personal communication).

Our finding that Ipl1 and Sli15 colocalize with kinetochore proteins before but not after bi-orientation in yeast suggests that this model might also be applicable to yeast kinetochores, which at first glance appear to lack "inner centromere" regions. We suggest that Ipl1-Sli15 associates with chromatin adjacent to kinetochores and that chromatin compaction brings the kinase complex into direct contact with kinetochores. Bi-orientation pulls kinetochores away from the surrounding chromatin and might thereby remove them from Ipl1-Sli15 (Figure 7D). We suggest that the chromatin surrounding kinetochores in yeast is equivalent to the inner centromere region of more complex centromeres. It is equally possible that the unraveling of chromatin in the vicinity of centromeres caused by the tension produced through bi-orientation has a key role in turning off Ipl1 (Figure 7D). If the Ipl1-Sli15 kinase complex were only active when associated with compacted chromatin, then chromatin unraveling could turn off the kinase and hence prevent turnover of kinetochore-SPB connections. It is interesting in this regard that Ipl1 clearly interacts closely with nucleosomes, because it is responsible for phosphorylating histone H3 (Hsu et al., 2000).

It is unclear how Ipl1-Sli15 promotes turnover of kinetochore-SPB connections. One possibility is that Ipl1 destabilizes kinetochore-microtubule attachment so that a new attachment is generated. This possibility is consistent with the previous finding that a hitherto uncharacterized activity capable of connecting centromeric DNA to microtubules in vitro is inhibited by ATP in an Ipl1-dependent manner (Biggins et al., 1999). This factor may be the target of Ipl1 at kinetochores, and its identification will therefore be important for understanding further the mechanism by which Ipl1 regulates kinetochore function.

Our model explains a curious property of Aurora B-INCENP kinase complexes. Both in animal cells and yeast, the kinase complex dissociates from chromosomes at the metaphase to anaphase transition and associates with mitotic spindles. This property is responsible for their having been named chromosome passengers (see Adams et al., 2001a). If Ipl1 destabilizes the connection between kinetochores and SPBs in the absence of centromere tension, then it is crucial that the kinase be removed from centromeric chromatin when destruction of cohesion by separase releases tension at the metaphase to anaphase transition. Dissociation of Ipl1-Sli15 or Aurora B-INCENP from centromeric chromatin at the onset of anaphase would prevent it from destabilizing the connection between kinetochores and spindle poles during anaphase.

Our hypothesis predicts that Ipl1 should cause the frequent detachment of kinetochores from microtubules in the absence of tension. This might trigger the Mad2-dependent mitotic checkpoint, which is thought to sense occupancy of kinetochores by microtubules and delays destruction of securin (Amon, 1999; Rudner and Murray, 1996). This predicts that Ipl1-Sli15 might be responsible for triggering the Mad2-dependent checkpoint in Cdc6-depleted cells (Stern and Murray, 2001), which are incapable of replicating their chromosomes and incapable of inactivating Ipl1. This is indeed the case. Securin destruction is delayed in an Ipl1-dependent fashion in such cells (Biggins and Murray, 2001), though whether this is caused by chromatid detachment induced by Ipl1-Sli15 or due to novel tension-sensing mechanism of the mitotic checkpoint is presently unclear. The Mad2-dependent arrest of *glc7* mutants in yeast (Bloecher and Tatchell, 1999; Sassoon et al., 1999) might also be caused by elevated chromatid detachment promoted by Ipl1-Sli15.

The suggestion that Ipl1 is required for activating the mitotic checkpoint when chromosomes fail to come under tension (Biggins and Murray, 2001) is not inconsistent with our proposal that it also regulates the stability of kinetochore-SPB connections. Indeed, if the Ipl1 activity were sensitive to tension of chromatin surrounding centromeres, then it would be ideally suited to perform both of these functions.

A mechanism for ensuring bi-orientation is crucial for mitosis and must have been present in the common ancestor of all eukaryotic cells. It is therefore likely that Ipl1/Aurora B kinases have a similar function in animal and plant cells. Of course, this does not imply that this is the sole function of these kinases, but it is very possibly a crucial and conserved one. It is conceivable that ensuring bi-orientation is Ipl1's sole essential function in yeast, which in retrospect possibly facilitated the detection and characterization of this process using yeast as a model system.

Experimental Procedures

All strain construction, tagging of yeast proteins, yeast cultures, centrifugal elutriation, cell cycle synchronization by α factor treat-

ment and wash out, FACS analysis of DNA contents, observation of Tet-GFP dots, sample preparation for timelapse microscopy, indirect immunostaining, chromosome spread, and Southern blots were as described previously (Piatti et al., 1995; Tanaka et al., 2000; Toth et al., 2000) unless otherwise stated. Yeast cells were cultured in YEP-Glc at 25°C unless otherwise stated. For timelapse, Deltavision optical sectioning deconvolution microscopy was used in addition to the system previously described (Tanaka et al., 2000). For timelapse microscopy at 35°C, cells were incubated at the temperature for one hour before imaging, and the temperature was maintained by an objective heater during imaging. To make yeast cells in the stationary phase, lawns of the cells on culture plates were left for 2 days at 25°C. Tagging of yeast proteins with GFP, YFP, RFP, CFP, HA, and Myc epitopes was done at their C terminus at the original loci of their genes (fusion genes were expressed from their original promoters) except *SPC42-GFP* which was integrated at *ura3* locus. See Supplemental Data, Section S6 for plasmids used in the study.

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Note Added in Proof

When we were preparing our manuscript, centromere movements in *ipl1* mutants (similar to ones shown in Figure 1B) were also reported by He et al. (He, X., Rines, D.R., Espelin, C.W., and Sorger, P.K. (2001). Molecular analysis of kinetochore-microtubule attachment in budding yeast. *Cell* 106, 195–206).