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Fission Yeast Bub1 Is a Mitotic Centromere Protein Essential for the Spindle Checkpoint and the Preservation of Correct Ploidy through Mitosis

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Abstract. The spindle checkpoint ensures proper chromosome segregation by delaying anaphase until all chromosomes are correctly attached to the mitotic spindle. We investigated the role of the fission yeast bub1 gene in spindle checkpoint function and in unperturbed mitoses. We find that bub1 is essential for the fission yeast spindle checkpoint response to spindle damage and to defects in centromere function. Activation of the checkpoint results in the recruitment of Bub1 to centromeres and a delay in the completion of mitosis. We show that Bub1 also has a crucial role in normal, unperturbed mitoses. Loss of bub1 function causes chromosomes to lag on the anaphase spindle and an increased frequency of chromosome loss. Such genomic instability is even more dramatic in Δbub1 diploids, leading to massive chromosome missegregation events and loss of the diploid state, demonstrating that bub1 function is essential to maintain correct ploidy through mitosis. As in larger eukaryotes, Bub1 is recruited to kinetochores during the early stages of mitosis. However, unlike its vertebrate counterpart, a pool of Bub1 remains centromere-associated at metaphase and even until telophase. We discuss the possibility of a role for the Bub1 kinase after the metaphase–anaphase transition.

Key words: Bub1 • centromere • mitosis • Schizosaccharomyces pombe • spindle checkpoint

Accurate chromosome segregation through mitosis requires the proper attachment of kinetochores to the spindle before anaphase begins. Kinetochore capture by microtubules is an event which is primarily governed by chance and is therefore prone to error (for review see Nicklas, 1997). The spindle checkpoint ensures high fidelity chromosome segregation by delaying anaphase until all chromosomes are correctly attached to the spindle (for review see Elledge, 1996; Rudner and Murray, 1996; Wells, 1996; Allshire, 1997; Hardwick, 1998).

Genetic analyses in budding yeast have identified some of the genes involved in the spindle checkpoint pathway. Mutations in the three MAD (mitotic arrest-deficient) genes, MAD1-3, and the three BUB (budding uninhibited by benzimidazole) genes, BUB1-3 result in hypersensitivity to spindle poisons as mutant cells are unable to arrest mitotic progression in response to spindle damage (Hoyt et al., 1991; Li and Murray, 1991). Among these checkpoint components are protein kinases and phosphoproteins suggesting that checkpoint function requires a phosphorylation-based transduction pathway. Bub1 is a protein kinase that can bind and phosphorylate Bub3 (Roberts et al., 1994). Mad1 can bind Mad2 (Chen, R.-H., and K.G. Hardwick, unpublished observations) and is hyperphosphorylated upon checkpoint activation (Hardwick and Murray, 1995). The Mps1 kinase is required for the spindle checkpoint and appears to directly phosphorylate Mad1 (Hardwick et al., 1996; Weiss and Winey, 1996). A combination of genetics and biochemical analyses placed BUB1, BUB3, and MPS1 upstream of MAD1 and MAD2 whereas BUB2 and MAD3 would act downstream of these genes (Hardwick and Murray, 1995; Hardwick et al., 1996). A dominant allele of BUB1 has recently been identified which activates the spindle checkpoint when overexpressed. Analysis of its overexpression in mad, bub, and mps1 mutants suggests that the Bub1 and Mps1 kinases act together at an early step in the checkpoint pathway, upstream of all the other Bub and Mad proteins (Farr and Hoyt, 1998).

In addition to gross alterations in spindle structure, the budding yeast spindle checkpoint can also respond to low doses of microtubule depolymerizing drugs, to defects in-
duced by mutations in centromere-binding proteins or in centromeric DNA, and to aberrantly segregating centromeres (for review see Rudner and Murray, 1996). All of these defects could interfere with kinetochore–microtubule attachment and a sensing system at kinetochores would allow the checkpoint to monitor the whole process of spindle assembly. Consistent with this idea, a phosphopeptope recognized by the monoclonal antibody 3F3 is only present at unattached kinetochores in mammalian somatic cell lines (Gorbsky and Ricketts, 1993). Furthermore, the vertebrate homologues of Mad2, Bub1, Mad3 and Bub3 all localize to unattached kinetochores (Chen et al., 1996; Li and Benzer, 1996; Taylor and McKeon, 1997; Taylor et al., 1998). It is not clear precisely how the spindle checkpoint monitors chromosome attachment. Checkpoints components could sense free microtubule binding sites at kinetochores or the tension exerted within the kinetochore by spindle poleward forces or a combination of both.

The most likely target of the spindle checkpoint is the anaphase-promoting complex (APC) or cyclosome. The APC acts as a ubiquitin-protein ligase to specifically mark proteins consequently targeted for destruction by the proteasome (for review see King et al., 1996). Activation of the APC triggers anaphase by degrading inhibitors of sister chromatid separation like budding yeast Pds1 and fission yeast Cut2 (Cohen-Fix et al., 1996; Funabiki et al., 1996) and allows cells to exit mitosis by degrading mitotic cyclins and regulators of spindle structure such as Ase1 (Juang et al., 1997). Recently, it was shown that Mad2 interacts directly with Sip1/Cdc20 (Hwang et al., 1998; Kim et al., 1998), which is thought to be a substrate-specific activator of APC-dependent proteolysis (He et al., 1997; Li et al., 1997; Visintin et al., 1997). It has been proposed that when the spindle checkpoint is activated Mad2, most likely in a complex with Mad1 and Mad3 (Hwang et al., 1998), represses the activity of Cdc20 and thus inhibits the metaphase–anaphase transition. The molecular mechanism of this repression is not understood.

The centromere/kinetochore complex is a key element in the process of mitosis. As well as the regulatory function of monitoring chromosome attachment to the spindle, the kinetochore carries out a number of mechanical functions. These include sister chromatid cohesion, attachment of sister centromatis to the spindle microtubules and the subsequent chromosome movements of metaphase and anaphase. In most eukaryotes, centromeric DNA consists of long arrays of repetitive DNA packaged into transcriptionally silent, recombination cold, late-replicating, heterochromatin. The exact function of heterochromatin is unknown but kinetochores in higher eukaryotes generally form within centromeric heterochromatin (Karpen and Allshire, 1997). In Schizosaccharomyces pombe three regions of the genome are known to assemble transcriptionally silent, heterochromatin-like structures. These are the centromeres, the telomeres, and the silent mating-type loci (Allshire et al., 1994; Nimmo et al., 1994; Thon et al., 1994). Mutations in clt4, rik1, and swi6 genes were shown to affect repression at the silent mating-type loci, at centromeres, and to a limited extent at telomeres (Lorentz et al., 1992; Ekwall and Ruusala, 1994; Allshire et al., 1995). The swi6 gene encodes a structural component of heterochromatin which localizes to centromeres, telomeres, and the mating-type region and functional Rik1 and Clr4 are required for this localization (Ekwall et al., 1995, 1996). Although Swi6 is present at telomeres it does not appear to play a crucial role in telomere function since mutations in the swi6 gene have little effect on telomere silencing and do not affect telomere length nor telomere clustering (Allshire et al., 1995; Ekwall et al., 1996). In addition, deletion of the swi6 gene induces an elevated loss rate of both linear and circular minichromosomes, suggesting that Swi6 acts primarily at the centromere. Indeed, the lack of a functional swi6 gene causes centromeres to lag on anaphase spindles (Ekwall et al., 1995). From these observations, it has been proposed that cells devoid of a functional swi6 gene assemble a compromised centromere (Ekwall et al., 1996).

In order to uncover new centromere proteins, we looked for loci that genetically interact with swi6 through a synthetic lethal screen. One of the mutants identified was found to bear a mutation in a gene homologous to budding yeast BUB1. We show that S. pombe bub1– is required to detect alterations in spindle structure or defects in kinetochore function. Activation of the checkpoint results in the recruitment of Bub1 to centromeres and the introduction of a delay in the completion of mitosis. Through genetic and cytological observations we show that the loss of the bub1 gene causes defects in chromosome segregation, providing clear evidence that Bub1 function is also required in a normal, unperturbed mitosis. Immunofluorescence analyses indicate that Bub1 is systematically recruited to centromeres during the prophase and prometaphase stages of normal mitosis. In metaphase, a fraction of the Bub1 pool is released from centromeres but surprisingly, some remains centromere associated until cytokinesis raising the possibility of a role for Bub1 at kinetochores past the metaphase–anaphase transition.

Materials and Methods

Strains, Media, Transformation and Genetic Techniques

All the strains used in this study are listed in Table I. Media were essentially as described by Moreno et al. (1991) and Allshire et al. (1994). Synthetic minimal medium is PMG. PMG with 20 µM thiamine. When appropriate, media were supplemented with 0.1 g/liter of leucine (L), uracil (U), adenine (A), and histidine (H). When required, Phloxin B was added to the media (2.5 mg/l) to stain colonies containing dead cells. YES medium was supplemented with 0.1 g/liter of leucine, adenine, and histidine. YES medium was supplemented with a limiting amount of adenine (0.01 g/liter) to allow the development of the red and pink colors of ade6-210 and ade6-216 colonies. Transformation and genetic techniques were as described (Moreno et al., 1991). Minichromosome loss rates were measured as described (Allshire et al., 1995). Briefly, cells from white colonies formed on a YES 1/10A plate were harvested, diluted in water, plated on YES 1/10A, and
then incubated 4 d at 32°C. The rate of minichromosome loss per division is the number of colonies with a red sector covering at least half of the colony divided by the total number of white colonies plus half-sectored colonies.

**DNA Manipulations and Plasmid Constructions**

DNA manipulations were performed according to standard procedures. Sequencing was done by Genome express S.A. (Grenoble, France) on double-stranded templates using an Applied Biosystems 373XL sequencer (Foster City, CA). The fission yeast B1 genomic library made in pUR19 (S. pombe ura4* gene as selection marker) was provided by A.M. Carr (Medical Research Council Cell Mutation Unit, Brighton, UK).

**Construction of pbub1-6HA.** A 540-bp fragment was generated by PCR using oligonucleotides bub1-3′ in (5′-GAATTCGCGCCGCAAA-1777TTTCTTTTTTGTGC-3′) and bub1-Xba out (5′-ACTTCTTC-ACTTCTCTC-3′). This fragment contains a XbaI site at the 5′ end, the last 178 codons of bub1* without the stop codon and a NotI site and an EcoRI site at the 3′ end. A 220 bp fragment was amplified using primers bub1-3′ out (5′-GAATTCCTAAATTTCGCTAGAGG-3′) and bub1-3′ out (5′-GTCATGATATTCAGGGTGG-3′). This fragment contains an EcoRI site at the 5′ end, a STOP codon followed by 200 bp of bub1* untranslated regions, and a NotI site at the 3′ end. The two PCR products were cut with EcoRI, ligated together, and then cut with XbaI and NsiI and cloned between the NdeI filled-in/HindII and PstI sites of pBluescript SK. The entire EcoRI site at the 5′ end of pbub1-6HA was cut with the single ClaI site lying within bub1* sequences and transformed into strain 77. Southern blotting was used to identify a strain with the expected structure.

**Isolation of the bub1-118 Mutant**

Strain 57 was mutagenized with ethylmethane sulfonate as described (Moreno et al., 1991). Mutagenized cells were plated on PMGLUA plates and incubated at 32°C until colony formation. Colonies were then replica-plated onto PMGLUAthi phloxinB and PMGLUA phloxinB and incubated at 32°C for 2–3 d. Colonies that stained dark red on the thiamine-containing plates were selected. Mutant strains were backcrossed at least three times. From ~120,000 colonies screened, seven mutants were isolated. All seven mutants were recessive and defined five genes named ssl1 to ssl5 (swi6 synthetic lethal). The ssl5 gene was renamed bub1. It is represented by a single allele, ssl5-118 which was renamed bub1-118.

**Cloning of bub1**

bub1-118 was found to be tightly linked to ade6 on chromosome III. To isolate the bub1* gene, strain 139 was transformed with the genomic DNA library, selecting for adenine prototrophy. Ade+ transformants were screened for ability to grow onto thiamine containing medium. Among the complementing plasmids, p135bub1* had the smallest genetic insert and was used for subcloning.

**Elutriation and H1 Kinase Assays**

Cultures were grown overnight in YES to 5 × 10^6 cells/ml and then loaded onto a JE-5.0 Beckman elutriation rotor (Palo Alto, CA). Small G2 cells were then shifted to 18°C and time points taken at 1-h intervals. Cells were fixed with 70% ethanol, before washing with PEM (100 mM Pipes, pH 6.9, 1 mM EGTA, 1 mM MgSO4) and staining with 4′,6-diamidino-2-phenylindole (DAPI) and calcofluor. Cell samples for kinase assays were then spun in a microfuge at 6,000 rpm for 5 min to remove beads and insoluble material, before protein concentrations were measured (Bio-Rad assay; Hercules, CA) and equalized. 1 μl of extract was added to a 10 μl kinase assay containing 80 mM Na-β-glycerophosphate, 50 mM KCl, 15 mM MgCl2, 20 mM EGTA, 2 mM Na vanadate, 1 mM DTT, 0.5% Triton, 1 mM PMSF, and 1 μg/ml of leupeptin, pepstatin, and chymostatin. Extracts were then spun in a microfuge at 6,000 rpm for 5 min to remove beads and insoluble material, before protein concentrations were measured (Bio-Rad assay; Hercules, CA) and equalized. 1 μl of extract was added to a 10 μl kinase assay containing 80 mM Na-β-glycerophosphate, pH 7.4, 15 mM MgCl2, 20 mM EGTA, 2 μg histone H1 (Boehringer Mannheim, Mannheim, Germany), 1 μCi of γ-32P-ATP,
200 μM ATP, and 1 mM DTT. Kinase assays were incubated for 15 min at 30°C, stopped by adding an equal volume of 2× SDS sample buffer, and then separated by 15% SDS PAGE. Gels were stained, fixed, and then dried and the relative levels of H1 kinase activity determined by autoradiography.

**Cytological Techniques**

Immunostaining was as described previously (Ekwall et al., 1996) with some modifications. Briefly, cells were fixed in 1.2 M sorbitol, 1.7% paraformaldehyde for 40 min. Incubation with anti-HA antibodies and secondary antibodies were performed for 3–16 h in PEMBAL in which BSA was replaced with 5% (wt/vol) nonfat dry milk. Monoclonal anti-HA 16B12 (Berkeley Antibody, Berkeley, CA) was used at 1:400 dilution, affinity-purified rabbit anti-HA Y-11 (Santa Cruz, CA, USA) at 1:50, and anti–α-tubulin monoclonal antibody TAT1 (Woods et al., 1989) at 1:20. Fluorescence microscopy was done using a Leica DMRXA microscope (St. Gallen, Switzerland). Images were captured with a Princeton charge-coupled device camera (Princeton, NJ) and processed with the Metamorph software. Figures were mounted using Adobe Photoshop 4.0 (Adobe Systems, San Jose, CA). In the montages from Fig. 4, different exposure times were used for the Bub1 signal, depending on the brightness of the subject. In particular, the exposure time or signal intensity were reduced for prophase and prometaphase cells to minimize halo formation around intense Bub1 signals. Length measurements were done using the Metamorph software (Universal Imaging, West Chester, PA).

**Results**

**Isolation of the bub1+ Gene**

In order to identify genes involved in mitotic centromere function, we performed a screen for mutants inviable in the absence of the centromere protein Swi6. Seven mutants were isolated defining five complementation groups. The bub1-118 mutation was found to be tightly linked to ade6 on chromosome III and this linkage was exploited to clone the bub1+ gene (Materials and Methods). Bub1+ resides within a 3,769-bp BamHI-SphI fragment which was fully sequenced (GenBank/EMBL/DDBJ accession number AF064796). A start codon was found at position 433, followed by an uninterrupted ORF encoding a predicted 1,044-amino acid protein with a calculated molecular mass of 113,363 D and a pI of 6.22. Database searches using the BLAST program (Altschul et al., 1990) revealed that the predicted peptide was similar to the budding yeast spindle checkpoint serine/threonine protein kinase encoded by BUB1 (Roberts et al., 1994). A significant match was also found with Bub1 homologues from Drosophila (P = 6.5 × 10^{-30}), human (P = 3.1 × 10^{-28}), and mouse (P = 2.5 × 10^{-28}). Fig. 1A shows the alignment of fission yeast Bub1 with *S. cerevisiae* and mouse proteins. The best conserved region resides in the COOH terminus kinase domain. In particular, lysine 762 which was shown to be essential for kinase activity and Bub1 function in budding yeast is conserved in all three Bub1 proteins, and in the human Bub1-related protein and budding yeast Mad3 (Fig. 1B). In fact it was this region of homology which led one of us (K.G. Hardwick) to first identify the fission yeast *bub1*+ gene in BLAST searches of sequence databases. The molecular function of this domain remains unclear: it is close to, but nevertheless distinct from the recently defined Bub3 interaction domain (Taylor et al., 1998) (Hardwick, K.G., unpublished observations on Mad3).

**Fission Yeast Bub1 Is an Essential Component of the Spindle Checkpoint**

A null allele was made by replacing the entire *bub1*+ ORF either with the *ura4*+ or LEU2 genes. The resulting haploid strains are viable showing that *bub1*+ is not essential for cell viability. In budding yeast, Δbub1 strains are viable

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**Figure 1.** Sequence comparison of Bub1 homologues. (A) Alignment of Bub1 protein sequences from *S. pombe* (sp), *S. cerevisiae* (sc), and mouse (m). Asterisks, identical residues; dots, similar residues; shaded area, the extent of the kinase domain. (B) Sequence alignment of the NH2-terminal domains of the Bub1 and Mad3 homologues from *S. cerevisiae* (sc), human (h), mouse (m), and *S. pombe* (sp). Boxes, identical or similar residues.
but hypersensitive to microtubule-depolymerizing agents such a nocodazole or benomyl owing to the loss of a functional spindle checkpoint (Hoyt et al., 1991; Roberts et al., 1994). As shown in Fig. 2A, the bub1-118 and ∆bub1 strains at 32°C are unable to form colonies on medium containing 7.5 μg/ml benomyl whereas the wild-type control strain still does. A ∆swi6 strain was also included in the experiment. Cells devoid of Swi6 are known to be hypersensitive to benomyl presumably as a result of an altered interaction of weakened kinetochores with spindle microtubules (Ekwall et al., 1996). Interestingly, the ∆bub1 strain showed the same pattern of benomyl sensitivity as the ∆swi6 strain (Fig. 2A). Sensitivity to a spindle poison can arise either from a structural defect in the spindle/kinetochore or from the loss of the spindle checkpoint. The two classes of defects should be distinguishable since the former are checkpoint proficient and should be able to halt cell cycle progression in response to spindle damage whereas the latter should not. To investigate whether ∆bub1 and ∆swi6 have a functional mitotic checkpoint we asked whether mutant strains can arrest their cell cycle in the absence of a spindle. To prevent spindle formation, we used nda3KM311, a cold-sensitive mutation in the β-tubulin gene. When shifted to the restrictive temperature, nda3KM311 cells accumulate with condensed chromosomes, single spindle pole body, a high level of histone H1 kinase activity, and unseparated sister chromatids (Hirao et al., 1984; Kanbe et al., 1990; Moreno et al., 1989; Funabiki et al., 1993). We synchronized cultures of the different nda3 mutant strains by elutriation, and Fig. 2B shows that when shifted to the cold, ∆swi6 nda3KM311 cells arrest with high levels of H1 kinase activity whereas the kinase activity drops rapidly in ∆bub1 nda3KM311 cells. The drop in kinase activity is accompanied by a wave of seption (Fig. 2C), confirming that the ∆bub1 nda3 KM311 cells are progressing through the cell cycle. The effects of such cell cycle progression in the absence of a spindle are readily apparent: several of the checkpoint-defective cells display the classic cut (cell untimely torn) phenotype because they fail to segregate their DNA to the two daughter cells prior to septation (Fig. 2D). In stark contrast to this the ∆swi6 nda3KM311 cells do not separte and remain arrested with hypercondensed chromosomes (data not shown). An elutriated nda3KM311 control culture behaved like the ∆swi6 nda3KM311 strain (data not shown).

From this experiment we concluded that ∆bub1 cells are deficient for the spindle checkpoint whereas the checkpoint is functional in ∆swi6 cells. To further investigate the consequences of the loss of a functional bub1+ gene, we asked whether sister chromatid segregation separate when ∆bub1 nda3KM311 cells are shifted to the cold. Cells from bub1+ nda3KM311 and ∆bub1 nda3KM311 strains were grown to early log phase at 32°C and then transferred to 18°C. Aliquots of the cultures were fixed at 1, 5, and 15 h and then processed for FISH using a probe detecting all three centromeres (cenFISH). Fig. 2E shows an nda3KM311 cell displaying three separate cenFISH signals. Those cells represented only a fraction of cells with condensed chromatin (Table II) because the chromosomes have to diffuse away from each other to show separate cenFISH signals. No more than three spots could be detected in bub1+ nda3
KM311 cells even 15 h after the shift to 18°C, showing that sister chromatid cohesion is maintained in those cells. By contrast, cells with more than three spots were readily detected in ∆bub1 nda3KM311 (Fig. 2 E, right). They first appeared 1 h after the temperature shift and rose throughout the course of the experiment (Table II). In cells with more than three spots the DNA always appeared decondensed, consistent with an exit from mitosis. It should be mentioned that the proportion of cells with separated sisters is likely to be underestimated since, as mentioned above, individual chromosomes or chromatids must diffuse away from each other to produce separate cenFISH signals. Although not quantitative, this experiment clearly demonstrates that sister chromatid separation occurs in ∆bub1 cells in the absence of a spindle.

The above experiments show that Bub1 is an essential component of the fission yeast spindle checkpoint. In the absence of a mitotic spindle, cells with a nonfunctional bub1 gene fail to maintain sister chromatid cohesion and high levels of H1 kinase activity, and as a result they exit mitosis with unsegregated chromosomes.

*bub1* Function Is Required for High Fidelity Segregation of Chromosomes in Normal Mitosis

As shown above, Bub1 function is essential to prevent chromosome segregation in the absence of a spindle. Next, we asked whether Bub1 is also required for normal mitosis. This was assayed by measuring the fidelity of chromosome segregation. The rate of chromosome loss was estimated by scoring the mitotic loss rate of the 530-kb linear minichromosome Ch16 (Matsumoto et al., 1990) using the half-sectoring assay method (Allshire et al., 1995). In wild-type cells, Ch16 loss rate is less than 0.1% of cell divisions (Allshire et al., 1995). As a functional bub1* gene was present on Ch16, we constructed Ch16∆bub1, a Ch16 derivative in which the bub1* gene was deleted. Chromosome loss assays demonstrated that Ch16∆bub1 is as stable as the original Ch16 in an otherwise wild-type background (Table III). By contrast, the minichromosome was lost in 3.5% of cell divisions in ∆bub1 cells, showing that the loss of bub1* function causes a 70-fold increase in Ch16 loss over wild-type.

Similarly, loss of *bub1* function induced an elevated loss rate of CM3112, a circular minichromosome bearing a functional centromere derived from chromosome III (Matsumoto et al., 1990). The loss rate was up to 20% (Table III), a value similar to the loss rate of acentric or ars plasmids in fission yeast (Heyer et al., 1986; Matsumoto et al., 1990). Therefore, the mitotic segregation of CM3112 is almost completely disrupted in cells lacking *bub1* function.

To investigate whether the segregation of regular chromosomes was also affected, we looked at the rate of breakdown of a diploid homozygous for the *bub1* deletion. Chromosome missegregation events in a diploid generate aneuploid cells. As fission yeast cells are highly sensitive to aneuploidy (Niwa and Yanagida, 1985), aneuploids are unstable and return rapidly to the haploid state. Thus the rate of diploid breakdown can be used to estimate the rate of whole chromosome loss (Allshire et al., 1995). Diploids were forced by intragenic complementation between the ade6-210 (red) and ade6-216 (pink) alleles. Ade* cells were harvested from a plate lacking adenine and plated onto YES medium containing a limiting amount of adenine to allow the development of colored (haploid) colonies. As shown in Fig. 3 A, cells from the *bub1+/bub1* strain formed pure white (diploid) colonies and very few colored (haploid) colonies (the rate of breakdown is <10⁻³/cell division, Allshire et al., 1995). By contrast, cells from the ∆bub1/∆bub1 strain never formed white colonies but exclusively pink (ade6-216), red (ade6-210), or mixed pink/red haploid colonies. The extreme instability of the diploid precluded any precise measurement of the rate of breakdown. However, since white colonies never formed in the absence of selection for Ade* cells, one can conclude that *bub1* function is essential for chromosome stability in diploid cells.

We next investigated whether defects in chromosome segregation could be seen by cytological observation of mitotic cells. Strains 77 (*bub1*) and 393 (∆bub1) were grown to early log phase at 26°C, fixed, and then processed for immunofluorescence microscopy. DAPI and anti-tubulin antibody staining visualized nuclear chromatin and microtubules, respectively. The most striking observation in ∆bub1 mutants was the presence of unsegregated chromosomes at anaphase (Fig. 3 B, top left). In ~10% of late anaphase cells (Table IV), a chromatin mass remained near the spindle midzone whereas the bulk of chromatin had already reached the spindle poles. Lagging chromosomes were never observed in wild-type anaphase B cells.

A similar experiment was performed with diploid strains.

### Table II. Loss of Sister Chromatid Cohesion in nda3-KM311–arrested ∆bub1 Cells

<table>
<thead>
<tr>
<th>Strain Background</th>
<th>Time at 18°C</th>
<th>Number of cells examined</th>
<th>Number of cells with 1, 2, 3, and &gt;3 cenFISH signals*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>379</td>
<td>nnda3KM311</td>
<td>1</td>
<td>418 399 16 3 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>421 273 115 33 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>422 179 179 64 0</td>
</tr>
<tr>
<td>401</td>
<td>nnda3KM311</td>
<td>1</td>
<td>431 397 30 3 1</td>
</tr>
<tr>
<td></td>
<td>∆bub1</td>
<td>5</td>
<td>428 300 71 42 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>408 241 96 46 25</td>
</tr>
</tbody>
</table>

*One cenFISH signal was defined as a well-individualized spot of fluorescence; doubles were scored as a single signal.

### Table III. Effect of bub1 Loss of Function on the Fidelity of Minichromosome Transmission through Mitosis

<table>
<thead>
<tr>
<th>Strain Background</th>
<th>Chromosome loss per division</th>
<th>Fold increase in loss rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>530-kb linear Ch16∆bub1</td>
<td>&lt;0.05 (0 out of 2,187)</td>
<td>1</td>
</tr>
<tr>
<td>502  ∆bub1</td>
<td>3.5 (81 out of 2,302)</td>
<td>70</td>
</tr>
<tr>
<td>428  ∆bub1</td>
<td>20.4 (470 out of 2,302)</td>
<td>12.75</td>
</tr>
</tbody>
</table>
were fixed and processed for tubulin (green phase and processed for immunofluorescence. As were inoculated into complete medium, grown to early log producing pure white colonies whereas the Bernard et al. 

Cells from \( D_bub1 \) and \( D_bub1 \) diploids were forced by intragenic complementation between the red \( ade6-210 \) and pink \( ade6-216 \) alleles of the \( ade6 \) locus. \( Ade^+ \) cells were harvested from medium lacking adenine and plated onto YES 1/10 ade medium to allow the development of red and pink colors. The \( bub1 \) strain readily broke down to the haploid state, producing red, pink, and some transmission through mitosis. Figure 3

**Figure 3.** \( bub1 \) function is essential for high fidelity chromosome transmission through mitosis. (A) The diploid state can not be maintained in the absence of a functional \( bub1 \) gene. Diploids were forced by intragenic complementation between the red \( (\text{red}) \) and pink \( (\text{pink}) \) alleles of the \( ade6 \) locus. \( Ade^+ \) cells were harvested from medium lacking adenine and plated onto YES 1/10 ade medium to allow the development of red and pink colors. The \( bub1 \) strain readily broke down to the haploid state, producing red, pink, and sectorless colonies. (B) Chromosomes lag on the anaphase spindle in \( D_bub1 \) cells. Exponential growing cells in YES medium at 26°C were fixed and processed for tubulin (green) and DAPI (red, pseudocolor) staining. Right: In wild-type haploid and diploid anaphases, the chromatin masses are at the spindle poles. Left: \( \Delta_bub1 \) haploid and diploid cells with chromosomes remaining in the midzone of the anaphase spindle. Bar, 10 \( \mu \)m.

Cells from \( \Delta_bub1 \) and \( \Delta_bub1/\Delta_bub1 \) diploids were inoculated into complete medium, grown to early log phase and processed for immunofluorescence. As \( \Delta_bub1 \) diploids were extremely unstable, the cell population was composed of a majority of haploid cells and very few diploid cells. Nevertheless, the latter could be identified unambiguously by their larger size. Among late-anaphase \( \Delta_bub1/\Delta_bub1 \) cells, 45% (52 out of 116) showed lagging chromosomes (Fig. 3 B, bottom left) whereas cells with lagging chromosomes were rarely (5 out of 600) observed in \( \Delta_bub1 \) late-anaphase cells.

The above results clearly demonstrate that \( bub1 \) function is required for high fidelity chromosome segregation during normal mitosis. It is unclear why diploid cells are so sensitive to the loss of \( bub1 \) function. One possibility is that diploid mitosis relies more heavily on the spindle checkpoint than haploid mitosis simply because in a diploid there are more chromosomes and hence more kinetochores to be captured by the mitotic apparatus.

**Cellular Localization of Bub1**

Strain 411 was constructed in which the \( bub1 \) gene is appended with six copies of the HA epitope. The strain displayed the same benomyl sensitivity as a \( \Delta_bub1 \) strain, showing that the tagged protein was functional (data not shown). To localize the protein in the cell, two anti-HA antibodies were used: a mouse monoclonal antibody (mHA) and an affinity-purified rabbit polyclonal serum (rHA). The monoclonal always gave the best staining and allowed the finest observations whereas rHA was used for double staining with the anti-tubulin antibody to identify the cell cycle stage. Whatever the anti-HA antibody used, no staining was seen on untagged fixed cells (data not shown). Fig. 4 A shows cells stained with rHA and tubulin. A series of frames are shown which recapitulate the stages of the cell cycle from interphase through mitosis. In interphase cells (panel a), rHA staining was diffused through the nucleus with some faint spots around the nuclear periphery. When cells enter mitosis (panel b), rHA systematically produced a single bright spot of fluorescence colocalizing with the prophase spindle (spindle length < 1 \( \mu \)m). In prometaphase cells (spindle length < 2 \( \mu \)m), two bright Bub1 spots were seen, always positioned along the spindle axis (panels c and d).

In most cases when cells approached metaphase (spindle length > 2 \( \mu \)m and unsegregated DNA) there was an abrupt reduction in the Bub1 staining intensity (Fig. 4 A, panel e), although intense signals were sometimes observed. Among 150 metaphase cells examined, five retained an intense staining pattern (one, two, or three bright spots) but the vast majority (145) showed weakly stained foci (Fig. 4 A, panel e) and a diffuse labelling of the chromatin. Therefore, in a normal mitosis the intense staining pattern of Bub1 is restricted to prophase and prometaphase and is lost when cells reach metaphase. In anaphase cells (Fig. 4 A, f and g), some of the signal appeared to trail behind the separating chromatin masses but

### Table IV. Frequency of Abnormal Anaphases in \( \Delta_bub1 \) Mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Background</th>
<th>Number of late-anaphase cells analyzed</th>
<th>Late-anaphase cells with lagging chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>77</td>
<td>Wild-type</td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>393</td>
<td>( \Delta_bub1::ura4^+ )</td>
<td>406</td>
<td>52 (12.8%)</td>
</tr>
<tr>
<td>277</td>
<td>( bab1-118 )</td>
<td>404</td>
<td>38 (9.4%)</td>
</tr>
</tbody>
</table>

*Late-anaphase cells were defined as cells with a spindle length greater than 5 \( \mu \)m.

Cells were classified as having lagging chromosomes when DAPI-stained material was observed on the spindle, at least 1.5 \( \mu \)m away from one spindle pole. Parentheses, fraction of anaphase cells with lagging chromosomes.
with centromeres throughout mitosis. Montage of bub1-HA cells stained with mHA (red) and the digoxigenin-labeled centromere probe (green). Blue, DAPI staining. Left to right: interphase (a), prophase (b–d), prometaphase (e), metaphase (f), anaphase A (g), anaphase B (h and i), and cytokinesis (j). Bottom, merged image where yellow is produced upon colocalization of red (Bub1) and green (centromeres) signals. Bars, 5 μm.

We next looked at the localization of Bub-HA relative to centromeres (Fig. 4 B). Cells were stained with mHA and centromeres were detected by cenFISH. In interphase fission yeast cells, all three centromeres are clustered close to the spindle pole body at the nuclear periphery (Funabiki et al., 1993). mHA in those cells (Fig. 4 B, panel a) produced multiple faint spots at the nuclear periphery. Most cells examined (41 out of 49) did not show any colocalization of Bub1 with the cenFISH signal. In prophase cells, three major patterns of cenFISH staining were observed (Fig. 4 B, panels b–d). The most common pattern (39 out of 60) was a single cenFISH signal colocalizing with the HA spot (Fig. 4 B, panel b). Less frequently (19 out of 60) two FISH signals were seen with the HA spot localized in between the two FISH signals (Fig. 4 B, panel c). Rarely (2 out of 60), the three centromeres could be detected separately and in those cases they were located around the HA spot (Fig. 4 B, panel d). In cells with two intense HA signals (Fig. 4 B, panel e), the distance between the two spots was always short (mean value 0.47 ± 0.06 μm). For these cells, the major pattern (37 out of 47) was a single cenFISH signal between the two HA spots. In some cells (10 out of 47), two cenFISH signals were observed, overlapping with the HA signals (data not shown). In metaphase cells (Fig. 4 B, panel f), the intense pattern of staining was lost but some punctate staining was still present and colocalized with the centromeres. In cells having completed anaphase (Fig. 4 B, panels g–i), some HA staining colocalized with the centromeres at the spindle poles but the bulk of the signal was not centromere-associated and formed a string-like structure between the separating chromatin masses (Fig. 4 B, panel h) which eventually disappeared in late anaphase B cells (Fig. 4 B, panel i). In seapted cells (Fig. 4 B, panel j) the interphase pattern reappeared.

To summarize, Bub1 in interphase is distributed in several foci at the nuclear periphery which are distinct from centromeres. When cells enter mitosis Bub1 is recruited to the centromeres, which are still clustered close to the SPBs at this early stage, and the staining becomes much more intense. In prometaphase, Bub1 usually forms two masses aligned along the spindle with the centromeres located in between. In metaphase, Bub1 appeared more diffuse but a fraction of Bub1 is still associated with the centromeres. In anaphase A and B the bulk of Bub1 is distinct from centromeres and follows the movement of the chromatin masses but a fraction of Bub1 remains centromere-associated until telophase.

Next we looked at the localization of Bub1 in cells arrested in prometaphase in response to the absence of a spindle. Cells from strain 415 (nda3KM311 bub1-6HA) were grown to early log phase at 32°C, shifted to 18°C for 8 h, and then fixed and processed for anti-HA immunofluorescence and cenFISH. After 8 h at restrictive temperature, cells were arrested with condensed chromosomes. In some instances, the chromosomes had diffused away from each other, allowing individual chromosomes to be seen by DAPI staining (Fig. 5). The cell at the top of the image...
shows three intense Bub1-HA signals colocalizing with the centromeres. Strikingly, some cells displayed paired foci of Bub1-HA staining rather than well-defined spots. In the bottom of the two cells shown in Fig. 5, all three spots were actually doublets. This pattern of staining was routinely seen in nda3-arrested cells but most often, only one or two chromosomes displayed paired Bub1 foci. Cells with three single spots represented ~63%, cells with one doublet and two spots 24%, two doublets one spot 25%, and three doublets 2%. The appearance of a doublet was not due to sister chromatid separation since the centromeric signal remained single, located in between the paired Bub1 foci. Such a staining pattern is consistent with Bub1 being associated with sister kinetochores. The mean distance between the two signals in a doublet was 0.42 ± 0.04 μm, which is strikingly similar to the distance separating the two Bub1 foci during the prometaphase stage of cycling cells. This suggests that the paired Bub1 structures observed in cycling cells are also formed by the close proximity of the sister kinetochores from all three chromosomes which are aligned on the spindle axis.

**Bub1 Is Required to Detect and Correct Defects in Centromere Function**

The bub1-118 mutant was identified in a screen for co lethality with Δswi6. The Swi6 protein is a structural component of fission yeast heterochromatin. As pointed out in the introduction, the Swi6 protein has only a minor role if any at telomeres but appears crucial for normal centromere function since the lack of a functional swi6+ gene is correlated with a defective movement of centromeres at anaphase and an elevated rate of chromosome loss (Allshire et al., 1995; Ekwall et al., 1996). Therefore, the lethality of the Δswi6Δbub1 double mutant suggests that the Bub1 checkpoint pathway is required to monitor defects in centromere function. If this is indeed the case, the lethality of the double mutant is expected to arise from a complete failure to segregate chromosomes. To test this prediction, we performed a cytological analysis of Δbub1 cells in which the synthetic lethality was induced by depletion of Swi6.

Strain 57 carries the swi6+ ORF under the control of the nmt promoter (nmtswi6) which is repressible by addition of thiamine to the culture medium (Maundrell, 1990). In the absence of thiamine, Swi6 is expressed and the strain behaves as a swi6+ strain. Addition of thiamine into the medium represses nmt and after 8–10 generations, Swi6 is depleted and defective anaphases appear (Table V) with a frequency similar to that observed in a Δswi6 strain grown at 32°C (data not shown). Strain 399 carries the Δbub1 allele in a nmtswi6 background. When Swi6 is produced, the frequency of defective anaphases was ~7.4% (Table V), close to the value obtained for the bub1-deleted strain. 20 h after the addition of thiamine, when Swi6 was depleted, the fraction of abnormal anaphases represented up to 50% of late anaphase cells. The image shown in Fig. 6 shows a field of cells in which all anaphase cells display lagging chromosomes and/or obvious unequal segregation of the chromatin masses. Therefore, the lethality of the Δswi6Δbub1 double mutant is likely to be a consequence of massive chromosome missegregation events. As a functional bub1+ gene is required for the viability of a Δswi6 cell, this suggests that Bub1 is somehow able to compensate for the centromere defect induced by the lack of Swi6. How can this be achieved? To address this question, we looked at Bub1 localization in a Δswi6 background. Cells from strain 429 (Δswi6 bub1-6HA) were grown to early log phase at 26°C and processed for anti-tubulin and anti-HA immunofluorescence. The Bub1 staining patterns in prophase and prometaphase were similar to that observed in a wild-type background (Fig. 7 A, top and middle) showing that the lack of Swi6 does not grossly affect the localization of Bub1. However, a large fraction of metaphase cells displayed an intense punctate staining (Fig. 7 A, bottom). Examination of 103 metaphase Δswi6 cells (spindle length

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**Figure 5.** Bub1 localizes at kinetochores in nda3KM311-arrested cells. Cells from strain 415 (nda3KM311 bub1-HA) were grown to early log phase at 32°C and shifted to 18°C for 8 h. Fixed cells were stained with mHA (red) and centromeric DNA was detected by FISH (green). Blue, DAPI staining. Bottom right, merged image. Two cells are shown. In some instances, Bub1 staining produces three doublets (the cell at the bottom). The merged image reveals that centromeric DNA is located precisely in between the paired dots, consistent with Bub1 being located on each sister kinetochore. Bar, 10 μm.
greater than 2 μm and unsegregated DNA) revealed that 49 (47.5%) were devoid of intense Bub1-HA staining but 54 (52.5%) displayed one, two, or three intense Bub1-HA signals. This is in sharp contrast with what was observed in a swi6- background where only five metaphase cells from 150 examined (3.4%) retained an intense Bub1-staining pattern.

Examination of Bub1-stained postmetaphase cells did not reveal any significant difference between swi6- and Δswi6 strains. Of interest was the Bub1 staining status of lagging chromosomes in Δswi6 anaphase cells. In all cells examined (100), lagging chromosomes did not show any intense Bub1 staining but a rather diffuse labelling of the lagging chromatin (Fig. 7B). The weak, diffuse signal on the lagging chromosomes is not due to poor immunofluorescence since, like in wild-type anaphases, Bub1 can be seen leading the separated main chromatin masses (Fig. 7B).

Taken together, these observations are consistent with the idea that, in early mitosis, Bub1 is recruited to centromeres not yet bound to microtubules but this ability is lost past the metaphase–anaphase transition since aberrantly segregating centromeres at anaphase do not recruit Bub1.

Next we asked whether the dramatic recruitment of Bub1 in Δswi6 metaphases could be correlated with the introduction of a cell cycle delay. As shown in Table VI, the fraction of metaphase cells is doubled in a Δswi6 background. This increase in metaphase cells is statistically highly significant (P < 0.01 by the Chi squared test). Therefore, cells harboring a compromised centromere spent twice the normal amount of time in metaphase. It should be noted that there is also an increase in the fraction of late mitotic cells in Δswi6 compared to wild type. Although these data are at the borderline of statistical significance (P < 0.05) they suggest that the duration of anaphase and/or telophase is also increased in Δswi6 cells.

From these observations, we conclude that cells in which centromeres are weakened by the absence of the Swi6 protein are delayed in metaphase and this delay correlates with the persistence of an intense punctate Bub1 staining. This suggests that misattached kinetochores activate the checkpoint through the recruitment of Bub1 and as a consequence anaphase is delayed.

The above observations suggest that Bub1 is able to slow cell cycle progression when it is recruited to centromeres. In wild-type cycling cells, intense Bub1 staining is observed in prophase and prometaphase. Thus, if Bub1 acts to control the timing of normal mitosis, one might expect Δbub1 cells to pass more quickly through mitosis. As shown in Table VI, there is a modest decrease in prophase and prometaphase cells in the Δbub1 strain as compared
to wild type, but the difference is not statistically significant. Therefore, the loss of bub1 function does not appear to grossly alter the timing of mitosis.

**Discussion**

We have shown that fission bub1 encodes a mitotic centromere protein which is an essential component of the spindle checkpoint. Vertebrate homologues of several checkpoint proteins, namely Mad2, Mad3, Bub1, and Bub3 have all been shown to localize to unattached kinetochores leading to the idea that microtubule-free kinetochores attract checkpoint components which in turn inhibit or delay cell cycle progression. Our data fully support this idea. Immunofluorescence analysis reveals that Bub1 is massively recruited to centromeres during the early stages of mitosis when kinetochores are not yet captured by microtubules, and when microtubule polymerization is prevented by a mutation in β-tubulin. In cells lacking the centromere protein Swi6, kinetochores are thought to be less efficient in capturing microtubules, and we found that in those cells the intense Bub1 staining persists in metaphase. Conversely, in wild-type cells the Bub1 staining is diminished in metaphase when all kinetochores have been captured. Thus, intense Bub1 staining at centromeres correlates well with the presence of microtubule-free kinetochores. There is also a good correlation between the centromeric recruitment of Bub1 and the introduction of a cell cycle delay. The prometaphase arrest of nda3 cells at 18°C is dependent upon a functional bub1 gene and is characterized by a strong Bub1 staining at kinetochores. Similarly, cells devoid of a functional swi6 gene show metaphases with intense Bub1 staining and spend twice the normal amount of time in metaphase. Therefore, our data suggest that microtubule-free kinetochores attract Bub1 which in turn delays the onset of anaphase.

Although the above is true when kinetochores or spindle function is compromised, the situation is less clear in an unperturbed mitosis since the lack of a functional bub1+ gene does not appear to shorten the early stages of mitosis. It is possible that kinetochore capture in mitosis is normally a very efficient process which is completed well before full activation of the APC. However, we found that in cells lacking bub1+ function ~13% of anaphases displayed lagging chromosomes, suggesting that stable kinetochore capture had not been achieved in a significant fraction of metaphases. In addition, it has been reported that overexpression of a dominant-negative form of mouse Bub1 in HeLa cells shortens mitosis by 25 min (Taylor and McKeon, 1997). Live analysis of mitosis in individual fission yeast cells will be necessary to address this proposed mitotic timing function for Bub1 more carefully.

Overexpression of Bub1 in budding yeast results in a nuclear accumulation, but unfortunately the protein could not be detected in wild-type cells (Roberts et al., 1994). The mouse Bub1 protein is recruited to kinetochores in early mitosis but dissociates in metaphase and is thus absent from kinetochores later in mitosis (Taylor and McKeon, 1997). Similarly in S. pombe, kinetochores are brightly stained with Bub1 in prophase and prometaphase and the intense signal is then lost in metaphase. However, some staining remains centromere associated in metaphase and throughout the final stages of mitosis, raising the possibility that Bub1 might have a role at kinetochores in anaphase. Such staining is reminiscent of that recently reported for p55Cdc20, the vertebrate homologue of budding yeast Cdc20 and fission yeast Slp1, both of which have been proposed to be effectors of the spindle checkpoint (Hwang et al., 1998; Kallio et al., 1998; Kim et al., 1998). Cdc20/Slp1 are essential genes required for the proteolytic destruction of a number of proteins including regulators of sister-chromatid separation (Pds1/Cut2) and the mitotic cyclins. Their precise roles and interaction with the APC remain unclear, but it has been reported (Kallio et al., 1998) that p55Cdc20 can be immunoprecipitated with components of the APC (Cdc27) and the spindle checkpoint (Mad2). Further biochemical work is necessary to determine whether this is true in fission yeast and if so whether Bub1 is also present in such complexes.

One explanation for the anaphase localization of Bub1 at kinetochores could be that it also has a nonessential, structural function within the kinetochore. For example, Bub1 might increase the kinetochore’s affinity for microtubules thereby promoting kinetochore capture and strengthening the attachment of chromosomes to the spindle. Lagging chromosomes in cells lacking bub1+ could arise from the combined effects of the lack of a functional checkpoint and a weakened kinetochore. Alternatively, a late role for Bub1 could also be restricted to a checkpoint function. Intriguingly, cells deleted for swi6 do show an increased proportion of anaphase cells, suggesting that they activate a checkpoint which operates past the metaphase–anaphase transition. For instance, lagging chromosomes in anaphase cells could activate a checkpoint to delay cytokinesis. A cytokinesis checkpoint has recently been reported in budding yeast (Muhua et al., 1998). However, the existence of such a checkpoint in fission yeast and any involvement of Bub1 remain speculative. In addition, lagging chromosomes in Δswi6 cells were not intensely Bub1 stained, suggesting that unattached kinetochores are unable to activate the spindle checkpoint past the metaphase–anaphase transition.

The existence of lagging chromosomes in Δswi6 cells raises the question of the effectiveness of the checkpoint

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**Table VI. Distribution of Mitotic Cells in Δswi6 and Δbub1 Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Background</th>
<th>Number of cells analyzed</th>
<th>Cells in prophase and prometaphase</th>
<th>Cells in metaphase</th>
<th>Cells in anaphase and telophase</th>
</tr>
</thead>
<tbody>
<tr>
<td>77</td>
<td>Wild-type</td>
<td>3,583</td>
<td>69 (1.9%)</td>
<td>62 (1.7%)</td>
<td>312 (8.7%)</td>
</tr>
<tr>
<td>423</td>
<td>Δswi6::his1+</td>
<td>3,090</td>
<td>42 (1.4%)</td>
<td>115 (3.7%)</td>
<td>322 (10.4%)</td>
</tr>
<tr>
<td>393</td>
<td>Δbub1::ura4+</td>
<td>3,047</td>
<td>46 (1.5%)</td>
<td>67 (2.2%)</td>
<td>305 (10%)</td>
</tr>
</tbody>
</table>

*Prophase and prometaphase were defined as cells with a spindle length shorter than 2 μm.
†Metaphase cells were defined as cells with unsegregated DNA and a spindle length greater than 2 μm and shorter than 5 μm.
‡Anaphase and telophase cells were defined as cells with segregated DNA and spindle length greater than 5 μm. Parentheses, fraction of each cell population.
in preventing missegregation events. The survival of Δswi6 cells is dependent upon a functional bub1 gene showing that the checkpoint is required to correct the defect induced by the lack of Swi6. However, the rescue is only partial since a fraction of anaphase cells show lagging chromosomes. Why do these missegregations events escape the checkpoint? Experiments in budding yeast have shown that mutations in the essential kinetochore component Ndc10 abrogated the spindle checkpoint (Tavormina and Burke, 1998), leading to the idea that a kinetochore has to be formed to recruit checkpoint components. It is possible that the lack of Swi6 produces a heterogenous population of centromeres with respect to kinetochore function. One class would have compromised centromeres with some residual kinetochore and checkpoint function and the other class would have disrupted kinetochores which, like ndc10 kinetochores, would remain undetected by the checkpoint and would generate lagging chromosomes at anaphase. This scenario could also explain why we did not observe any recruitment of Bub1 to the centromeres of lagging chromosomes. Further experiments are thus needed to investigate a possible role for Bub1 during the final stages of mitosis.

In light of recent results, it has been suggested that mutational inactivation of the spindle checkpoint might play an important role in the progression of human cancer. Some cancers are associated with a chromosomal instability phenotype (CIN) leading to aneuploidy. It has been proposed that checkpoint defects might lead to aneuploidy (Hartwell and Kastan, 1994) and that this would then be instrumental in tumorigenesis through the loss of tumor suppressor genes (Orr-Weaver and Weinberg, 1998). Cahill and colleagues showed that CIN in colorectal cancers is correlated with the loss of the spindle checkpoint and, in a number of cases, is associated with a mutation in hBUB1, the human homologue of budding yeast BUB1 (Cahill et al., 1998). These results suggest that aneuploidy is due to the lack of a functional checkpoint. Δbub1 spores in budding yeast grow very slowly and often fail to form colonies, but when propagated this growth phenotype reverts possibly due to the accumulation of suppressor mutations (Roberts et al., 1994). To our knowledge a quantitative rate of chromosome loss has not been reported for such budding yeast strains. Overexpression of the dominant-negative mouse Bub1 construct may have affected mitotic timing, but it did not appear to have a significant effect on chromosome segregation (Taylor and McKeon, 1997). We have shown that haploid S. pombe Δbub1 strains lose a 530-kb linear minichromosome 70 times more frequently than wild-type strains, and that homozygous bub1Δ diploids are extremely genetically unstable. Thus, our fission yeast results strongly support the idea of a causal relationship between the lack of a functional bub1+ gene, the loss of the spindle checkpoint, and the appearance of chromosome instability and aneuploidy in human cancers.

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